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Impact of intra-vineyard soil heterogeneity on Malbec. Vine growth, yield and wine elemental composition and sensory profile

Impacto de la heterogeneidad de suelo intra parcelaria en Malbec. Crecimiento, rendimiento, composición elemental y perfil sensorial de sus vinos

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Abstract

In Mendoza, viticulture is increasingly expanding into mountainous regions, taking advantage of cooler temperatures. High-altitude vineyards, characterized by greater soil heterogeneity, can significantly impact grapevine growth, development, elemental uptake, and wine sensory attributes. Despite its relevance, the effects of intra-vineyard variability on wine organoleptic quality and elemental composition remain underexplored in the existing literature. This study investigated a high-altitude vineyard planted with *Vitis vinifera* L. cv. "Malbec", focusing on two contrasting soil depth profiles: shallow soil (SS) and deep soil (DS). The DS exhibited a finer texture, higher water retention and greater cation exchange capacity than the SS. Additionally, DS contained higher concentrations of Mn, while SS was richer in Ca. Vegetative growth and yield varied according to soil type and vintage. Wines from DS showed higher [Mn], consistent with the soil, and increased [Fe] and [Cu] compared to SS wines, possibly due to indirect effects. Significant differences were observed in wine organoleptic properties, with SS wines exhibiting greater color intensity, astringency, and structure. Certain aromas, such as cherry and plum were negatively correlated with [Mn]. These findings highlight the influence of vineyard soils on the elemental composition and sensory profiles of wines, providing valuable insights into terroir characteristics for management strategies.

Keywords

cationic profile • edaphic variability • organoleptic wine properties • phenotypic expression • soil type • terroir

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Resumen

En Mendoza, la viticultura está en expansión creciente hacia áreas montañosas para aprovechar temperaturas más frescas. Los viñedos de altura, que están caracterizados por una mayor heterogeneidad del suelo, pueden influir significativamente en el crecimiento, desarrollo, absorción de elementos, y atributos sensoriales de las uvas y vinos. A pesar de su relevancia, los impactos de la variabilidad a nivel intra parcelario, en la calidad organoléptica y la composición elemental de los vinos, han sido poco explorados en la literatura existente. Este estudio investigó un viñedo de altura plantado con *Vitis vinifera* L. cv. "Malbec", centrándose en dos sectores con profundidad contrastante de suelo: suelo superficial (SS) y suelo profundo (DS). DS presentó una textura más fina, mayor capacidad de retención de agua y mayor capacidad de intercambio catiónico en comparación con SS. Además, DS mostró mayores concentraciones de Mn, mientras que SS tuvo más [Ca]. El crecimiento vegetativo y el rendimiento variaron según el tipo de suelo y la temporada de cultivo. Los vinos de DS presentaron mayor [Mn] en concordancia con el suelo y mayor [Fe] y [Cu] en comparación con los vinos de SS, posiblemente debido a efectos indirectos. Se encontraron diferencias significativas en las propiedades organolépticas de los vinos, con una mayor intensidad de color, astringencia y estructura en los vinos de SS. Algunos aromas, como cereza o ciruela, se correlacionaron negativamente con [Mn]. Estos hallazgos destacan la influencia de los suelos del viñedo en la composición elemental y los perfiles sensoriales del vino, contribuyendo a la comprensión de las características del terroir para estrategias de manejo.

Palabras clave

perfil catiónico • variabilidad edáfica • propiedades organolépticas del vino • expresión fenotípica • tipo de suelo • terruño

Introduction

In Mendoza, Argentina's primary wine-growing region, vineyards planted near the Andes mountain in the Uco Valley, at altitudes ranging from 900 to 1,500 m above sea level, have expanded rapidly in recent years. Malbec is the most widely planted red grapevine cultivar in Argentina, covering 42,999 ha, with 85.1% of this area located in Mendoza province (30). According to the literature, high-altitude vineyards are generally defined as those located within a broad elevation range of 350 m to 2,900 m a. s. l. (46), with the primary goal of achieving optimal temperatures for grapevine cultivation. As a result, high-altitude viticulture is gaining significance due to its potential to produce high-quality wines in regions increasingly affected by global warming (3).

As vineyards are planted closer to the mountains in search of optimal temperatures, they also experience other environmental changes, such as fluctuations in ambient humidity, wind patterns, and increased exposure to ultraviolet-B radiation (UV-B) (3, 8). Soil composition constitutes one critical factor to be considered in mountainous environments (21). Soils play a fundamental role in balancing the vegetative and reproductive development of grapevines, influencing berry quality and the sensory profile of the resulting wine (56). In foothill areas, soils are shaped by alluvial and fluvial processes, forming alluvial cones with variations in soil depth, texture, and rock volume, which lead to pronounced soil heterogeneity at an intra-vineyard scale (≤ 1 ha) (21, 41, 50). This- high degree of variability can significantly affect grapevine cultivation and wine quality (11). Differences in soil depth can result in physical and chemical variations, such as changes in texture, water-holding capacity, and cation exchange capacity (CEC) (50), all of which impact root development, water uptake and nutrient absorption (60). These factors, in turn, influence vegetative growth, yield, and the quality and sensory profile of berries and wines (61). Considering Malbec cultivar, Roig-Puscama *et al.* (2021) reported that strong soil heterogeneity within a single vineyard induces changes in the xylem structure of grapevine main stems, interpreted as an adaptive response to differences in soil water retention capacity. Soils with low water retention can induce water stress, leading to higher levels of abscisic acid (ABA) and, consequently, increased total polyphenol content in berries and wines under heterogeneous soil conditions (47). This variability can produce wines with distinct styles from the same

vineyard (11). Intra-vineyard variability challenges viticulturists and winemakers who seek uniform fruit parcels for specific products (12). However, it also offers an opportunity for winemakers to differentiate their products by leveraging the unique characteristics of soil heterogeneity.

Soil heterogeneity influences mineral composition, affecting uptake and accumulation of elements in grapevines. Regional-scale studies have demonstrated a correlation between the elemental composition of soils, berries and wines, facilitating the identification of a wine's geographical origin (32, 39). Berry and wine elemental profile primarily reflects soil characteristics, shaped by its distinct geological features (2). However, the accumulation of these elements can vary depending on the plant material (31), while changes in soil fertility may influence the ripening process and sugar accumulation in berries (29). Variations in elemental content in berries can also affect oxidation-reduction reactions during vinification, leading to differences in organoleptic properties and, ultimately, wine quality (55). Despite this, the relationship between soil elemental composition and sensory attributes of wine remains poorly understood (36).

Previous studies on the effects of soil in vineyards have primarily been conducted at a regional scale, making it difficult to disentangle key influencing factors such as climate and topography (25, 32, 59). Moreover, there is limited understanding of how soil elemental concentrations at the intra-vineyard scale affect the elemental composition of wine and, consequently, its organoleptic characteristics.

This study aimed to examine the influence of two soil types with contrasting properties, specifically depth, texture, and the presence of boulders, on vegetative growth, yield, elemental composition, and the sensory profile of wines at an intra-vineyard scale. Importantly, the objective was not to propose management strategies for homogenizing vigor and yield based on soil type. Instead, the analysis was conducted within a high-altitude Malbec vineyard, where plant material, management practices, and climate were controlled as fixed factors.

Material and methods

Study site

The study was conducted over three growing seasons (2017-2019) in a high-altitude commercial vineyard located within the Geographical Indication (GI) "Paraje Altamira" (Zuccardi Valle de Uco winery, 33°46'20.29'' S; 69°9'14.62'' W; 1,100 m a. s. l.), Mendoza, Argentina. This vineyard, situated in the foothills of the Andes mountains, is characterized by significant soil heterogeneity.

Plant material and vineyard characteristics

The study focused on a 2.5-ha parcel planted in 2009 with own-rooted *Vitis vinifera* cv. Malbec, derived from massal selection. The vines were 8 years old at the start of the study. The vineyard was trained using the Double Guyot system, with each vine pruned to two five-bud canes (long fruit-bearing shoots) and two-bud spurs (short shoots for renewal), totaling 14 buds per plants. Rows were oriented north-south, with a planting density of 1.8 m between rows and 0.8 m between plants. Shoots were vertically positioned using foliage wires, and the vineyard was equipped with anti-hail nets and drip irrigation. Fertigation consisted of potassium (potassium nitrate), nitrogen (urea) and phosphoric acid, applied at a rate of 25-7-12 N-P-K units over five weeks between flowering and fruit set. Pathogen management included micronized sulfur (7.2 kg ha^{-1} in four applications per season), and three $CuSO_4$ applications per season, with increasing concentration of 1% for the first two applications and 1.5% for the third. All management practices were applied uniformly across soil types.

Experimental design

Sectors with contrasting soil depths were identified using electromagnetic conductivity (EMC) maps generated with an EM38MK2 ground meter (Geonics Ltd., Canada). Measurements were taken at depths of 0.75 m and 1.5 m, identifying six conductivity

classes ranging from 1.06 to 5.24 mS m^{-1}. The two extreme classes were selected using GIS software (48), and soil properties were confirmed through trench excavations. Two distinct soil types were identified. Shallow soils (SS) contained boulders (>0.3 m in diameter) occupying ~85% of the soil profile, with an alluvial layer depth of 0.2-0.3 m. Deep soils (DS) consisted of alluvial sediments with a sandy-loam texture and an average depth of 2 m. The depth and structure of both soil types are shown in figure 1. Six experimental units (plots) were established per soil type, totaling 12 plots. Each plot covered 184 m^2 and included 128 plants (figure 1).

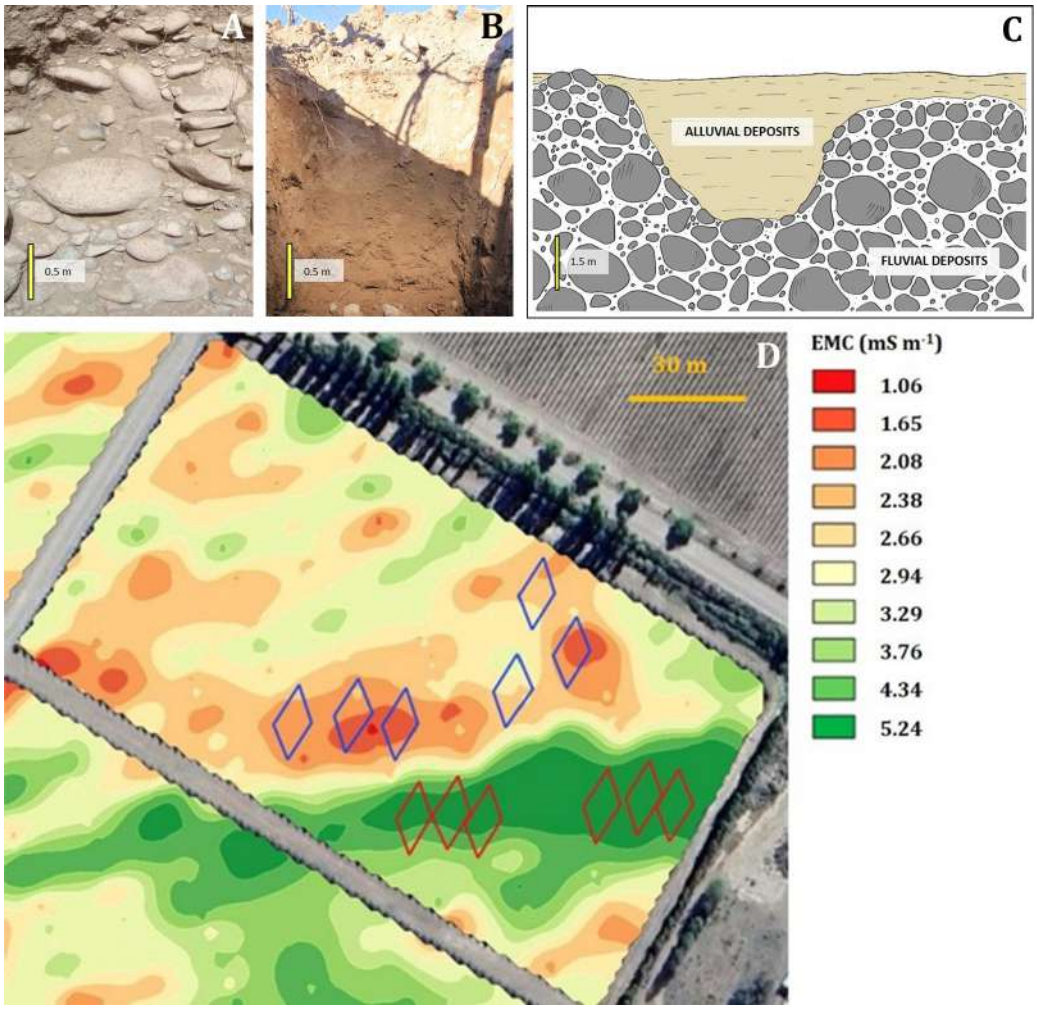


Figure 1. Vertical profile of soil structure and depth for SS (A) and DS (B). Schematic representation of soil profile found in Paraje Altamira (C). Electromagnetic conductivity (EMC) maps measured with the EM38 MK2 ground meter and EMC values. Experimental blocks are showed with parallelogram: reds for SS and blues for DS (D).

Figura 1. Perfil vertical de la estructura y profundidad del suelo para SS (A) y DS (B). Representación esquemática del perfil de suelo encontrado en Paraje Altamira (C). Mapas de conductividad electromagnética (EMC) medidos con el medidor de suelo EM38 MK2 y los valores de EMC. Los bloques experimentales se muestran con un paralelogramo: rojos para SS y azules para DS (D).

Soil sampling and analysis

Soil sampling was conducted in 2017 at the start of the study during trench excavations used to define soil types. Composite soil samples were collected from each plot at a depth of 0.20-1 m to minimize surface contaminants and capture the root zone. Soil texture was analyzed using the hydrometer method described by Bouyoucos (1951) and cation exchange capacity (CEC) was determined following Richards (1954). Water-holding capacity, including saturated water point (Ws), field capacity (Wc) and permanent wilting point (Wp), was estimated using pedotransfer functions based on soil texture classes (14, 49). Elemental composition was determined by acid digestion following Funes-Pinter (2018) Dried soil samples were treated with a mixture of HNO_3 (65%), HCl (37%), HCl_4 (65%), and H_2O_2 (30% vol), followed by centrifugation and dilution. Elemental analysis was performed using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS) with a PerkinElmer SCIEX, ELAN DRC-e equipment (Thornhill, Canada), to quantify Mg, Ca, Na, K, Mn, Fe, Cu, Al, Zn, Li, Rb, and Cd concentrations.

Vegetative growth, yield components

Vegetative growth and yield observations were assessed annually over three growing seasons (2017-2019). Three healthy and homogeneous plants per plot were randomly selected for monitoring. During the growing season, at the berry pea-size phenological stage (fruit diameter ~5 mm, classified by Baggiolini (1952), measurements were taken for shoot length, internode length, and shoot diameter, key indicators of vine vigor. For this purpose, the north-directed cane in the row was selected on each plant, corresponding to the five-bud canes left during pruning, as described in the vineyard characteristics section. Shoot diameter was measured with a caliper at the midpoint of the first internode, and average values were recorded for each plant. At harvest, yield components were evaluated, including the number of bunches per plant, bunch weight, yield per plant, berries per bunch, and berry weight. During winter dormancy, vegetative growth was further assessed by measuring total pruning weight per plant, another key vigor indicator. These measurements were performed on the same plants monitored during the growing season. The yield to pruning weight ratio was expressed as the Ravaz index (27). Additionally, stem water potential (Ψ_{stem}) was measured weekly from flowering to mid-veraison each season using a Scholander pressure chamber, following Scholander (1965). Fully expanded leaves were covered with aluminum foil at least one hour before measurement to ensure equilibration, and Ψ_{stem} values were recorded to assess plant water status.

Harvest and vinification procedure

Harvesting was conducted at commercial maturity, defined at a sugar concentration of 24 °Brix (1° Brix = 1 g of sugar per 100 mL of grape juice). Harvest dates varied by soil type (table S1). All plants in each plot were harvested, and grapes were collected in 16 kg boxes to minimize berry breakage. Quality control at the winery involved discarding dehydrated grapes and those showing signs of Botrytis infection. Micro-vinifications were performed for each plot following the standardized protocols of Zuccardi Valle de Uco Winery. Grapes were destemmed, sulphited, and placed in 50 kg stainless steel tanks, then inoculated with a proprietary yeast poll (confidential data). Maceration and alcoholic fermentation occurred over 12 days at 25-28°C. Wines were then transferred to 20 L plastic tanks for malolactic fermentation, with pH adjusted to 3.75 using tartaric acid and free sulfur dioxide corrected to 45 mg L^{-1}. Finally, wines were bottled in 0.75 L glass bottles and stored at 20°C in darkness until analysis.

Elemental composition of wines and descriptive sensory analysis

The elemental composition of wine samples was analyzed using ICP-MS, following a modified version of the soil analysis protocol. Five milliliters of each wine sample were transferred into a 15 mL tube, and 0.5 mL of 65% HNO_3 was added. The mixture was vortexed for 15 seconds, microwaved for 15 seconds at 600 W, and diluted with 9.5 mL of ultrapure water. Subsequently, 1 mL of the diluted sample was mixed with 1 mL of 65% HNO_3, sonicated for 30 minutes, and combined with 0.5 mL of H_2O_2. The mixture was heated in a water bath at 60°C for 60 minutes, then diluted with 9.5 mL of ultrapure water, and used for elemental analysis.

Additionally, wines from each plot were evaluated through quantitative descriptive sensory analysis (QDA), following the protocol described by Lawless and Heymann (2010). Two bottles of wine from each plot were analyzed. A professional panel of eight tasters from Argentina's National Institute of Viticulture (INV) established sensory descriptors for each soil type, covering visual, aroma, and taste attributes. These included 11 aroma descriptors, 11 taste descriptors, and 5 visual descriptors (table 1).

Sensory evaluations were conducted in individual booths. Each panelist used a structured tasting sheet with an unstructured scale from 1 (very low intensity) to 5 (very high intensity). Wine samples (30 mL) were served at room temperature in ISO-standard tasting glasses (ISO 3591-1977), covered with plastic lids and coded for blind evaluation.

Table 1. General sensory descriptors in Malbec wines from both types of soil during tasting consensus.

Tabla 1. Descriptores sensoriales generales encontrados en los vinos Malbec de ambos tipos de suelo durante el consenso realizado por el panel de cata.

Visual	Aroma		Taste/Mouthfeel	
Clarity	Mineral note	Caramel sauce	Acid	Unctuous
Color intensity	Strawberry	Peppers	Sweet	
Red Hue	Plum	Vanilla	Astringent	
Violet hue	Cherry	Chocolate	Hot	
Garnet hue	Blackberry	Jam/Liqueur	Bitter	
	Violets	Global intensity	Structure	

Statistical analysis

Soil physicochemical variables and elemental composition data were analyzed using non-parametric tests($p \leq 0.05$, Kruskall-Wallis), as the data did not meet the normality and homoscedasticity assumptions required for parametric tests. Vegetative growth and yield components were analyzed by multifactorial ANOVA (soil type and growing seasons; Fisher's LSD, $p \leq 0.05$). All statistical analyses were performed using InfoStat software (23).

Multiple Factor Analysis (MFA) and biplot graphics were used to explore relationships among elemental composition, sensory descriptors, and qualitative variables such as soil type and vintage. The analysis integrated two datasets, the elemental content matrix and the sensory variable matrix, into a unified framework, using the FactoMineR package in R (1, 34). Two biplots were generated: the first considered soil type as an active variable and vintage as a supplementary variable, highlighting the impact of soil type on the analysis. The second combined both, the elemental and sensory datasets. Aromatic Persistence, Varietal Typicity, and Global Quality descriptors were excluded, following Abdi *et al.* (2013), as these variables reflect taster preferences. Additionally, the biplot emphasized the 10 variables with the highest contribution percentages.

Results

Soil physicochemical traits and grapevine growth

The analysis revealed significant differences in physicochemical properties between the two soil types, except for pH. Shallow soils (SS) contained 18.6% more sand than deep soils (DS), while DS had 97.8% more clay and 88.8% more silt. Additionally, DS exhibited a 78% higher CEC and greater water retention capacity at all measured points (Ws, Wc, and Wm), with increases of 16.9%, 28.4%, and 33.4%, respectively (table 2, page 7).

Table 2. Soil water holding capacity (as indicated by Ws, saturated water point; Wc, field capacity; Wp, permanent wilting point), extractable soil water (RU), soil texture (clay, silt, and sand) and soil cation exchange capacity (CEC).

Tabla 2. Capacidad de retención de agua del suelo (indicado por Ws, punto de saturación. Wc, capacidad de campo; Wp, punto de marchitez permanente), agua extraíble del suelo (RU), textura del suelo (arcilla, limo y arena) y capacidad de intercambio catiónico del suelo (CEC).

Values are means and different letters within each factor and column indicate statistically significant differences (p≤ 0.05, Kruskall-Wallis test).

Los valores son medias, y letras diferentes dentro de cada factor y columna indican diferencias estadísticamente significativas (p≤ 0,05, prueba de Kruskal-Wallis).

Treatments	Ws (g%g)	Wc (g%g)	Wp (g%g)	RU (mm)	Clay (%)	Silt (%)	Sand (%)	CEC (meq%g)	pH
Deep soil	32.59 a	15.33 a	8.10 a	7.82 a	10.64 a	17.33 a	71.98 b	15.27 a	6.74 b
Shallow soil	27.89 b	11.94 b	6.07 b	4.38 b	5.38 b	9.18 b	85.40 a	8.58 b	6.79 ab
***P*-value**	0.0001	0.0001	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0735

Regarding vegetative growth and yield components, DS plants generally showed higher vegetative growth indices, with pruning weight (153%), shoot length (29%), internode length (25%), and shoot diameter (19%) exceeding those of SS vines. No significant differences were observed in yield, number of berries per bunch, or average berry weight between the two soil types. However, DS plants produced 12% more bunches per plant, while SS plants had 25% higher average bunch weight. The Ravaz index indicated an imbalance in DS plants, with values below 5, largely influenced by the 2017 season, suggesting that DS plants exhibit greater vegetative growth relative to berry production. In contrast, SS plants demonstrated more balanced values (table 3, page 8).

The season significantly influenced all analyzed variables. Pruning weight per plant in 2017 was 84% higher compared to the average of the 2018 and 2019 seasons. Yield per plant in 2017 was 50.6% lower than in 2019, which recorded the highest yield of the analyzed period. The 2019 season saw an average increase of 9 more bunches per plant, along with a higher average bunch weight, a greater number of berries per bunch, and a higher average berry weight compared to 2017. The interaction between soil type and season was significant for pruning weight, yield, number of bunches per plant, bunch weight, and berry weight. Pruning weight was consistently higher in DS plants across all years, with the largest difference observed in 2017, where DS had 187% higher pruning weight than SS. Conversely, yield responses varied by soil type and season. In 2017, SS yielded 43% less than DS, while in 2019, DS produced 33% more yield than SS. The number of bunches per plant also fluctuated based on soil type across the three years. In 2017, DS had 21.4% fewer bunches per plant than SS, but in 2018 and 2019, DS plants produced 29.2% and 27.1% more bunches, respectively. Bunch weight was significantly lower in DS during 2017 and 2018, with reductions of 28% and 38%, respectively. Berry weight only showed significant differences in 2018, where DS was 21% lower than SS.

Table 3. Multifactorial ANOVA of vegetative growth and yield components of plants growing in shallow (SS) and deep soils (DS), during 2017-2019 seasons.

Tabla 3. ANOVA multifactorial del crecimiento vegetativo y componentes de rendimiento de plantas en suelos cortos (SS) y profundos (DS), durante las temporadas de 2017-2019.

Treatments	Pruning weight (g)	Shoot length (m)	Internode length (cm)	Shoot width (mm)	Yield (kg pl^{-1})	Bunches per plant	Bunch weight (g)	Berries per bunch	Berry weight (g)	Ravaz index
Deep soil	710.84 a	118.75 a	5.06 a	7.17 a	1.20 a	22.76 a	48.60 b	47.22 a	1.46 a	2.20 b
Shallow soil	281.37 b	91.90 b	4.04 b	6.05 b	1.27 a	20.31 b	60.71 a	55.92 a	1.56 a	5.35 a
2017	712.54 a	117.39 a	6.15 a	7.37 a	0.85 c	17.55 b	45.55 b	47.63 b	1.44 b	1.79 b
2018	398.13 b	100.80 b	2.96 c	6.25 b	1.14 b	20.25 b	55.27 a	46.64 b	1.48 ab	4.40 a
2019	377.64 b	97.78 b	4.54 b	6.21 b	1.72 a	26.81 a	63.15 a	60.44 a	1.60 a	5.14 a
Deep soil*2017	1056.41 a	129.02 a	6.91 a	7.98 a	0.62 d	15.44 d	38.16 d	36.94 b	1.46 b	0.67 d
Shallow soil*2017	368.67 c	105.77 b	5.38 b	6.77 b	1.08 bc	19.65 bc	52.94 bc	58.31 a	1.42 b	2.90 cd
Deep soil*2018	585.26 b	119.23 a	3.27 de	6.93 b	1.02 c	22.83 b	42.48 cd	44.94 ab	1.41 b	1.87 cd
Shallow soil*2018	211.00 d	82.37 c	2.65 e	5.57 c	1.25 bc	17.67 cd	68.05 a	48.33 ab	1.79 a	6.93 a
Deep soil*2019	490.83 b	108.00 b	4.99 bc	6.61 b	1.97 a	30.00 a	65.17 ab	59.78 a	1.50 b	4.05 bc
Shallow soil*2019	264.44 cd	87.56 c	4.09 cd	5.82 c	1.48 b	23.61 b	61.14 ab	61.11 a	1.46 b	6.23 ab
p(soil)	<0.0001	<0.0001	0.0046	<0.0001	0.5917	0.0353	0.0012	0.0695	0.0797	0.0003
p(season)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0007	0.0326	0.0696	0.0032
p(soil*season)	<0.0001	0.0931	0.5587	0.1403	0.0039	0.0004	0.0044	0.1730	0.0028	0.2308

The Values are means (n = 18) and different letters between each factor indicate statistical differences ($p \leq 0.05$, LSD Fisher).
Los valores son medias (n = 18), y letras diferentes entre cada factor indican diferencias estadísticas ($p \leq 0,05$, prueba LSD de Fisher).

Elemental composition of soil and wine

There were no significant differences in the total elemental concentrations between DS and SS soils. However, specific elements showed notable variations: SS contained higher levels of calcium [Ca], while DS had greater concentrations of manganese [Mn] and potassium [K]. The average concentrations of major elements in both soil types followed the order Mg > Ca > K, and for trace elements, Fe > Al > Na > Mn > Zn > Cu > Rb > Li > Cd (table 4, page 9) .

Considering wines, no significant differences were observed in total elemental concentrations between soil types. However, wines from DS-grown plants exhibited higher levels of iron [Fe], manganese [Mn], and copper [Cu] compared to those from SS-grown plants. The concentrations of major elements in the wines followed the order K > Mg > Ca. For trace elements, wines from DS plants showed the order Na > Fe > Al > Mn > Rb > Cu > Li > Cd, whereas wines from SS plants followed Na > Al > Fe > Rb > Mn > Cu > Li > Cd. Notably, [Zn] concentrations in wines were below the detection limit for both soil types (table 4, page 9).

Descriptive sensory evaluation of wine

The visual descriptors indicated that soil type influenced the intensity of certain characteristics. SS wines exhibited greater color intensity and a more pronounced violet hue than DS wines (table 5, page 9-10). In terms of aromas, SS wines displayed more intense mineral notes, as well as plum aromas, compared to DS wines (table 5, page 9-10). For taste descriptors, SS wines were characterized by greater astringency, structure, varietal typicity, and overall quality than DS wines. In contrast, DS wines had a more pronounced sensation of acidity (table 5, page 9-10).

Table 4. Nonparametric analysis of variance (Kruskal Wallis) for elemental content in soil and Malbec wines (mg L^{-1}).

Tabla 4. Análisis de varianza no paramétrico (Kruskal-Wallis) para el contenido elemental en suelos y vinos Malbec (mg L^{-1}).

Treatments	K	Ca	Mg	Fe	Mn	Cu	Zn
Soil							
Deep	10.04 a	31.61 b	162.74 a	44.71 a	1.35 a	0.07 a	0.15 a
Shallow	8.33 b	54.13 a	154.51 a	42.93 a	1.06 b	0.10 a	0.14 a
Wine							
Deep	1072.46 a	14.61 a	21.42 a	1.20 a	0.71 a	0.06 a	-
Shallow	1018.63 a	14.56 a	20.39 a	0.68 b	0.52 b	0.04 b	-
***P*-value**							
Soil	0.0260	0.0043	0.2403	0.6991	0.0152	0.6991	0.3939
Wine	0.9734	0.8951	0.8432	0.0046	0.0001	0.0479	-

	Na	Al	Li	Rb	Cd	Total
Deep	1.89 a	18.25 a	0.02 a	0.05 a	8.00E-04 a	270.89 a
Shallow	1.67 a	15.69 a	0.02 a	0.04 a	7.30E-04 a	273.21 a
Wine						
Deep	11.65 a	1.10 a	0.04 a	0.61 a	1.10E-03 a	1123.86 a
Shallow	12.90 a	0.99 a	0.04 a	0.57 a	5.70E-04 a	1069.32 a
***P*-value**						
Soil	0.1039	0.0649	0.5887	0.1320	0.3615	>0.9999
Wine	0.3215	0.3913	0.4876	0.1872	0.1419	0.9474

Values are means and different letters within each factor and column indicate a statistically significant difference (p≤0.05, Kruskall Wallis).

Los valores son medias, y letras diferentes dentro de cada factor y columna indican una diferencia estadísticamente significativa (p≤0,05, prueba de Kruskal-Wallis).

Table 5. Intensity of visual, aroma, taste and mouthfeel descriptors of Malbec wines.

Tabla 5. Intensidad de los descriptores visuales, aromáticos, sabor y sensación en boca de los vinos Malbec.

Visual	Clarity	Color intensity	Red hue	Violet hue	Garnet hue	
Deep soil	4.80 a	4.17 b	3.63 a	4.00 b	3.79 a	
Shallow soil	4.70 a	4.49 a	3.65 a	4.31 a	3.58 a	
***P*-value**	0.1676	0.0003	0.7851	0.0037	0.0833	
Aromas	**Mineral**	**Strawberry**	**Plum**	**Cherry**	**Blackberry**	**Violets**
Deep soil	2.69 b	3.36 a	3.69 b	3.37 a	3.53 a	3.61 a
Shallow soil	3.23 a	3.29 a	3.89 a	3.13 a	3.57 a	3.70 a
***P*-value**	<0.0001	0.5818	0.0453	0.0579	0.7253	0.4758
Taste Mouthfeel	**Acidity**	**Sweet**	**Astringency**	**Heat**	**Bitter**	**Structure**
Deep soil	3.56 a	2.79 a	3.00 b	3.04 a	2.93 a	3.07 b
Shallow soil	3.30 b	2.94 a	3.38 a	3.12 a	2.75 a	3.64 a
***P*-value**	0.0084	0.1563	<0.0001	0.4019	0.1119	<0.0001

Values are means and different letters between each factor indicate statistical differences (p ≤ 0.05, LSD Fisher).

Los valores son medias, y letras diferentes entre cada factor indican diferencias estadísticas (p ≤ 0,05, prueba LSD de Fisher).

Aromas	Caramel	Paprika	Vanilla	Chocolate
Deep soil	2.73 a	2.69 a	2.59 a	2.57 a
Shallow soil	2.56 a	2.70 a	2.74 a	2.62 a
***P*-value**	0.2183	0.8834	0.2051	0.6836
Taste Mouthfeel	**Oiliness**	**Harmony**	**Aromatic persistence**	**Varietal typicity**
Deep soil	3.07 a	3.29 a	3.59 a	3.70 b
Shallow soil	3.14 a	3.33 a	3.55 a	3.96 a
***P*-value**	0.4182	0.6253	0.6461	0.0022

Aromas	Liqueur jam	Global intensity
Deep soil	2.96 a	3.56 a
Shallow soil	2.83 a	3.49 a
***P*-value**	0.3754	0.4842
Taste Mouthfeel	**Global quality**	
Deep soil	3.57 b	
Shallow soil	4.02 a	
***P*-value**	<0.0001	

Values are means and different letters between each factor indicate statistical differences ($p \leq 0.05$, LSD Fisher).

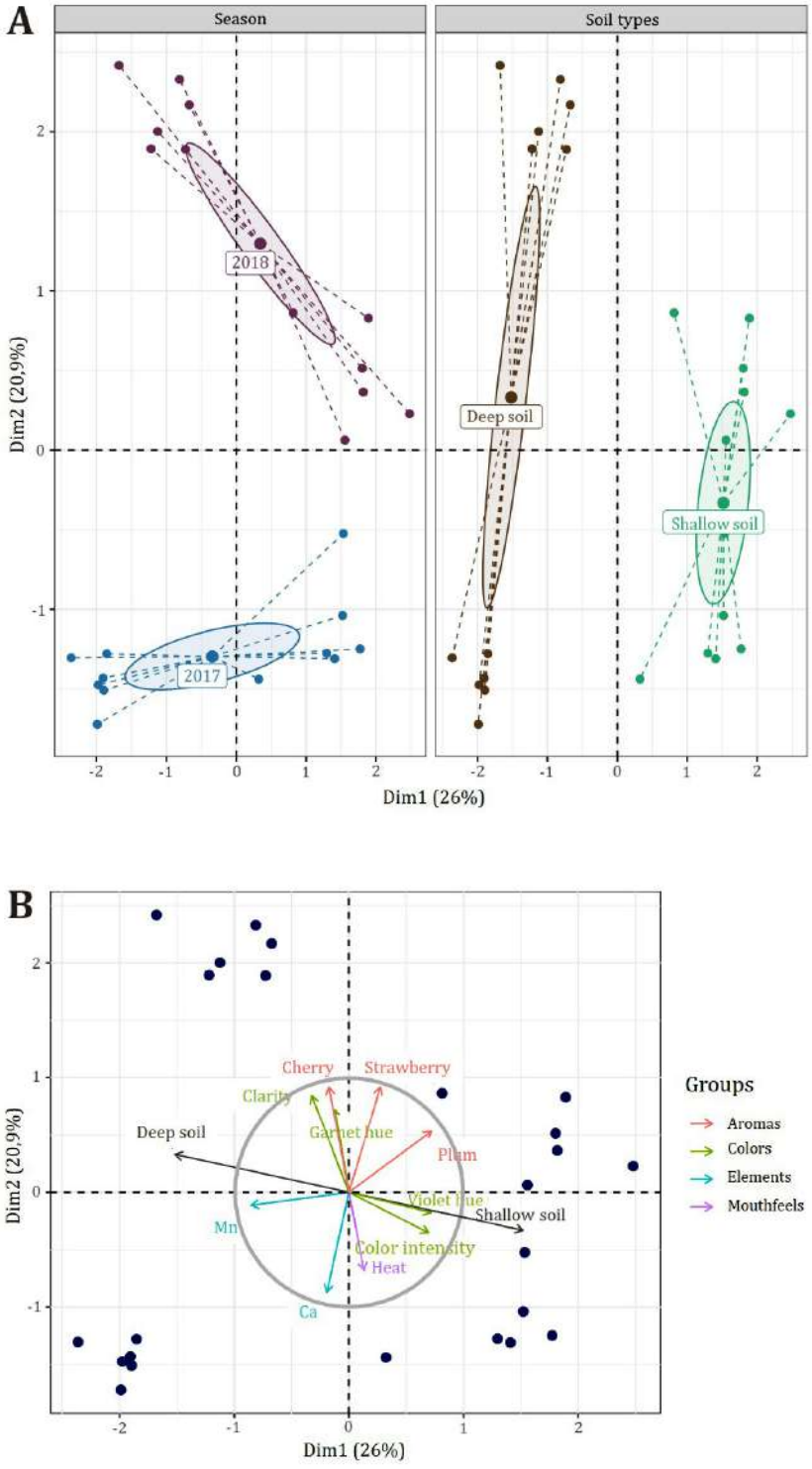
Los valores son medias, y letras diferentes entre cada factor indican diferencias estadísticas ($p \leq 0,05$, prueba LSD de Fisher).

The MFA revealed that wines from DS showed greater variability across vintages, suggesting a stronger influence of interannual conditions on this soil type. In contrast, wines from SS exhibited more consistent characteristics, highlighting the dominant effect of soil type on sensory variables over climatic variations (figure 2 A, page 11).

Among the sensory and elemental variables with the highest contributions were Cherry, Strawberry, Clarity, Garnet hue, Plum, Violet hue, Color intensity, Heat, Mn, and Ca (Figure S3). The analysis combining elemental composition and sensory data highlighted key relationships between quantitative and qualitative variables, such as soil type and vintage. Variables positioned closer to the gray circle (value 1) in the biplot contributed significantly to the model, emphasizing their relevance in the main dimensions. For example, [Ca] was more influenced by vintage than soil type, with 2017 showing a higher accumulation of this element in wines. Conversely, [Mn] was more strongly associated with deep soils, regardless of vintage (figure 2 B, page 11).

In terms of visual descriptors, wines from SS were characterized by higher color intensity and more pronounced violet hues, whereas wines from DS exhibited subtler visual attributes, such as clarity. Considering aromatic descriptors, SS wines were particularly noted for their plum aromas.

Some sensory descriptors, however, appeared to be more influenced by vintage than soil type, likely due to climatic conditions. For instance, cherry and strawberry aromas, as well as the visual descriptor Garnet hue were more closely associated with climatic conditions, especially the 2018 vintage. In contrast, the sensation of heat (linked to higher alcohol content) was primarily associated with the 2017 vintage.



(A) Influence of soil type (active variable) and vintage (supplementary variable) on Dim1 and Dim2, showing greater interannual variability in DS wines compared to SS wines. (B) Biplot integrating elemental and sensory descriptors, highlighting key contributors.

(A) Influencia del tipo de suelo (variable activa) y la añada (variable suplementaria) en Dim1 y Dim2, mostrando una mayor variabilidad interanual en los vinos de DS en comparación con los de SS. (B) Biplot que integra descriptores elementales y sensoriales, destacando los principales contribuyentes.

Figure 2. Multiple Factor Analysis (MFA).

Figura 2. Resultados del Análisis Multifactorial (MFA).

Discussion

Our findings indicate that soil characteristics, such as depth, texture and fertility, have differential effects on vegetative growth, as well as on the elemental composition and sensory profile of Malbec wines. However, yield components did not show a consistent pattern based on soil type, exhibiting variability across seasons. Indicators of vegetative growth, including pruning weight, shoot length, internode length, and shoot width, were strongly correlated with soil depth, with DS displaying superior values across all vigor indicators. These differences in vegetative growth can likely be attributed to variations in water availability, as DS retained higher water content than SS, and these growth indicators are known to be highly sensitive to water deficit (52). This finding is consistent with previous studies conducted in similar areas of the Mendoza foothills, where vines planted in deeper soils exhibited improved growth due to better water retention (42).

Surprisingly, no significant differences in yield were observed between soil types when analyzing the average values across the three studied seasons. However, a strong interaction was found between vigor and production indicators, which varied by season. The 2017 season saw significantly higher vigor in DS plants across all analyzed variables. However, the most productive plants during this season were those in SS, likely due to a greater number of bunches and more berries per bunch. This suggests improved fruit set, reduced berry shatter, and/or lower bunch abortion rates in SS compared to DS plants. As noted earlier, no bunch thinning was performed in the analyzed treatments. Notably, the 2017 season was particularly humid compared to the historical average (Figure S1), which could have influenced the vigor of DS plants, creating an imbalance between vigor and fertility. The imbalance is important, as the Malbec cultivar is prone to shatter and millerandage in vigorous plants (17).

In contrast, the 2018 season was approximately 40% drier than the historical average (Figure S1). The impact of this drier season was primarily observed in reduced internode growth in plants from both soil types, with consistently low values and no significant differences between soils. Yield per plant also showed no differences between soil types. However, SS plants had fewer bunches per plant but higher bunch weights due to greater berry weight, while DS plants produced more bunches per plant but with smaller berries. The drier conditions likely affected bud fertility, fruit set, and bunch necrosis (26) in SS plants, resulting in fewer bunches. During bud primordia development, low water availability may cause floral primordia to dedifferentiate into tendrils, reducing bud fertility and ultimately the number of bunches in the following season (53).

In 2019, the same trend in vigor indicators as in 2018 was observed, although internode length values were higher. This season had slightly more rainfall than the historical average, and the higher yield in DS plants was primarily due to a greater number of bunches per plant. Since both soil types had the same number of buds per vine during pruning, this suggests that soil conditions significantly influence final yield. Reduced bud fertility in SS plants, evidenced by fewer bunches per plant, likely explains the lower yield in this soil type. Additionally, some buds in SS plants failed to break, further contributing to the reduced yields. While soils with higher water-holding capacity often correlate with increased yields (12), studies by van Leeuwen *et al.* (2004) have reported reduced bunch weights in stony soils compared to clay soils. Limited water absorption reduces vegetative growth and production (41, 58), but our results showed no significant differences in yield per plant, number of berries per bunch, or berry weight between soil types. Although differences in bunches per plant and bunch weight were observed across vintages, no clear trend was found. While SS plants had larger bunches in 2017, DS plants exhibited this trait in 2018 and 2019. These variations in yield across vintages are likely due to climatic factors, as the same number of buds was retained during pruning.

Bunches per plant and bunch weight are influenced by various factors, including climatic conditions, soil electrical conductivity, and agricultural management practices (42). The equal number of buds left in plants (14 per plant) in both soil types may have contributed to the observed results. Previous studies suggest that the Malbec cultivar develops compensatory mechanisms between vegetative growth and production, acclimating to the edaphic environment by enhancing hydraulic conductivity in SS. This adaptation may help

Malbec overcome the low water retention capacity in SS (49), which could play a significant role in balancing production across different soil types. However, the Ravaz index, calculated from yield and pruning weight data, indicated that DS plants exhibited excessive vegetative growth relative to production. This finding aligns with previous research (41), which showed that Malbec plants in DS demonstrated greater vegetative growth in relation to yield. In contrast, other studies have reported that SS plants have lower vegetative-to-yield ratios (59). Despite these differences in the Ravaz index between soil types, our study found no significant differences in yield for the Malbec cultivar under the conditions analyzed.

Our study revealed that the elemental profile of soil at intra-vineyard level varies with depth. Soil analysis showed that DS, characterized by a higher proportion of fine particles, increased CEC, and greater water retention, provides a potentially less stressful environment for grapevine growth compared to SS (16). Although SS exhibited significantly higher [Ca], consistent with the high $CaCO_3$ content in the fluvial strata of the Andes (20, 21), no differences in pH were detected between the two soil types, which could influence nutrient absorption (13). The higher [Mn] and [K] observed in DS are consistent with its higher clay content (43). Consequently, DS soils are enriched in Mn and K, whereas SS soils have higher [Ca], potentially influencing nutriment uptake and accumulation in berries, and subsequently in wine, due to antagonistic or synergistic nutrient interactions (43). For example, high potassium levels in soil may enhance manganese uptake (5), while elevated calcium levels may inhibit it (37).

Wines from DS soils showed higher [Mn], [Cu] and [Fe]. Elevated concentrations of Mn in DS soils were also reflected in the wines produced from these soils. Similar findings have been reported by Kment *et al.* (2005), who found that wines from deep, clay-rich soils had higher [Mn] than those from SS (12). Campbell and Nable (1988) explained that plant [Mn] levels are directly influenced by xylem flow (25). Given this and the greater water-holding capacity of DS soil, DS plants likely experience increased [Mn] availability through mass flow, which would explain the higher concentrations of this element in DS wines. Although these observations were made on a regional scale, previous studies have identified Mn as a key element distinguishing wines based on their origin (39).

This study highlighted the significant impact of soil heterogeneity at the intra-vineyard level (micro-scale) on wine, resulting in differential elemental profiles. Although high [Cu] and [Fe] in wines did not correlate with higher concentrations of these elements in DS, the increased [Cu] can be attributed to a potentially greater interception of $CuSO_4$ applications by the canopy, as DS plants have a larger exposed leaf surface. The elevated [Fe] detected in wines may be influenced by several factors, including differential adsorption of dust particles on the epicuticular wax of berries and physiological differences in Fe uptake and allocation within the plant. While the higher vegetative growth in DS plants may alter Fe dynamics, the specific mechanism leading to elevated Fe levels in berries and wines remains unclear. This potential relationship between vegetative growth and Fe accumulation warrants further investigation to confirm a direct causal link. Additionally, our study demonstrated that visual, aroma, and taste descriptors of wine differ depending on soil type at intra-vineyard level. Grape harvest was standardized across all soil types at 24° Brix to ensure uniform maturity levels and minimize sensory differences related to ripeness. Visual descriptors showed that SS wines had greater color intensity and a more pronounced violet hue, consistent with previous findings (41), reporting higher total anthocyanin concentrations in Malbec berries grown under similar SS conditions. Bramley *et al.* (2011) similarly observed greater color intensity in Cabernet Sauvignon wines from shallow soils. The quantity and composition of anthocyanins in wines directly affect color intensity and tonality, and are influenced by factors such as water restrictions, yield, plant vigor, temperature, and soil characteristics like texture and stone volume (18, 45). Furthermore, wine color is also influenced by pH, as lower pH values favor the proportion of anthocyanins in the form of flavylium cation (22). However, during the winemaking process, the pH was standardized to 3.75 for all wines, which likely minimized potential color differences related to pH.

Water deficit, depending on intensity and the phenological stage, can increase anthocyanin and tannin content in berries (60). This effect is primarily attributed to increased production of abscisic acid (ABA) in response to water stress, which stimulates anthocyanin biosynthesis (7). The higher color intensity observed in SS wines aligns with the more stressful water conditions typically associated with this soil type (Figure S2).

Additionally, the greater color intensity may be influenced by vegetative expressions in each soil type, and high-altitude vineyard conditions. Indicators such as pruning weight, shoot length, internode length, and shoot width suggest that DS plants exhibit greater vegetative growth , which could result in increased berry shading. In contrast, the reduced vegetative growth in SS plants likely leads to greater exposure of the berries to solar radiation.

Previous studies conducted in a nearby vineyard demonstrated that Malbec berries exposed to higher UV-B radiation accumulated higher concentrations of total anthocyanins than those exposed to reduced UV-B (8). The intensified violet hue observed may result from copigmentation effects, in which molecular associations between anthocyanins and other organic molecules, such as myricetin or caffeic acid, induce a color shift towards violet (9). McDonald *et al.* (1998) noted that the concentration of flavonols, such as myricetin, may vary depending on geographical origin and could be higher in thick-skinned berries. Furthermore, Berli *et al.* (2008) reported increased flavonols, such as quercetin, myricetin, and kaempferol, in vineyards exposed to higher UV-B radiation. These compounds, powerful antioxidants, accumulate either through direct stimulation of their synthesis or indirectly by increasing berry skin thickness, enhancing their concentration (53). However, further studies are required to validate these findings, as berry skin thickness and specific flavonol concentrations were not measured in this study.

Regarding aromas, SS wines exhibited more intense plum aromas, which are commonly associated with fruit characteristics (44). This finding aligns with Bramley *et al.* (2011), who reported higher concentration of volatile compounds associated with fruity aromas in SS wines. Additionally, Chapman *et al.* (2005) found that wines from vineyards subjected to water deficit tend to have more pronounced fruity aromas and flavors. Furthermore, SS wines were characterized by a higher intensity of mineral notes, which the tasting panel described as a "wet stone" aroma. Although this specific descriptor is not explicitly listed in the aromatic wheel of Noble *et al.* (1987), it is generally referred to as a mineral note. Panelists described these aromas as reminiscent of crushed stones, rocks, wet cement, chalk, gravel, or limestone (26). While such descriptors are often associated with wines from stony soils and are sometimes referred to as "rich in minerals", it is important to note that berries have limited direct chemical interaction with soil minerals, and minerals themselves are odorless, so they cannot contribute directly to wine aroma (35). Our study found no differences in total elemental content between the two soil types or the wines they produced, suggesting that the mineral aroma in wine does not stem from a higher elemental content. Tominaga *et al.* (2003) suggested that benzene methane thiol (benzyl mercaptan) could contribute to mineral aromas in wine, but further research is needed to better understand the origins of these sensory characteristics.

Concerning taste descriptors, SS wines exhibited greater astringency and structure, suggesting water stress plays a significant role in promoting tannins concentration (flavanol polymers) in berries, which are primarily responsible for astringency sensations (4). This observation is supported by Figure S2, which shows that SS plants experienced moderate water stress compared to DS plants. This was previously found by Mezzatesta *et al.* (2022), who reported higher total polyphenol content in SS berries. Wine structure is influenced by three key components: tannins, acidity, and alcohol (18). Although the acidity sensation in SS wines was significantly lower than in DS wines, the heightened astringency may have contributed to a more pronounced structure in SS wines. Wine typicity refers to the specific varietal organoleptic traits, which can be modified by the unique characteristics of the terroir (18). In the case of SS wines, the attributes of this soil favored the varietal expression of Malbec, enhancing plum notes with a slight tendency towards violet aromas, typical of this cultivar (18). Finally, the higher overall quality attributed to Malbec SS wines likely stems from the combination of sensory attributes distinguishing these wines. In general, sensory analyses support the assertion by Bramley and Hamilton (2007) that wines originating from delineated areas within the same parcel, uniformly managed according inherent vigor and yield propensity, exhibit distinct sensory profiles.

According to this study, evidence suggests that the significant heterogeneity of intra-vineyard soils generates distinct sensory profiles, with soil type playing a predominant role. SS soils were linked to vineyards experiencing water stress, leading to distinct sensory profiles. Identifying and distinguishing soil types within a vineyard and adjusting harvesting practices accordingly could result in wines with diverse sensory profiles.

MFA highlighted the interactions between the edaphic characteristics and interannual climatic conditions in shaping elemental wine composition and sensory attributes. The greater variability observed in DS wines across vintages emphasizes the sensitivity of this soil type to climatic conditions, such as differences in precipitation and water stress between 2017 and 2018 (Figures S1 and S2). In contrast, wines from SS exhibited more consistent sensory characteristics, suggesting that soil properties largely influenced climatic factors.

The variation in [Ca] across vintages, with higher accumulation in 2017, could be attributed to the elevated precipitation during that season, which likely favored the availability and transport of Ca to the berries (62). In contrast, the [Mn], more strongly associated with DS irrespective of vintage, may be linked to the higher clay content in these soils, which, combined with their greater water retention capacity, influences plant absorption and accumulation of this element (45). These differences in elemental composition reflect soil-climate interactions and contribute to the observed variations in sensory descriptors.

In sensory terms, wines from SS stood out for their higher color intensity and fruity aromas, such as plum, which were likely amplified by the moderate to severe water stress experienced in these soils. In contrast, wines from DS, while exhibiting subtler visual attributes (clarity) displayed greater variability in descriptors like cherry and strawberry, particularly in 2018, a drier season that may have heightened these attributes. Additionally, Mn, a key element influencing wine color due to its ability to form stable complexes with amino acids and polyphenols (56), showed a negative association with color intensity and violet hue. This contrasts with the findings of Mantilla *et al.* (2018), who reported greater color intensity in Shiraz wines with higher Mn levels. This discrepancy underscores how soil properties modulate the impact of climatic conditions on wine quality, emphasizing the importance of differentiated management strategies to optimize both productivity and sensory profiles.

Finally, the associations of descriptors such as "Garnet hue" or "Heat" with specific climatic conditions highlight vintage significance in shaping the final wine profile, particularly in soils like DS, which are more responsive to interannual variations. These observations reinforce the need to understand soil-climate dynamics to interpret and predict how these interactions influence wine quality and terroir expression.

Conclusions

The significant intra-vineyard soil heterogeneity in high-altitude plantations located in the foothills of the central Andes influence both elemental and sensory profiles of wines. While Malbec yield was not distinctly affected by soil type, probably given climatic variations across growing seasons, there were notable differences in vegetative expression between shallow and deep soils. Although we confirmed that wine elemental profile is influenced by soil characteristics, further studies should stablish its direct impact on sensory attributes. This is crucial for winemakers aiming to diversify their blends or produce distinct wines from the same vineyard under the single parcel concept.

It is important to clarify that this study does not propose management strategies to increase or decrease vine vigor based on soil type. Instead, we aimed to examine the effect of relatively homogeneous but contrasting soil sections on grapevines and wine quality. The findings may contribute to developing management strategies in Argentinian Malbec vineyards that optimize parcel shapes and sizes according to soil type, facilitating the production of wines with unique elemental and sensory profiles on a small scale. Moreover, further research on Malbec phenotypic plasticity and its relationship with elemental composition and sensory properties will have significant implications for both viticulture and oenology, offering valuable insights for the winemaking industry.

Supplemmentary material:

https://docs.google.com/document/d/1KBl4hw-zz1HOfLjLNbTlLK12dSA2q-pd/edit?usp=sharing&ouid=111310786017351827239&rtpof=true&sd=true

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Influence of *Apis mellifera* in-hive conditions on germination capacity of rapeseed pollen (*Brassica napus*)

Influencia del ambiente interno de la colmena de *Apis mellifera* sobre la capacidad germinativa del polen de colza (*Brassica napus*)

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Abstract

Brassica napus L. (rapeseed, canola) ranks third in worldwide importance among oilseeds. The production of hybrid rapeseed seed requires an androsterile female parent; therefore, fertilization is possible through pollinators carrying viable pollen from an androfertile line. However, hives employed for hybrid seed pollination may transport pollen that could lead to contamination if the androfertile lines in successive fields differ. The *in vitro* germinability of pollen exposed to in-hive conditions was evaluated. Samples of rapeseed pollen obtained from potted plants were placed in four hives of *Apis mellifera* L. In-hive conditions are unfavorable for rapeseed pollen germinability. Brood areas with the highest temperatures showed no germinated pollen grains within 24 h. Starting at 48 h, germinability decreased significantly, with germinated grains showing atrophied tubes. At 72 h, pollen placed away from brood areas lost germinability. Therefore, to minimize contamination risks, hives should remain outside the new production plot for at least 72 h.

Keywords
canola • germinability • honey bee • pollination

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Resumen

Brassica napus L. (colza, canola) es la tercera en importancia mundial de las oleaginosas. La producción de semilla híbrida de colza requiere de una línea parental androestéril; por lo tanto, su fertilización es posible a través de los polinizadores que portan polen viable desde una línea androfértil. Sin embargo, las colmenas dedicadas a la polinización de semilla híbrida pueden transportar polen que podría contaminar la producción si las líneas androfértiles de los campos sucesivos son diferentes. Se evaluó la germinabilidad *in vitro* del polen expuesto a las condiciones ambientales dentro de la colmena. A partir de plantas de colza cultivadas en macetas, se obtuvieron muestras de polen que se colocaron dentro de cuatro colmenas de *Apis mellifera* L. Las condiciones dentro de las colmenas son desfavorables para la germinabilidad de los granos de polen de colza. En el área de cría con las temperaturas más altas en 24 h no se registraron granos de polen capaces de germinar. A partir de las 48 h, la germinación decreció significativamente y los granos germinados mostraron tubos atrofiados. El polen ubicado más lejos del área de cría mantuvo su germinabilidad por menos de 72 h. Por consiguiente, y con el fin de reducir los riesgos de contaminación, las colmenas deben mantenerse fuera de la nueva parcela de producción al menos 72 h.

Palabras claves
colza • germinabilidad • abeja melífera • polinización

Introduction

The productivity of cultivated plants, especially those that bear fruit, seed, or grain, is highly correlated with pollen production and viability. This viability can be strongly influenced by non-optimal environmental conditions, such as drought, heat, and solar radiation (13, 24, 25), causing reduced fruit set and yield, as reported for rapeseed crops exposed to high temperatures during flowering (2, 33).

Brassica napus L. (rapeseed, canola) ranks first among Brassicaceae and third among oilseeds, after palm and soybean (19, 31). The seed production of hybrid rapeseed requires an androsterile female parent and pollinators that transport viable pollen from an androfertile line (6, 23, 32). Female line productivity, pollen production, and floral-synchrony management between parental lines are key aspects for maximizing yield. Under this production scheme, the potential contamination with foreign pollen poses a major risk. In rapeseed, cross-pollination decreases with the increase in distance between receptive stigma and pollen source (11). Therefore, in hybrid seed production, the spatial distribution of plots is carefully defined to prevent the androsterile line from being fertilized by foreign pollen (32). Typically, such production considers natural pollen transfer by wind, water, or insects rather than human-mediated unintentional transfer (21, 30).

Unlike naturally occurring pollen transfer, the anthropic movement of pollen has scarcely been studied. In the case of entomophily, there is limited information on the duration of pollen viability on insect bodies (12, 20). Colonies dedicated to pollination in hybrid seed production are commonly moved from one field to another in the same crop. Pollen in hives or on bees could contaminate production if the androfertile lines in successive fields are different. Pollen already processed by bees (corbicular pollen, pressed pollen, or bee bread) is of no concern; rather, pollen grains that remain on their bodies or are free in the hive are in question (20).

The production of hybrid rapeseed seed requires large quantities of pollinators in a relatively short period, specifically during crop flowering. Native pollinators are highly efficient in pollen transfer, but their low and unpredictable population density requires incorporation of human-managed pollinators. The species most commonly used for pollinating crops, including rapeseed, is *Apis mellifera* L. (32).

Despite its global presence, honey bees maintain consistent colony traits, notably brood nest temperature (14). For proper development, *A. mellifera* larvae need a stable temperature, which adults keep between 32 and 36°C (4, 5, 8, 27, 28, 29). Unlike temperature, worker bees have limited control of hive humidity. The optimal humidity varies across the brood nest and fluctuates with external conditions. Because temperature regulation takes priority, it inevitably impacts humidity levels (9, 10).

Pollen grains on bee bodies are exposed to in-hive conditions. Temperature can affect canola pollen germinability during pre-anthesis (17), and germinability and pollen tube length can be reduced when germinated at 33°C (18). Beekeepers must follow strict hive movement rules to prevent potential contamination, even without clear evidence of whether the pollen in the hives retains its germination capacity. To date, there are no records of rapeseed pollen germinability under actual in-hive conditions. This study aimed to determine how long rapeseed pollen maintained germination capacity in the hive, helping to establish safe intervals before moving hives between hybrid rapeseed fields, thus minimizing the risk of contamination.

Materials and Methods

Experimental site

To assess *in vitro* pollen germinability under hive conditions, experiments were conducted once each spring (October/November) of 2017, 2018, and 2019 at the Laboratorio de Estudios Apícolas, Universidad Nacional del Sur, Bahía Blanca, Argentina (-38.694944, -62.253293). Four Langstroth-type *A. mellifera* hives were selected each year, with nine frames per brood chamber. The seven central frames held brood, while the two outer frames stored honey. No signs of disease were observed. The queen was ovipositing prolifically.

Pollen samples

Pollen samples were obtained from 20 Hyola 433 rapeseed plants grown in 10-liter pots. When plants reached full bloom, mature flower buds (close to opening) were labeled. The following day, at 7:30 AM (-3 GMT), 80 of these marked flowers were harvested, ensuring that anthers had been fully developed, as pollen is most fertile immediately after the flower opens (16). Anthers from all harvested flowers were pooled into one sample. Three random anthers were placed in 60 tubes (0.5 ml) and crushed with a histological needle to release pollen. Tubes were left open in the hives, secured with two pins to prevent movement due to bee activity (figure 1).

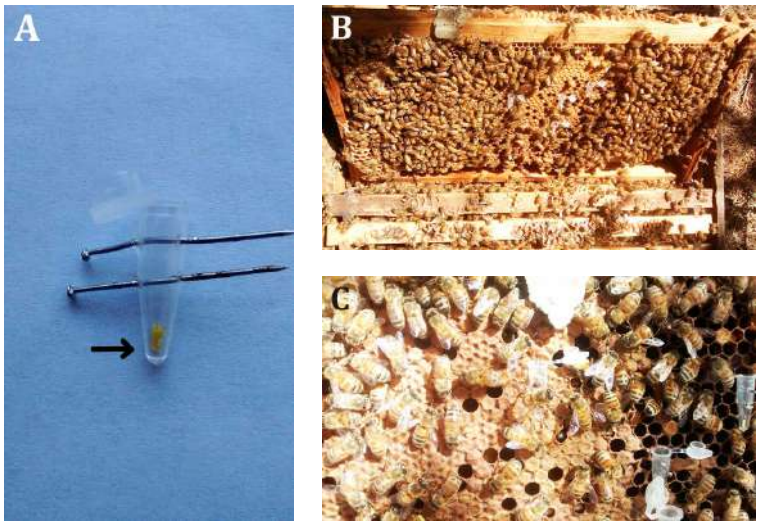


Figure 1. A: Tube with *Brassica napus* anther tissue and pollen samples (arrow) secured with pins to prevent movement from bee activity; B: Distribution of pollen samples in an *Apis mellifera* hive; C: Detail of pollen samples on a brood frame (Frame 5).

Figura 1. A: Tubo con tejido de anteras y muestras de polen (flecha) de *Brassica napus*, sujetado con alfileres para evitar su movimiento por la actividad de las abejas; B: Distribución de muestras de polen dentro de una colmena de *Apis mellifera*; C: Detalle de muestras de polen en un cuadro de cría (Cuadro 5).

Treatments

Out of 60 samples, the germinability of 12 freshly harvested pollen samples was analyzed as the 0 h treatment. The remaining 48 tubes were randomly grouped in fours and placed in three different frames in each hive, with 12 samples per hive per year. Frame 5, in the center of the brood chamber, had abundant brood. Frame 7, located between the edge and the center, had brood in the center and honey and pollen on the margins. Frame 9, on the outer edge, contained only honey (figure 2). Each year, temperature sensors (Onset HOBO UA-002-64 Temperature/Light Data Logger 64K spa) were placed to record in-hive conditions every hour. During 2019, two sensors (HOBO Onset H08-032-IS Temperature/HR Data Logger 64K HOBO), one in the brood area and another in the honey reserves, were added to record humidity hourly in one of the hives. Over three consecutive days, one tube per frame was removed every 24 h from each of the hives, totaling 12 tubes per day, for analysis.

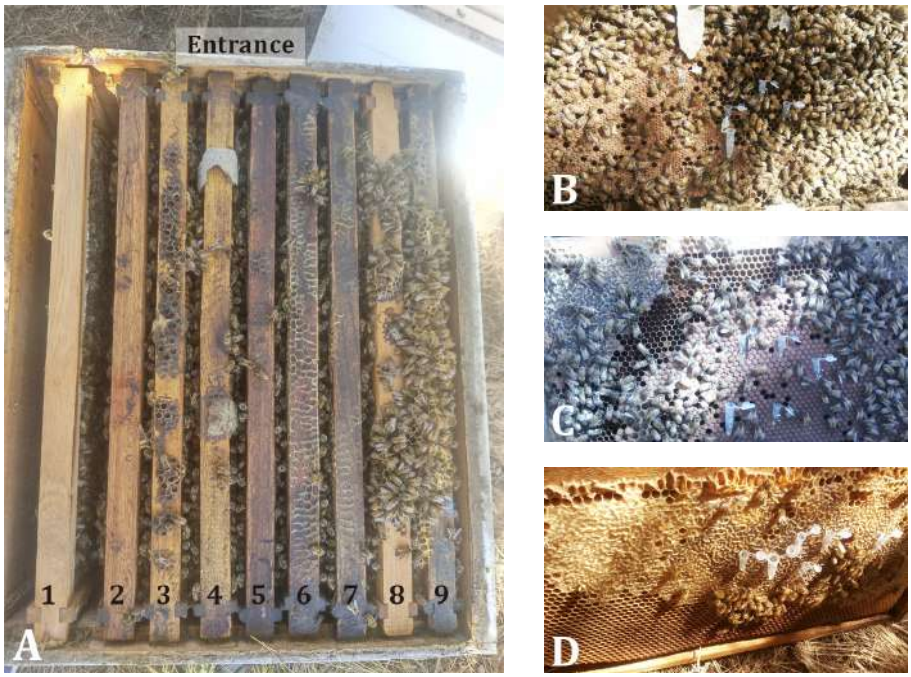


Figure 2. A: Identification of frames in one of the four *A. mellifera* hives used for the experiment; B: Frame 5, center of the brood chamber, completely covered by brood; C: Frame 7, with brood in the center and stored honey and pollen on the edges; D: Frame 9, outer edge of the brood chamber only with stored honey.

Figura 2. A: Identificación de los cuadros de una de las cuatro colmenas de *A. mellifera* utilizada para el ensayo; B: Cuadro 5, centro de la cámara de cría, cubierto completamente por cría; C: Cuadro 7, compuesto por cría en el centro, miel y polen en los bordes; D: Cuadro 9, externo de la cámara de cría compuesto por reservas de miel.

Technique to determine germinability

The hanging drop technique was used to assess the percentage of *in vitro* pollen germination (25). *Brassica napus* pollen grains were incubated in a culture medium at 25°C and 90% relative humidity for two hours. A drop of culture medium was placed on a glass slide, and pollen samples were added. The glass slide was then inverted in a sealed humid chamber with wet absorbent paper at the bottom. After two hours, a cover slip was placed over the drop, and 500 pollen grains per sample were counted under a microscope at 400X magnification. Germination was determined by the percentage of grains with a pollen tube longer than grain diameter (15).

Culture medium

The culture medium used to measure *in vitro* pollen germinability, originally described for sunflower by Astiz (2012), proved suitable for rapeseed pollen. It contained 150 g/L polyethylene glycol 6000 (PEG6000) in distilled water, 100 g/L sucrose, 240 mg/L calcium nitrate, and 100 mg/L boric acid. The pH was maintained between 6.5 and 7.0, adjusted with 0.1 N sodium chloride. PEG6000, which is inert to pollen metabolism and unable to enter cells (22), was included to enhance the development of pollen tubes by regulating plasma membrane permeability and providing stability to the pollen tube membrane (22).

Statistical analysis

The experimental design consisted of randomized complete blocks with four replicates. Data were subjected to analysis of variance (ANOVA), and if differences were detected at p-values < 0.05, means were compared using Fisher's LSD test. Statistical analyses were performed using Infostat software (7).

Results

In-hive temperature and humidity

Over the three years and during the experiments (three days), differences in hourly temperature were observed among the hive sectors studied ($p < 0.0001$; $n = 72$). Frame 5 had the highest temperatures (around 35°C), followed by Frame 7 and 9 (figure 3).

Throughout the period of pollen exposure to in-hive conditions, humidity showed no significant differences between brood and honey storage areas ($p = 0.1099$; $n = 138$). In the area with stored honey, the average (± SD) relative humidity was 42.09% (±10.47), and in the brood area it was 44.23% (± 5.29).

Bars show mean values (±SD).

Equal letters indicate no significant differences (p > 0.05) between the groups analyzed.

Las barras muestran valores medios (± DE).

Letras iguales indican que no se detectaron diferencias significativas (p > 0,05) entre los grupos analizados.

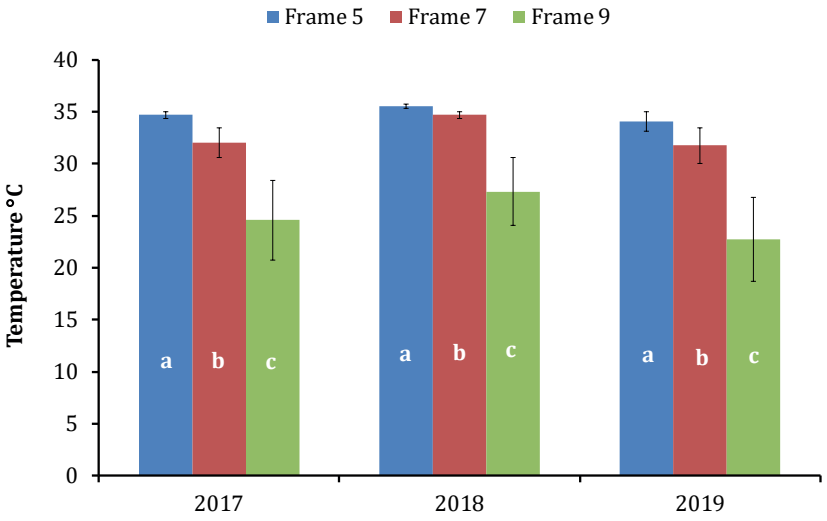


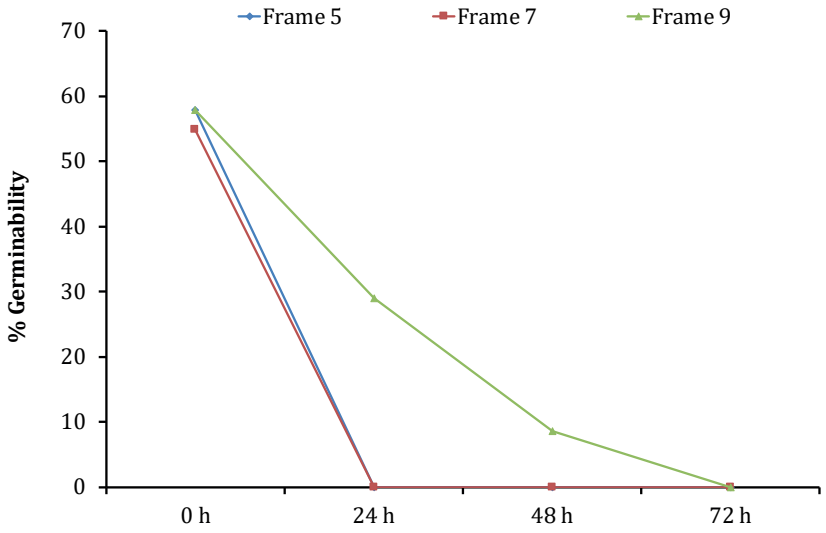
Figure 3. In-hive temperature variation in Frames 5, 7, and 9.

Figura 3. Variación de la temperatura dentro las colmenas experimentales, Cuadro 5, 7 y 9.

Pollen germinability

In all years, the highest germination percentages ($\bar{X} = 57.9\%$) were obtained with freshly collected pollen (0 h) ($p < 0.01$). In-hive conditions reduced rapeseed pollen germinability, with pollen in brood areas losing its germination ability within 24 h. No significant differences were found between brood frames (5 and 7) ($p > 0.05$).

Pollen in Frame 9, near honey reserves, retained its germination capacity longer than that in the brood areas ($p < 0.05$) (figure 4). Across all trials, germination capacity dropped similarly, with some pollen in Frame 9 still viable at 48 h, but falling below 20%. After 72 h, no pollen germinated, in most cases with a significant reduction occurring within 24 h.



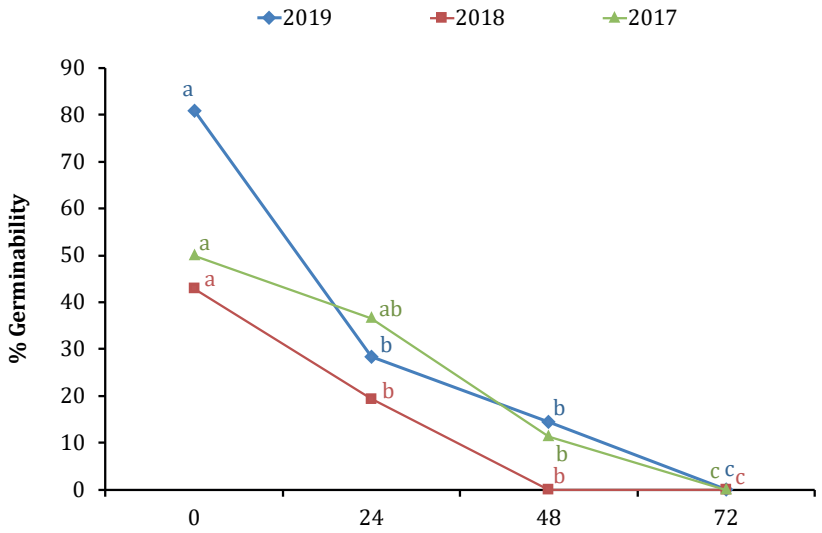
The curve corresponding to Frame 5 overlaps with that of Frame 7.

La curva correspondiente al Cuadro 5 se superpone con la del Cuadro 7.

Figure 4. Mean percentages of *in vitro* germinability of rapeseed pollen grains after storage in different locations in an *A. mellifera* hive.

Figura 4. Porcentajes promedios de la germinabilidad *in vitro* de los granos de polen de colza luego de permanecer en diferentes ubicaciones dentro de una colmena de *A. mellifera*.

Temperature averages in Frame 9 were 24.6 ± 3.8°C, 27.3 ± 3.2°C, and 22.7 ± 4.0°C in 2017, 2018, and 2019, respectively. Despite these differences, pollen germinability decreased substantially after 48 h of exposure to in-hive conditions (figure 5). Pollen germination capacity was completely lost after 72 h.



Equal letters indicate no significant differences ($p > 0.05$) between the groups analyzed.

Letras iguales indican que no se detectaron diferencias significativas ($p > 0,05$) entre los grupos analizados.

Figure 5. Percentage of pollen germinability in Frame 9 in 2017, 2018, and 2019.

Figura 5. Porcentaje de germinabilidad de los granos de polen en el Cuadro 9 en 2017, 2018 y 2019.

Fresh pollen had the thickest and most developed pollen tubes. After 24 h in the hive, pollen grains had thinner, shorter, and convoluted pollen tubes. After 48 h, pollen tubes were atrophied (figure 6).

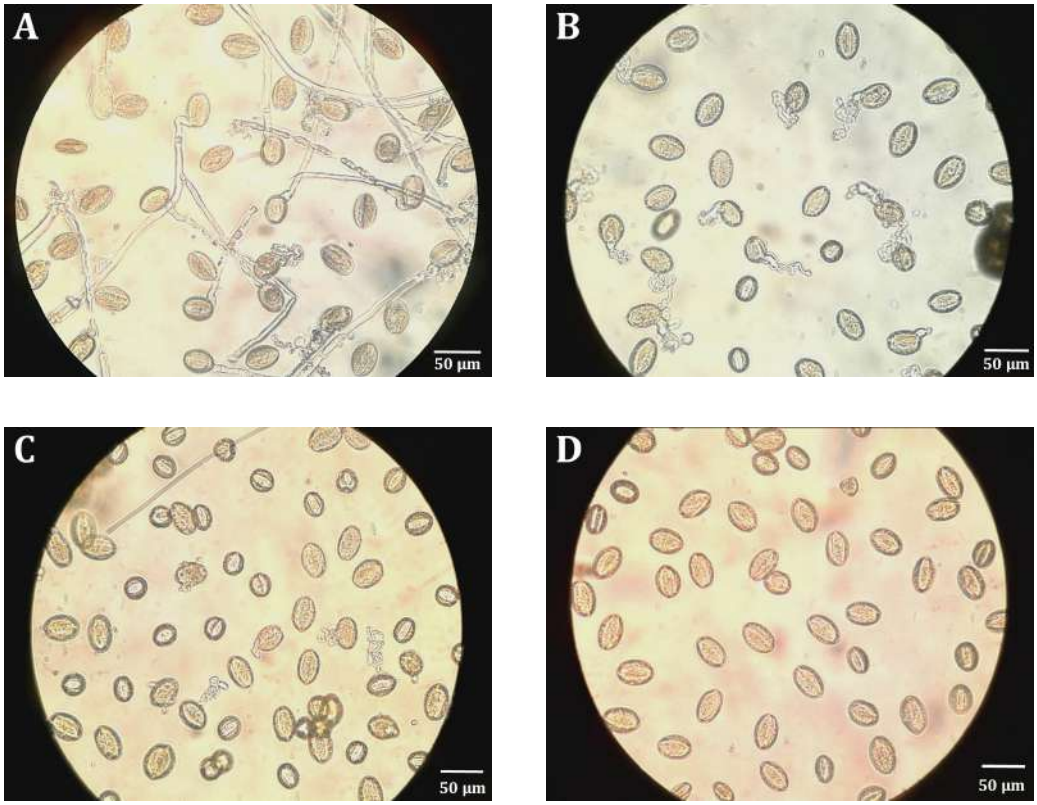


Figure 6. Development of pollen tubes from rapeseed pollen grains in Frame 9 after different in-hive exposure periods (400X). A: 0 h; B: 24 h; C: 48 h; D: 72 h.

Figura 6. Desarrollo de los tubos polínicos de granos de polen de colza en el Cuadro 9 luego de diferentes períodos de exposición en una colmena (400X). A: 0 h ; B: 24 h; C: 48 h; D: 72 h.

Discussion

The outer frames of the brood chamber showed wider temperature variations and lower average temperatures than those of the central frames. This was expected because the brood is mainly in the center, while the outer frames, typically filled with honey, have less temperature regulation from bees. Honey acts as an insulator against external temperature variations, and nurse bees concentrate on controlling the temperature of brood frames. Humidity levels showed no significant differences between honey and brood areas, consistent with findings reported by Alburaki & Corona (2021) and Human (2006).

Pollen grains in the brood area lost their germination capacity after 24 h of exposure to in-hive conditions, markedly reducing the risk of contaminating new seed production plots. Temperature affects pollen not only during transport to the stigma but also during its Temperature affects pollen not only during transport to the stigma but also during its development in the anther (17, 24, 26). Notably, pre-anthesis temperature affects canola pollen germinability (17), with significant reductions in germinability and pollen tube length at 33°C (18). Our findings support this, as the temperature of the brood area remained close to 35°C.

Pollen tubes from fresh pollen and those exposed to in-hive conditions showed similar results to those reported by Young *et al.* (2004), who found that pollen germinated at 35°C had abnormal growth, being thinner and shorter than those germinated at 23°C. These abnormalities in pollen tubes could hinder proper ovule fertilization and, even if successful, would be in significant disadvantage compared to fresh pollen grains in the new plot.

Conclusions

In-hive conditions at the experimental site significantly reduced the germination capacity and pollen tube development of rapeseed. To minimize contamination risks, hives should remain outside the new production plot for 72 h.

These studies provide a starting point for understanding the potential risks of *Apis mellifera* hives carrying viable pollen and contaminating hybrid rapeseed seed production plots. Further research is recommended to confirm the required waiting period before relocating hives between plots. Similar studies on other seed crops would also be beneficial.

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Water quality assessment of streams and rivers for irrigation in Southern Continental Patagonia

Evaluación de la calidad del agua para irrigación en ríos y arroyos de la Patagonia Austral Continental

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Abstract

This work aimed to analyze and classify the suitability of freshwater sources for irrigation in three large hydrographic regions of Southern Continental Patagonia: Coyle, Serrano, and Gallegos. In these regions, there is a lack of information on the irrigation suitability of surface waters. For this, 74 surface water locations were sampled from 42 watercourses in Santa Cruz province and Magallanes region in Argentina and Chile, during dry and wet seasons between 2017 and 2019. The concentration of ions of agricultural interest was evaluated in the laboratory. The pH ranged between 6.1-9.5 with little seasonal variability. The prevailing ions were Ca^{2+} Mg^{2+} and HCO_3^-, while the lower cation concentration was K^+. The Sodium Adsorption Ratio was 0.58 ± 0.21 during winter and 0.46 ± 0.15 in summer. Most waters in the region have electrical conductivity values below 250 µS/cm and may be categorized as low-salinity waters. We determined no significant hazards for crops, vegetables, and pasture production in terms of the combined salinity and sodicity indicators. However, a potential negative impact on soil structural stability mainly due to Na^+ concentration must be considered for the implementation of suitable irrigation projects.

Keywords
agriculture • hydrochemistry • hydrology

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Resumen

En este trabajo se analizó y clasificó la aptitud de fuentes de agua para riego en tres regiones hidrográficas de la Patagonia Austral (Argentina): Coyle, Serrano y Gallegos. Estas regiones carecen de antecedentes sobre la aptitud de sus aguas para la irrigación. A fin de proporcionar esta información, se analizaron en laboratorio muestras de aguas superficiales de 74 locaciones en 42 cursos de la provincia de Santa Cruz en Argentina y de la región de Magallanes en Chile, durante las estaciones seca y húmeda del año, entre 2017 y 2019. Se evaluó la concentración de cationes y aniones de interés agrícola. Las aguas mostraron un rango de pH entre 6,1 y 9,5 con poca variabilidad estacional. Los cationes predominantes fueron Ca^{2+} y Mg^{2+} y menor en K^{+}, siendo HCO_{3^-} el principal anión. El SAR se encontró entre 0,58 ± 0,21 durante el invierno y 0,46 ± 0,15 en verano. Con excepción de algunas muestras, la mayoría de las aguas tienen valores de conductividad eléctrica inferiores a 250 µS/cm y pueden catalogarse como aguas de baja salinidad. No se detectaron peligros significativos para la producción de cultivos, hortalizas y pastos en términos de los indicadores combinados de salinidad y sodicidad. Sin embargo, existe un potencial impacto negativo en la estabilidad estructural del suelo debido principalmente a la concentración de Na^{+} que debe tenerse en cuenta para la implementación de proyectos de riego.

Palabras clave
agricultura • hidroquímica • hidrología

Introduction

Patagonia occupies a vast territory in southern Argentina and Chile. This includes various heterogeneous ecological areas, mainly because of the diverse edaphoclimatic characteristics that determine the predominance of arid, semi-arid, and very arid bioclimatic zones (1). The grassy and shrub steppes on plateaus and glaciofluvial valleys represent the main features of the landscape. The main socioeconomic activities are extensive sheep and cattle farming and agriculture in irrigated valleys. These environments have been enduring constant degradation for little more than a century since the initial European settlement at the end of the 19th century. This process still occurs mainly because of the combination of poor agricultural management practices, livestock overgrazing, and recurrent drought events. In this context, plant communities and agro-productive activities are severely limited by water deficit.

Consumptive use for agricultural production represents the greatest demand for freshwater in the world, with an estimate of 70% globally by 2020 (27) and in Argentina (10). This use is also one of the most inefficient due to overexploitation, lack of reuse, contamination with agrochemicals, low irrigation efficiency, and flooding (24).

Irrigating natural grasslands in river valleys of Southern Patagonia has exerted increasing pressure on the consumptive use of surface water, mainly due to the frequent drought events in recent decades. In arid and semi-arid regions of Patagonia, this practice can significantly improve natural grass yields up to 10-20 times, mainly in wetlands (4). Irrigation to supplement the rainfall in the warm months and the snow melting in early spring arises as an alternative during critical stages of the grasslands growing season and cultivated pastures (15) in traditional dry farming lands. However, irrigation may produce negative environmental impacts. Soil sodicity, salinity or ion toxicity caused by poor management irrigation practices in hazardous situations (25) are some of the greatest environmental pressures of agriculture worldwide (11). Because of these potential negative effects, it is important to better understand of how water quality influences the management of irrigated agriculture.

Successful irrigation projects involve appropriate quantification and distribution of the required water and adequate control of its quality (5, 11, 25). Currently, only salinity and sodicity hazards combined have caused 23.5% of the total land degradation by irrigation in Argentina, which represents 500.000 ha (9). Therefore, regular monitoring of water quality for irrigation in this region becomes relevant to support decisions on sustainable

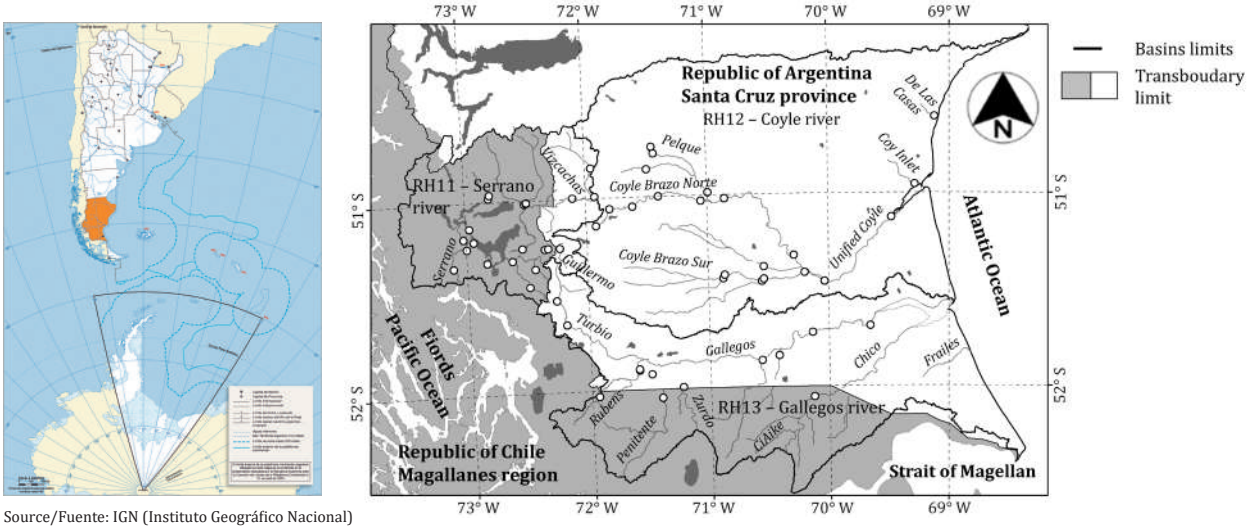
use, management, and conservation of both water and soil (15). Although there has been considerable interest in this topic around the globe, there is a lack of studies in Patagonia.

In this study, we analyze and classify the suitability of freshwater sources for irrigation in the southernmost region of the Southern Continental Patagonia. It was carried out from a set of widely used indicators for the detection of sodicity and salinity hazards. We analyzed 148 surface water samples from 42 watercourses in the Santa Cruz province (Argentine) and Magellan region (Chile) during the dry and wet seasons between 2017 and 2019. We evaluated the following indicators: Sodium Adsorption Ratio (SAR), adjusted SAR, Residual Sodium Carbonate, Soluble Sodium Percentage, Standardized Electrical Conductivity, Total Dissolved Solids, Effective Salinity and Potential Salinity.

Materials and methods

Study site

The study area focused on three major hydrographic regions (HR) in Southern Continental Patagonia, covering 57,406.4 km^2 of which 76.1 % is located in Argentina, and 23.9% in Chile (figure 1). The first region, the Serrano River basin (HR11), covers 8,638.8 km^2 with 23.6% located in Argentina, in the upper basin (6). This transboundary system with a predominantly nivo-glacial mixed regime drains into the Pacific Ocean, exhibiting an average annual flow of 2,964.3 hm^3. About 164.0 hm^3/year (5.5%) is produced in the Argentine side. The second region, the Gallegos River basin (HR13), is another transboundary territory that flows into the Atlantic Ocean, with an extension of 19,289.1 km^2, with 63.2 % in Argentina. It also has a predominantly nivo-pluvial mixed flow regime and produces an annual surface runoff of slightly over 1,000 hm^3. The last region, the Coyle River basin (HR12), extends across 29,424.0 km^2 exclusively in Argentina. Characterized by a mainly nival flow regime it produces an annual runoff of 39.9 hm^3.



Source/Fuente: IGN (Instituto Geográfico Nacional)

Figure 1. Sampling sites in rivers, streams, and creeks of Southern Continental Patagonia.
Figura 1. Sitios de muestreo en ríos y arroyos de la Patagonia Austral Continental.

Data collection and analysis of Argentine waters

In Southern Patagonia, the main demand for complementary irrigation of crops, pastures, and natural grasslands occurs between late winter and early spring (September and October, corresponding to a wet season for HR12 and HR13, and a dry season for HR11) and late summer (February and March). Fifty-five surface water samples from different locations along 28 watercourses in the Santa Cruz province were collected during the dry and wet seasons. A total of 110 samples were obtained between 2017 and 2019 (figure 1, page 30). Both sampling moments represent opposite moments of the annual hydrograph.

Watercourses were classified as permanent, intermittent, or ephemeral types according to their annual discharge and the percentage of annual exposure of the channel bed (23). We used a quantitative watercourses classification adapted from Jowett (2020) to contextualize and facilitate the interpretation of results: creeks (mean annual discharge <1 m^3/s), streams (<5 m^3/s), and rivers (>5 m^3/s).

On-site equipment handling procedures, water sampling methods, conditioning for conservation and transport were implemented in accordance with the protocols for water quality sampling suggested by USGS (26). The concentration of ions of agricultural interest was evaluated in the laboratory. Sodium (Na^+) and Potassium (K^+) were determined by flame photometry according to standards SM-3500-Na B and SM-3500-K B. Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) were analyzed by complexometric titration with EDTA at pH 12 using murexide as the indicator for the first case, and at pH 10 with Eriochrome® Black T for the second, according to standard SM-2340-C. The presence of Chloride (Cl^-) was determined through the standard SM-4500-Cl^- B; Sulfate (SO_4^{2-}) through precipitation with Barium and by turbidimetry monitoring according to SM-4500-SO_4^{-2} E standard. Carbonate (CO_3^{2-}) and Bicarbonate (HCO_{3-}) were determined through titration with 0.1 N hydrochloric acid using phenolphthalein and helianthin as indicators, according to standard SM-2320 B. The pH and specific electrical conductivity -ECw- were determined *in situ* according to SM-4500-H^+ B and SM-2510 B standards, through a calibrated portable probe. The total dissolved solids (TDS) were determined through a gravimetric method on the dry residue according to the SM-2540 C standard.

Data collection and analysis of Chilean waters

Water quality data publicly available from the Chilean governmental authority (7) was used for 14 creeks, streams, and rivers in 19 different locations, in the same time periods sampled in the Argentine sector. Major cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) were determined by atomic absorption spectroscopy according to the standard SM-3111 B. The anions Cl^-, SO_4^{2-}, CO_3^{2-}, and HCO_{3-}, the pH and EC were determined through the same procedures used for the Argentine samples.

Data processing and water quality analysis

All 148 water samples analyzed in the present work (55 from Argentina + 19 from Chile, for each season) showed less than 5% absolute average error in the electroneutrality condition from major ion concentrations. Water types were classified according to their chemical composition and dynamics based on Piper (21).

Four indicators were used to evaluate the sodium hazard. First, the Sodium Adsorption Ratio (SAR), widely used for the suitability of typical irrigation waters (14, 31), constitutes a strong predictor of the soil exchangeable sodium percentage (11, 30). This standard SAR equation was adjusted (SAR_{adj}) when alkaline waters contained relatively high concentrations of Mg^{2+}, Ca^{2+} as well as carbonates (CO_3^{-2}) and bicarbonates (HCO_{3-}). This situation could raise the relative proportion of Na^+ in solution concentrations after precipitation of carbonate salts with Ca^{2+} and Mg^{2+} (3, 8, 12, 14, 25, 31), particularly in soils of arid environments subject to high evapotranspiration or evaporation rates (1). Weiner (2013) suggests the application of SAR_{adj} to water samples with >200 mg/l HCO_3 and pH>8,5. Among different approaches for its calculation, we used one suggested by Lesch and Suarez (2009). A third indicator used was the Residual Sodium Carbonate (RSC), defined by Eaton (1950). Finally, we used the Soluble Sodium Percentage (SSP) to complement the SAR. This is useful for characterizing water hardness (30) to anticipate the long-term negative effects of Na^+ on the soil (22).

Four indicators were used to evaluate the salinity hazard. First, the Standardized Electrical Conductivity -ECw- (in µS/cm) and Total Dissolved Solids -TDS- (in mg/l) were analyzed. Both are highly correlated with the total concentration of soluble salts (31) and, consequently, widely used for the interpretation of the saline hazard in irrigation waters (30). Even in terms of potential sodicity hazard, SAR is best interpreted when analyzed together with ECw (14). Second, the Effective Salinity (ES) defined by Marín *et al.* (2002), is useful when some less soluble salts precipitate in the form of carbonates or sulfates in contact with the soil. Under such circumstances, ECw tends to overestimate the impact of the real salinity. Finally, the Potential Salinity (PS) indicator was used. This is often recommended when soil moisture content drops below 50%, and chlorides and sulfates are the last salts to remain in solution (16, 20). This is a common situation in the summer for Southern Patagonian environments.

The results were analyzed using arithmetic means and standard deviations in different sample groupings, according to hydrographic regions. The Shapiro-Wilks normality test ($p<0.05$) was conducted before the arithmetic analysis. Specific relationships were established between analytical results and seasonal flows by Pearson's linear correlation coefficient at $p<0.05$.

Results and discussion

Descriptive analysis

Water temperature plays a critical role in numerous physical and chemical processes essential for the aquatic environment including gas and some ionic compounds solubility, biodegradability of substances, toxicity of chemicals, metabolic activity, nutrient cycles, and primary production (28). During the sampling campaigns, the mean water temperature ranged between 4.9 and 7.6°C in winter and 8.2 to 11.5°C in summer (table 1, page 33). Extreme values ranged from 0.2 to 14.8°C during winter, with the lowest values occurring in the mountain range (HR11 and southwestern HR12). In contrast, summer water temperatures ranged from 2.1 to 19.9°C, with the highest values found in small creeks and streams in the center of the HR12 and HR13 basins.

Another major controlling variable of chemical processes in aquatic environments is pH (28). All regional waters showed a pH range between 6.1 and 9.5 with little seasonal variability and without a relationship with their flow regimes or annual discharge. The proximity to the western mountain range narrowed pH values to 7.7-7.9 in streams and rivers of the HR11 and tributaries in the upper watersheds of the HR13 basin. Waters become slightly more alkaline in HR12 and eastern watersheds of the HR13 basin (for example in intermittent Los Frailes and ephemeral Coy Inlet streams), with a pH range of 8.1-9.4.

The prevailing cations in waters of the three large hydrographic regions (HR) were Ca^{2+} and Mg^{2+} with a slight dominance of the former (figure 2, page 34). Na^{+} had similar concentrations to Mg^{2+} in HR11, prevailing only in a few cases, as in the Don Guillermo stream (concentration over 25.0% between major cations when expressed in meq/l). The lowest concentration between cations was K^{+}, with a mean value of 3.8% in meq/l in winter and 2.4% in summer. This distribution of proportions among cations is consistent with most rivers in the world (17). HCO_3^- was the predominant cation resulting in the Ca(Mg) HCO_3 water type in the Argentine side of HR11 (figure 2, page 34). Data was insufficient for such analysis in the Chilean side of this basin.

Except for Cl^-, the average concentrations of different ions tend to be slightly lower during summer compared to winter, with the occurrence of annual peak flows in HR11. In both seasons the proportions of these ions tend to remain unchanged. As stated by Mosley and Row (1981), this suggests the dilution of solutes by run-off and a relatively low concentration of elements contributed by the subsurface flow. This process can be associated with a faster transit of rainwater toward the waterways in the wet season without interacting with soil solutes. This is particularly evident in creeks and streams, rather than rivers.

Table 1. Statistics for salinity and sodicity hazard indicators obtained from the analysis of surface water samples in the most important courses of the Serrano (HR11), Coyle (HR12), and Gallegos (HR13) river basins, between 2017 and 2019.

Tabla 1. Estadísticos de indicadores de riesgo de salinidad y sodicidad obtenidas del análisis de muestras de aguas superficiales en los cursos más importantes de las cuencas de los ríos Serrano (HR11), Coyle (HR12) y Gallegos (HR13), entre 2017 y 2019.

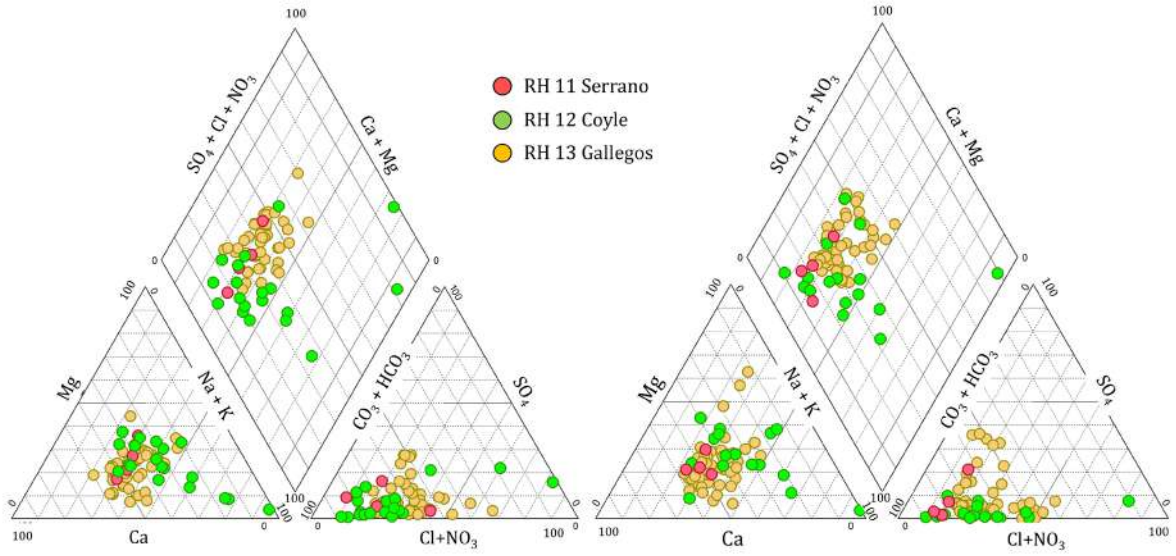
Indicator	Late winter / Early spring			Late summer		
	HR 11	HR 12	HR 13	HR 11	HR 12	HR 13
Water temperature (°C)	5.4 ± 4.2 (77.2)	7.6 ± 2.8 (37.1)	4.9 ± 2.6 (53.7)	10.2 ± 4.2 (41.2)	8.2 ± 4.0 (48.3)	11.5 ± 2.6 (22.5)
pH	7.7 ± 0.5 (5.9)	8.5 ± 0.2 (2.8)	7.9 ± 0.5 (6.7)	7.7 ± 0.4 (5.8)	8.4 ± 0.5 (5.7)	7.5 ± 0.7 (9.7)
SAR [Ec.1] [a]	0.58 ± 0.21 (36.6)	3.79 ± 8.91 (235.1)	0.78 ± 0.42 (53.9)	0.56 ± 0.15 (26.9)	6.66 ± 20.64 (310.0)	0.84 ± 0.52 (61.7)
SAR_{adj} [Ec.2] [b] ***	nd **	7.87 ± 15.02 (190.9)	1.55 ± 0.53 (34.3)	nd **	17.15 ± 34.41 (200.6)	1.46 ± 0.72 (49.4)
RSC [Ec.3] [c] (meq/l)	0.26 ± 0.39 (148.3) *	1.04 ± 1.82 (176.0)	-0.04 ± 0.34 (878.7)	0.03 ± 0.30 * (960.0)	1.31 ± 3.34 (256.0)	-0.07 ± 0.33 (470.2)
SSP [Ec.4] [d] (%)	20.2 ± 8.8 (43.8)	54.2 ± 9.3 (17.1)	31.3 ± 5.5 (17.7)	18.1 ± 6.6 (33.9)	53.0 ± 12.9 (24.4)	30.5 ± 6.5 (21.4)
ECw [e] (µS/cm)	122.1 ± 69.8 (57.1)	554.6 ± 986.6 (177.9)	209.6 ± 195.7 (93.3)	168.8 ± 130.9 (77.6)	1,249.4 ± 3.609.8 (288.9)	245.8 ± 217.7 (88.6)
TDS [f] (mg/l)	96.8 ± 49.3 (50.9) *	343.2 ± 639.5 (186.3)	145.0 ± 131.0 (90.3)	113.3 ± 46.4 * (41.0)	749.3 ± 8.91 (235.1)	165.6 ± 141.0 (85.1)
ES [Ec.5] [g] (meq/l)	0.6 ± 0.2 (36.7) *	4.3 ± 10.8 (248.3)	0.9 ± 0.8 (87.4)	0.6 ± 0.4 * (68.7)	11.1 ± 36.6 (330.5)	1.1 ± 1.1 (94.3)
PS [Ec.6] [h] (meq/l)	0.4 ± 0.1 (36.1) *	2.9 ± 7.7 (261.3)	0.8 ± 0.8 (102.7)	0.3 ± 0.1 * (52.2)	10.4 ± 36.8 (355.4)	0.8 ± 0.9 (118.7)

Data are presented as mean values ± standard deviation and the percentage of variation coefficient between brackets.

[a] Sodium Adsorption Ratio; [b] Adjusted Sodium Adsorption Ratio; [c] Residual Sodium Carbonate; [d] Soluble Sodium Percentage; [e] Specific Electrical Conductivity; [f] Total Dissolved Solids; [g] Effective Salinity; [h] Potential Salinity; * Only valid cases in the Argentine sector due to unavailable data from the Chilean sector in transboundary basins HR11 and HR13. ** No data in range for calculation (HCO_3 >200 mg/l). *** Only cases with HCO_3 >200 mg/l.

Los datos se presentan como valores medios ± desviación estándar y el coeficiente de variación porcentual entre paréntesis.

[a] Relación Adsorción de Sodio; [b] Relación Adsorción de Sodio ajustada; [c] Carbonato de Sodio Residual; [d] Porcentaje de Sodio Soluble; [e] Conductividad Eléctrica Específica; [f] Sólidos Totales Disueltos; [g] Salinidad Efectiva; [h] Salinidad Potencial; * Solo casos válidos en el sector argentino debido a faltante de datos en el sector chileno en las cuencas transfronterizas RH11 y RH13. ** Sin datos en el rango de cálculo sugerido (HCO_3 >200 mg/l). *** Solo casos con HCO_3 >200 mg/l.



Data available only for Argentine basins. / Datos solo disponibles para la porción Argentina de las cuencas transfronterizas.

Figure 2. Piper diagram for winter waters (left) and summer waters (right).
Figura 2. Diagrama de Piper para muestras de agua de invierno (izquierda) y de verano (derecha).

Also, in HR13, most of the waters did not have a prevalent cation between Ca^{2+} and Mg^{+2}. Na^{+} was the second most important cation (30.5 and 31.1% in meq/l for winter and summer, respectively) while Mg^{2+} occupied the third place (22.7 and 23.6% in meq/l for winter and summer, respectively). Few samples showed a dominance of chlorinated water type in both seasons. This occurred, mainly, in small steppe creeks and streams near the seacoast, like in Ci-Aike (over 40% in meq/l Cl^{-}) and Los Frailes (over 50% in meq/l Cl^{-}), both of intermittent stream type (figure 2). A similar pattern of concentrations occurred with the remaining anions without seasonal variation, in which the bicarbonate type dominated. The hydrochemical facies of these waters are a combination of the $Ca(Mg)HCO_3$ type and a mixed type (figure 2).

There was no dominant type among the cations in HR12 samples. However, Na^{+} was the most prevalent (over 40.0% in meq/l), followed by Ca^{2+} (between 30.9 and 31.9% in meq/l) and Mg^{2+} (23.1 and 26.0% in meq/l). There was a tendency for Na^{+} to prevail in short ephemeral coastal watercourses towards the east of this HR, with extreme values of 74.7% in meq/l in De Las Casas and 94.1% in meq/l in Coy Inlet. Bicarbonate waters are dominant in this region which determined the existence of facies mainly of the $Ca(Mg)HCO_3$ type and the $Na(K)HCO_3$ mixed type (figure 2). Only few samples were corresponded to a chlorinated type.

Although large rivers cross the extensive Patagonian steppes, like Coyle and Gallegos, with a high evapotranspiration rate during summer, there was no evident change in ion concentrations along river courses to the sea. Likewise, there is no clear dominance of Ca^{2+} or Mg^{2+} in the upper basins or tributaries. This suggests a relatively uniform lithology along watercourses that determines a homogeneous distribution of major element concentrations (17).

Sodicity (alkali) hazard

The soluble salts present in the soil or in the irrigation water contribute to the increase the salinity of the soil solution. Similarly, when these salts involve exchangeable Na^{+}, they contribute to the increase of Na^{+} relative saturation, given its more persistent nature in soils (25). Excess of sodium salts represents a toxicity hazard for sensitive plant species, it negatively affects the soil permeability and hydraulic conductivity and, therefore, it alters soil structure aggregation with the consequent unavailability of water for crops intake (15, 31). Na^{+} hazard in soils is more complex to establish than the water sodicity hazard because of several interacting factors, such as soil texture (28), electrical conductivity, and rate of sodium adsorption during soil watering (25).

Serrano (HR11) and Gallegos (HR13) regions exhibited lower levels of SAR than Coyle (HR12). In HR11, the mean SAR ranged from 0.58 ± 0.21 during late winter and 0.56 ± 0.15 in summer (table 1, page 33). This is a small difference despite the contrasting seasonal flows in rivers and streams, which can reach mean annual values as low as 0.01 - 2.0 m^3/s (Don Guillermo and Chorrillo streams) and 2.5 - 30.0 m^3/s (Vizcachas, Baguales, Las Chinas and Paine rivers) or higher, up to 120.0 - 380.0 m^3/s (Grey and Serrano rivers), all of them being of permanent type. The only extreme values were observed in Don Guillermo stream waters with SAR ranging between 0.92 and 1.06, depending on the season. Low mean SAR values were also found in HR13, ranging from 0.78 ± 0.42 in winter and 0.84 ± 0.52 in summer, with a few exceptions in the San José creek, a minor tributary located in the upper portion of the system (SAR=2.40). Relatively high values were observed in Los Frailes and Ci Aike creeks (SAR=2.61), which represent intermittent courses in the eastern portion of HR13. Regardless of the ECw, these SAR values were always located in the S1 category, which represents a low sodium hazard for irrigation (figure 3) (25, 31).

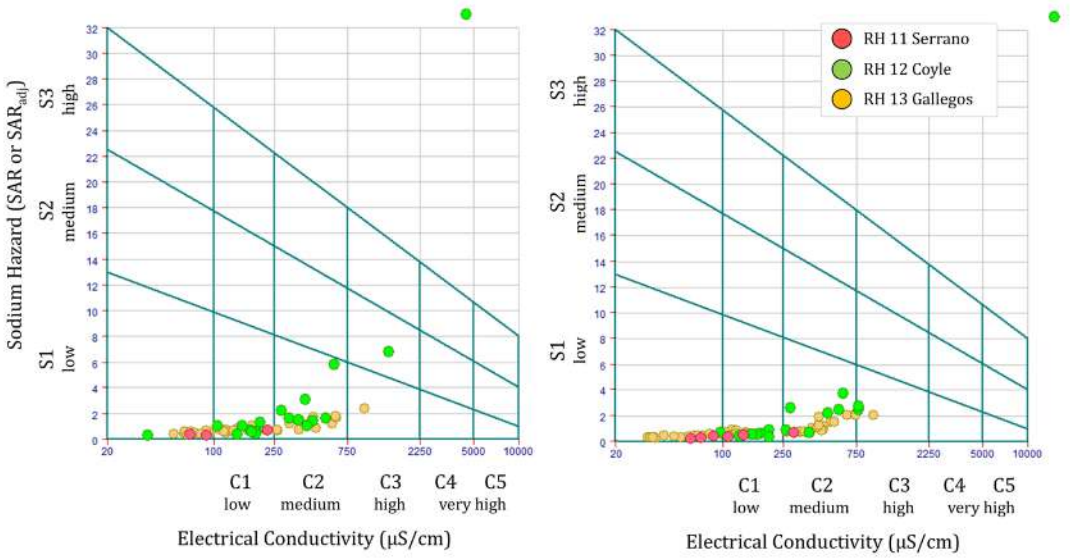


Figure 3. Salinity and sodicity combined hazards in surface waters from the three HR in the winter (left) and in the summer (right), through the Wilcox plot (1955).

Figura 3. Riesgos combinados de salinidad y sodicidad en aguas superficiales de las tres RH, según el esquema de Wilcox (1955), durante invierno (izquierda) y verano (derecha).

HR12 showed the highest mean regional SAR values, with great spatial variability. The average SAR ranged between 3.79 ± 8.91 for winter and early spring and 6.66 ± 20.64 for summer, with spatial variabilities between 235 to 310%, respectively. The highest SAR values were recorded in the ephemeral waters of Coy Inlet Creek, located at the mouth of the Coyle River, where it meets the sea (39.9 and 83.9 for winter and summer, respectively), and in the Fabre creek, located in the central section of HR12 (5.9 and 3.8 for winter and summer, respectively). Coy Inlet creek water exhibited an extremely high sodium hazard (>S4), exceeding the scale proposed by USDA (25). Furthermore, a few streams such as the Fabre creek and the De Las Casas stream reached a medium sodium hazard category S2 (figure 3). This condition, combined with fine-textured soils, high cation exchange capacity, and restricted drainage, typical situations in this region, represents a high risk for several crop species (31). Excluding these extreme cases, the mean SAR of waters in this basin ranged from 1.55 ± 1.52 in winter to 1.34 ± 0.94 in summer.

In general terms, there were significant strong positive correlations between the mean season flows and SAR values for HR11 and HR13. The predominant nivo-glacial mixed regime type in HR11 rivers, streams, and creeks showed two hydrograph peaks from late winter to mid-spring, and a maximum in late summer. In HR13, the nivo-pluvial mixed flow regime presented two hydrograph peaks: one moderate from late autumn to early winter, and a maximum from late winter to mid-spring. In both cases, seasonal peak flows correlated with higher SAR values in terms of m^3/s determined in gauging stations. For HR11, the correlation was 0.847 (r^2= 0.717, p-value <0.05) in 32 valid cases, while for HR13 correlation was 0.825 (r^2= 0.681, p-value <0.01) in 49 cases. A valid case consisted of the existence of a flow record at the same site as a sample collection. No statistical significance was detected for HR12 water samples between seasons (0.639, r^2= 0.406, p-value=0.114). The predominantly nival regime produces a strong peak flow between late winter to mid-spring, with minimum flows during the rest of the year, and most creeks and small streams dry out during the warmest months.

No HR11 samples met the requirements proposed by Weiner (2013) to implement the SAR_{adj}. Although the mean SAR_{adj} value was 25% higher in water samples from HR13 with >200 mg/l HCO_3 than the mean standard SAR values both remained in the S1 sodicity hazard category. In HR12 water samples SAR_{adj} emphasized the sodium character of waters with high concentration of bicarbonates, especially in creeks and streams such as Coy Inlet, Fabré, and De Las Casas, all of them ephemeral types. The sodium hazard categories in these sites were between S2 and S4 (from high to very high), with extreme SAR_{adj} values up to 44.8 in winter and 87.4 in summer, slightly above the standard SAR indicator. Despite these cases, most samples were classified, in terms of SAR_{adj}, within the S1 category (low hazard) with an average of 11% higher than standard SAR values.

When irrigation water contains enough carbonates and bicarbonates to precipitate Ca^{2+} and Mg^{2+} calcium and magnesium, a small proportion of Na^{+} may be enough to cause initial symptoms of soil sodification (8). Applying the classification suggested by Wilcox *et al.* (1954), mean RSC values less than 1.25 meq/l in both seasons and from the three hydrographic regions determined that waters are safe for irrigation (table 1, page 33). HR12 waters showed a mean RSC value of 1.04 ± 1.82 in winter and 1.31 ± 3.34 in summer. However, Fabré creek, sections of Brazo Norte of Coyle river and the unified Coyle river (convergence of all its tributaries in the HR12) had marginal waters (between 1.25 and 2.5 meq/l). De Las Casas stream and Coy Inlet creek, both of ephemeral type, showed RSC>2.5 meq/l, rendering them unsuitable for irrigation.

Most waters in the HR11 basin had SSP values below 35.0%, qualifying as good to excellent quality for irrigation according to Wilcox (1955), with no potential hazard for soil physical properties or plant growth (22). The average SSP for these waters was between 20.2 ± 8.8% in winter and 18.1 ± 6.1 in summer (table 1), with an exceptional SSP value of 53.5% in the lower Serrano river. A similar situation was observed in HR13 (mean SSP of 31.3 ± 5.5 in winter and 30.5 ± 6.5 in summer) and HR12 waters, which showed the highest mean SSP with 54.2 ± 9.3 in winter and 53.0 ± 12.9 in summer (table 1, page 33). In both HR, most water samples qualified as good to permissible for irrigation purposes. HR12 waters showed the highest SSPs average in the region.

Salinity hazard

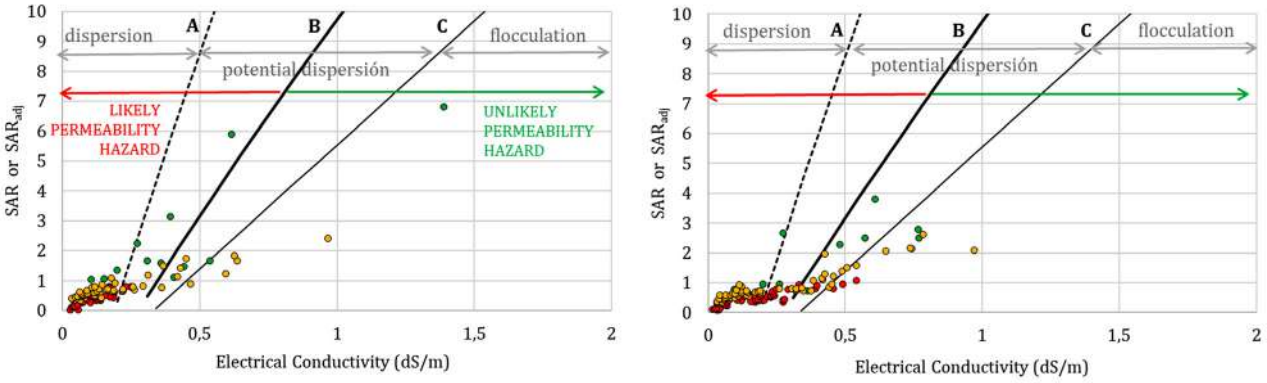
Diagnosis and classification of the total concentration of soluble salts in irrigation waters, may be adequately expressed in terms of ECw (25, 30). Except for a few samples, most waters in the region had ECw values below 250 µS/cm and may be categorized as low salinity waters (C1), according to Wilcox (1955). Such waters can be used for crop irrigation in a great variety of soils without great risks of developing salinity problems. This is particularly evident in rivers and streams of HR11, with an average of 122.1 ± 69.8 µS/cm in winter, and 168.8 ± 130.9 µS/cm in summer (table 1, page 33). Few cases in this basin, such as Don Guillermo (Argentine) and Chorrillo (Chile) streams, showed higher sodium hazard levels up to the C2 category (ECw<750 µS/cm), (figure 3, page 35). This corresponds to medium salinity waters, which can be used for irrigation of crops with moderate tolerance to salinity in soils with good drainage (30). Increasing ECw levels tend to mitigate negative sodium effects on soils but it can simultaneously induce crop stress by degrading the quality of the available water via salinization (14).

Similarly, in HR13 waters mean ECw was 209.6 ± 195.7 µS/cm and 245.8 ± 217.7 µS/cm in winter and summer, respectively. About 69% of samples were below 250 µS/cm in both seasons (C1) and 28% were within C2 (<750 µS/cm) (figure 3, page 35). Los Frailes and Ci Aike (two small intermittent streams in the final stretch of the HR13 near the seacoast) and San José (a small creek of the permanent type in the upper basin that receives strong discharges from extractive coal mining since the 1960s) reached a C3 category (from 750 to 2,250 µS/cm).

Coy Inlet and De Las Casas intermittent streams, in HR12, were the only C4 waters in the region, with an ECw of 4,440 and 14,760 µS/cm, a high salinity not suitable for irrigation of intolerant crops. Excluding these sections from a global analysis, mean ECw values in this basin ranged from 276.9 ± 160.8 µS/cm in winter to 348.7 ± 232.7 µS/cm in summer (table 1, page 33), qualifying closer to HR11 and HR13 waters although slightly higher. A 48.6% water of samples from H12 were C1, 37.1% were C2 and 8.6% were C3 (figure 3, page 35).

The relationship between sodicity and salinity hazards is more complex in soil than in water. While increasing salinity and sodicity in water involve crop irrigation restrictions, the long-term negative impact on soil occurs when increasing sodicity coincides with decreasing salinity (3, 25, 30). High Na^+ concentration leads to soil sodicity, increasing the susceptibility to crusting, runoff, erosion, and poor aeration, as well as the deterioration of soil hydraulic properties which can be counteracted with increasing salinity (21).

In contrast to the relatively low sodium hazard of waters for crop irrigation, the SAR analysis indicates an important potential for negative impacts on soil stability. In general, most water samples were found at the likely permeability hazard threshold based on the SAR/ECw relationship (figure 4). During early spring, with the beginning of complementary irrigation season, 100% of water samples in HR11, 89.5% in HR12, and 90.2% in HR13 demonstrated a possible permeability hazard. More than 50.0% of the total water samples in the region were under the curve of high risk of dispersion probability (curve A). During summer, the HR12 waters maintained a similar proportion of samples in the potential (likely) probability hazard category (89.5%), with a slight decrease in the samples from HR11 (91.2%) and HR13 (84.3%).



A, B, C, threshold functions of potential impacts on soil. / A, B, C, funciones umbral de impacto potencial en el suelo.

Figure 4. Relationship between SAR and ECw of irrigation water for prediction of soil structural stability in the three HR during late winter and early spring (left) and late summer (right). Adapted from ANZECC and ARMCANZ (2000) and Feitz and Lundie (2002).

Figura 4. Relación entre SAR y ECw en agua de riego para la predicción de la estabilidad estructural del suelo en las tres RH, entre fines de invierno y principio de primavera (izq.) y finales del verano (der.). Adaptado de ANZECC and ARMCANZ (2000) y Feitz and Lundie (2002).

There were statistically significant strong positive correlations between seasons and the ECw for both HR11 and HR13, with higher values during summer. In the case of HR11, the correlation value was 0.693 (r^2= 0.481, p-value <0.01) and for HR13, correlation was 0.819 (r^2= 0.671, p-value <0.01). No statistical significance was detected for HR12 water samples between seasons despite a high correlation coefficient (0.847, r^2= 0.717, p-value=0.128).

The average ES values for the HR11 and HR13 samples were 0.6 ± 0.2 meq/l and 0.9 ± 0.8 meq/l respectively, during late winter and early spring, and 0.6 ± 0.4 meq/l and 1.1 ± 1.1 meq/l during summer (table 1, page 33). Except for Los Frailes and Ci Aike (lower HR13) and San José (upper HR13) with some seasonal sodicity and salinity restrictions, the effective salinity hazard was low for all the remaining water samples (ES<3 meq/l), according to Palacios and Aceves (1970). ES values in these cases were not particularly high (ES<5 meq/l), but enough to be classified as conditioned waters for use in irrigation (20). The Potential Salinity (PS) indicator shares the same ranges that ES (PS<3 meq/l are good irrigation waters, PS between 3-15 meq/l conditioned waters and PS>15 meq/l not recommended waters for use). All the waters analyzed in HR11 and HR13 were classified with the PS indicator similarly to ES (table 1, page 33).

With average values above 4.3 meq/l in winter and 11.1 meq/l in summer, the ES in HR12 waters were slightly higher than those found in HR11 and HR13. This situation is comparable to the PS indicator, with averages of 2.9 and 10.4 meq/l for both HR, respectively. In general, there is a high proportion of good water for irrigation in the region (ES and PS<3 meq/l) except for few isolated streams and creeks with conditioned-type waters (ES and PS between 3-15 meq/l) such as De Las Casas (lower basin), Cañadón Fabré (middle basin), some sections of the Pelque stream (upper basin) and the main course of the Coyle river (the most important one of the HR12 in terms of annual flow). PS and ES values >15 meq/l were only registered in the Coy Inlet creek (lower HR12), which makes water not recommended for irrigation.

Conclusions

Results from this study indicate that most water samples from the three basins pose no significant salinity and sodicity hazards for irrigating crops, vegetables, and pastures. Exceptions include a few temporary streams and creeks. However, a significant proportion of water samples showed a potential negative impact on soil structural stability, from the beginning of the irrigation season (late winter to early spring) to the end of the growing season (late summer). Both saline and sodium hazards of irrigation water may re-transform the pre-existing soil solution through interactions with soil physics and chemistry mainly by precipitation of salts. These potential hazards must be considered during the planning and operating of irrigation schemes in arid and semiarid regions. This is particularly important where overuse through inefficient irrigation practices is common. The potential combined negative effects of the use of these waters for irrigation, in relation to regional soils, need further studies for the implementation of suitable irrigation projects.

Supplemmentary material

https://docs.google.com/document/d/1CHISvNHhFHmVz4kenuVC0O89W1BHCAva/edit?usp=sharing&ouid=111310786017351827239&rtpof=true&sd=true

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Methodological analyses for determining thermal requirements of grape varieties in Tandil, Argentina

Análisis de metodologías para determinar los requerimientos térmicos de variedades de vid en Tandil, Argentina

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Abstract

This study evaluated the thermal demand of different grapevine varieties (Cabernet Franc, Merlot, Semillón, and Tannat) in Don Bosco, Tandil, Buenos Aires, Argentina. Phenology was evaluated during four productive cycles, identifying periods from budburst to flowering onset (BB-FO), flowering onset to veraison onset (FO-VO), and veraison onset to maturity (VO-M). The National Meteorological Service of Argentina provided daily maximum and minimum air temperatures. Six thermal sum methods were used: methods 1.1, 1.2, and 1.3 depended on base temperature for vine development (10°C); methods 2.1 and 2.2, considered base temperature and optimum temperature (25°C); and method 3, considered base, optimum, and threshold temperature (35°C). These methods were evaluated using the standard error of the thermal sum. Methods 2.2 and 3 best fit all four varieties, allowing adequate estimates of cumulative daily heat summation.

Keywords
degree-days • *Vitis vinifera* L. • phenology • temperate - humid climate

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Resumen

El presente estudio tuvo como objetivo evaluar los requerimientos térmicos de diferentes variedades de vid (Cabernet Franc, Merlot, Semillón y Tannat) en viñedos plantados en Don Bosco, Tandil, Buenos Aires, Argentina. Se evaluó cronológicamente el desarrollo fenológico durante cuatro ciclos productivos, identificando las fechas de ocurrencia de los eventos y delimitando la duración de los subperíodos en días: inicio de brotación a inicio de floración, inicio de floración a inicio de envero, y envero a madurez. Las temperaturas máximas y mínimas diarias del aire se obtuvieron de los registros del Servicio Meteorológico Nacional. Se emplearon seis métodos de suma térmica: los métodos 1.1, 1.2 y 1.3, que se basan exclusivamente en la temperatura base para el desarrollo de la vid (10°C); los métodos 2.1 y 2.2, que, además de la temperatura base, consideran también la temperatura óptima (25°C); y el método 3, que incorpora los parámetros anteriores junto con la temperatura umbral (35°C). Estos métodos se evaluaron utilizando el error estándar de la suma térmica. Los métodos 2.2 y 3 demostraron el mejor ajuste para las cuatro variedades. Concluimos que los métodos 2.2 y 3 son más precisos en la estimación de la suma térmica diaria para todas las variedades estudiadas.

Palabras clave
grados-días • *Vitis vinifera* L. • fenología • clima templado - húmedo

Introduction

Among climatic factors, temperature plays a fundamental role in crop development. Temperature controls vegetative growth and development. Thermal weather models predicting plant development confirm its crucial role in phenology (54).

Grapevine (*Vitis vinifera* L.) is one widely cultivated fruit crop (30). Optimal production largely depends on local climatic conditions in each growing region (5). In modern viticulture, identifying phenological phases for each cultivar is crucial for effective crop management (21, 41). In recent decades, several investigations have focused on predicting grapevine budburst, flowering, and ripening (2, 15). Phenological characterization and quantification of thermal units for each phase allow harvest date estimations, suggesting site potential for viticulture (36). Thermal quantification, known as thermal sum (39) and typically expressed as degree-days (C° d) is widely used to account for temperature effects on plant development (40, 43). Daily thermal sum (DTS) considers average ambient temperature and basal temperature (BT) for each species. Grapevine has a DTS of 10°C. When the value after subtracting BT from average temperature is positive, degree-days are accumulated (16, 17, 25). This accumulated C°d can be calculated on a daily or monthly basis, for each phenological stage (20). Villaseca *et al*. (1986) indicated 850 to 950 C°d for early maturing table grape varieties and 1150 to 1350 C° d for late maturing cultivars in Chile. Chronological times between phenological stages vary with variety, climate, and geographic location (48). Thus, before introducing grape varieties in new regions, environmental effects on phenology should be considered for better technological management (4).

Unfortunately, research on grape phenology in southeastern Buenos Aires, Argentina, is limited, especially when compared to other regions of the country, such as Mendoza (14). In this province, the hilly area of Tandil constitutes a promising region for viticulture. According to Godoy and Gancedo (2022) hilly areas adequately satisfy the thermal needs of Merlot and Cabernet Franc, being Tandil an emerging region. European grape varieties of different cycles and origins are being evaluated in Tandil. According to the National Institute of Viticulture of Argentina (2022), vineyards in Buenos Aires grew by 200% between 2011 and 2021.

Several authors consider a BT of 10 °C to characterize varietal thermal requirements (29, 32, 35, 39). However, this varies with phenology. The optimal growth temperature ranges from 25 to 30°C (9). Above 30°C, growth decelerates, ceasing at approximately 38°C (38). Research on optimal temperature for each developmental stage indicates optimal average daily temperature for budburst above 10-12°C, between 18 and 22°C for flowering, between 22 and 26°C from flowering to veraison, and between 20 and 24°C from veraison to ripening (38).

Methods to calculate DTS are related to cardinal temperatures for plant development or based on air temperature (53). The former are grouped according to whether they only rely on BT or include optimum or upper threshold temperatures for plant development. The latter are grouped according to whether they rely on mean air temperature or also consider minimum and maximum temperatures (40, 51).

Defining phenological stages allows for rationalizing and optimizing cultural practices in vine cultivation (26). This study evaluated thermal requirements of Cabernet Franc, Merlot, Semillón, and Tannat varieties, by different calculation methods in the hilly region of Tandil, Buenos Aires province.

Materials and methods

The research was conducted at a commercial property called "Viñedo y Bodegas Cordón Blanco," in Tandil, southeastern Buenos Aires province (37°22'28" S, 59°06'23" W, altitude of 271 m) (figure 1). The 1.8-hectare vineyard was planted in 2008 with 1.8 m between rows, 1.2 m between plants, and 4,700 plants per hectare. It is subdivided into 40 x 25 m plots, with one vine variety per plot. We assessed four plots with Cabernet Franc, Merlot, Semillón, and Tannat. Soil is clayey and climate is temperate-humid with mild summers. Average annual temperature is 14.2°C, and average annual rainfall is 827 mm. Considering vine growing cycle from September to March, average rainfall is 539.5 mm, and average maximum and minimum temperatures are 23.9°C and 9.8°C, respectively (data provided by the National Meteorological Service of Argentina, SMN).

The vineyard was agronomically managed by pruning and phytosanitary controls. The four varieties were grafted onto a '101-14 Mgt' rootstock, a *V. riparia/V. rupestris* hybrid, easy to root and graft, and resistant to *Daktulosphaira vitifoliae* (phylloxera) (31). Starting in 2013, monitoring began from budburst to berry softening during 2013 - 2014, 2016 - 2017, 2018 - 2019, and 2019 - 2020 seasons. Determinations started from the fifth growth cycle, with a fully established crop, selecting five plants from different points within each plot, considered replicates. Phenology related to thermal summation was evaluated chronologically by identifying dates for each event, determining subperiods, in days.

Figure 1. Aerial view of the "Cordón Blanco" vineyard, located in Tandil, Buenos Aires province, Argentina, in September 2021.

Figura 1. Vista aérea del viñedo "Cordón Blanco", ubicado en Tandil, Buenos Aires, en septiembre del 2021.

Main phenological stages (24) were identified via Biologische Bundesanstalt Bundessortenamt Chemise (BBCH) scale, including sub-stages 05 (10-15% budburst) to 09 (end of budburst), 65 (flowering), 81 (veraison onset), and 85 (softening of berries, start of grape maturation). Budburst was determined when 50% of buds had visibly burst, at sub-stage 07 in the BBCH scale. Sub-stage 81 (veraison onset) was established by berry color change. Following this phenological scale, varietal growth cycles were evaluated up to sub-stage 85, until berry softening.

Minimum (Tmin) and maximum (Tmax) air temperatures and daily rainfall were obtained from the SMN. Daily mean air temperature (Tm) was calculated as the arithmetic mean between daily Tmin and Tmax. As described, thermal requirements were calculated as the sum of degree-days (°C d) for each phenological sub-period and from budburst to ripening. DTS (C° d) was determined via six different methods: method 1.1 (12), method 1.2 (52), method 1.3 (45, 47), method 2.1 (34), method 2.3 (28), and method 3 (49, 55). These were grouped according to whether they used only BT (methods 1.1, 1.2, and 1.3), *i.e.* the temperature at which the vine metabolic process is minimum (10°C), the optimum temperature (OT) (methods 2.1 and 2.2), or the three cardinal temperatures: BT, OT and upper threshold (TT) (method 3). Table 1 shows all six methods.

Table 1. Daily thermal sum (DTS) methods used, equation, calculation concerning temperature parameters, and references.

Tabla 1. Método de suma térmica diaria (STD) utilizado con la fórmula final, su base de cálculo en relación con las temperaturas empleadas y la bibliografía asociada a cada método.

DTS method	Calculation basis	References
1.1 (Tm - 10°C) × 1 day	If Tm < 10 °C, then Tm = 10°C	Gilmore and Rogers (1958) Villa Nova *et al.* (1972) Streck *et al.* (2007a, 2007b) Paula *et al.* (2008) Moura *et al.* (2007)
1.2 (Tm - 10°C) × 1 day	If Tmin ≤ 10 °C, then Tmin = 10°C	
1.3 DTS = {[(Tmin - 10°C) + [(Tmax - Tmin) / 2]} × 1 day; DTS = {[(Tmin - 10°C²] / [2(Tmax - Tmin)]} × 1 day; DTS = (0) × 1 day	When Tmin > 10°C When Tmin < 10°C When Tmax < 10°C	
2.1 (Tm - 10°C) × 1 day	If Tm < 10°C, then Tm = 10°C; If Tm > 25°C, then Tm= 25°C	
2.2 (Tm - 10 °C) × 1 day	If Tmin ≤ 10°C, then Tmin = 10 °C; If Tmax > 25°C, then Tmax = 25°C	
3 DTS = [(Tmax – 10°C)0,5] × 1 day; DTS = (0) × 1 day; DTS = (Tm - 10°C) × 1 day; DTS = {(25°C - 10°C) × [(35°C - Tm) / (35°C - 25°C)]} × 1 day; DTS = {(35°C - 25°C) × [(35°C - Tm) / (35°C - 25°C)]} × 1 day	When Tm < 25°C and Tmin < 10°C; When Tmax < 10°C; When Tm < 25°C and Tmin > 10°C; When Tm > 25°C and Tmax < 35°C; When Tm > 25°C and Tmax > 35°C, being that if Tmax > 35°C, then Tmax = 35°C	Tomazetti *et al.* (2015) Zeist *et al.* (2017)

Tmax is maximum temperature, Tm is mean temperature, and Tmin is minimum temperature. Cardinal temperatures are the lower basal temperature (10°C), the optimum temperature (25°C), and the upper threshold temperature (35°C).

Tmax se refiere a la temperatura máxima, Tm a la temperatura media y Tmin a la temperatura mínima. Las temperaturas cardinales son: Temperatura base inferior (10°C), óptima (25°C) y umbral superior (35°C).

For BT, OT, and TT, 10, 25, and 35°C were adopted respectively (55) (table 1, page 44). Cumulative thermal sum (CTS) for the whole cycle and each subperiod included individual DTS, *i.e.* CTS = ΣDTS (12, 44). Years were considered replicates and varietal CTS was assessed by calculating the standard error of the mean (SE). The SE of the CTS was obtained after calculating the standard deviation of the mean CTS for each year. The thermal requirements needed to complete the cycle and each phenological stage were subjected to ANOVA and compared by Tukey's test, with Infostat software (10) at $p \geq 0.05$.

Results and discussion

During the four years, absolute minimum and maximum temperatures fluctuated between -6.5°C and 39.3°C, respectively (table 2). Temperatures below BT (10°C) were recorded in all production cycles (figure 2, page 46), primarily between budburst and flowering onset (BB-FO). During November, coinciding with FO, average maximum temperature was 25°C in all four periods, with an average minimum temperature of 10°C. These conditions fell within the range between BT (10°C) and OT (25°C) (figure 2, page 46). In December, average maximum temperature reached 29°C during FO-VO and 20°C when calculated as mean temperature considering the four growth cycles. Finally, temperatures above TT (35°C) were recorded in all four production cycles, particularly during 2013-2014, on December 23, 24, 28, and 29, and January 6, 15, 17, and 18. Noticeably, total rainfall recorded in 2013 was 762.6 mm, below the historical average of 30 years (889 mm), while in 2014 was 1402 mm, significantly above historical average for Tandil. Table 2 shows 442 mm total rainfall in the last cycle (2019-2020), and during winter before BB, minimum temperature of -7.8°C and rainfall of 18 mm.

Table 2. Phenological evaluation of "Cabernet Franc", "Merlot", "Semillón" y "Tannat" (*Vitis vinífera* L.) in Tandil, Buenos Aires province, from September to February.

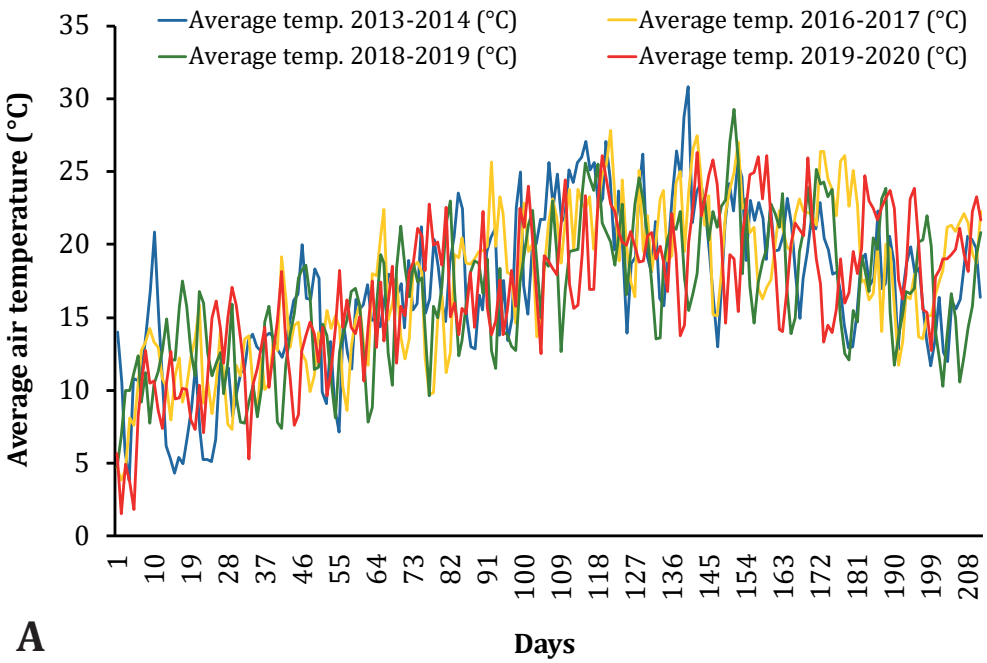
Tabla 2. Periodos de evaluación fenológica de las 4 variedades de *Vitis Vinifera* L. plantadas en Tandil, Buenos Aires.

Rainfall (mm), mean (Tm), absolute maximum (Tmax), and minimum (Tmin) temperatures (°C) recorded in each growth cycle.

Precipitaciones (mm), Temperaturas medias (Tmed), máximas (Tmax) y mínimas (Tmin) absolutas (°C) registradas en cada ciclo de crecimiento.

Period (Sept - Feb.)	Rainfall (mm)	Absolute Tmax (°C)	Absolute Tmin (°C)	Tm (°C)
2013/2014	770.1	39.3	-5.6	17.1
2016/2017	536.0	37.4	-4.2	17.4
2018/2019	697.1	34.2	-2.5	16.7
2019/2020	442	35.5	-6.5	16.9

The complete cycle from BB to M lasted, on average, 161 days for Cabernet Franc, 162 days for Merlot, 161 days for Tannat, and 154 days for Semillón. No differences were observed between varieties ($p > 0.05$). Table 3 (page 47) details phenological dates for all four seasons.



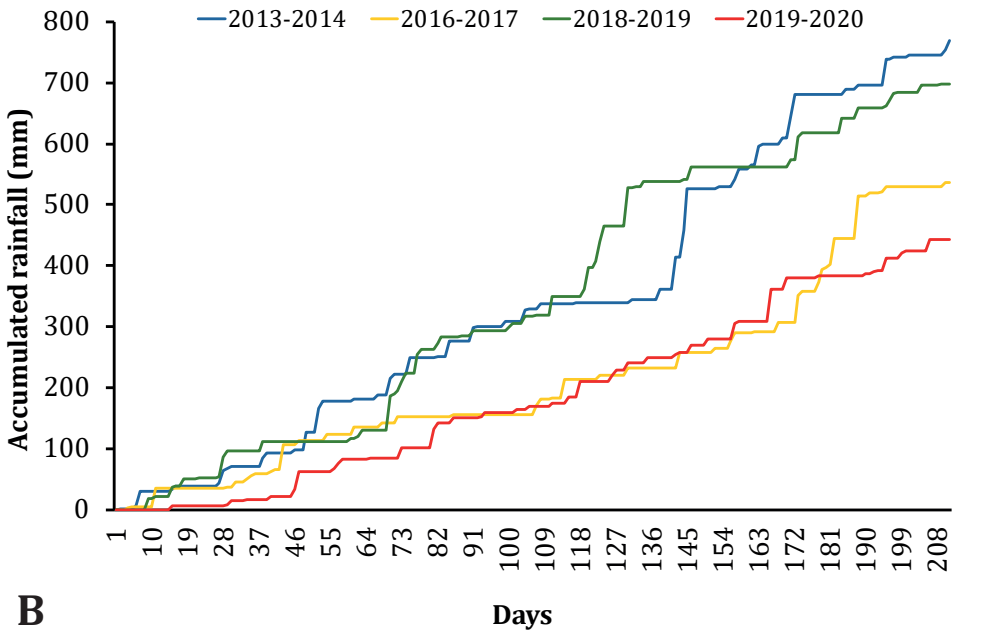


Figure 2. A. Average air temperature (°C) and **B.** Accumulated rainfall (mm) from September 1 to the end of March in four seasons, for Cabernet Franc, Merlot, Semillón, and Tannat grapevines (*Vitis vinifera* L.) in Tandil, Buenos Aires province, Argentina.

Figura 2. A. Temperaturas medias del aire (°C) y **B.** Precipitaciones acumuladas (mm), desde el 1 de septiembre hasta finales de marzo en cuatro temporadas, para las variedades de vid Cabernet Franc, Merlot, Semillón y Tannat (*Vitis vinifera* L.) en Tandil, provincia de Buenos Aires, Argentina.

Table 3. Phenological dates for Semillón, Cabernet Franc, Merlot, and Tannat varieties, recorded during four growing seasons: 2013 - 2014, 2016 - 2017, 2018 - 2019, and 2019 - 2020, in Tandil, Buenos Aires province.

Tabla 3. Fechas de los estadios fenológicos observados en las variedades: Semillón, Cabernet Franc, Merlot y Tannat, registrados durante 4 ciclos de cultivo: 2013 - 2014, 2016 - 2017, 2018 - 2019 y 2019 - 2020, en Tandil, Buenos Aires.

Cv. Semillón	**BB**	**FO**	**VO**	**M**
2013/2014	Sept 16	Nov 24	Jan 27	Feb 24
2016/2017	Sept 15	Nov 24	Jan 25	Feb 20
2018/2019	Sept 24	Nov 26	Jan 28	Feb 28
2019/2020	Oct 7	Nov 18	Jan 27	Feb 24
Cv. Cabernet Franc	**BB**	**FO**	**VO**	**M**
2013/2014	Sept 9	Nov 16	Jan 30	Feb 20
2016/2017	Sept 12	Nov 21	Jan 30	Feb 24
2018/2019	Sept 17	Nov 26	Jan 28	Feb 23
2019/2020	Sept 21	Nov 18	Jan 27	Feb 24
Cv. Merlot	**BB**	**FO**	**VO**	**M**
2013/2014	Sept 9	Nov 16	Jan 31	Mar 1
2016/2017	Sept 12	Nov 29	Jan 25	Feb 24
2018/2019	Sept 17	Nov 26	Jan 28	Feb 24
2019/2020	Sept 30	Nov 16	Jan 27	Feb 28
Cv. Tannat	**BB**	**FO**	**VO**	**M**
2013/2014	Sept 9	Nov 18	Jan 27	Feb 26
2016/2017	Sept 12	Nov 24	Jan 23	Feb 25
2018/2019	Sept 24	Nov 26	Feb 5	Feb 28
2019/2020	Sept 30	Nov 18	Jan 27	Feb 26

Budburst (BB), flowering onset (FO), veraison onset (VO), and berry softening (M).

Los eventos fenológicos registrados fueron inicio de la brotación (IB), inicio de la floración (IF), inicio del envero (IE), y ablandamiento de yemas (M).

Methods 2.2 and 3 showed the most precise heat summation adjustments considering the whole cycle in Tannat, Semillón, and Cabernet Franc, given lower standard errors (SE) obtained in the FO-VO subperiod (table 4, page 48). These methods did not statistically differ from each other ($p > 0.05$) but did differ from method 1.2 for all varieties (table 5, page 48-49). Considering method 2.2, CTS for Tannat, Semillón, and Cabernet Franc were 1219, 1182, and 1198°C d, respectively, with SE values of 13.5, 31.5, and 11.78°C d. In contrast, when using method 3, CTS were 1152, 1124, and 1135 C° d, respectively, with SE values of 28.7, 44.8, and 28.1 C° d (table 5, page 48-49). For Merlot, method 3 provided the best adjustment (lowest SE), with 1145 C° d CTS and an SE of 30.4 C° d. For Cabernet Franc and Tannat, medium and medium-late maturing varieties respectively, method 3 also showed a statistical difference of 1.3 ($p < 0.05$) compared to method 2.2 (table 5, page 48-49).

Thermal demands for each subperiod were also calculated after the six thermal summation methods (figure 3, page 49 and figure 4, page 50). Noticeable trends were observed among thermal sums for the four varieties, particularly in the first subperiod (BB-FO), where methods 2.2 and 1.2 had higher CTS values than the other methods (figure 3, page 49). These methods assume that when minimum temperatures are lower than or equal to BT, minimum temperature equals BT and overestimates DTS and CTS, especially in cold months with temperatures below BT. Method 2.1 resulted in the lowest SE in Tannat and Semillón, with CTS values of 251.2 ± 16.6 C° d and 260.3 ± 37.9 C° d, respectively.

Table 4. Mean duration of each subperiod (BB-FO, FO-VO, and VO-M, in days), + standard deviation.

Tabla 4. Duración media de cada subperiodo en días, con su desvío estándar, IB-IF (días), IF-IE (días), IE-M (días).

Cv. Semillón	BB-FO (days)	FO-VO (days)	VO-M (days)
2013/2014	68.4 ± 1.1	64.5 ± 2.4	26.0 ± 1.4
2016/2017	68.8 ± 2.7	65.0 ±3.7	25.4 ±3.4
2018/2019	61.2 ± 2.4	62.6 ± 1.8	29.0 ± 2.0
2019/2020	45.0 ± 3.1	68.0 ± 4.3	26.0 ± 3.7
Cv. Cabernet Franc	**BB-FO (days)**	**FO-VO (days)**	**VO-M (days)**
2013/2014	68.4 ± 1.2	74.6 ± 3.0	21.2 ± 1.7
2016/2017	69.5 ±1.1	69.7 ±0.5	25.6 ±0.9
2018/2019	70.4 ± 1.8	62.7 ± 1.3	25.8 ± 0.5
2019/2020	58.0 ± 4.3	68.2 ± 3.5	28.3 ± 0.5
Cv. Merlot	**BB-FO (days)**	**FO-VO (days)**	**VO-M (days)**
2013/2014	68.6 ± 1.1	76.0 ± 0.7	27.8 ± 0.8
2016/2017	76.4 ±4.7	58.2 ±1.1	29.0 ±1.4
2018/2019	71.8 ± 3.5	61.2 ± 3.7	26.2 ± 1.3
2019/2020	50.0 ± 5.7	70.7 ± 3.2	31.7 ± 0.6
Cv. Tannat	**BB-FO (days)**	**FO-VO (days)**	**VO-M (days)**
2013/2014	70.0 ± 3.5	69.0 ± 3.4	30.0
2016/2017	73.3 ± 1.3	60.3 ±0.5	31.8 ± 1.8
2018/2019	62.5 ± 3.3	70.0 ± 0.5	24.0 ± 2.0
2019/2020	47.2 ± 3.6	69.7 ± 1.7	32.5 ± 2.1

The table the average duration of each subperiod in days, along with its standard deviation: BB-FO (days), FO-VO (days), VO-M (days).

La tabla expresa la duración media de cada subperiodo en días, con su desvío estándar, IB-IF (días), IF-IE (días), IE-M (días).

Table 5. Average cumulative thermal sum (CTS) calculated for each subperiod (BB - FO, FO - VO, and VO - M) and the BB-M period for the four varieties by six methods of calculation of daily thermal sum (DTS).

Tabla 5. Suma térmica acumulada (STA) media calculada para cada subperíodo (IB - IF, IF - IE, IE - M) y para el período IB - M, para las 4 variedades, utilizando los 6 métodos de cálculo de la suma térmica diaria (STD).

DTS method	Varieties	Average CTS for BB-M (C° d)	Average CTS for BB-FO (C° d)	Average CTS for FO-VO (C° d)	Average CTS for VO-M (C° d)
1.2	Merlot	1383 a	362.1	720.1	301.6
1.3	Merlot	1299 ab	309.4	691.1	292.0
1.1	Merlot	1245 ab	259.9	688.9	296.9
2.1	Merlot	1235 ab	260.5	681.0	294.2
2.2	Merlot	1189 b	349.8	592.4	262.2
3	Merlot	1145 b	268.8	606.5	269.4
1.2	Semillón	1362 a	355.4	686.2	320.7
1.3	Semillón	1261 ab	292.6	658.1	310.7
1.1	Semillón	1226 ab	260.3	656.1	310.2
2.1	Semillón	1215 ab	260.3	648.1	307.6
2.2	Semillón	1182 b	343.2	562.9	276.0
3	Semillón	1124 b	264.0	574.8	285.4
1.2	Cab. Franc	1381 a	369.0	737.9	274.3

Different letters in columns indicate statistical differences ($p > 0.05$).

Valores en las columnas seguidos de la misma letra, no difieren entre sí por la prueba de Tukey ($p > 0,05$).

1.3	Cab. Franc	1275 ab	299.2	706.9	269.2
1.1	Cab. Franc	1234 bc	262.3	704.0	267.8
2.1	Cab. Franc	1218 bc	258.1	696.1	264.9
2.2	Cab. Franc	1198 bc	357.6	605.5	234.4
3	Cab. Franc	1135 c	272.4	613.5	248.9
1.2	Tannat	1399 a	349.8	791.1	258.4
1.3	Tannat	1310 ab	302.2	759.9	248.4
1.1	Tannat	1259 bc	251.2	757.2	250.5
2.1	Tannat	1150 bc	251.2	650.1	249.3
2.2	Tannat	1219 bc	339.0	654.0	225.7
3	Tannat	1152 c	259.1	678.2	230.7

Different letters in columns indicate statistical differences ($p > 0.05$).

Valores en las columnas seguidos de la misma letra, no difieren entre sí por la prueba de Tukey ($p > 0,05$).

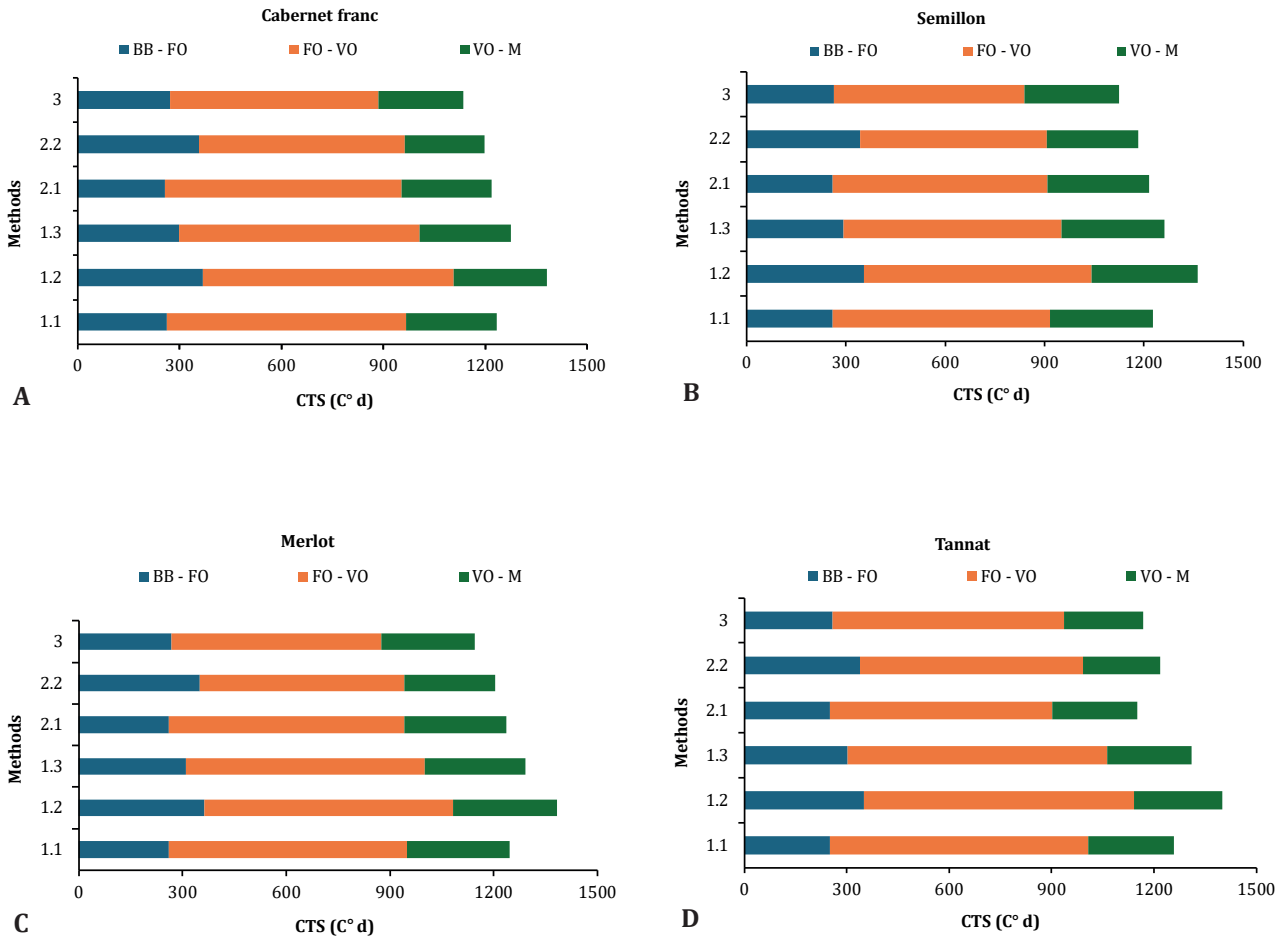


Figure 3. Cumulative thermal sum (CTS, C° d) calculated for each subperiod: budburst to flowering onset (BB-FO), flowering to veraison (FO - VO), and veraison to berry softening (VO - M), by six methods (1.1, 1.2, 1.3, 2.1, 2.2, and 3) estimating STD in Cabernet Franc, Semillón, Merlot, and Tannat, in Tandil, Buenos Aires province.

Figura 3. Suma térmica acumulada (STA, °C d) calculada para cada subperíodo: inicio de la brotación - inicio de la floración (IB - IF), inicio de la floración - inicio del envero (IF - IE), inicio del envero - ablandamiento de bayas (IE - M), utilizando seis métodos (1.1, 1.2, 1.3, 2.1, 2.2, y 3), estimando la STA en las variedades Cabernet Franc, Semillón, Merlot y Tannat, en Tandil, provincia de Buenos Aires.

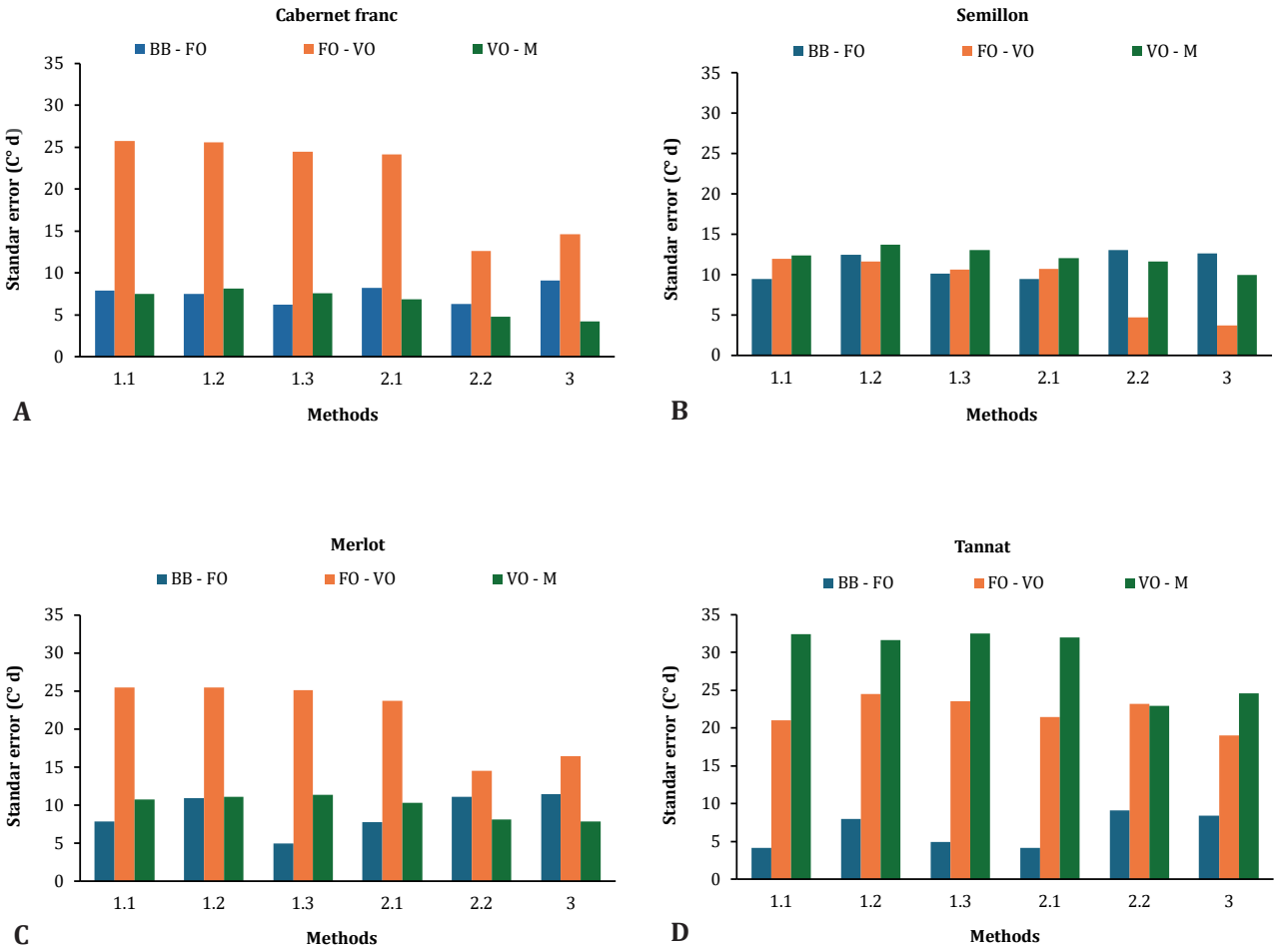


Figure 4. Standard error (SE) of cumulative thermal sum (CTS, C° d) for each subperiod: budburst to flowering (BB - FO), flowering to veraison (FO - VO) and veraison to berry softening (VO - M). Six methods (1.1, 1.2, 1.3, 2.1, 2.2, and 3) estimated daily thermal sum (DTS) in Cabernet Franc, Semillón, Merlot, and Tannat in Tandil, Buenos Aires province.

Figura 4. Error estándar (ES) de la suma térmica acumulada (STA, °C d) para cada subperíodo: inicio de la brotación - inicio de la floración (IB - IF), inicio de la floración - inicio del envero (IF - IE), inicio del envero - ablandamiento de bayas (IE - M). Seis métodos (1.1, 1.2, 1.3, 2.1, 2.2 y 3) estimaron la suma térmica diaria (STD) en las variedades Cabernet Franc, Semillón, Merlot y Tannat en Tandil, provincia de Buenos Aires.

In Semillón, no difference was found among methods ($p > 0.05$), whereas in Tannat, however, method 2.1 differed from methods 1.2 and 2.2 ($p < 0.05$). In Cabernet Franc, methods 1.3 and 2.2 yielded similar SE values (6.2°C d), with CTS 300 C° d and 357 C° d, respectively, while in Merlot method 1.3 yielded a lower SE (figure 4), and no differences with methods 2.2 and 3 ($p > 0.05$). Overall, methods 2.2 and 1.2 had significantly ($p > 0.05$) higher CTS values for this subperiod (BB - FO), while method 2.1 yielded the lowest CTS for Tannat and Semillón. Additionally, Tannat had a significantly different CTS calculated using method 2.1 from the estimated using methods 1.2 and 2.2 ($p < 0.05$). Although these variations between methods showed no differences for most varieties, they suggest that calculation method influences CTS estimation. Considering Cabernet Franc and Merlot, BB occurred on September 15 and 17, respectively, averaging four observation cycles (table 3, page 47). In other regions of South America, such as southern Brazil, BB for Merlot and Cabernet Franc

occurs around September 13 (26). Additionally, during 2018-19 and 2019-20, a two-week delayed BB was observed compared to previous years (table 3, page 47). This delay could be attributed to the lower temperatures recorded toward the end of winter, along with lower precipitation (figure 2, page 46), considering varietal BB phenological pattern is primarily influenced by air temperature (26).

The longest subperiod (63 - 91 days) with the highest thermal sum requirement was FO-VO, in all four varieties. Minimum SE for this subperiod was recorded for methods 2.2 and 3. Considering Merlot, Cabernet Franc, and Tannat, no statistical differences were observed with the other methods (figure 4, page 50). However, for Semillón, methods 2.2 and 3 showed differences in SE (figure 4, page 50) and CTS (table 4, page 48) compared to methods 1.1, 1.2 and 1.3 ($p < 0.05$). Merlot had CTS ± SE of 592.2 ± 14.5 C° d with method 2.2 and 606.5 ± 16.4 C° d with method 3. Semillón, showed 562.9 ± 4.7 C° d with method 2.2 and 574.8 ± 3.7 C° d with method 3. Cabernet Franc had CTS 605.5 ± 12.6 C° d with method 2.2 and 613.5 ± 14.6 C° d with method 3. Finally, CTS in Tannat was 662.5 ± 19.0 C° d with method 3. Considering Tannat, all six methods showed high SE (figure 4, page 50).

The VO-M subperiod showed no statistical differences among methods ($p > 0.05$) or the four varieties. However, method 3 yielded the best fit in three varieties (figure 4, page 50). In detail, CTS ± SE for Merlot, Cabernet Franc, and Semillón were 269.4 ± 7.9 C° d, 248.9 ± 4.2 C° d, and 285.4 ± 10.0 C° d respectively. Instead, in the VO-M subperiod, Tannat showed higher SE than the other varieties while, with method 2.2, it obtained the minimum SE (figure 4, page 50) with CTS of 225.7 ± 24.6 C° d. This late variety had a delayed VO in the last two cycles (table 3, page 47). Between February 20 and March 1, 50% of the plants of all varieties (table 3, page 47) were at sub-stage 89. Harvesting occurred later, particularly in the intermediate to late varieties. As latitude increases, more days are needed to reach a particular phenological stage (3).

Methods 1.1, 1.2, and 1.3 generated higher SE values for the four varieties. This lower methodological efficiency in representing thermal accumulation has been previously reported in southern Brazil (49). These three methods, based only on BT, showed higher variability (high SE) in CTS estimation than methods considering OT (methods 2.1 and 2.2) and TT (method 3). In other crops like wheat, Rosa *et al*. (2009) found that methods incorporating daily minimum and maximum air temperature to cardinal temperatures, improved phenological simulation. Our results indicate greater accuracy in calculating the thermal sum via methods including OT and TT in addition to BT. Therefore, we discourage methods based solely on the BT, especially considering that, during summer, higher temperatures can affect plant metabolism (45, 49).

These results suggest that, concerning grapevine development and climate change scenarios, simulations should use methods 2.1, 2.2, and 3. This is particularly relevant for Buenos Aires and its frequent extreme climatic events (6). Future scenarios predict increasing average air temperature in several regions of the planet (19), with negative consequences for viticulture (52). This acceleration of climate change invalidates old phenological calculations based on number of days (8, 22). Recent studies have shown significant correlations between temperature increase and earlier beginning of several stages in grapevine (1), with shortened phenological stages (3, 20, 41). Some prominent phenological changes suggest a significantly reduced anthesis-ripening duration (26). Besides reduced vine growth and yields, other possible undesirable implications for the wine industry include changes in wine quality (11, 37, 50, 52). In this context, considering that grapevine phenology is a crucial indicator of environmental impacts (7), accurate estimations of thermal requirements based on cardinal temperatures can be beneficial, regardless of the time required to satisfy them (33).

Considering methods 2.2 and 3, both demonstrating the lowest SE in the CTS, the varieties evaluated exhibited no statistical differences in thermal requirements during FO-VO and VO-M. Both methods estimated SE from 0.77 to 1.66 days for FO-VO and from 0.47 to 1.47 days for VO-M for Cabernet Franc, Merlot, and Semillón, indicating relatively low dispersion compared to the other four methods, where SE was twice higher. For Tannat, SE was two days, using these methods and approximately four days with the remaining four methods, in both subperiods. In BB-FO, the lowest SE was 1.18 days by method 2.2 for Cabernet Franc, while SE for the other varieties, was higher.

Our results provide information on thermal requirements of four grapevine varieties at different phenological stages in the region of Tandil, center Buenos Aires, Argentina. The use of degree-days improved phenological prediction, compared to other approaches considering days between phenological events (23). Considering the onset of autumn in our region, high humidity levels and low evapotranspiration may negatively affect fruit quality. Therefore, harvest should occur before the end of March. Future research should determine thermal requirements for maturity and harvest, contributing to climate risk mitigation and definition of optimal harvest time.

Conclusion

According to method 3, Merlot, Cabernet Franc, Semillón, and Tannat grown in Tandil require 1145, 1135, 1125, and 1153 C° d, respectively, to reach BBCH sub-stage 85.

The period between budburst and flowering onset showed the highest variability in thermal sum when comparing six methods for thermal requirement determination.

We conclude that methods 2.2 and 3 most accurately estimate cumulative daily heat summation in these varieties, in Tandil, using optimum temperature and upper threshold temperature for *Vitis vinifera* L.

Method 2.2 was accurate in this specific region, given the low frequency of temperatures above 35°C during the periods evaluated. Considering changes in climatic events in the center of Buenos Aires, we recommend models capable of recording daily variations in maximum and minimum temperatures. Further research will determine thermal requirements of different grapevine varieties in this region, especially between veraison and harvest.

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Dry mass production, nutrient accumulation and decomposition rate of cover crops intercropped with a *Theobroma cacao* full-sun system

Producción de materia seca, acumulación de nutrientes y tasa de descomposición de fitomasa de cultivos de cobertura intercalados con *Theobroma cacao* en un sistema a pleno sol

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Abstract

Cover crops play a crucial role in promoting soil protection, enhancing organic matter content, facilitating nutrient cycling, and improving overall soil quality. The objective of this study was to evaluate the biomass production, nutrient accumulation, and decomposition rate of cover crops intercropped with *Theobroma cacao* trees in a full-sun system. The research was conducted in Ilhéus, Bahia state, Brazil. The experimental design employed randomized blocks with three treatments, four decomposition times, and four replications. The treatments consisted of three cover crops: 1) pigeon pea (*Cajanus cajan*); 2) brachiaria (*Urochloa decumbens*); and 3) spontaneous vegetation. Decomposition rates were evaluated using litter bags at specific intervals: 0, 47, 94, 116, and 136 days after field deposition. Dry biomass production and nutrient accumulation by the cover crops were also measured. Spontaneous vegetation and brachiaria treatments exhibited the highest potassium accumulation, while no significant differences were observed among the treatments for the other evaluated nutrients. Moreover, spontaneous vegetation and brachiaria demonstrated higher decomposition rates, with 16.7% and 26.7% of the deposited material remaining at the end of the 136-day study period, respectively. In contrast, the decomposition rate of pigeon pea proved to be slower, with a remaining dry mass of 38.3%, indicating longer persistence in the soil, and consequently a greater half-life time. The cover crops investigated in this study are regarded as promising options for intercropping with cocoa, as they exhibit an average dry mass production of 10 Mg ha^{-1}. This value falls within the desired range for conservationist systems. When selecting species for intercropping, it is crucial to consider the decomposition rates these plants. This consideration ensures that the soil surface remains covered for an extended duration, leading to enhanced conservation and improvement of the soil's physical, chemical, and biological properties. Soil conservation can be effectively achieved by choosing cover crop species with slower decomposition rates, thereby contributing to the overall health and quality of the soil.

Keywords
cocoa monoculture • soil cover • Fabaceae • Poaceae

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Resumen

Los cultivos de cobertura desempeñan un papel crucial en la promoción de la protección del suelo, el aumento del contenido de materia orgánica, la facilitación del ciclo de nutrientes y la mejora de la calidad general del suelo. El objetivo del presente estudio fue evaluar la producción de biomasa, la acumulación de nutrientes y las tasas de descomposición de los cultivos de intercalados con árboles de cacao. La investigación se llevó a cabo en Ilhéus, estado de Bahía, Brasil. El diseño experimental empleó un diseño de bloques aleatorizados con tres tratamientos, cuatro tiempos de descomposición y cuatro repeticiones. Los tratamientos consistieron en tres cultivos de cobertura: 1) pigeon pea (*Cajanus cajan*), 2) braquiaria (*Urochloa decumbens*) y 3) vegetación espontánea. Las tasas de descomposición se evaluaron utilizando bolsas de descomposición a intervalos específicos: 0, 47, 94, 116 y 136 días después de la deposición en el campo. Se evaluó la producción de biomasa seca y la acumulación de nutrientes por los cultivos de cobertura. La producción promedio de biomasa seca fue de 10 Mg ha^{-1}. Los tratamientos de vegetación espontánea y braquiaria mostraron la mayor acumulación del nutriente potasio. La vegetación espontánea y la braquiaria demostraron tasas de descomposición más altas, con 16,7% y 26,7% de material remanente después de 136 días de estudio. Por el contrario, la descomposición del guandú resultó en una persistencia más prolongada, con una materia seca restante de 38,3%, en consecuencia, un mayor tiempo de vida media. Los cultivos de cobertura investigados en este estudio se consideran opciones prometedoras para la intercalación con cacao, ya que exhiben una producción de materia seca promedio de 10 Mg ha^{-1}. Este valor se encuentra dentro del rango deseado para los sistemas conservacionistas. Al seleccionar especies para la intercalación, es crucial considerar las tasas de descomposición de estas plantas. Esta consideración asegura que la superficie del suelo permanezca cubierta durante un período prolongado, lo que conduce a una mejora en la conservación y las propiedades físicas, químicas y biológicas del suelo. Al elegir especies de cultivos de cobertura con tasas de descomposición más lentas, se puede lograr una conservación efectiva del suelo, contribuyendo así a la salud y calidad general del mismo.

Palabras clave
monocultivo de cacao • cobertura del suelo • Fabaceae • Poaceae

Introduction

The cocoa tree (*Theobroma cacao* L.) is a plant species native to the Amazon and cultivated in tropical countries of South America, West and Central Africa, India, and Southeast Asia, holding significant economic importance in several countries (17). World cocoa production is concentrated in a few key countries, such as Ivory Coast, Ghana, Indonesia, Nigeria, Ecuador, Cameroon, and Brazil, which collectively account for 88% of global production. Ivory Coast is the largest contributor, producing approximately 39% of the total (14). Brazil stands as the largest cocoa producer in South America and the seventh-largest producer globally, having reported a production of 280 thousand tons in 2021 with a planted area of 617 thousand hectares (14).

Cocoa cultivation in Brazil is predominantly concentrated in four states: Bahia, Pará, Espírito Santo and Rondônia, with Bahia being the leading producer, accounting for 100,864 tons in 2020 (4). Moreover, cocoa farming represents the most important economic activity in the southern region of Bahia. Cocoa is predominantly grown in an Agroforestry System in southern Bahia, where the cocoa tree is cultivated in the understory of the native Atlantic Forest, locally referred to as "*cabruca*" (33). Another cultivation system that has been gaining prominence is monoculture, also known as full-sun cultivation. In this case, the cocoa tree shading is temporary, only occurring in the initial growth phase, and then the entire crop cycle occurs in full sun. This system is used in countries considered as the largest cocoa producers in the world (34), and has been gaining ground in Brazil, including in non-traditional regions for cocoa cultivation.

Cover crops can be used to promote maintained soil quality and conservation in the full-sun cocoa system. The association of perennial fruit trees with cover crops is already a consolidated agricultural practice (12, 25, 27, 28, 30, 42), however, it has not yet been studied in consortium with cocoa trees in a full-sun system, warranting the need for studies to validate the production of phytomass, nutrient accumulation and the decomposition rate of cover crops. Among the various benefits, these plants can provide soil protection through litter accumulation, promote nutrient cycling, increase biological activity, enhance infiltration, and improve water storage in the soil (5, 25, 27, 29, 42), as well as increase the production of commercial crops, as verified for citrus (19), and banana (20, 29).

Crop residue accumulation on the soil surface is influenced by the decomposition rate of cover crops, which in turn is regulated by the physical and chemical conditions of the soil, the material composition that is supplied, the presence of edaphic fauna, microbial activity of the soil, and precipitation (47). In a study conducted in the Cerrado biome of Goiânia, Brazil, the decomposition rates for pigeon pea (*Cajanus cajan* L.) were found to be 62% 60 days after the deposition of litter bags in the field (38). Also in the Cerrado biome of Piauí state, Brazil, the decomposition rate at 314 days after cutting was 83% for *Urochloa eminii* (Mez) Davidse (sub. *U. ruziziensis* (R.Germ. & C.M.Evrard) Crins) and 79% for pigeon pea (41). The dry matter production and decomposition of *Zea mays* and *U. eminii* (sub. *U. ruziziensis*) straw were additionally evaluated in an integrated crop-livestock system. The obtained dry mass was 6.6 Mg ha^{-1} and the half-life time was 115 days. At the end of the study, 36% of the crop residue was on the soil, with a loss of 4.23 Mg ha^{-1} of dry matter (36).

Cover crops are widely used in intercrops with fruit trees, especially species of brachiaria, and pigeon pea as an option for Poaceas and Fabaceas, respectively. However, the use of cover crops in full-sun cocoa systems has not yet been studied. Therefore, the present study was carried out with the hypothesis that pigeon pea (*Cajanus cajan*) in consortium with full-sun cocoa exhibit an accelerated decomposition rate compared to brachiaria (*Urochloa decumbens* (Stapf) R.D. Webste) and spontaneous vegetation. The objective of the present study was to evaluate the phytomass production, nutrient accumulation, and decomposition rate of cover crops intercropped with cocoa trees cultivated in a full-sun system.

Materials and methods

Characterization of the study area

The experiment was conducted at the cocoa research center (CEPEC-CEPLAC), in Ilhéus, Bahia state, Brazil. The site was located at coordinates 14°47'55" S and 39°02'01" W. According to the Köppen climate classification, the region has an Af-type hot and humid tropical forest climate, without a distinct dry season. The average annual precipitation exceeds 1,300 mm, distributed throughout the year, with an average temperature of 23°C and relative humidity of 80%. Climatic data, including temperature and precipitation, were recorded at the CEPLAC/CEPEC/SERAM meteorological station during the experiment (figure 1, page 58).

The regional topography is characterized as undulating, with an altitude of 60 m. The experimental area soil is classified as a Typic Hapludalfs (34). The soil particle size distribution was 320 g kg^{-1} sand, 338 g kg^{-1} silt and 342 g kg^{-1} clay. The chemical properties of the soil before implementing the experiment are presented in table 1 (page 58).

Experimental area history

The experimental area (2000 m^2) was initially maintained until 2016 in an agroforestry system of cocoa with *Erythrina* spp. This previous system was then subjected to clearcutting to implement a monoculture cocoa. All plant residues were removed from the site, and subsoiling was carried out to a depth of 0.50 m, followed by harrowing to incorporate phosphate fertilization (144 kg ha^{-1} of P_2O_5) applied in the form of single superphosphate. Liming was not used due to low active acidity and adequate levels of Ca and Mg in the soil. The area remained fallow until 2019, during which time spontaneous vegetation growth occurred.

Source: Meteorological station of CEPLAC/ CEPEC/SERAM.

Fuente: Estación meteorológica de CEPLAC/CEPEC/ SERAM.

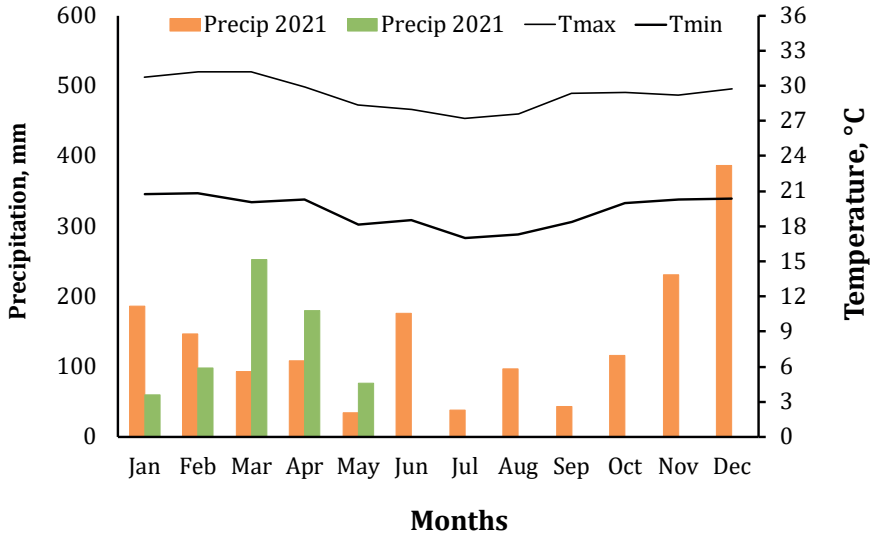

Figure 1. Meteorological data from the study period (December 2021 to May 2022).

Figura 1. Parámetros meteorológicos del período de estudio (diciembre de 2021 a mayo de 2022).

Table 1. Soil chemical attributes before the experiment implemented in the 0-20 cm layer.

Tabla 1. Indicadores químicos del suelo antes de que se implementara el experimento en la capa de 0-20 cm.

pH	H+Al	Al	Ca	Mg	SB	T	P	B	S	K	Cu	Fe	Mn	Zn	V	m
	---------- $cmol_c\ dm^{-3}$ ----------						---------- $mg\ dm^{-3}$ ----------								---%---	
6.1	4.2	0	7.5	2.1	9.9	9.9	40	0.65	7	78	4.9	84	489	10	70	0

H+Al: potential acidity; Al: aluminum; Ca: calcium; Mg: magnesium; P: phosphorus; SB: sum of bases; T: CEC a pH 7; S: sulfur; K: potassium; Cu: copper; Fe: iron; Mn: manganese; Zn: zinc; B: boron; V: base saturation; m: aluminum saturation.

H+Al: acidez potencial; Al: aluminio; Ca: calcio; Mg: magnesio; P: fósforo; SB: suma de bases; T: CIC a pH 7; S: azufre; K: potasio; Cu: cobre; Fe: hierro; Mn: manganeso; Zn: zinc; B: boro; V: saturación de bases; m: saturación de aluminio.

Then all vegetation was cut (full mowing with brush cutter) in July 2019, and soil surface was maintained with straw. Seedlings (6 months old) produced by cuttings from plagiotropic branches of the cacao CEPEC 2002 clone were planted in holes with dimensions 0.40 x 0.40 x 0.40 m, with spacing of 1.5m between plants and 4 m inter-rows.

Experimental design and treatments

The experiment was conducted in a randomized complete block design with four replications. The experimental plots had an area of 9 m x 12 m, totaling 108 m^2, with 18 cocoa trees by plot. One harrowing operation was performed in all inter-rows of the cacao trees, followed by manual sowing and incorporation of cover crop seeds. The cover crops implemented in March 2020 were: 1) brachiaria (*U. decumbens*); (2) crotalaria (*Crotalaria breviflora* DC); and (3) spontaneous vegetation. The following seed quantities were used: brachiaria: 3.5 kg ha^{-1} and crotalaria: 15 kg ha^{-1}. The spontaneous vegetation consisted of germinating the existing seed bank on the site, without the addition of external seeds.

In March of 2021, the evaluation year of this study, the crotalaria treatment was replaced by pigeon pea variety IAPAR 43, utilizing a seed rate of 45 kg ha^{-1}. There was no need to replant brachiaria and spontaneous vegetation treatments in 2021, as the plants persisted in the plots. The spontaneous vegetation treatment consisted of local native species, predominantly including *Commelina benghalensis* L. (10%); *Bidens pilosa* L. (3%); *Cyperus odoratus* L. (6%); *Euphorbia heterophylla* L. (5%); *Rhynchospora nervosa* (Vahl) Boeckeler (6%); *Megathyrsus maximus* (Jacq.) B.K. Simon & S.W.L. Jacobs (40%); and *Sorghum bicolor* subsp. *verticilliflorum* (Steud.) de Wet ex Wiersema & J.Dahlb. (30%), with the latter two species having the greatest occurrence.

The experiment evaluated three cover crop treatments and their decomposition rates: (1) brachiaria; (2) pigeon pea; and (3) spontaneous vegetation. The decomposition periods refer to the days (0, 47, 94, 116 and 136) that the cover crop residues remained in the field.

Determination of dry mass and nutrient accumulation of cover crops

The cover crop biomass was sampled in July 2021, four months after sowing the pigeon pea treatment. A 0.5 m × 0.5 m metallic square was randomly thrown into each plot (10) and all plant material within the square was cut close to the ground. The collected plant material was dried in an oven at 65°C for 72 hours to determine the dry mass production. Next, the nutritional composition of the dried plant samples was analyzed to evaluate the nutrient accumulation in the cover crops. The nitrogen (N); phosphorus (P); potassium (K); calcium (Ca); magnesium (Mg); sulfur (S); iron (Fe); zinc (Zn); copper (Cu); manganese (Mn) and boron (B) concentrations were determined (17). The nutrient accumulation was calculated by multiplying the dry mass and the respective nutrient concentrations in the cover crop biomass (21, 41).

The cover crop shoot management was performed with a brush cutter, and the residues were maintained on the soil surface. The mowings were repeated in the following months from July 2020: September 2020, November 2020, January 2021, March 2021, July 2021, September 2021, November 2021, January 2022 and March 2022. Two annual fertilizations were performed on the cocoa tree after cutting the cover crops at a dose of 50 kg ha^{-1} of N per application in the form of urea in July and January of each year (2020 and 2021). No other fertilizations were carried out, neither for the cocoa tree nor the cover crops.

Decomposition rate of cover crops

The plant material collected in July 2021 was separated according to each cover crop treatment and then dried in an oven. Afterwards, it was fragmented into pieces of approximately five centimeters. Portions of 11 g of the fragmented plant material were weighed and packed in litter bags. The litter bags were made with nylon fabric, with a mesh size of 2 mm and dimensions of 0.20 m x 0.20 m. Four litter bags for each cover crop treatment were distributed in the rows of cocoa trees in each experimental plot in direct contact with the soil surface. The decomposition rate of the cover crop residues was evaluated in the field during December 2021 to April 2022. The following litter bag collection times were considered: 0, 47, 94, 116 and 136 days after deposition in the field. A litter bag was collected from each treatment after each period. The material was removed from the litter bag, and washed in distilled water under a screen through a 0.053 mm mesh to remove soil particles. The material was then dried in a forced-air circulation oven at 65°C until reaching constant weight (45). Finally, the material was weighed to obtain the remaining dry mass. The remaining mass percentage (R%) was calculated using the relationship between the final dry weight (Wf) and the initial dry weight (Wi), according to the expression: R%= (Wf/Wi) x 100. The exponential model proposed in equation (1) was used to describe the decomposition rate of the residues (44).

$$X = X_0 . e^{-kt} \quad (1)$$

were:
X = amount of dry phytomass remaining after a period of time t, in days
X_0 = initial amount of dry phytomass
k = residue decomposition constant

The half-life time was calculated using the value of k, which represents the time required for the decomposition of half of the initial plant residues. This was obtained through the simple exponential linearization model (18), calculated by equation (2).

$$T_{\frac{1}{2}} = \frac{0.69315}{k} \quad (2)$$

Chemical characterization of residues after decomposition

The material remaining in the litter bag collected after the final decomposition period was washed and dried in an oven at 65°C for chemical characterization. Three subsamples of each treatment were separated, ground and the total nitrogen content was determined by the Kjeldahl method (13). Additionally, the lignin and cellulose concentrations were determined using the acid detergent fiber (ADF) method (48).

Statistical analysis

The data analysis was conducted considering the following factors: treatment (cover crops); the decomposition period and characterization of the residues after decomposition; the collection time of the litter bags; and the interaction of these factors. The data were analyzed for homogeneity of error variances by the Cochran test and normality by the Lilliefors test, followed by analysis of variance (ANOVA); mean comparison in significant cases was applied by the Tukey's test ($p<0.05$), using the RStudio program, version 3.5.0 (R-Development Core Team 2019).

Results and discussion

The precipitation in the period which included the growth of cover crops (March to June 2021) was 412mm and the temperature ranged from 31°C to 18°C (figure 1, page 58).

Dry mass production and nutrient accumulation

Higher dry mass production was observed in the brachiaria treatment (11.9 Mg ha^{-1}), followed by spontaneous vegetation (10.3 Mg ha^{-1}) and pigeon pea (7.9 Mg ha^{-1}) (figure 2).

Equal letters indicate no difference between treatments by Tukey's test ($p<0.05$). The bars represent the standard error of the means.

Letras iguales indican que no hay diferencias entre tratamientos según la prueba de Tukey ($p<0,05$). Las barras indican los valores del error estándar de las medias.

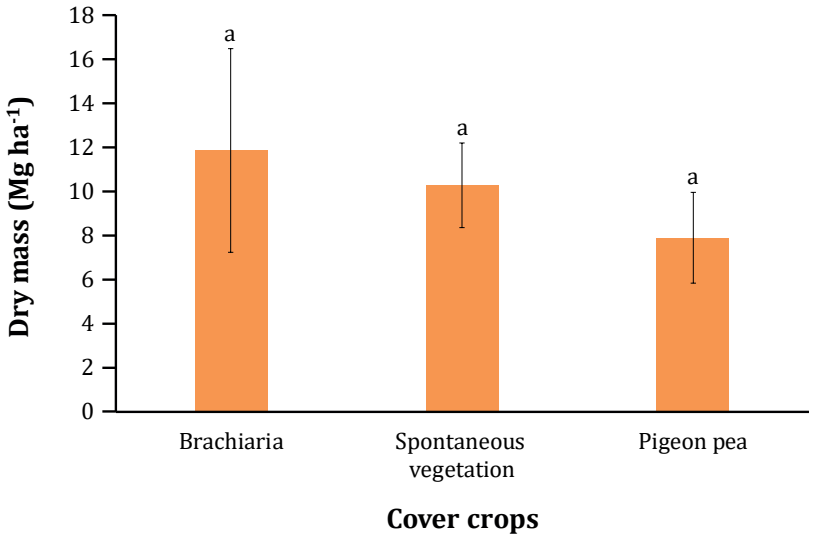

Figure 2. Dry mass production (March - July 2021) of cover crops intercropped with cocoa trees.

Figura 2. Producción de materia seca (Marzo - Julio 2021) de cultivos de cobertura intercalados con cacao.

The dry matter production in the three treatments is considered adequate for conservationist agricultural systems, as the values were between 6 Mg ha^{-1} and 12 Mg ha^{-1}, which is the average amount necessary to provide sufficient soil coverage and ensure beneficial effects on the physical, chemical, and biological attributes of the soil, especially in tropical climate regions (1, 43).

It is important to note that the dry mass production is influenced by the location and time of cultivation, climatic conditions, soil fertility, species used, and cutting age (24, 39). These factors (table 1, page 58 and figure 2, page 60) were also responsible for the dry mass production obtained in the present study. A study (23) in the municipality of Diamantina, Minas Gerais state, Brazil, implementing a no-tilling system using cover crops observed a dry mass of 4.54 Mg ha^{-1} for spontaneous vegetation and 4 Mg ha^{-1} for pigeon pea, constituting lower values than those observed in the current study (10.3 and 7.9 Mg ha^{-1} for spontaneous vegetation and pigeon pea, respectively). These same authors reported a production of 11.2 Mg ha^{-1} for *Urochloa decumbens*, which is a similar result to that found in the present investigation. Regarding the spontaneous vegetation treatment, the high dry mass production observed in this study can also be attributed to the occurrence of two grasses with great biomass production capacity, *Megathyrsus maximus* (sub. *Panicum maximum* L.) and *Sorghum bicolor* subsp. *verticilliflorum* (sub. *S. arundinaceum*), present in the experimental area. Furthermore, the adequate precipitation conditions during the growth of cover crops (figure 1, page 58) were a key factor in the dry mass achieved (24, 28, 39).

When evaluating different cover crop species in an orange orchard in Cruz das Almas, Bahia state, Brazil, Carvalho *et al.* (2021) reported the highest dry matter production of 11 Mg ha^{-1} for *Urochloa decumbens* and *U. eminii*. In this same study, spontaneous vegetation had the lowest dry matter production, which differs from the results of the current investigation. The discrepancy in the performance of spontaneous vegetation between the two studies could be attributed to differences in factors such as soil fertility, climatic conditions, and the specific species composition of the spontaneous vegetation (23, 28, 39).

Significant differences regarding nutrient accumulation (table 2) were only observed for potassium (K^+). The spontaneous vegetation treatment exhibited the highest K^+ accumulation, reaching 359 kg ha^{-1}, which was significantly greater than the pigeon pea treatment, presenting the lowest K^+ accumulation at 88 kg ha^{-1}. A substantial K^+ accumulation was also observed in the brachiaria treatment, although it did not differ significantly from the other treatments. The results indicate a direct relationship between potassium accumulation by the treatment and dry matter production (figure 2, page 60 and table 2). The K^+ accumulation in the spontaneous vegetation was approximately five times greater than that of the pigeon pea treatment. The high dry matter production and considerable K^+ accumulation in the spontaneous vegetation suggest that this treatment may provide similar nutrient cycling benefits as those observed with purposefully cultivated cover crops species (39). Therefore, when spontaneous plant communities possess the characteristics of high biomass production and nutrient accumulation, they can be considered viable alternatives for cover cropping, as they can deliver the benefits of implanted species without the establishment cost.

Table 2. Nutrient accumulation (March-July 2021) of cover crops intercropped with cocoa trees.

Tabla 2. Acumulación de nutrientes (Marzo-Julio 2021) de plantas de cobertura intercaladas con cacao.

Cover crops	N	P	K	Ca	Mg	S	Fe	Zn	Cu	Mn	B
	kg ha^{-1}										
Spontaneous vegetation	224	49	359a	91	53	18	1.05	0.47	0.10	1.54	0.15
Pigeon pea	228	23	88b	32	13	7	0.57	0.17	0.05	0.39	0.10
Brachiaria	167	43	211ab	49	41	24	1.63	0.52	0.08	0.96	0.12

Averages followed by the same letter in the columns do not differ from each other, ns: not significant by Tukey's test ($p<0.05$). N: nitrogen; P: phosphorus; K: potassium; Ca: calcium; Mg: magnesium; S: sulfur; Fe: iron; Zn: zinc; Cu: copper; Mn: manganese; B: boron.

Promedios seguidos por la misma letra en las columnas no difieren entre sí, ns: no significativo por la prueba de Tukey ($p<0,05$). N: nitrógeno; P: fósforo; K: potasio; Ca: calcio; Mg: magnesio; S: azufre; Fe: hierro; Zn: zinc; Cu: cobre; Mn: manganeso; B: boro.

Forage grasses are reported to exhibit great potential for K^+ absorption and subsequent accumulation, which is then returned to the soil following the decomposition of plant residues (6). Grasses in no-tillage systems can leverage their deep root system, remove nutrients from depth and subsequently return them to the soil surface through decomposition of their plant residues (36). Studies indicate that brachiaria grasses are highly efficient in K^+ cycling, corroborating the observations made in the present study (22, 40, 45). It is noteworthy that high values regarding nitrogen (N) accumulation were observed across the treatments, ranging from 167 to 228 kg ha^{-1}, although no statistically significant differences were detected (table 2, page 61). The ability of cover crops to accumulate N is primarily dependent on the specific species used (32). In a study conducted in the state of Piauí, Brazil, the N accumulation observed for pigeon pea was similar to the values reported in the current study (260 kg ha^{-1}); however, the accumulated N value for *Urochloa eminii* (sub. *U. ruziziensis*) was higher compared to the present findings (253 kg ha^{-1}) (41).

Decomposition rate and half-life ($T_{1/2}$)

The decomposition of cover crop residues decreased exponentially over time. During the 136-day evaluation period, the pigeon pea treatment exhibited the lowest loss of dry mass in its residues. In contrast, the brachiaria and spontaneous vegetation treatments showed greater degradation of their residues, with similar patterns observed between these two treatments (figure 3).

Different lowercase letters indicate differences between treatments for remaining dry mass on the same day of decomposition, by Tukey's test ($p<0.05$).

Diferentes letras minúsculas indican diferencias entre tratamientos para materia seca remanente, el mismo día de descomposición, utilizando la prueba de Tukey ($p<0,05$).

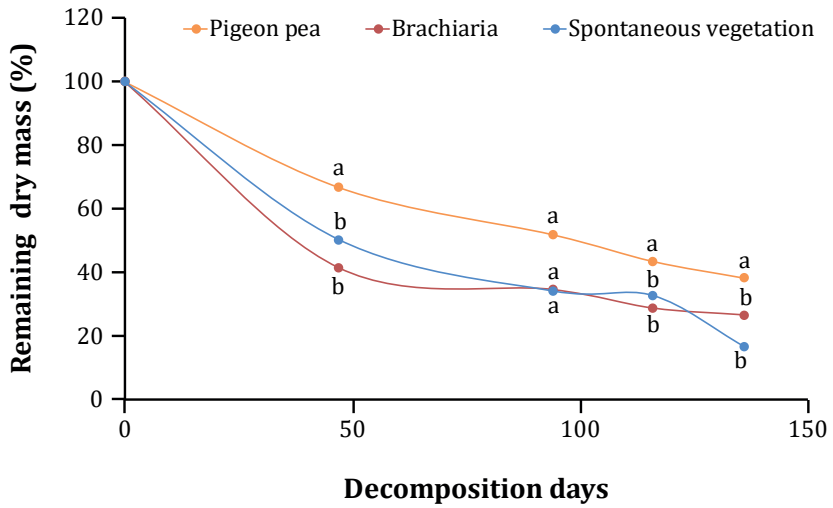

Figure 3. Remaining dry mass (%) of cover crops, intercropped with cocoa trees.

Figura 3. Materia seca restante (%) de cultivos de cobertura, intercalados con árboles de cacao.

The highest decomposition rates were observed for brachiaria and spontaneous vegetation treatments, with only 41.5% and 50.4% of residues remaining, respectively, in the first 47 days after deposition in the field (figure 3). These treatments differed significantly from the pigeon pea treatment for this evaluation period, which exhibited a higher residue remaining percentage of 66.8% (figure 3). By the end of the evaluation period (136 days), the spontaneous vegetation and brachiaria treatments continued to display the highest decomposition rates, with only 16.7% and 26.7% of the deposited material remaining, respectively. These values differed from the pigeon pea treatment, which had the highest remaining dry mass on the soil of 38.3% (figure 3).

In evaluating the decomposition rate of cover crops in the Cerrado biome, it was observed that the pigeon pea treatment exhibited a higher remaining dry mass (37%) after 154 days of deposition compared to spontaneous vegetation (23%) and brachiaria (21%) (46),

corroborating what was observed in the present study. However, a contradictory result was observed in a study conducted in the Cerrado, Góias state, Brazil. In this aforementioned study, high decomposition rates were reported for brachiaria (48%) and pigeon pea (65%) 150 days after cutting (16), constituting contrasting results to what was observed in our study, especially for pigeon pea. The authors suggest that the elevated decomposition rate in this case may have been driven by the soil being maintained in a moist condition through irrigation during the decomposition period.

The spontaneous vegetation and brachiaria treatments displayed the highest decomposition coefficient (k) of 0.011496 g g^{-1} d^{-1} and 0.009154 g g^{-1} d^{-1}, respectively. Consequently, these treatments also exhibited the shortest half-life times ($T_{1/2}$) of 60 and 75 days, indicating that half of the residues had decomposed by those time points (table 3).

Table 3. Decomposition coefficient (k) and half-life time ($T_{1/2}$) of cover crops intercropped with cocoa.

Tabla 3. Coeficiente de descomposición (k) y tiempo de vida media ($T_{1/2}$) de cultivos de cobertura intercalados con cacao.

Cover crops	k (g g^{-1} d^{-1})	$T_{1/2}$ (days)
Pigeon pea	0.006869	100
Brachiaria	0.009154	75
Spontaneous vegetation	0.011496	60

The pigeon pea exhibited a lower decomposition coefficient (k), resulting in an estimated $T_{1/2}$ of 100 days. Various factors influence the decomposition rate of plant residues, with the chemical composition being one of these factors. The higher the C/N ratio and higher cellulose, hemicellulose, lignin, and polyphenols levels in the plant constituents lead to slower decomposition of phytomass (2, 37). In a study conducted to evaluate the decomposition rate of cover crops in the Cerrado region during the 2001/2002 period, it was observed that the $T_{1/2}$ for brachiaria was 78 days, while for pigeon pea it was 101 days (45). This finding aligns with the results obtained in the present study. The same authors also mention that Fabaceae plants in uncovered Cerrado soil exhibited slower decomposition compared to grasses.

The extended persistence of pigeon pea residues in the soil can be attributed to its composition, characterized by a significant proportion of lignified stems that impede rapid decomposition. This assertion is supported by the analysis conducted on plant residues after the deposition period in the field, as outlined in table 4 (page 64). Pigeon pea and spontaneous vegetation exhibited the highest lignin contents, which differed significantly from brachiaria, showing the lowest lignin contents. It is worth noting that cover crops cut during flowering stages tend to have higher hemicellulose and lignin concentrations (8). The same authors also reported higher lignin concentrations during flowering for *Cajanus cajan*, a species that displayed a slower decomposition rate. The same occurred in this study for pigeon pea, which exhibited elevated lignin concentrations (table 4, page 64) and a reduced decomposition rate (figure 3, page 62). In contrast, the spontaneous vegetation presented high lignin content and a rapid decomposition rate.

The chemical composition of plant residues, including lignin, cellulose, hemicellulose, and polyphenols, as well as the C/N ratio and the lignin:N ratio, play a significant role in the decomposition process. Lignin presents a challenge to decomposition due to its resistance and impermeability to microbial attack in plant tissues (7, 9). In a study conducted by the same authors, the lignin contents of cover crops species were assessed, with the highest concentration observed in pigeon pea *cv*. mandarin. Conversely, *Urochloa eminii* exhibited the lowest lignin concentration. This composition, contributes to the slower decomposition rate of pigeon pea and the faster decomposition rate of brachiaria. The degradation process is hindered by the presence of lignin because only a limited number of microorganisms possess the necessary enzymes to break down its chemical bonds (15).

Table 4. Nitrogen, cellulose, and lignin concentrations in cover crop residues after 136 days deposited in the field.

Tabla 4. Concentración de nitrógeno, celulosa y lignina en residuos de cultivos de cobertura después de 136 días depositados en el campo.

Cover crops	Nitrogen	Celullose	Lignin
	g kg^{-1}		
Brachiaria	1.25 b	323.2 a	153.7 b
Pigeon pea	1.37 b	429.6 a	277.9 a
Spontaneous vegetation	2.02 a	292.7 a	285.9 a

Means followed by the same letter in the columns do not differ from each other by the Tukey's test ($p<0.05$).

Los promedios seguidos por la misma letra en las columnas no difieren entre sí por la prueba de Tukey ($p<0,05$).

In addition to lignin, nitrogen also plays a significant role in the decomposition process. The spontaneous vegetation treatment presented the highest nitrogen contents, which differed from the other treatments, as indicated in table 4. Higher nitrogen concentrations in plant tissues enable microorganisms to oxidize amide bonds (NH_2) of organic molecules. This process provides energy for microbial growth and facilitates decomposition (3). The presence of higher nitrogen levels in the spontaneous vegetation treatment justifies the observed remaining dry mass at the end of the study, which was similar to that of the brachiaria treatment, despite having similar lignin contents to pigeon pea. The increased nitrogen levels in spontaneous vegetation promoted greater microbial activity in the plant tissue, thereby facilitating decomposition even in the presence of lignin levels.

Conclusions

The cover crops investigated in this study are regarded as promising options for intercropping with cocoa, as they exhibit an average dry mass production of 10 Mg ha^{-1}. This value falls within the desired range for conservationist systems. When selecting species for intercropping, it is crucial to consider the decomposition rates of these plants. This consideration ensures that the soil surface remains covered for an extended duration, leading to enhanced conservation and improvement of the soil's physical, chemical, and biological properties. Soil conservation can be achieved by choosing cover crop species with slower decomposition rates, in turn contributing to the overall health and quality of the soil.

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Challenges in germination of *Neltuma caldenia* in semi-arid regions: optimization of germination protocols, influence of saline stress and seed quality

Desafíos en la germinación del *Neltuma caldenia* en regiones semiáridas: optimización de protocolos de germinación, influencia del estrés salino y evaluación de la calidad de las semillas

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Abstract

Global climate change presents challenges to arid and semi-arid ecosystems, impacting native species such as *Neltuma caldenia*, endemic to Argentina. This underscores the importance of understanding germination processes for both conservation programs and the restoration of degraded areas. We aimed to evaluate the germination rate of *N. caldenia* seeds from the south Espinal, using various scarification methods (chemical, mechanical and physical), and temperatures (25-30°C). Additionally, we investigate the effects of accelerated aging (0-96 h at 45°C and 100 relative humidity) and different saline solution concentrations during germination (0-0.6 M NaCl). Our results show that all scarification treatments effectively break seed dormancy while temperature significantly affects germination rates. Prolonged storage (0 to 96h) decreased seed viability. Moderate NaCl levels (0-0.2 M) did not affect germination, but higher concentrations inhibited it completely, with a threshold of -1.81 MPa osmotic potential. Understanding the impact of environmental stressors on seed germination can inform the development of effective conservation strategies among these climate change pressures.

Keywords
Fabaceae • *Prosopis* • caldén • dormancy • scarification • optimal temperature

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Resumen

El cambio climático global presenta desafíos para los ecosistemas áridos y semiáridos, impactando a especies nativas como *Neltuma caldenia*, endémica de Argentina. Esto resalta la importancia de comprender los procesos de germinación tanto para programas de conservación como para la restauración de áreas degradadas. Nuestro objetivo fue evaluar la tasa de germinación de semillas *de N. caldenia* del sur del Espinal, utilizando varios métodos de escarificación (químico, mecánico y físico) y temperaturas (25-30°C). Además, investigamos los efectos del envejecimiento acelerado (0-96 h a 45°C y 100% de humedad relativa) y de diferentes concentraciones de solución salina durante la germinación (0-0,6 M NaCl). Nuestros resultados muestran que todos los tratamientos de escarificación rompen eficazmente la latencia de las semillas, mientras que la temperatura afecta significativamente las tasas de germinación. El almacenamiento prolongado (0 a 96h) disminuyó la viabilidad de las semillas. Niveles moderados de NaCl (0-0,2 M) no afectaron la germinación, pero concentraciones más altas la inhibieron completamente, con un umbral de -1,81 MPa de potencial osmótico. Comprender el impacto de los factores de estrés ambiental en la germinación de semillas puede informar el desarrollo de estrategias de conservación efectivas ante estas presiones del cambio climático.

Palabras clave
Fabaceae • *Prosopis* • caldén • latencia • escarificación • temperatura óptima

Introduction

Global climate change (GCC), biodiversity loss, and environmental degradation present profound challenges for ecosystems, especially in arid and semi-arid regions (24). These changes exceed the physiological thresholds of many native plant species, including those in the *Neltuma* genus (formerly *Prosopis*), posing severe risks to their survival. Species persistence in such regions relies on traits like seed dormancy, germination temperature, and water stress tolerance. Understanding germination and early survival of plants under these conditions is critical for future conservation. The 'Decade on Ecosystem Restoration' of the United Nations (2021-2030) emphasizes the urgent need to address these environmental challenges. This is especially relevant for species like *Neltuma caldenia* (Burkart) C.E. Hughes & G.P. Lewisthe, known as 'caldén', which is endemic to the Espinal region of Argentina and impacted by GCC and deforestation.

One main constraint for seed germination in arid and semi-arid regions is seed dormancy. In these ecosystems, over 80% of native shrub species have seeds that will not germinate unless dormancy is broken. Dormancy is an adaptive strategy that prevents a viable seed from germinating under favorable conditions until specific triggers are met. This trait may arise from the structures surrounding the embryo, which inhibit germination even when conditions are suitable for non-dormant seeds (36). The presence of seeds with various dormancy levels enables temporal distribution of offspring, offering protection in unpredictable and variable environments, a particularly relevant aspect in arid and semi-arid regions (36). Studies have reported that *N. caldenia* seeds exhibit physical dormancy imposed by the seed coat (31, 36). Germination speed is crucial for species establishment and may vary based on the scarification treatment used. Utello *et al.* (2023) identified several scarification strategies to break dormancy in central Espinal *N. caldenia* seeds, with mechanical and chemical scarification showing the most success. However, physical scarification with boiling water yielded low germination rates, around 20%. Emergence rates for *N. caldenia* vary among studies, depending on scarification method and seed origin (21, 31, 36). Nonetheless, information specific to the La Pampa province, in the southern Espinal region of Argentina, where *N. caldenia* forests predominate, remains scarce. Dormancy type and degree can vary significantly across species within a genus and among populations within a species, influenced by maternal effects during seed formation (11). Thus, studying this trait in local seed provenances is important for understanding germination patterns.

Once dormancy is overcome, temperature becomes a critical factor in the seed germination process, highlighting ideal establishment times. Optimal germination temperatures for other *Neltuma* species from arid regions range from 20 to 40°C, varying by species and origin (4). However, this has not yet been evaluated for *N. caldenia*. Seed banks are vital for preserving genetic material for future conservation and restoration, yet storage conditions also influence germination. Assessing seed vigor, critical for this purpose, requires evaluating seed viability under simulated long-term storage conditions, such as accelerated aging (AA) tests (1). AA testing, though common in agronomic seeds, is less common for native species, despite its relevance for predicting viability thresholds in germplasm conservation (14). Fontana *et al.* (2016) were the first to apply the AA technique to the *Neltuma* genus, suggesting that seed vigor may be influenced by geographic origin and environmental conditions.

Salinity tolerance during germination is crucial for plant establishment in *N. caldenia*-inhabited environments. Soil salinity can hinder germination, particularly during dry years when saline conditions may increase due to GCC (22). Many native and endemic species in arid and semi-arid regions employ strategies to tolerate or avoid environmental filtering at different development stages. Salt stress tolerance has been confirmed in the genus, supporting its potential for restoring soils degraded by salinity (22, 33, 34). While *N. caldenia* has not been studied for salt tolerance, its presence in central-west La Pampa, where saline soils and salt flats are common, suggests it may possess similar tolerance mechanisms (8, 13).

In this study, we aim to evaluate the germination rate of *N. caldenia* seeds from southern Espinal in La Pampa province using various scarification methods and identify optimal germination temperatures. Additionally, we also examine the effects of accelerated aging and salinity on germination quality under laboratory conditions.

Materials and methods

Study area

The sampling zone is situated in the Caldenal district, within the Phytogeographic Province of Espinal, characterized by a temperate and dry climate with predominantly summer rainfall (6, 23). The collection of *N. caldenia* pods took place in La Pampa province (37°24'10.91'' S; 63°40'22.93'' W), where the average annual precipitation is 500 mm and the average temperature is 15°C (17). Permission to access and use native flora was obtained from provincial authorities in compliance with the Convention on Biological Diversity and the Nagoya Protocol.

Seed collection, conditioning and storage

Mature pods from 10 to 20 plants were manually harvested between February and April of 2018 and 2019, following FAO Forest Seed Handling Guide Guidelines (35). Seeds were extracted in the laboratory, using tweezer, selecting only those exhibiting no visible signs of deterioration. Selected seeds were then stored in paper envelopes at room temperature (20±2°C) for 2 to 14 months until the final experiment.

Germination of *N. caldenia*

Various scarification methods were tested and the optimal germination temperature was determined. Before each trial, seeds were disinfected following the protocol for *N. alpataco* (4). Briefly, seeds were soaked in 70% (v/v) ethanol for 15 minutes, followed by a 20 minutes immersion in 30% (v/v) NaClO solutions (48 g of active chlorine/L) and followed by three rinses with distilled water.

Dormancy breaking

Physical (PS), mechanical (MS), and chemical scarification (CS) methods were assessed following guidelines in the FAO Forest Seed Handling Guide (35) and the ISTA International Rules for Seeds Testing (18). PS involved soaking the seeds in water at 100°C until reaching room temperature. For MS, a small incision was made in the seed coat with tweezers avoiding

damage to the embryo. CS required immersing the seeds in sulfuric acid (98%) for 10, 20, 30, and 40 min (CS10, CS20, CS30, and CS40, respectively), followed by rinsing with distilled water, as proposed for other Norpatagonian *Neltuma spp* (4). Based on the obtained results, mechanical scarification was selected for the remaining experiments to be conducted, as it is a more environmentally sustainable methodology and avoids the potential interference of acid with other treatments.

Optimal germination temperature

Seeds were evaluated at temperatures ranging from 25°C to 45°C with a germination chamber, based on previous findings identifying optimal temperatures for *Neltuma spp* (4). The optimal temperature was considered as the one yielding the highest germination percentage in the shortest time.

Determination of seed vigor through accelerated aging (AA) test

The methodology from Fontana *et al.* (2016) for *Neltuma alba* was employed to assess seed vigor. Seeds were exposed to 45°C and 100% relative humidity in a germination chamber for durations of 0 (control), 24, 48, 72, and 96 h. Glass jars containing 100 ml of distilled water with a mesh above the water level were used to support the seeds during this procedure. Then, seeds were mechanically scarified and disinfected for germination.

Effect of salinity on germination

Seeds, previously scarified and disinfected, were arranged for germination in Petri dishes containing moistened cotton and filter paper with NaCl solutions at the following concentrations: 0 (control - T0), 0.05 (T1), 0.1 (T2), 0.2 (T3), 0.4 (T4), and 0.6 M (T5). These were converted to osmotic potential (Ψo) using the van't Hoff relationship (27):

$$\Psi o = (MPa) = - CiRT$$

where
Ψo = the osmotic potential in MPa
C = the concentration in mol/L
i = the dissociation constant of NaCl (*i.e.* 1.8)
R = the gas constant (0.0083 L/atm/mol/K)
T = the temperature in Kelvins.

The obtained osmotic potentials were: 0 (T0), -0.22 (T1), -0.45 (T2), -0.90 (T3), -1.81 (T4), -2.71 (T5) MPa. As temperature can influence osmotic potential it is recommended to perform these tests at the optimal temperature for each species (9); in the case of *N. caldenia* it was 30°C. After 14 days of treatment, the inhibitory effect of salts on the development of surviving seedlings was evaluated by measuring the length of the radicle and hypocotyl (cm). Subsequently, seedling vigor index (SVI), percentage phytotoxicity for roots and hypocotyls (RPT and HPT, respectively) and the tolerance index for roots and hypocotyls (RTI and HTI, respectively) parameters were calculated (26).

Germination conditions

Seeds from all experiments were placed on moistened cotton and filter paper in Petri dishes and then incubated in germination chambers at 30°C, except for the optimal germination temperature assay and the accelerated aging test. All germination experiments were conducted in darkness, as has been done by other authors with *N. caldenia* and other species of the genus (4). The trials were randomized and included 10 replications of 10 seeds each (N=100), with controls. Germination was defined as the emergence of a radicle at least 2 mm long (37), and daily germination counts were recorded for up to a week or until no further germination occurred.

Seed viability

The viability of seeds that appeared healthy but failed to germinate for all experiments was verified using tetrazolium testing (5). A solution of 2,3,5-triphenyl tetrazolium chloride 1% (p/v) in a phosphate buffer at pH 7.4 was prepared. The seeds were immersed in the solution for 24 hours at room temperature and then cut in half to observe viability. Stains were analyzed based on the color patterns described by Craviotto *et al.* (2011).

Germination evaluation

The germination parameters evaluated after one week of daily observations in the various germination assays (scarifications, optimal temperature, accelerated aging, and saline stress) were:

Germination Capacity (GC) (3), which is the total germination percentage at the end of the experiment and it is calculated as shown in table 1, in equation E1, where *G* is the number of germinated seeds at the end of the experiment and *n* is the total number of seeds in the test.

Table 1. Equations used to calculate the germination parameters evaluated in this study.
Tabla 1. Ecuaciones utilizadas para calcular los parámetros germinativos evaluados en este estudio.

The table presents the equations for Germination Capacity (GC), Mean Germination Time (MTG), germination speed index (GSI), Velocity Coefficient (VC), and Uniformity Factor (U), along with their respective units of measurement.

La tabla presenta las ecuaciones para la Capacidad Germinativa (GC), el Tiempo Medio de Germinación (MTG), el índice de velocidad germinativa (GSI), el Coeficiente de Velocidad (VC) y el Factor de Uniformidad (U), junto con sus respectivas unidades de medida.

Equation	Formula	Units of measure
E1	$GC = \sum\left(\frac{G}{n}\right) * 100$	Percentage (%)
E2	$MGT = \sum(n_i t_i)/\sum n_i$	Days
E3	$GSI = \frac{G1}{AG1} + \frac{G2}{AG2} + \ldots \frac{Gn}{AGn} = \sum(Gi/AGi)$	Number of seeds per day
E4	$VC = \frac{\sum n_i}{\sum(n_i * t_i)} * 100$	-
E5	$U = \frac{\sum(g - \sum t_i)^2 n_i}{\sum n_i - 1}$	-

Mean Germination Time (MTG) with the formula proposed by Martínez-Gonzáles *et al.* (2022), (E2):

where

T = germination time

ti = number of days of assay

ni = number of seeds germinated on day *i*

Germination Speed Index (GSI) or Maguire's Index (19), which is expressed as the number of germinated seeds per day (E3):

where

G1 = the number of seeds that germinated on day 1 (not cumulative)

G2 = the number of seeds that germinated on day 2 (not cumulative)

Gn = the number of seeds that germinated on day *n* (not cumulative, end of the experiment)

AG1 = the cumulative number of germinated seeds on day 1

AG2 = the cumulative number of germinated seeds on day 2

AGn = the cumulative number of germinated seeds on day *n* (end of the experiment)

Germinative energy (GE)(4) is the percentage of daily cumulative germination at the highest germination rate.

Specifically, for defining an efficient scarification protocol in terms of velocity and uniformity, the following parameters were also assessed:

Velocity Coefficient (VC) (31) is an index based on the number of germinated seeds inversely related to the time and the number of seeds germinated per day. It is a measure of the distribution of germination over time in relation to the number of germinated seeds and is expressed in the equation E4:

where

VC = velocity coefficient

n = number of seeds germinated on day *i*

t = number of days since sowing.

Uniformity Factor (U) (31) is proposed as a measure of the variance in germination time or the germination over time (E5):

where

U = uniformity factor

g = mean germination time

ti = number of days after sowing

ni = number of seeds germinated on day *i*.

Additionally, the time to reach the maximum accumulated germination (Tmax) was considered, which indicates the day from which no further germinations occurred.

Statistical treatment of data

The study employed a completely randomized experimental design. Data analysis was conducted using the open-source statistical analysis software InfoStat (30). Treatment differences were assessed using ANOVA with Tukey's test, or the non-parametric Kruskal-Wallis test when assumptions were not met (*i.e.* optimal germination temperature, accelerated aging test and effect of salinity on germination). Results were presented as mean ± standard error (SE) of the replications. The Pearson Correlation Coefficient assessed the relationship between germination parameters in the dormancy interruption assay (see Dormancy interruption) following reference ranges by Schober *et al.* (2018): very low correlation for r^2 less than 0.00, low for 0.10-0.39, moderate for 0.40-0.69, strong for 0.70-0.89, and very strong for 0.90-1.

Results and discussion

Seed germination protocol optimization

Dormancy breaking

In the control group of *N. caldenia* seeds, 56% did not germinate but were viable based on the tetrazolium test, indicating dormancy. Conversely, 42% germinated, and 2% did not germinate and were non-viable. Scarification enabled germination of all viable seeds, regardless of treatment, confirming physical dormancy imposed by the seed coat, as reported by Utello *et al.* (2023).

The evaluated germination parameters revealed a strong to very strong positive correlation between GC and GSI parameters (r^2=0.99 and 0.80, respectively). The correlation indicates that treatments improved both the germination percentage and speed (table 2, page 73). This improved germination-uniformity, with the CS10 and MS treatments exhibiting less dispersion, is consistent with previous findings (31). Furthermore, due to rapid germination, the obtained Germination Energy (GE) values were similar to those of CG and thus were excluded from further analysis.

Scarification with sulfuric acid for up to 30 minutes (CS30) resulted in a GC greater than 95%, but longer exposure negatively affected germination. However, the GC values obtained were considerably higher than those reported in seeds from other provenance, which ranged between 75% and 30% (31, 36). Although acid treatments (CS20, CS30, CS40) did not significantly affect the GC, they impacted GSI, MGT and Tmax. A negative or low correlation was observed among these parameters (r^2= -0.83 to 0.53). This suggests that while a high GC was maintained, the temporal efficiency of the process was reduced.

Table 2. Germinative parameters evaluated in scarification of *N. caldenia* seeds.

Tabla 2. Parámetros germinativos evaluados en las escarificaciones de las semillas de *N. caldenia*.

Treatment	GC (%)	MGT (days)	T max (days)	GSI (n°seeds/day)	VC	U
CS10	98.0±1.3^{a}	1.1±0.1^{a}	1.5±0.2^{a}	9.3±0.3^{a}	92.1±3.6^{a}	7.5±0.3^{a}
CS20	96.0±1.6^{ab}	1.9±0.1 ^{bc}	3.3±0.3^{c}	6.3±0.2^{c}	54.1±4.1^{ed}	25.9±1.8^{c}
CS30	95.0±2.2^{ab}	1.6±0.2 ^{b}	2.8±0.4^{bc}	7.3±0.41^{bc}	68.1±4.1^{dc}	26.9±2.0^{c}
CS40	89.0±3.1^{b}	2.1±0.1 ^{c}	3.9±0.2^{d}	5.2±0.4^{d}	46.7±2.0^{e}	24.4±1.5^{bc}
PS	100.0±0.0^{ab}	1.2±0.1 ^{a}	2.6±0.2 ^{bc}	8.2±0.2^{b}	76.3±3.8^{bc}	13.9±0.4^{b}
MS	100.0±0.0 ^{a}	1.2±0.1 ^{a}	2.0±0.0^{a}	9.2±0.2^{a}	87.1±2.9^{ab}	6.0±0.1^{a}
Control	42.0±5.6 ^{c}	1.3±0.1 ^{a}	2.0±0.3^{ab}	3.5±0.5^{e}	71.2±9.6^{bc}	17.5±2.9^{b}
p-value*	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.003

Germinative Capacity (GC), Mean Germination Time (MGT), Time of maximum germination (Tmax), Germinative Speed Index (GSI), Velocity Coefficient (VC) and Uniformity Factor (U) for chemical scarification treatments with sulfuric acid for 10 (CS10), 20 (CS20), 30 (CS30) and 40 (CS40) min, physical scarification (PS), mechanical scarification (MS) and the control.

Results were expressed as the mean ± standard error (SE) of the repetitions.

*different letters are not significantly different (*p*-value > 0.05).

Capacidad Germinativa (GC), Tiempo Medio de Germinación (MGT), Tiempo máximo de germinación (Tmax), Índice de Velocidad Germinativa (GSI), el Coeficiente de Velocidad (VC) y el Factor de Uniformidad (U) para los tratamientos de escarificación química con ácido sulfúrico durante 10 (CS10), 20 (CS20), 30 (CS30) y 40 (CS40) min, escarificación física (PS), escarificación mecánica (MS) y el control.

Los resultados se expresaron como la media ± error estándar (EE) de las repeticiones.

*letras distintas son significativamente diferentes (*p*-valor > 0,05).

Prolonged acid immersion decreased GSI and increased MGT and Tmax. This led to a more dispersed germination pattern, indicated by a decrease in VC (<92) and an increase in U (>7.5). Utello *et al*. (2023) used sulfuric acid scarification for 15 minutes on *N. caldenia* seeds from another province in the central Espinal region. Their results exhibited similar germination speed and duration values to our study at the longest exposure times (CS30 and CS40). The same trend was observed in the control seeds analyzed by Utello *et al*. (2023), with a Tmax five times higher than that of the control seeds in our study, despite a similar GC. This difference may stem from seed morphology, storage conditions, chemical composition, or seed coat thickness, influenced by regional environmental conditions (4, 21). Longer acid exposure than 10 minutes led to oxidative stress and reduced radicle elongation, consistent with Utello *et al.* (2023). The decline in germination rates may result from acid infiltration into seed tissues, raising temperatures and potentially harming the embryo (36).

Both mechanical and physical scarification treatments effectively broke dormancy in *N. caldenia* seeds, with no significant difference in MGT. MS was more efficient for germination speed, resulting in more uniform germination with increased VC, comparable to CS10. Utello *et al.* (2023) reported a 90% improvement in *N. caldenia* germination using a mechanical method. However, their physical scarification yielded germination rates approximately four times lower than those in our study, with a GSI 2.5 times higher. Zeberio and Pérez (2020) observed no germination when applying a combination of mechanical and physical scarification to *N. caldenia* seeds from the northern Monte region. In contrast, our study yielded significantly higher germination rates by applying similar scarification methods separately.

Timing, speed, homogeneity, and synchrony of germination are essential for understanding seed vigor and stress performance. Homogeneous germination supports synchronized seedling establishment, which benefits agriculture and restoration. While varied timing aids survival in wild populations, synchronized germination in managed environments promotes consistent and resilient growth (15). In this regard, the shorter duration chemical scarification method (CS10) and mechanical scarification were statistically more efficient than the other treatments. Furthermore, mechanical treatments for seed germination represent an effective and sustainable approach.

Optimal germination temperature

Table 3 (page 74), summarizes the evaluations of germination parameters at different temperatures. Germination rates remained near 100% up to 40°C but decreased at 45°C, where no seeds germinated. The tetrazolium staining indicated that these seeds were non-viable. GSI values showed significantly faster germination at 30 and 35°C. Thus, the optimal temperature range for *N. caldenia* in the south-central region of Espinal was between

30 and 35°C. Similar ranges have been reported for related species. Boeri *et al.* (2019) found an optimum germination temperature of 30°C for *N. alpataco*, while Villagra *et al.* (2017) suggested 35°C for both *N. alpataco* and *N. argentina*. In this sense, the optimal germination temperature varies according to species and geographic distribution.

Table 3. Optimal germination temperature.

Tabla 3. Temperatura óptima de germinación.

Treatment	GC (%)	MGT (days)	GSI (n° seeds/day)
25°C	100.0±0.0 [a]	1.26±0.0 [a]	9.2±0.2 [a]
30°C	99.0±1.0 [a]	1.04±0.0 [b]	10.6±0.6 [b]
35°C	100.0±0.0[a]	1.05±0.0 [b]	9.8±0.1 [ab]
40°C	98.0±1.3 [a]	1.7±0.15 [c]	6.6±0.3 [c]
45°C	0.0 [b]	0.0 [d]	0.0 [d]
p-value*	<0.0001	<0.0001	<0.0001

Values of Germinative Capacity (GC), Mean Germination Time (MGT) and Germinative Speed Index (GSI) obtained for *N. caldenia* germinations from 25 to 45°C.

The results were expressed as the mean ± standard error (SE).

* different letters are not significantly different (*p*-value > 0.05).

Valores de Capacidad Germinativa (GC), Tiempo Medio de Germinación (MGT) e Índice de Velocidad Germinativa (GSI) las germinaciones de *N. caldenia* de 25 a 45°C.

Los resultados se expresaron como la media ± error estándar (EE).

* letras distintas son significativamente diferentes (*p*-valor > 0,05).

In the study region, the highest precipitation occurs during the warm semester (October to March), accounting for 69% of the annual total. During these months, average maximum temperatures range between 28 and 36°C (29). In this sense, the optimal germination temperature of *N. caldenia* coincides with the period of highest precipitation in the region. However, climate change has led to a significant increase in temperature amplitude, which may alter the optimal conditions for germination and seedling survival.

Seed vigor

Accelerated aging (AA) of *Neltuma caldenia* seeds significantly reduced germination rates over time (table 4, page 75). The highest GC occurred within the first 24 hours of AA. This was followed by a 15% decline between 48 and 72 hours compared to the control. MGT remained stable for up to 72 hours. However, at 96 hours, GC decreased by 50%, accompanied by a twofold increase in MGT. Fontana *et al*. (2016) applied this method to *N. alba* seeds from northern Argentina and observed 50% lethality within 48 hours of storage. This suggests that *N. caldenia* seeds may demonstrate greater resilience to high-temperature and humidity conditions, potentially due to higher vigor. Seed vigor can vary by species and is influenced by environmental factors such as light, temperature, soil moisture, and nutrients (15). GSI decreased significantly with longer AA durations, resulting in germination rates 2.17 times lower than the control (table 4, page 75). This decline indicates potential physiological and biochemical changes, such as reduced plasma membrane integrity, molecular alterations in nucleic acids, decreased enzymatic activities during seed senescence, and delayed germination (25).

Effect of salinity on germination

The inhibitory effects of salinity on germination, due to ionic toxicity and osmotic stress impeding water uptake by the embryo, are well documented in various *Neltuma* species. Table 5 (page 75), summarizes these effects on *N. caldenia* germination. Germination capacity remained unaffected at osmotic potentials up to -0.90 MPa (T1-T3). However, it decreased significantly under higher osmotic stress, with total inhibition at the most severe level (T5) and no viable seeds according to the tetrazolium test. The germination response of *N. caldenia* under saline conditions resembles that of salt-tolerant plants, halophytes, showing resistance up to a critical concentration followed by a sharp decline.

Similar patterns were observed in *N. alpataco*, with reduced germination at comparable osmotic potentials (32). However, studies on *Strombocarpa strombulifera* and *N. alba* report a higher saline tolerance, with GC above 80% at -1.2 and -2.2 MPa, respectively (22). Additionally, *N. chilensis* showed 56% germination at -2.7 MPa, highlighting species-specific adaptations to salinity within the genus (34). This variation underscores the diverse salinity responses within *Neltuma*, illustrating the complex nature of salinity adaptation.

Table 4. Accelerated Aging (AA) test.

Tabla 4. Prueba del envejecimiento acelerado (AA).

Treatment (h)	GC (%)	MGT (days)	GSI (n° seeds/day)
0	100.0±0.0 [a]	1.2±0.3 [a]	9.8±0.1 [a]
24	100.0±0.0 [a]	1.3±0.1 [a]	9.2±0.2 [a]
48	88.0±3.9 [ab]	1.3±0.0 [a]	7.3±0.2 [b]
72	85.0±4.0 [b]	1.1±0.1 [a]	8.1±0.5 [ab]
96	50.0±8.2 [c]	2.3±0.1 [b]	2.4±0.4 [c]
p-value*	<0.0001	<0.0001	<0.0001

Germinative Capacity (GC), Mean Germination Time (MGT) and Germinative Speed Index (GSI) obtained for the AA test of *N. caldenia* seeds for 0, 24, 48, 72 and 96 h.

Results were expressed as the mean ± standard error (SE) of the repetitions.

*different letters are not significantly different (*p*-value > 0.05).

Capacidad Germinativa (GC), Tiempo Medio de Germinación (MGT) e Índice de Velocidad Germinativa (GSI) obtenidos para el ensayo de AA de las semillas de *N. caldenia* durante 0, 24, 48, 72 y 96 h.

Los resultados se expresaron como la media ± error estándar (EE) de las repeticiones.

* letras distintas son significativamente diferentes (*p*-valor > 0,05).

Table 5. Values of Germinative Capacity (GC), Mean Germination Time (MGT) and Germinative Speed Index (GSI) obtained for seeds subjected to treatments T0, T1, T2, T3, T4 and T5 with different osmotic potentials (Ψo) induced by NaCl.

Tabla 5. Valores de Capacidad Germinativa (GC), Tiempo Medio de Germinación (MGT) e Índice de Velocidad Germinativa (GSI) obtenidos para las semillas sometidas a los tratamientos T0, T1, T2, T3, T4 y T5 con diferentes potenciales osmóticos (Ψo) inducidos por NaCl.

Ψo (MPa)	GC (%)	MGT (days)	GSI (n° seeds/day)
0 (T0)	99.0±3.0[a]	1.0±0.0[a]	9.8±0.4[a]
-0.22 (T1)	98.0±1.3 [ab]	1.1±0.0 [ab]	9.2±0.1 [ab]
-0.45 (T2)	97.0± 0.6 [ab]	1.0±0.0 [a]	9.3±0.2 [ab]
-0.90 (T3)	98.0±2.0 [ab]	1.4±0.1[bc]	8.3±0.5 [bc]
-1.81 (T4)	83.0±4.9 [b]	2.7±0.2 [c]	3.0±0.1 [c]
-2.71 (T5)	0.0±0.0	0.0±0.0	0.0±0.0
p-value*	<0,0001	<0,0001	<0,0001

The results were expressed as the mean ± standard error (SE) of the repetitions.

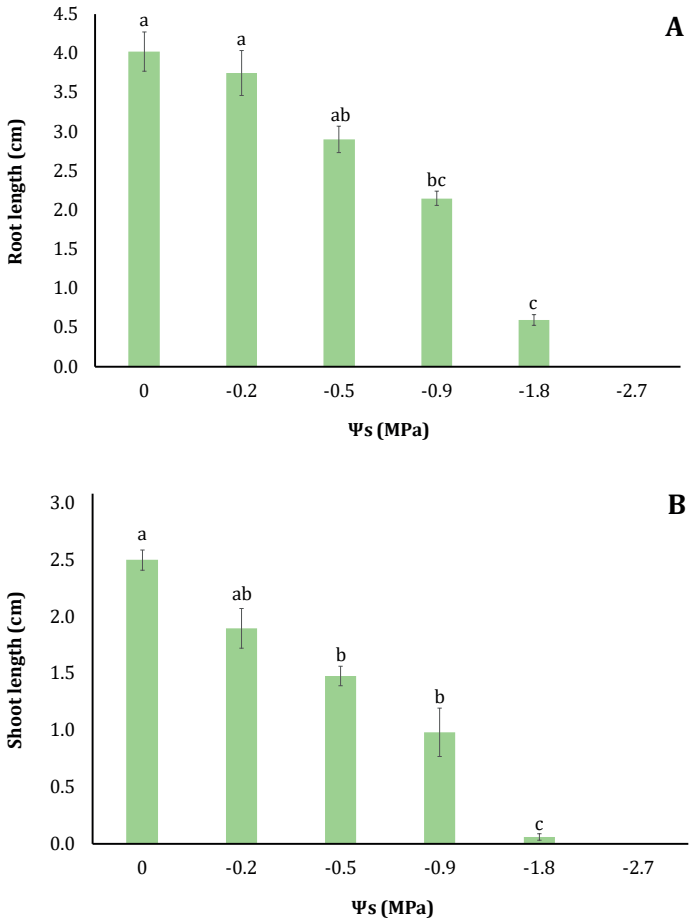
* different letters are not significantly different (*p*-value > 0.05).

Los resultados se expresaron como la media ± error estándar (EE) de las repeticiones.

* letras distintas son significativamente diferentes (*p*-valor > 0,05).

MGT and GSI were unaffected at lower salinity levels (T1, T2). However, they decreased significantly at higher salinity (T3, T4), likely due to delayed seed imbibition from low water potential, as observed in *N. alba* (22). Westphal *et al.* (2015) reported a similar germination delay in *N. chilensis* under saline conditions (NaCl 450-600 mM), requiring 5 days longer than controls to reach maximum germination. Similarly, *N. caldenia* seeds needed 4 days to reach maximum germination at high NaCl levels (T4), twice the duration of the control.

To quantify the impact of the osmotic pressure on seedling growth, both radicle and shoot lengths were measured. Increased salinity significantly reduced root and shoot lengths (figure 1, page 76). Root length showed no significant reduction at -0.22 and -0.45 MPa but declined beyond T3, reaching 6.7 times less than control length at -1.81 MPa (figure 1A, page 76). Shoot length declined from the lowest NaCl concentration, approaching minimal values at -1.81 MPa (figure 1B, page 76). These results indicate that NaCl inhibits shoot growth more than root growth, potentially due to endogenous abscisic acid (ABA), a phytohormone that reduces shoot growth and moderates root elongation under osmotic stress (2). Similar findings in *S. strombulifera* showed increased ABA levels and reduced shoot growth under high humidity and NaCl (12).



The results are expressed as the mean, and the bars indicate the standard error (SE) of the repetitions. Means with the same letter are not significantly different (p-value > 0.05).

Los resultados se expresaron como la media y las barras indican el error estándar (EE) de las repeticiones. medias con letra común no son significativamente diferentes (p-valor > 0,05).

Figure 1. Influence of osmotic treatments induced by NaCl (0 to -2.7 MPa) on the development of roots (A) and hypocotyls (B), recorded in cm of *N. caldenia*.

Figura 1. Influencia de los tratamientos osmóticos inducidos por NaCl (0 a -2,7 MPa) en el desarrollo de las raíces (A) y de los hipocótilos (B) de *N. caldenia*, registrado en cm.

The reduction in shoot and root growth led to a decline in SVI (table 6, page 77), which remained statistically similar to the control up to an osmotic pressure of -0.22 MPa. From treatment T1 onwards, SVI progressively decreased, with vigor dropping below half at -1.81 MPa. Root and shoot tolerance indices showed similar patterns, exceeding 50% up to -0.45 MPa, while salinity at T3 and T4 induced more severe phytotoxic effects on shoots. Total toxicity was observed for roots and shoots at T5.

Given that *N. caldenia* inhabits saline soils and salt flats during wet seasons, it may possess adaptive mechanisms to salinity, as our findings indicated. However, tolerance at the germination stage does not ensure similar tolerance in seedling growth (8, 13). While seeds tolerated up to -0.90 MPa during germination, 14-day-old seedlings were more sensitive, showing toxic effects at -0.45 MPa. This heightened sensitivity could limit seedling recruitment and survival in variable salinity environments. Beyond tolerable salinity, reductions in radicle and seedling growth are likely due to NaCl toxicity and impaired nutrient absorption (7). As soil salinity fluctuates with precipitation, it is essential to consider both germination capacity and salinity effects on seedling growth to inform effective conservation and restoration strategies.

Table 6. Seedling Vigor Index (SVI), Root Phytotoxicity (RPT), Hypocotyl Phytotoxicity (HPT), Root Tolerance Index (RTI) and Hypocotyl Tolerance Index (HTI) obtained for seedlings subjected to treatments T0, T1, T2, T3, T4 and T5 with different osmotic potentials (Ψo) induced by NaCl.

Tabla 6. Índice de Vigor de Plántula (SVI), Fitotoxicidad de la raíz (RPT) y de hipocótilo (HPT), Índice de Tolerancia de la raíz (RTI) e índice de Tolerancia del hipocótilo (HTI) obtenidos para las plántulas sometidas a los tratamientos T0, T1, T2, T3, T4 y T5 con diferentes potenciales osmóticos (Ψo) inducidos por NaCl.

The results were expressed as the mean ± standard error (SE) of the repetitions.

*means with common letter are not significantly different (*p*-value > 0.05).

Los resultados se expresaron como la media ± error estándar (EE) de las repeticiones.

*medias con letra común no son significativamente diferentes (*p*-valor > 0,05).

Treatment (MPa)	SVI	RPT (%)	HPT (%)	RTI (%)	HTI (%)
0 (T0)	646.4±26.4 [a]	16.4±2.9 [a]	7.4±2.5 [a]	100.00±3.5 [a]	100.0±6.2 [a]
-0.23 (T1)	553.3±42.8 [ab]	29.3±5.4 [ab]	28.3±6.4 [ab]	70.62±5.4 [ab]	72.1±6.6 [ab]
-0.45 (T2)	432.7±25.1 [bc]	44.4±3.2 [bc]	43.7±3.2 [bc]	55.54±3.1 [bc]	56.2±3.2 [bc]
-0.91 (T3)	303.9±18.5 [cd]	59.4±1.7 [cd]	63.2±7.6 [c]	40.6±1.7 [cd]	37.1±8.0 [c]
-1.81 (T4)	57.2±9.5 [d]	88.5±1.2 [d]	97.6±1.1 [d]	11.4±1.2 [d]	2.4±1.1 [d]
-2.72 (T5)	0.0±0.0	100.0±0.0	100.0±0.0	0.0±0.0	0.0±0.0
p-value*	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Conclusions

This study demonstrates the effectiveness of scarification techniques in promoting *N. caldenia* seed germination, with both mechanical and chemical methods successfully breaking seed dormancy. The seeds showed high vigor, with germination rates strongly affected by temperature, although prolonged storage reduced vigor, especially after accelerated aging. These findings underscore the need for appropriate storage practices to preserve seed viability. Additionally, *N. caldenia* seeds displayed salinity tolerance levels during germination comparable to or greater than those of other salt-tolerant species within its genus. Optimizing germination protocols and understanding the effects of salinity are essential steps toward formulating robust conservation and management strategies. Optimizing germination protocols and understanding salinity impacts are key to developing effective conservation and management strategies. Addressing these factors supports environmental restoration and habitat preservation, contributing to the sustainable use of *N. caldenia*, a notable species of the Espinal ecosystem under significant environmental pressure.

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Remaining in rural areas: towards a rural entrepreneur's analysis framework

Quedarse en las áreas rurales: Hacia un marco de análisis del emprendedor rural

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Abstract

This research employs an empirical approach to understand aspects defining why young people remain in their rural territories. Utilising a rural region of Caldas, Colombia as a case study and based on an agricultural education program for entrepreneurship, information from 368 rural young people was obtained. The study explored a conceptual model shaped by four dimensions and 34 variables. Using a Probit method, we identify significant variables regarding permanence in rural areas. We identify 11 key variables that determine the categories of socio-demographic profile, profile of entrepreneur characteristics, and category of motivations and territory. Our study contributes to *literature* on rural entrepreneurship from an empirical approach. Additionally, we propose a new analytical framework to address major problems in agriculture and rural territories, particularly in developing countries, such as Latin America.

Keywords
rural entrepreneurship • rural territories permanence • rural youth • entrepreneurship educational programs

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Resumen

Este artículo emplea una perspectiva empírica para comprender los aspectos que definen por qué los jóvenes permanecen en sus territorios rurales. Utilizando un estudio de caso de una región rural de Colombia y con base en un programa de educación agrícola para el emprendimiento, se obtuvo información de 368 jóvenes rurales. El estudio exploró un modelo conceptual formado por cuatro dimensiones y 34 variables. Además, mediante un método Probit buscamos identificar las variables significativas sobre la permanencia en el área rural. En los resultados demostramos la existencia de 11 variables clave como determinantes en las categorías de perfil sociodemográfico, perfil de características del emprendedor y categoría de motivaciones y territorio. Nuestro estudio contribuye a la ampliación de la literatura sobre emprendimiento rural, desde un enfoque empírico y la propuesta de un nuevo marco analítico para abordar uno de los problemas más relevantes del sector agrícola y de los territorios rurales, especialmente en países en desarrollo como América Latina.

Palabras clave

emprendimiento rural • permanencia en territorios rurales • jóvenes rurales • programas educativos de emprendimiento

Introduction

The rural population, especially in developing countries faces an environment that has historically been characterized by certain restrictions on access to services, markets, technologies, and other public goods, these factors provide challenges for the design and promotion of public policies focused on the development of rural areas (19). However, various public policies and programs in developing countries aim to address these challenges, such as policies supporting and promoting agribusiness through strengthening strategies, financing, and marketing (35). There is promotion of educational programs for skills development and learning with a focus on the rural youth, and the promotion of entrepreneurship (18). These programs intend to make territorial permanence more attractive and address the problem of rural migration, especially youth migration (10).

In this sense, rural entrepreneurship (RE) gains importance given its implications from the productive, economic, social, and environmental point of view within rural territories. Rural Entrepreneurship is an important strategy to promote rural development. Entrepreneurship based on the sustainable use of local resources for creating new economic activities can help reduce unemployment and poverty, whilst generating alternatives for rural societies (11). Furthermore, rural entrepreneurship promotion is seen as a strategy to enhance the rural economy (26) and confront the migration problem of rural youth. These strategies are focused on the diversification of the local productive structure, value addition, the transition towards the service sector, and consideration of territorial characteristics (5, 16).

In this study we consider whether rural entrepreneurship is an exit or a result strategy, being a product of the behaviour, characteristics and actions of the rural entrepreneur as a promoter of the business project. Approaches on RE do not necessarily put the entrepreneur at the centre of the process as a dynamic and complex actor, with characteristics that could represent possible typologies of the entrepreneur. Therefore, we conceptually and empirically contribute to the research question: What aspects drive the rural entrepreneur to stay within the rural territory? That is, we consider that it is not the permanence over time of the business and the entrepreneurship project based on a set of strategies (22), but a greater understanding of what allows the permanence of the person in their territory and the vision they have of staying in the rural territory over time.

Despite rural education programs in entrepreneurship, the reasons for entrepreneurs remaining in the countryside might be the lack of better job alternatives, and the drive for needs (14); advantageous market opportunities (32), or certain perceptions regarding institutional support (34). There is a need to understand the role of the family, its historical perspective and its entrepreneurial culture in influencing rural youth and their interests in

staying in rural areas. Therefore, it is necessary to identify what factors may determine the desire to stay within the territory. In this sense, the objective of this study is to determine the factors explaining the desire of rural entrepreneurs to stay within the rural territory, based on an analysis of the dimensions: Socio-demographic profile, Profile of entrepreneur characteristics, Entrepreneurship skills, Motivations, and Territory.

Construction of the conceptual and empirical model

In this study, we propose a conceptual model interrelating four dimensions (table 1). The main output of the model is the interest of young rural entrepreneurs in staying in their rural territory. The conceptual model is determined by dimensions usually found separately within the literature. Therefore, we propose their integration, generating a new conceptual model. Previous literature considers incorporating the sociodemographic profile on rural entrepreneurship analysis because sociodemographic variables have been positively associated with entrepreneurial intentions of rural young people (4), as well as the determination of innovative behaviour (28).

Table 1. Dimensions and analysis variables of the conceptual model.
Tabla 1. Dimensiones y variables de análisis del modelo conceptual.

Dimensions analysis	Key aspects addressed in each dimension	
Socio-demographic profile	1	Gender
	2	Program educative rural
	3	Age range
	4	Existence of family businesses
	5	Rural geographic location
	6	Active entrepreneurship
	7	Duration of the entrepreneurship
	8	Conformation of the entrepreneurship
Profile of entrepreneur characteristics	9	Interest in rural entrepreneurship
	10	Entrepreneurship for local and community impact
	11	Proactivity for rural entrepreneurship
	12	Interest in participating in training
	13	Interest in environmental impact and natural resources
	14	Innovative rural business ideas
	15	Perception of rural opportunities for entrepreneurship
	16	Perception of lack of resources as an impediment
	17	Perception of viability of non-agricultural projects

Entrepreneurship skills	**18**	Digital and computer skills
	19	Skills to identify new business opportunities and agricultural projects
	20	Skills to plan strategies and plans in the short, medium and long term
	21	Project leadership capabilities
	22	Ability to take risks
	23	Skills in working in groups
	24	Capabilities to do financial analysis in Microsoft Excel
	25	Capabilities to try again
	26	Ability to relate well with actors in the region
Motivations and territory	**27**	The municipality where you live motivates you for entrepreneurship
	28	Family impulse for entrepreneurship
	29	In the rural municipality it is possible to learn new things and access knowledge
	30	Perception of local institutional support
	31	Perception of culture and local entrepreneurial tradition
	32	Perception of prestige and reputation of the rural municipality
	33	Perception of people's business mindset
	34	Perception of supportive inter-institutional relationship

Secondly, for the most effective design of public policies on the repopulation of rural municipalities, the socio-demographic characteristics of rural entrepreneurs must be explored, since they tend to leave their territory (9). Furthermore, regarding the entrepreneurial characteristics dimension, our model proposes a profile approach directly related to the analysis around the rural entrepreneur's desire in staying in the countryside. Therefore, we propose, addressing aspects such as the entrepreneur's interest in impacting their rural and community environment, as well as impacting the use of local natural resources (28).

Our conceptual model considers the existence of two internal and external environments that interrelate a set of perceptions and variables, these contribute to understanding the desire of rural young people to stay in the countryside. The dimension of capabilities of the rural entrepreneur is one of the most addressed topics in the literature, a factor which we incorporate into the conceptual model of analysis. Aspects such as management, creativity, leadership, digital skills in rural entrepreneurship stand out as influencing the entrepreneur in the identification and recognition of business ideas (12). Finally, we include the integration of the motivation and territory dimension, based on a set of key variables that allow us to understand if the rural geographical space is a perceived viable environment by the rural entrepreneur to undertake a certain project (23).

Materials and methods

Study area context

Caldas is in the Colombian coffee zone, is a region with 27 rural municipalities (figure 1, page 84). In this region, the University of Caldas has led a public-private alliance "The University in the Field and in the Territory", which carries out educational programs for rural youth allowing the development of agricultural entrepreneurship capabilities, facilitating the people involved to stay in their rural territories. In 2023, these educational programs involved around 1,100 rural youth throughout the entire geographic study area. This area is characterised by the influence of coffee production and industry, with various agroclimatic conditions and productive systems, creating entrepreneurship opportunities in agricultural, livestock, agro-industrial and tourism.

Figure 1. Study area.
Figura 1. Area de estudio.

The research adopted a quantitative approach, with the data collection conducted in 2023. An online survey was created, with four domains and 34 variables (table 1, page 82-83); Socio-demographic profile, Profile of entrepreneur characteristics, Entrepreneurship skills, and Motivations and territory. Through a list of 1,100 previous students from agricultural programs for rural youth, a random sample of 368 people was obtained, which corresponds to a response rate of 33.45%. To advance the process, all students were informed of the program, whilst also attaching the form with the questions and the respective institutional letter of invitation to participate in the study. The questions on the survey were related to the proposed variables (table 1, page 82-83), in addition to various response options of nominal, ordinal, and dichotomous nominal types (table 2, page 85-86).

Method of information analysis

Data Analysis was carried out using a Probit model, which is a discrete choice model, where the endogenous variable presents two alternatives 0 and 1 (1). In this way, the dependent variable (Y) is related to the intention of rural youth to stay in the countryside. For our analysis, two values were assumed: 1 if the rural youth want to stay in the rural territory and 0 otherwise.

The econometric analysis in this study follows the stages developed by Cuevas-Reyes *et al.* (2020), and the theoretical underpinnings proposed by Aldrich & Nelson (1984). The Probit model uses a normal cumulative distribution function, where the probabilistic model is estimated by the maximum likelihood method and obtains the marginal change. Furthermore, the marginal change of the density function of the standard normal distribution is evaluated at a defined point and the parameter to be evaluated (17), as expressed in equation 1.

$$\frac{\partial P_i}{\partial X_{ki}} = \frac{\partial \phi(X_i\beta)}{\partial X_{ki}} = \phi(X_i\beta)\beta_k \quad (1)$$

Table 2. Description of explanatory variables.
Tabla 2. Descripción de variables explicativas.

Variable	Variable type	Description
x_1	Nominal	1=female 2=male
x_2	Nominal	1= technical 2= technological
x_3	Ordinal	1=14-17; 2=17-20; 3=20-25; 4= more than 25
x_4	Dichotomous nominal	0=Not, 1=yes
x_5	Ordinal	1= Small rural village; 2= Rural municipality; 3= Area near the rural municipality; 4=Area far away from rural municipality
x_6	Dichotomous nominal	0=Not, 1=yes
x_7	Ordinal	0= Does not have; 1=Less than a year; 2=1-3 years; 3=more than 5 years
x_8	Ordinal	1=individual; 2= With family; 3= maximum 3 people; 4= association or cooperative
x_9	Dichotomous nominal	0=No, 1=yes
x_{10}		0=No, 1=yes
x_{11}		0=No, 1= yes
x_{12}		0=No, 1= yes
x_{13}		0=No, 1= yes
x_{14}		0=No, 1= yes
x_{15}		0=No, 1= yes
x_{16}		0=No, 1= yes
x_{17}		0=No, 1= yes
x_{18}		0=No, 1= yes
x_{19}		0=No, 1= yes
x_{20}		0=No, 1= yes
x_{21}		0=No, 1= yes
x_{22}		0=No, 1= yes
x_{23}		0=No, 1= yes
x_{24}		0=No, 1= yes

x_{25}	Dichotomous nominal	0=No, 1= yes
x_{26}		0=No, 1= yes
x_{27}		0=No, 1= yes
x_{28}		0=No, 1= yes
x_{29}		0=No, 1= yes
x_{30}		0=No, 1= yes
x_{31}		0=No, 1= yes
x_{32}		0=No, 1= yes
x_{33}		0=No, 1= yes
x_{34}		0=No, 1= yes

The empirical model that represents the dependent variable Y (Staying in the field) and the independent variables (X) that influence the decision to staying in the field, was the following:

$$Y = \beta_0 + \beta_1 X_{1i} + \beta_2 X_{2i} + \cdots + \beta_k X_{ki} + u_i \quad (2)$$

where
Y = binary value aggregation variable
β_i = coefficients to be estimated
Xki = explanatory variables of the model (table 1, page 82-83)
ui = stochastic error.

In addition, the Wald test was used to evaluate paremeter individual significance. Overall goodness of fit was assessed by the McFadden's R2 and the LR statistic or likelihood ratio. Finally, the results were obtained by using Data Analysis and Statistical package (2012).

Results and discussion

Descriptive statistics of socio-demographic profile

Based on the socio-demographic approach addressed in our study (table 3, page 87), the descriptive statistics revealed that the tendency of rural youth to emigrate from rural territories is greater (57.33%), despite the majority having agricultural educational training at the technical level. The population results at younger ages (14 to 17 years), were similar across different areas of the rural geographic space. Therefore, it can be highlighted that the percentages of rural young people who currently have a business are lower, thus presenting a relationship with the low existence of family businesses. However, in contrast, it could be stated that of the percentage of young people who have active rural entrepreneurship (33.96%), the preferred trend for forming the business is with the family itself (36.68%). Against this, there is evidence that the local rural roots of family businesses can generate localized advantages and the construction of links that influence the desirability of forming these types of ventures (3).

Table 3. Descriptive statistics showing socio-demographic variables of rural youth.
Tabla 3. Estadísticas descriptivas a partir de variables socio-demográficas del joven rural.

Variables		N	Percentages
Interest in staying in rural areas (Variable y)	Yes	157	42.66%
	Not	211	57.33%
Gender	Male	163	44.29%
	Female	205	55.70%
Rural educational program	Technical	323	87.77%%
	Advanced technological	45	12.22%
Age	14 to 17 years	233	63.31%
	17 to 20 years	91	24.72%
	20 to 25 years	20	5.45%
	25 + years	24	6.52%
Existence of family business	Yes	125	33.96%
	Not	243	66.03%
Current geographic location	Small rural village	83	22.55%
	Rural municipality	55	14.94%
	Area near the rural municipality	147	39.94%
	Area far away from the rural municipality	83	22.55%
Current agricultural entrepreneurship	Yes	102	27.71%
	Not	266	72.28%
Duration of the entrepreneurship	Does not have	271	73.64%
	Less than a year	43	11.68%
	Between 1 to 3 years	40	10.86%
	More than 5 years	14	3.80%
Conformation of the entrepreneurship	Individual	94	25.54%
	With family	135	36.68%
	Associated with maximum 3 people	46	12.50%
	Within an association or cooperative	93	25.27%

Econometric model

The results of the econometric model reveal that of the total variables analysed, 11 of them are statistically significant ($p<0.5$) in relation to the four analysis dimensions of our model (table 4, page 88). The gender variable (x1) was significant at 90% ($p<0.1$) but with a negative sign, meaning that the probability of staying in the territory decreases by 11.7% if the gender is female compared to male. In addition, the rural geographic location variable (x5) was also significant at 90%, therefore, if the rural youth is located far away from the rural municipality, then their probability of remaining in the rural territory is 6%. The results reveal that the duration of entrepreneurship (x7) related to the agricultural sector was statistically significant ($p<0.05$), which means that those rural young people who have been involved with a rural project for the longest period have a probability of permanence in their rural territories of 13.2%. Likewise, the conformation of entrepreneurship (x8) was significant ($p<0.05$), which implies that it is a determining variable of the socio-demographic profile for the interest of young people to stay in their rural territories. However, the result expressed a negative sign compared to the marginal effect of the variable (x8) on the dependent variable of the model.

Table 4. Variables that influence the probability of staying in rural territory.
Tabla 4. Variables que influencian la probabilidad de quedarse en áreas rurales.

Variable	Coefficient	z	P>z	dy/dx
x_1	-0.307664	-1.78	0.075**	-.1174504
x_2	0.3766111	1.11	0.268	.1437709
x_3	0.0526166	0.4	0.687	.0200863
x_4	0.1412129	0.74	0.459	.0542158
x_5	0.1572976	1.93	0.054**	.0600482
x_7	0.3461891	2.9	0.004*	.1321573
x_8	-0.1575732	-2.07	0.039*	-.0601534
x_9	0.4390554	2.17	0.03*	.1657988
x_{10}	0.550047	2.66	0.008*	.2075006
x_{11}	-0.1941587	-0.71	0.478	-.0752722
x_{12}	0.4532449	2.35	0.019*	.1716845
x_{13}	0.4197658	1.7	0.089**	.1539446
x_{14}	-0.2901887	-1.4	0.162	-.1106793
x_{15}	0.076936	0.38	0.703	.0292409
x_{16}	-0.3073476	-1.34	0.18	-.1198523
x_{17}	-0.0733074	-0.42	0.678	-.0279793
x_{18}	0.1799369	0.93	0.354	.0686063
x_{19}	0.2301191	0.98	0.327	.0865896
x_{20}	0.0367123	0.17	0.865	.0140079
x_{21}	0.0663933	0.3	0.765	.0252878
x_{22}	-0.2721225	-1.26	0.208	-.1035909
x_{23}	-0.2784167	-1.13	0.258	-.1080204
x_{24}	-0.0888142	-0.4	0.689	-.0340897
x_{25}	0.1415434	0.68	0.498	.0539372
x_{26}	0.0718927	0.28	0.777	.0272351
x_{27}	0.5487282	1.99	0.047*	.194402
x_{28}	0.2918432	1.35	0.176	.1084964
x_{29}	0.17006	0.61	0.545	.0636639
x_{30}	0.2445028	0.85	0.394	.0909187
x_{31}	-0.4674785	-1.55	0.122	-.1828304
x_{32}	-0.6652717	-1.81	0.071**	-.2603507
x_{33}	1.29848	3.94	0000***	.3819793
x_{34}	-0.1182813	-0.51	0.609	-.1174504
Constant	-2.143088	-3.57	0	

Own elaboration, dy/dx is the marginal effect of the variable x on the dependent variable y; dy/dx significance level: P<0.05*; P<0.1**, and P<0.001***. Pseudo R2=0.3362.

Elaboración propia, dy/dx es el efecto marginal de la variable x sobre la variable dependiente y; nivel de significancia dy/dx: P<0,05*; P<0,1**, y P<0.001***. Pseudo R2=0,3362.

Therefore, the probability of remaining in the territory for young people can decrease by 6% if the enterprise is formed within a family that may prefer to initially undertake the enterprise individually compared to doing so in other forms of groupings. When analysing the entrepreneur's characteristics profile, four key variables stand out as significant regarding the interest of rural young people in staying within their territories. Therefore, young people's interest in rural entrepreneurship (x9) stands out as a significant variable ($p<0.05$) with a 16.5% probability of staying in the territory. Likewise, rural entrepreneurship is seen to impact the local and community environment (x10), it is a variable that was found to be significant ($p<0.05$) and a probability of youth permanence of 20.7%. Within this second component of the analysis model, variables are significant ($p<0.05$), such as the interest in participating in training (x12) and the interest of rural entrepreneurship in the impact on the environment and natural resources, both with their respective probabilities regarding the permanence of young people in their rural territories.

Finally, our results reveal three key and statistically significant variables in the motivations and territory component. Initially, where the rural youth lives is a motivating factor to stay in the countryside ($p<0.5$). In addition, the perception of prestige and reputation of the rural municipality (x32) showed significance ($p<0.5$) and a negative sign. This implied that the place where the young person lives and its perception are key aspects since the probability of the young person remaining in that territory can decrease by 26% if the issue of local reputation is not well perceived. This has implications for training and support programs for rural entrepreneurship in certain rural regions. Additionally, within this component of motivations and territory, our results highlight the perception of the entrepreneurial personality of the inhabitant population of the rural municipality (x33). This variable emerges as highly significant ($p<0.5$) and the highest percentage probability on wanting to remain within their territory (38.1%). Therefore, this variable highlights the importance of what can be considered a local entrepreneurial culture as a factor that drives rural youth's interest in staying within their region, since various entrepreneurial people can make use of the local culture and its tradition of activities to seek to potentialize their ideas (29).

Model determining variables

Our results allow us to interpret that eleven variables influence the interests of rural young people in wishing to remain within their territories. These are based on our proposed dimensions apart from entrepreneurship skills (table 5). Our model contributes to the discussion of expanding the understanding of phenomena associated with rural entrepreneurship. However, we also hold the critical position that, the issue of rural population migration to cities has not received the deserved attention in the research literature and requires further empirical studies (9).

Table 5. Dimensions and determinant variables of the model.
Tabla 5. Dimensiones y variables determinantes del modelo.

Dimensions analysis	Key variable
Socio-demographic profile	x1= Gender
	x5= Rural geographic location
	x7= Duration of the entrepreneurship
	x8= Conformation of the entrepreneurship
Profile of entrepreneur characteristics	x9= Interest in rural entrepreneurship
	x10= Entrepreneurship for local and community impact
	x12=Interest to participate in trainings
	x13= Interest in environmental impact and natural resources
Entrepreneurship skills	None
Motivations and territory	x27= The municipality where you live motivates entrepreneurship
	x32= Perception of prestige and reputation of the rural municipality
	x33= Perception of people's business mindset

The categories and variables determining the permanence of rural youth in their territory based on entrepreneurship can be divided into an internal environment such as the dimensions of the profile and skills for entrepreneurship, as well as an external environment based on what determines the territory as a motivational factor. From an interrelation of both environments, our study disproves the hypothesis according to which the promotion of rural education programs for entrepreneurship constitutes a strategy that ensures the territorial permanence of rural youth as they can develop or strengthen capacities for entrepreneurship. In fact, according to the approaches of Galvão *et al.* (2020), educational programs for entrepreneurship are decisive for the rural population since they contribute to the involvement and local interaction of actors, generating a support ecosystem that facilitates the entrepreneur's action. However, in our study, the perception of institutional support and conditions of greater access to knowledge did not emerge as key variables. This could explain the interest of young people in staying within rural regions. Regarding the role of the family, the consideration of gender is an issue that cannot be ignored in the context of rurality. Our survey highlighted the highest percentage of female respondents. As Sidhu & Kaur (2006) propose, rural entrepreneurship is more beneficial for the current and multifunctional role of women, both for their function within the social system as generators of family income and their decision-making capacity in the family environment (15).

Additionally, our study presents results related to the profile characteristics of the rural entrepreneur, where four key variables are prominent. Our results are related to the approaches of Shivacharan *et al.* (2017), who highlight the importance of variables such as the interest of entrepreneurs in participating in training, which is related to a person with a tendency to search for information. Based on our results within the profile of characteristics of the entrepreneur, we agree that there is a tendency towards rural entrepreneurship to be seen as a vector of territorial development and a search for local sustainability in rural municipalities and a concern for the area (7, 20, 21). Therefore, we agree with educational programs in rural entrepreneurship strongly focusing on the role played by both the local place and the community (36). This means that the development of entrepreneurship capabilities by rural young people constitutes a factor of permanence, and implies the interrelation of other dimensions, such as the territorial dimension.

From the external environment of the rural entrepreneur, the perspective of the spatial-geographic role has been widely discussed in the scientific literature. However, few approaches associate the territorial issue with the permanence of the youth population from a vision of entrepreneurship especially in developing countries, even in Latin America. In this sense, considering motivations and territory, our results identify three key variables for analysing the model proposed in our study. The relationship between motivations and territory is important, as discussed by Modrego & Foster (2021), there are idiosyncratic territorial issues specific to rural entrepreneurship, which influence the perceptions of entrepreneurs and their possible decision-making. In fact, this geographical spatial dimension is considered an important element in the field of business culture compared to what is implied by the existence of visible success stories in the local area, which can motivate people to become rural entrepreneurs (32). This work is related to our results, which include variables associated with the perception of a local business mentality, and what the rural municipality implies as a motivating factor for the permanence of rural youth within. It could also be associated with the level of roots within the territorial culture (8).

Most studies do not consider the perception that young rural entrepreneurs have of their own municipal territory. However the study by Fanjul *et al.* (2023), refers to the existence of rural municipalities that can attract local people, and even neighbouring inhabitants, to the development of companies, which implies the importance of the geographical environment. Finally, our model shows the relevance of the interrelation of dimensions and environments, as even when rural young people have entrepreneurial skills, other aspects promote permanence in the territories. For this reason, this type of rural youth likely embodies certain local values and a sense of rurality, including the possibility of creating a local impact from their activity. There may be experiences not only of business development but also based on resource management, cultural and natural, where in the territory there is a tendency to build natural capital based on a certain sensitive perception about the interaction with the biophysical space (25).

Conclusions

Undoubtedly, the agricultural sector in developing countries, such as Latin America, faces numerous challenges and problems. Many of them are generated by the effects of the global and commercial environment, as well as by internal factors of the countries which have contributed to a declining situation for the sector. One of the most concerning problems is the migration of the young rural population towards urban and more densely populated areas. In response, countries such as Colombia have promoted a broad set of public policies, among which the implementation of agricultural educational programs stands out to strengthen the entrepreneurial capacities of this rural population. In addition, some public and private programs seek to financially support the emergence of rural enterprises.

However, the topic has not yet been sufficiently addressed in the literature, which constitutes the main contribution of our study. In this sense, we propose an analysis model of the rural entrepreneur, which seeks to understand the aspects that determine the interest of rural young people wanting to stay within their own rural territories. Furthermore, our findings contribute to a gap in the empirical analysis within the growing literature on rural entrepreneurship, where most studies present purely theoretical and conceptual approaches. We propose a conceptual model of analysis in which variables that relate an internal and external environment of the rural entrepreneur are considered. Furthermore, we consider several research opportunities on typology of rural entrepreneurs interested in developing a lifestyle within their rural territory. Finally, we consider that in the external environment of motivations and territory, the role of network links from rural entrepreneurs can be empirically explored, even between local actors at a meso level. However, not associated with entrepreneurship itself or business performance, but with the problem related to rural migration and the motivations for the entrepreneur to live within their rural environment.

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Impact of Cry1Ac soybean (*Glicine max*) on biological and reproductive cycles and herbivory capacity of *Spodoptera cosmioides* and *Spodoptera eridania* (Lepidoptera: Noctuidae)

Impacto de la soja (*Glicine max*) Cry1Ac sobre el ciclo biológico, reproductivo y la capacidad herbívora de *Spodoptera cosmioides* y *Spodoptera eridania* (Lepidoptera: Noctuidae)

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Abstract

Increasing populations of *Spodoptera cosmioides* (Walker) and *Spodoptera eridania* (Stoll) have recently been detected in soybean crops in central Argentina. Besides being polyphagous, these species tolerate the Cry1Ac insecticidal toxin, expressed by genetically modified *Bt* soybean (MON89788 x MON87701). Consequently, when facing big populations, farmers often apply insecticides. This study aimed to determine the effects of *Bt* soybean on the consumption, biological cycle, and reproduction of both *Spodoptera* species. Larval feeding on *Bt* soybean led to a shorter pupal period (23% less than control) and a decreased leaf-area consumption for *S. cosmioides* (14% less than the non-*Bt* soybean). In *S. eridania,* the larval stage, adult longevity, larva-to-adult, and oviposition periods were reduced (11, 23, 13, and 30% shorter than control, respectively). Despite these reductions, both Lepidoptera species completed their reproductive cycles. These valuable findings help us understand the biology of these potential pests in *Bt* soybean crops in Argentina.

Keywords
Glicine max (L.) • plant resistance • non-target pests • black armyworm • southern armyworm

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Resumen

En los últimos años, las poblaciones de *Spodoptera cosmioides* (Walker) y *Spodoptera eridania* (Stoll) se han incrementado en los cultivos de soja de la zona central de Argentina. Además de ser polífagas, estas especies son tolerantes a la toxina insecticida Cry1Ac expresada por la soja *Bt* genéticamente modificada (MON89788 x MON87701), por lo que los agricultores deben recurrir al control químico con insecticidas cuando se presentan altas densidades poblacionales. Este estudio tuvo como objetivo determinar el efecto de la soja *Bt* sobre el consumo, ciclo biológico y reproducción de ambas especies de *Spodoptera*. La alimentación larval con soja *Bt* determinó una menor duración del período pupal (23% menos que el tratamiento control) y una disminución en el consumo de área foliar en *S. cosmioides* (14% menos que la soja no *Bt*). *Spodoptera eridania* registró una menor duración del estado larval, longevidad de adultos, período larva-adulto y del período de oviposición (11, 23, 13 y 30% menos que el tratamiento control, respectivamente). Sin embargo, ambas especies de Lepidoptera completaron su ciclo reproductivo con éxito. Los resultados obtenidos en este trabajo son de gran utilidad para comprender la biología de estas especies, que tienen el potencial de convertirse en plagas importantes en los cultivos de soja *Bt* en Argentina.

Palabras clave

Glicine max (L.) • plantas resistentes • plagas no blanco • oruga cogollera negra • oruga cogollera del sur

Introduction

Genetically modified (GM) crops exhibiting insect-resistance are valuable tools in integrated pest management (IPM) systems (25). These crops express genes derived from the entomopathogenic bacterium *Bacillus thuringiensis* Berliner (*Bt*), producing (Cry) proteins with highly selective insecticidal activity. B*t* soybean expressing these insecticidal toxins is effective in controlling several major lepidopteran pests in agricultural environments, including *Anticarsia gemmatalis* (Hübner), *Chrysodeixis includens* (Walker), *Helicoverpa gelotopoeon* (Dyar) and *Rachiplusia nu* (Guenée) (44).

High efficacy of Bt soybean crops against pest populations and the consequent reduced insecticide use has significantly altered the agroecosystem. Consequently, the reduced interspecific competition after controlling *Bt* target species has facilitated the emergence of new phytophagous pest species; many of which could become economically significant (19, 30, 47). Recent reports mention increasing populations of *Spodoptera cosmioides* Walker and *Spodoptera eridania* Stoll (Lepidoptera: Noctuidae) in Argentinean soybean crops, including *Bt* cultivars (23, 29, 30, 32). Factors contributing to these phenomena include tolerance to the Cry1Ac protein, insecticide resistance and the ability to complete life cycles on the weed *Amaranthus* sp. (2, 5, 9, 24, 26, 30). *S. eridania* thrives in temperate regions like the Argentinean Pampas, with a developmental threshold of 11.9°C and an inability to complete its life cycle above 34°C. In contrast, *S. cosmioides* is adapted to warmer temperatures (from 13.2), prevailing in soybean and cotton crops in northern Argentina (31). Although soybean and cotton are the preferred hosts (11), caterpillars of both species are polyphagous, and develop on weeds and grain, fruit, and ornamental crops (14). These species also have greater herbivorous potential than other soybean defoliators, consuming vegetative structures, flowers, and pods (6, 21, 27, 38).

Understanding biological and reproductive pest cycles is essential for elucidating population dynamics and predicting potential crop populations. Most studies assessing the effects of the Cry1Ac protein on the development and foliar consumption of lepidopteran pests (4, 5, 8, 12) have been conducted in Brazil, with almost no equivalent in Argentina. This study hypothesized that the Cry1Ac protein affects biological performance, reproduction, and feeding behavior of both *Spodoptera* species. We investigated the impact of *Bt* soybean on foliar consumption and life cycle to assess pest potential in the central soybean region of Argentina.

Materials and methods

This study was conducted in the breeding chamber of the Plant Production Department at the Facultad de Ciencias Agrarias (Universidad Nacional del Litoral), in Esperanza City, Santa Fe province, Argentina.

Insect rearing

Spodoptera cosmioides and *S. eridania* larvae were collected in February 2019 from commercial soybean fields in Santa Fe province, near Franck (31°35'00"S60°56'00"W,31ma.s.l.)and SantaMaríaNorte(31°31'00"S61°08'00"W,44ma.s.l.). The caterpillars were transported to the breeding chamber in containers with soybean leaves and identified using taxonomic keys (43, 46). They were reared under controlled temperature (24 ± 2°C), relative humidity (60%), and photoperiod (14:10 h, light: dark) in transparent PVC boxes (26 cm long, 17 cm wide, and 7 cm high), covered with muslin caps for air circulation. An artificial diet consisting of corn flour, wheatgerm, yeast, water, agar, nipagin, benzoic acid, and ascorbic acid was provided until pupation (33). The emerged adults were placed in oviposition cages (50 cm length, 40 cm width, and 40 cm height), with paper sheets for oviposition. They were fed daily with an artificial adult diet (10) provided through soaked cotton. Eggs were collected daily and placed in 9 cm diameter Petri dishes with artificial feed for neonate larvae. Three days after hatching, larvae were transferred to PVC boxes for large-scale rearing with an artificial diet. This process continued until the F_2 generation, ensuring enough population for the study.

Plant material

Leaves for larvae feed were obtained from soybean cultivars RA 5715 IPRO (*Bt*) and RA 549 (non-*Bt*). Both cultivars are glyphosate-tolerant, but only the former expresses the Cry1Ac toxin. To ensure a continuous supply of leaves, both cultivars were periodically planted in 3x2 m plots under field conditions. Weeds were manually removed and soybean plants were kept disease-free by the eventual application of fungicides.

Effect of *Bt* soybean on the biological and reproductive cycle of *S. cosmioides* and *S. eridania*

A second instar (L2) larva of either *S. cosmioides* or *S. eridania* was placed on two soybean leaflets (from *Bt* or non-*Bt* soybean plants, depending on the treatment) inside 9 cm diameter Petri dishes lined with absorbent paper. Petioles were wrapped in cotton saturated with distilled water, maintaining humidity. *Bt* and non-*Bt* soybean leaflets were harvested at V6-V8 vegetative stage before anthesis, according to phenology by Fehr *et al.* (1977). The V6-V8 vegetative stage corresponds to maximum Cry1Ac expression in the *Bt* cultivar (45). Food and absorbent papers were renewed daily until pupation. We defined sex by observing the terminal portion of pupae (7) using a stereomicroscope set (Lancet Instruments, China) at 30× magnification. Thirty replicates were performed for each treatment (*Bt* and non-*Bt* soybean) and species (*S. cosmioides* and *S. eridania*). Once adults emerged, one couple was placed per oviposition container (17 cm height, 11 cm upper diameter, and 7 cm lower diameter), covered with a muslin cap facilitating air circulation and preventing adult escape. The same diet used for rearing was supplied to adults with soaked cotton (10). Fecundity was determined by daily collecting egg masses laid by females after mating. Egg masses were photographed using an Olympus SZ40 stereomicroscope (Olympus Corporation, Tokyo, Japan) at 40X for egg counting, considering any overlapping or superimposed eggs. Each egg mass was placed in a separate Petri dish (9 cm diameter) lined with absorbent paper and food for emerging neonates. Fertility (viable eggs) was estimated by the number of viable larvae hatched from each egg mass. The assays were conducted with 11 and 10 couples of *S. cosmioides* and 14 and 10 couples of *S. eridania* for the *Bt* and non-*Bt* soybean treatments, respectively.

The following variables were recorded: duration (in days) of larval, pupal and adult stages, the larva-to-adult period, pupal weight (g) using an OHAUS-PIONNER precision scale (± 0.0001 g), fecundity (number of eggs/female), fertility (number of hatched eggs), pre-oviposition (days from adult emergence to first egg laying), oviposition (days from the first to the last egg laying), and post-oviposition (days from last egg laying to death).

Effect of *Bt* soybean on leaf consumption by larvae of *S. cosmioides* and *S. eridania*

Leaf area consumption (cm^2) was determined using the same larvae and soybean cultivars (species and treatments) as when assessing the impact of *Bt* soybean on the biological and reproductive cycle. Fresh leaflets were provided daily as food, and the remaining unconsumed portions were scanned using an HP Deskjet F4280 multifunction printer. The consumed leaf area was quantified by image analysis with ImageJ® software (1). Adjusted leaf area loss due to dehydration was based on data from soybean leaflets not exposed to larvae.

Statistical analysis

Bioassays for each lepidopteran species were conducted independently under a completely randomized experimental design. Since the duration of larva, pupal, and adult stages, as well as the larva-to-adult period did not meet normality, non-parametric Kruskal-Wallis test ($\alpha \leq 0.05$) was performed. Pupal weight was analyzed by ANOVA and Tukey test ($\alpha \leq 0.05$). Foliar consumption

means were compared using an independent samples T-test ($\alpha \leq 0.05$). All statistical analyses were conducted using InfoStat software (13).

Results

Effect of *Bt* soybean on the biological and reproductive cycle of *S. cosmioides* and *S. eridania*

Feeding *S. cosmioides* larvae with *Bt* soybean leaves did not significantly affect larval duration compared to control, with 20.46 and 19.48 days, respectively (H= 0.64; p= 0.4160) (table 1). However, in *S. eridania*, significant differences were evidenced for larval length of 28.13 days when fed with *Bt* soybean leaves and 31.69 days when supplied with non-*Bt* soybean leaves (H= 7.43; p= 0.0062) (table 1).

Significant differences in the *S. cosmioides* pupal stage duration showed 10.65 and 13.96 days (H= 14.72; p= 0.0001), with *Bt* and non-*Bt* soybean, respectively. In *S. eridania*, differences were not significant (H= 3.77; p= 0.0434) (table 1). Regarding the adult stage, no significant differences were found in *S. cosmioides* (H= 0.04; p= 0.8485). However, *S. eridania* adults lived significantly longer on non-*Bt* soybean leaves (12.87 days), compared to *Bt* soybean (9.90 days) (H= 11.70; p= 0.0005) (table 1).

Table 1. Days of larval, pupal, and adult stages, larva-to-adult period, and pupae weight (g) (Mean ± SD) of *Spodoptera cosmioides* and *S. eridania*, fed *Bt* and non-*Bt* soybean leaves under controlled conditions.

Tabla 1. Duración (días) de los estadios larval, pupal y adulto, el período de larva a adulto y el peso de las pupas (g) (Media ± DE) de *Spodoptera cosmioides* y *S. eridania*, alimentadas con hojas de soja *Bt* y no-*Bt* en condiciones controladas.

Spodoptera cosmioides						
Treatment	**n**	**Larva (days)***	**Pupae (days)***	**Adult (days)***	**Larva-to-adult period (days)***	**Pupae weight (g)***
Bt	26	20.46 ± 3.37 **a**	10.65 ± 3.57 **a**	13.19 ± 4.92 **a**	44.31 ± 9.12 **a**	0.33 ± 0.08 **a**
non-*Bt*	23	19.48 ± 2.0 **a**	13.96 ± 3.93 **b**	13.26 ± 5.14 **a**	46.70 ± 7.73 **a**	0.36 ± 0.10 **a**
Spodoptera eridania						
Treatment	**n**	**Larva (days)***	**Pupae (days)***	**Adult (days)***	**Larva-to-adult period (days)***	**Pupae weight (g)***
Bt	29	28.13 ± 3.12 **a**	11.93 ± 1.31 **a**	9.90 ± 2.72 **a**	49.97 ± 4.89 **a**	0.18 ± 0.03 **a**
non-*Bt*	26	31.69 ± 4.74 **b**	12.9 ± 2.16 **a**	12.87 ± 2.97 **b**	57.45 ± 6.8 **b**	0.17 ± 0.03 **a**

* Different letters indicate significant differences among treatments. (Test: Kruskal Wallis, $\alpha \leq 0.05$). Pupal weight (Test: Tukey, $\alpha \leq 0.05$).

*Diferentes letras en las columnas indican diferencias significativas entre tratamientos (Prueba: Kruskal Wallis, $\alpha \leq 0,05$). Peso de pupa (Prueba: Tukey, $\alpha \leq 0,05$).

The larva-to-adult period for *S. cosmioides* was 44.31 days on *Bt* soybean and 46.70 days on non-*Bt* soybean, with no significant differences (H= 0.48; p= 0.4881). In contrast, *S. eridania* had a larva-to-adult period significantly longer on non-*Bt* soybean (57.45 days) compared to *Bt* soybean (49.97 days) (H= 13.89; p= 0.0002) (table 1, page 96).

Considering pupal weight, no significant differences were found for either species: *S. cosmioides* (F= 0.95; p= 0.3359) and *S. eridania* (F= 3.77; p= 0.0577) (table 1, page 96).

Larval feeding did not affect average number of eggs per female in either species. *S. cosmioides* had 3291.82 eggs on *Bt* soybean leaves and 3049.0 on non-*Bt* soybean leaves (H= 0.08; p= 0.7782). *S. eridania* had lower fecundity than *S. cosmioides,* with 841.79 eggs on *Bt* soybean and 830.70 eggs on non-*Bt* soybean (H= 0.01; p= 0.9068) (table 2). The Cry1Ac protein ingested by larvae did not affect fecundity in the studied species (table 2). Similarly, no significant differences were observed in fertility (percentage of hatched eggs) between treatment species (H= 0.04; p= 0.8327 for *S. cosmioides* and H= 2.14; p= 0.1432 for *S. eridania*) (table 2).

Table 2. Fecundity (number of eggs) and Fertility (% of hatched eggs) (Mean ± SD) of *Spodoptera cosmioides* and *S. eridania* fed *Bt* and non-*Bt* soybean leaves under controlled conditions.

Tabla 2. Fecundidad (número de huevos) y fertilidad (% de huevos eclosionados) (Media ± DE) de *Spodoptera cosmioides* y *S. eridania* alimentadas con hojas de soja *Bt* y no-*Bt* en condiciones controladas.

Spodoptera cosmioides			
Treatment	**n**	**Number of eggs***	**Hatched eggs (%)***
Bt	11	3291.82 ± 2089.99 **a**	50.44 ± 12.14 **a**
non-*Bt*	10	3049.0 ± 1855.57 **a**	52.08 ± 10.67 **a**
Spodoptera eridania			
Treatment	**n**	**Number of eggs***	**Hatched eggs (%)***
Bt	14	841.79 **±** 391.13 **a**	70.75 ± 16,34 **a**
non-*Bt*	10	830.70 ± 356,73 **a**	60.85 ± 12,21 **a**

*Different letters in columns indicate significant differences between treatments. (Test: Kruskal Wallis α ≤ 0.05).

* Diferentes letras en las columnas indican diferencias significativas entre tratamientos (Prueba: Kruskal Wallis α ≤ 0,05).

Pre and post-oviposition periods were similar for both species under both larval feeding treatments (table 3, page 98), (H= 1.32; p= 0.2395 and H= 0.85; p= 0.34 for *S. cosmioides,* respectively; H= 0.51; p= 0.4460 and H= 2.25; p= 0.1238 for *S. eridania*, respectively). However, significant differences were found in the oviposition period for *S. eridania*, with 4.29 and 6.13 days in adults emerging from larvae fed *Bt* and non-*Bt* soybean leaves, respectively (H= 4.62; p= 0.0293).

Effect of *Bt* soybean on leaf consumption by *S. cosmioides* and *S. eridania*

Total leaf area consumption by *S. cosmioides* was lower when larvae were fed with *Bt* soybean (T= -2.77; p= 0.0081). In contrast, *S. eridania* showed no significant differences in leaf area consumption between *Bt* and non-*Bt* soybean leaves (T= 0.05; p= 0.9585) (table 4, page 98).

Table 3. Pre-oviposition, oviposition and post-oviposition periods of *Spodoptera cosmioides* and *S. eridania* (Mean ± SD) fed *Bt* and non-*Bt* soybean leaves under controlled conditions.

Tabla 3. Períodos de preoviposición, oviposición y postoviposición de *Spodoptera cosmioides* y *S. eridania* (Media ± DE) alimentados con hojas de soja *Bt* y no *Bt* en condiciones controladas.

Spodoptera cosmioides				
Treatment	**n**	**Pre oviposition (days)**	**Oviposition (days)**	**Post-oviposition (days)**
Bt	11	4.18 ± 2.27 **a**	9.18 ± 3.12 **a**	1.09 ± 1.22 **a**
No *Bt*	10	2.91 ± 1.76 **a**	8.82 ± 2.93 **a**	1.55 ± 1.29 **a**
Spodoptera eridania				
Treatment	**n**	**Pre-oviposition (days)**	**Oviposition (days)**	**Post-oviposition (days)**
Bt	13	3.00 ± 1.24 **a**	4.29 ± 1.33 **a**	3.21 ± 1.19 **a**
No *Bt*	10	3.63 ± 1.85 **a**	6.13 ± 2.10 **b**	4.13 ± 1.46 **a**

* Different letters in columns indicate significant differences between treatments. (Test: Kruskal Wallis $\alpha \leq 0.05$).

* Diferentes letras en las columnas indican diferencias significativas entre tratamientos (Prueba: Kruskal Wallis $\alpha \leq 0,05$).

Table 4. Leaf area consumption of *Spodoptera cosmioides* and *S. eridania* (Mean ± SD) fed *Bt* and non-*Bt* soybean leaves under controlled conditions.

Tabla 4. Consumo de área foliar de *Spodoptera cosmioides* y *S. eridania* (Media ± DE) alimentados con hojas de soja *Bt* y no *Bt* en condiciones controladas.

Spodoptera cosmioides		
Treatment	**n**	**Consumption (cm^2) ***
Bt	26	343.11 ± 19.79 **a**
non- *Bt*	23	398.93± 21.04 **b**
Spodoptera eridania		
Treatment	**n**	**Consumption (cm^2) ***
Bt	29	149.70 ± 4.45 **a**
non-*Bt*	26	150.00 ± 3.75 **a**

*Different letters in columns indicate significant differences between treatments. (Test: T, $\alpha \leq 0.05$).

* Diferentes letras en las columnas indican diferencias significativas entre tratamientos. (Test: T, $\alpha \leq 0,05$).

Discussion

gM crops expressing Cry proteins are crucial for pest control. Besides killing susceptible species, these crops can have sublethal effects on tolerant species, through direct or indirect exposure, leading to broader ecological changes (40). *Spodoptera cosmioides* and *S. eridania* exhibit tolerance against the Cry1Ac protein (2) due to the type and quantity of receptor proteins in larval midgut membranes, low receptor affinity, or rapid protein degradation (35). Thus, insect exposure to stress factors like Cry1Ac protein expressed by *Bt* soybean may enhance fitness of the exposed population (17, 18). This explains why *S. eridania* individuals exhibited shorter durations in both larval and adult stages, and a reduced larva-to-adult period when fed soybean leaves expressing the Cry1Ac protein. In contrast, *S. cosmioides* only experienced a decrease in the pupal period when fed on insect-resistant GM soybeans (*Bt*).

Regarding larval cycle, our results for *S. cosmioides* agree with Bernardi *et al.* (2014) and Silva *et al.* (2019), who observed a similar duration for the last larval stage under the same treatments. In contrast, *S. eridania* showed significant differences between treatments with an average duration of 28.13 days on *Bt* soybeans and 31.69 days on non-*Bt* soybeans. These results are consistent with those reported by Bortolotto *et al.* (2014) and Rabelo *et al.* (2020), who observed a significant reduction of 2 days in the larval stage of *S. eridania* when fed GM soybeans expressing Cry1Ac.

Our results showed that *S. eridania* adults from *Bt* soybeans live 3 days less than those from the control group. In contrast, Silva (2013) and Bortolotto *et al.* (2014) reported a significant 3 days-increase in longevity of *S. eridania* males when reared on *Bt* soybean leaves. This discrepancy suggests that Cry1Ac might induce asynchronous adults' emergence between the two cultivars, potentially reducing mating chances in natural conditions. According to Jakka *et al.* (2014) and Murúa *et al.* (2019), the non-simultaneous emergence of adults in both cultivars could compromise the refuge strategy to avoid or delay resistance emergence. On the other hand, we found a shortened life cycle of *S. eridania* (7.48 days) when larvae were fed soybeans expressing Cry1Ac, as seen by Ramírez & Gómez (2010), who reported an average life cycle of 51.72 days for *S. eridania* with artificial diet.

Several studies have demonstrated that noctuid pupae weight can vary with temperature, host plants, and exposure to sublethal insecticide concentrations or *Bt* crop toxins (22). However, our results indicate that *Bt* protein did not affect pupal weight of either species. Additionally, feeding larvae with *Bt* soybean leaves did not affect the reproductive capacity of either *Spodoptera* species, as observed by Silva *et al.* (2016) and Sosa *et al.* (2020), in *S. cosmioides* for larvae fed with *Bt* soybean leaves. However, Páez Jerez *et al.* (2022) reported more eggs per female in *S. cosmioides* individuals fed *Bt* soybean. According to Specht & Roque-Specht (2019), fecundity in *S. cosmioides* is highly variable, with females capable of producing up to 5000 eggs/female, higher than for *S. eridania*, *S. albula*, *S. frugiperda* and *S. littoralis* (26, 27). In our study, average egg number per *S. eridania* female is consistent with Silva (2013), who reported similar fecundity in females reared on both *Bt* and non-*Bt* soybeans during larval stage, with averages of 881.35 and 911.85 eggs per female, respectively.

We found that exposure to the insecticidal protein Cry1Ac during larval stage shortened the oviposition period in *S. eridania*. Although the literature lacks specific data on the oviposition period of *S. eridania* fed on *Bt* cultivars, previous studies have reported variable oviposition periods ranging from 4.2 days to 6. 75 days when larvae were reared on non-*Bt* soybean leaves (14).

Biological fitness is the ability of an organism to compete successfully, pass on its genes to subsequent generations and influence population density and the potential to become a pest. However, insecticide exposure can have variable effects, enhancing or reducing performance, potentially leading to adverse impacts on survival, developmental rate, reproduction, and adult longevity (3). This phenomenon has been documented in several Lepidoptera species exposed to *Bt* protein (16). In our study, we observed a reduced pupal period in *S. cosmioides* and shortened larval, adult, and larval-to-adult cycles and oviposition periods in *S. eridania* when fed *Bt* soybean leaves.

Food quantity and quality directly influence host plant preference affecting biological, physiological, and behavioral features (11). While some studies have found no effects of Cry toxins on foliar consumption in lepidopterans (11), other research reports less leaf consumption due to Cry proteins in corn (5), as we found for *S. cosmioides*. According to Zurbrügg *et al.* (2010), glyphosate-resistant soybeans expressing the Cry1Ac toxin have more carbohydrates and lower protein content than non-transgenic cultivars. This variation in nutritional composition may influence insect food preference as seen in *S. cosmioides* when fed on Cry1Ac-expressing soybean.

Conclusion

Transgenic crops expressing Cry insecticidal proteins are valuable tools for controlling susceptible pests. However, they may also induce changes in life cycles, population dynamics, reproductive stages, feeding behavior, or longevity of non-target species. Understanding developmental and reproductive parameters of these non-target pests is essential for predicting population growth and species dynamics within agricultural systems. Our findings shed light on the biology of *S. cosmioides* and *S. eridania* in *Bt* soybean crops in Argentina, considering foliar consumption and herbivorous capacity. Since our experiments were conducted under controlled conditions, these investigations should further assess actual field damage caused by *Spodoptera* species in soybean crops.

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Preventive and curative effects of native yeasts on different *Botrytis cinerea* strains in "Superior Seedless" (*Vitis vinifera* L.) table grape cultured in Argentina

Efectos preventivos y curativos de levaduras nativas sobre diferentes cepas de *Botrytis cinerea* en uva de mesa "Superior Seedless" (*Vitis vinifera* L.) cultivada en Argentina

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Abstract

The fermenting grape must is a dynamic, stressful, and selective habitat where many yeast species compete. Specific yeasts isolated from this habitat can play a fundamental role in table grape biocontrol of fungal diseases. The present study evaluated 225 grapevine yeasts against four *Botrytis cinerea* strains isolated from "Superior Seedless" grapes, considering the possible antifungal action mechanisms. Eighteen enological yeasts (13 *Saccharomyces* and 5 non- *Saccharomyces*) showed preventive antifungal activity against the four native *B. cinerea* strains, with disease severity varying between 0 and 49.91%. These 18 strains also presented curative activity against at least one of the *B. cinerea* strains assayed (severity values between 0 and 45.99%). Considering action mechanisms, thirteen yeast strains inhibited mycelial growth of at least one *B. cinerea* strain during dual plating (antibiosis), "*killer*" activity, and volatile antifungal assays. Our results showed that 7 yeast strains affected conidial germination (CG) and germinal tube length (GTL) of at least one *B. cinerea* isolate. Two yeast strains occupied the same niche as 4 *B. cinerea* strains (NOI values > 0.90). All yeast strains exhibited at least two inhibitory action mechanisms against gray rot, except for BSc140 with one mechanism. The possibility of more than one mechanism per yeast strain makes biocontrol an effective tool to prevent and cure gray rot in table grapes.

Keywords
preventive • curative • oenological yeasts • *Botrytis cinerea* • table grape • modes of action

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Resumen

El mosto de uva en fermentación es un hábitat dinámico, estresante y selectivo donde compiten diferentes especies de levaduras. Levaduras enológicas pueden desempeñar un papel fundamental en el biocontrol de las enfermedades fúngicas de la uva de mesa. El presente estudio evaluó la eficacia de 225 levaduras vitícolas para controlar cuatro cepas nativas de *Botrytis cinerea* aisladas de uvas "Superior Seedless" y los posibles mecanismos de acción antifúngica. Dieciocho levaduras enológicas (13 *Saccharomyces* y 5 no *Saccharomyces*) mostraron actividad antifúngica preventiva frente a las cuatro cepas de *B. cinerea* presentando una severidad de la enfermedad que varía entre 0 y 49,91%. Estas 18 cepas presentaron actividad curativa contra al menos una de las cepas de *B. cinerea* ensayadas (valores de severidad entre 0 y 45,99%). Posibles mecanismos de acción: 13 cepas de levadura inhibieron el crecimiento micelial de al menos una de las cepas de *B. cinerea* ensayadas durante los ensayos dual (antibiosis), actividad "killer" y volátiles antifúngicos. Nuestros resultados mostraron que 7 cepas de levadura afectaron la germinación de los conidios (CG) y la longitud del tubo germinativo (GTL) de al menos uno de las cepas patogénicas. Dos cepas de levadura ocuparon el mismo nicho que las 4 cepas de *B. cinerea* (valores NOI > 0,90). Los presentes hallazgos indican que todas las cepas de levadura exhibieron al menos dos mecanismos de acción antifúngico para controlar la pudrición gris, excepto BSc140 (un mecanismo). La posibilidad de que las cepas de levadura puedan ejercer más de un mecanismo de acción hace que el biocontrol sea una herramienta más eficaz para prevenir y curar la pudrición gris en uva de mesa.

Palabras clave

preventivo • curativo • levaduras enológicas • *Botrytis cinerea* • uva de mesa • modos de acción

Introduction

San Juan is Argentina's main producer of *Vitis vinifera* L. table grapes. In 2023, the province produced 41264 tons of grapes for fresh consumption or raisins (6). Indoor and outdoor grapes suffer from a variety of fungal diseases. *Botrytis cinerea*, causing "gray mold", is one important postharvest decay of fresh fruit and vegetables (4). Treatments against fungal diseases can be preventive or curative. In Argentina, chemical products such as azoxystrobin + difenoconazole, benomyl boscalid + pyraclostrobin, and carbendazim have a preventive effect against this fungus in grapes. In Argentina, boscalid is registered as preventive and curative fungicide against *B. cinerea* (2). However, excessive use of synthetic fungicides concerns human health and environmental well-being. *B. cinerea* is a polycyclic pathogen that can develop resistance against chemical fungicides (1).

Grape must is a dynamic habitat with high selection pressure (stress), resulting from physical (osmolarity, low pH) and chemical conditions (limited nitrogen availability, high ethanol concentrations) rich in different competing microbial species (18). Yeasts isolated from environments subjected to various stresses, such as grape fermentation, are more likely to be effective antagonists (16). Unfortunately, information on yeasts with antifungal curative activity as novel biocontrol technique is limited. Only a few reports mention curative activity (24 h after pathogen infection) of yeasts like *Saccharomyces cerevisiae* (8), *Candida stellimalicola* (3), *Candida inconspicua*, *Pichia kluyveri* (22), *Pichia kudriavzevii* and *Rhodotorula glutinis* (9) against different fungal pathogens in various vegetables. No reports consider applying curative yeasts in white table grapes. Most studies assessed yeast preventive effects against only one *B. cinerea* strain (13, 19). Possible modes of action against reported pathogenic fungi include competition for nutrients and space, reduction in spore germination and germ tube length, and inhibition of fungal mycelial growth by diffusible and volatile metabolites (14, 20). Few reports describe preventive and curative effects against fungi (8, 9). We aimed to assess efficacy of 225 viticultural yeasts against four native *B. cinerea* strains, conducting preventive and curative assays. Possible preventive or curative antifungal mechanisms of selected yeasts against four *B. cinerea* isolates were also evaluated *in vitro.*

Materials and methods

Yeast isolates

The present study assayed two hundred and twenty-five grapevine yeasts belonging to 41 species (13). Seventeen native yeasts were previously isolated from table grapes, 9 from vineyard soil (Caucete, San Juan), and 199 from fermenting musts of different varieties from San Juan, Argentina. These yeasts were previously identified by morphological and molecular techniques (13).

Botrytis cinerea strains

Four *B. cinerea* strains (B11, B14, B15, B24) were previously isolated from "Superior Seedless" table grape from Mendoza, Argentina. Previous molecular identification was carried out using molecular markers based on PCR-RFLP. Amplification of the ribosomal intergenic spacer (IGS) was performed with PCR, and product restriction was carried out with CfoI, HaeIII, and HinfI enzymes (12).

Curative and preventive *in vivo* assays

Grape

Untreated grapes with chemical pesticides were washed, superficially disinfected with sodium hypochlorite 0.2% (v/v) for 3 min, and subsequently rinsed with distilled water to eliminate sodium hypochlorite. A single wound (3 mm diameter and 3 mm deep) was made at the equator of each fruit.

Preventive treatments

First, the antifungal effect of the 225 yeast strains against 4 *B. cinerea* strains was evaluated in *preventive* bioassays in the "Superior Seedless" table grape. A 20 µL of a yeast cell suspension (10^6 cells/mL) was inoculated in the artificially wounded area and treated 24 h later with 20 µL of a *B. cinerea* conidial suspension (10^4 conidia/mL).

Curative treatments

Wounded grapes were initially inoculated with *B. cinerea* suspension and 24 h later with yeast suspension with preventive antifungal activity (severity 50% or less). Microbial concentrations were as mentioned above.

Controls: a- A wounded grape initially inoculated with *B. cinerea* suspension (10^4 conidia/mL), b- A wounded grape initially inoculated with water, and c- A wounded grape inoculated with yeast alone.

Treatments were arranged in a completely random design, with 3 replicates and 9 grapes per replicate. Assays were performed three times. The fruit was stored for 10 d at 25°C and 90% RH.

After the bioassays, disease severity (%) considered as the average diameter of gray rot lesions (cm, using a digital caliper) was calculated as follows:

$$\textit{Disease Severity}\ (\%) = \frac{\textit{Average lesion diameter in grape wound inoculated with fungus and yeast (cm)}}{\textit{Average lesion diameter in fungal control (cm)}}$$

In preventive and curative assays, antagonistic yeasts reduced disease severity by 50% or more.

Evaluation of possible antifungal mechanisms of the selected preventive and curative yeasts against four *B. cinerea* strains (B11, B14, B15, B24) (18)

Dual culture assays (antibiosis)

Mycelial agar disks (5mm) obtained from the margin of 7-day-old fungus cultures were placed in the center of the dishes containing Czapeck-Agar (Sigma-Aldrich ®). Around the fungus, four aliquots (20 µL) of a yeast cell suspension (10^6 cells/mL) were spot-inoculated, 3 cm from the center. Plates were incubated at 25°C for 5 d, and subsequently, mycelial growth (cm) was measured with a digital caliper. Results are expressed as % of *B. cinerea* mycelial growth inhibition compared to control (100%) (21).

Detection of *killer* activity

Plates with YMB-MB-Phosphate Citrate Buffer- Agar (Britania®) at pH 4.5 were inoculated with 100 µL of *B. cinerea* conidia (10^4 conidia/mL) as lawn. After the plates solidified, spot inoculation of 20 µL of each yeast (10^6 cells/mL) was performed with an automatic pipette. Plates were incubated at 25 °C for 5 d in the dark. A clear zone around yeast colonies was recorded as positive (**+**).

Antifungal activity of volatile organic compounds (VOCs)

B. cinerea mycelial discs (5 mm diameter) were taken from margins of 7-day-old cultures. The mycelial disc was inoculated on the center in the base plate with Potato Dextrose Agar (PDA) (Britania ®) medium. Another base plate with YEPD-Agar medium was inoculated superficially with 100 µL of a yeast suspension (10^6 cells/mL). The two base plates were faced and sealed with plastic Parafilm® (13). Controls were performed by inoculating *B. cinerea* (without yeast cells) on PDA. The sealed plates were incubated at 25°C for 5 d. At the end of the assay, mycelial growth (diameter, cm) was measured with a digital caliper. Results are expressed as the % of mycelial growth inhibition of *B. cinerea* compared with fungal control.

Yeast effect on conidial germination (CG) and germinal tube length (GTL) of four *B. cinerea* strains in low-nutrient medium

A suspension of 100 µL yeast cells (10^6 cells/mL), 25 µL fungal conidia (10^4 conidia/mL), and 100 µL of 1% diluted (v/v) white grape must (Superior Seedless) were inoculated on sterile excavated slides (13). Controls consisted of *B. cinerea* conidia without yeast. In Petri dishes, the excavated slides were incubated at 25°C for 24 h in the dark at 80% RH. The CG results are expressed as percentage of germinated conidia compared with the control (observation of 100 conidia). Conidia were considered germinated when the germ tube length was larger or equal to the conidia. GTL (µm) was measured with an ocular micrometer calibrated in a 40X light microscope objective (observation of 30 conidia).

Niche Overlap Index (NOI)

Fungal conidia (20 µL, 10^4 conidia/mL) and yeast cells (20 µL, 10^6 cells/mL) were inoculated on separate plates containing distilled water agar (2% agar, pH 4.5) with different nutritional sources (10 mM). Carbon sources assayed are generally present in table grapes and represent niche size: proline, asparagine, rhamnose, alanine, melibiose, glycine, malic acid, glutamic acid, tyrosine, raffinose, arginine, lysine, fructose, methionine, mannitol, glucose, saccharose, citric acid, galactose and tartaric acid (14). Plates were incubated at 25°C for 7 d in the dark. NOI values were obtained as follows:

$$NOI = \frac{number\ of carbon\ sources\ used\ by\ both\ microorganisms\ (yeast\ and\ B.cinerea)}{number\ of\ compounds\ used\ by\ B.cinerea}$$

NOI values >0.90 represent occupation of the same niche (competitive exclusion), and values < 0.90 represent occupation of separate niches (coexistence) (23).

Statistical analysis

All experiments were performed in triplicate and thrice. SPSS® software was used for statistical analysis. ANOVA assumptions were examined before statistical analyses, and mean values were compared with Tukey's test at a p-value = 0.05. Percentages of wounds infected by *B. cinerea* and germination conidia were arcsine-square-root transformed before ANOVA. When ANOVA assumptions were not met, the non-parametrical Kruskal-Wallis test was used.

Results

Table grape bioassays (preventive and curative)

In preventive assays with artificially wounded grapes, 18 isolates of the 225 enological yeasts assayed reduced disease severity by 50% or more in 4 *B. cinerea* strains: B11, B14, B15, and B24 (table 1). Yeast strains belonged to different species, including *Saccharomyces cerevisiae* (BSc14, BSc16, BSc27, BSc60, BSc90, BSc96, BSc97, BSc102, BSc103, BSc112, BSc140, BSc206), *Saccharomyces chevalieri* (BSch26), *Torulaspora delbrueckii* (BTd156, BTd165), *Candida sake* (BCs54), *Hanseniaspora vineae* (BHv86), and *Debaryomyces vanrijiae* (BDv197). All 18 strains had been isolated from fermented musts. Antifungal preventive treatments with these yeasts reduced disease severity between 50.14 and 100%. Notably, *S. cerevisiae* BSc103 and *S. chevalieri* BSch26 inhibited total fungal growth of B14 and B15, respectively, during preventive assays (table 1 and figure 1, page 108).

Table 1. Disease severity (%) caused by *B. cinerea* strains (B11, B14, B15, and B24) in white table grapes treated with yeast strains in preventive and curative assays.

Tabla 1. Severidad de la enfermedad (%) causada por cepas de *B. cinerea* (B11, B14, B15 y B24) en uva de mesa blanca tratadas con cepas de levadura en ensayos preventivos y curativos.

Treatments	Severity							
	Preventive assay (%) *B. cinerea* strains				Curative assay (%) *B. cinerea* strains			
	B11	B14	B15	B24	B11	B14	B15	B24
S. cerevisiae BSc14	49.50 ^{b}	48.70 ^{b}	45.23 ^{c}	45.65 ^{b}	• ^{d}	• ^{c}	34.32 ^{a}	23.42 ^{a}
S. cerevisiae BSc16	48.36 ^{b}	45.36 ^{b}	47.85 ^{c}	12.42 ^{a}	• ^{d}	23.56 ^{a}	• ^{c}	12.98 ^{a}
S. cerevisiae BSc27	47.70 ^{b}	44.32 ^{b}	49.50 ^{c}	45.76 ^{b}	• ^{d}	23.77 ^{a}	• ^{c}	34.21 ^{b}
S. cerevisiae BSc60	48.9 1 ^{b}	45.98 ^{b}	34.65 ^{c}	45.32 ^{b}	• ^{d}	45.11 ^{b}	• ^{c}	34.37 ^{b}
S. cerevisiae BSc90	45.76 ^{b}	12.22 ^{a}	49.25 ^{c}	49.86 ^{b}	39.88 ^{c}	• ^{c}	• ^{c}	• ^{c}
S. cerevisiae BSc96	48.30 ^{b}	43.45 ^{b}	23.87 ^{b}	46.75 ^{b}	• ^{d}	23.99 ^{a}	• ^{c}	• ^{c}
S. cerevisiae BSc97	45.44 ^{b}	49.24 ^{b}	45.89 ^{c}	45.33 ^{b}	23.11 ^{b}	• ^{c}	45.93 ^{b}	• ^{c}
S. cerevisiae BSc102	48.57 ^{b}	45.13 ^{b}	49.86 ^{c}	45.34 ^{b}	• ^{d}	23.85 ^{a}	• ^{c}	23.96 ^{a}
S. cerevisiae BSc103	48.96 ^{b}	47.94 ^{b}	0 ^{a}	12.34 ^{a}	• ^{d}	• ^{c}	45.99 ^{b}	45.22 ^{b}
S. cerevisiae BSc112	45.44 ^{b}	49.20 ^{b}	49.11 ^{c}	45.65 ^{b}	0 ^{a}	• ^{c}	• ^{c}	• ^{c}
S. cerevisiae BSc140	48.91 ^{b}	45.63 ^{b}	45.99 ^{c}	49.83 ^{b}	• ^{d}	34.45 ^{b}	34.89 ^{a}	• ^{c}
S. cerevisiae BSc206	23.44 ^{a}	48.71 ^{b}	49.33 ^{c}	45.11 ^{b}	0 ^{a}	• ^{c}	• ^{c}	12.96 ^{a}
S. chevalieri BSch26	49.31 ^{b}	0 ^{a}	45.33 ^{c}	48.56 ^{b}	• ^{d}	34.56 ^{b}	45.94 ^{b}	• ^{c}
C. sake BCs54	49.91 ^{b}	45.63 ^{b}	23.32 ^{a}	47.84 ^{b}	• ^{d}	23.44 ^{a}	45.84 ^{b}	• ^{c}
H. vineae BHv86	49.32 ^{b}	33.92 ^{a}	47.32 ^{c}	45.80 ^{b}	• ^{d}	23.21 ^{b}	• ^{c}	23.93 ^{a}
T. delbrueckii BTd156	49.51 ^{b}	34.7 ^{a}	45.98 ^{c}	34.20 ^{b}	• ^{d}	23.12 ^{b}	• ^{c}	45.92 ^{b}
T. delbrueckii BTd165	48.22 ^{b}	45.21 ^{b}	49.31 ^{c}	45.41 ^{b}	• ^{d}	23.19 ^{b}	• ^{c}	45.95 ^{b}
D. vanrijiae BDv197	47.8 ^{b}	45.35 ^{b}	48.33 ^{c}	45.54 ^{b}	• ^{d}	34.4 ^{b}	• ^{c}	• ^{c}
Control ($H_2$0)	100 ^{c}	100 ^{c}	100 ^{d}	100 ^{c}	100 ^{d}	100 ^{c}	100 ^{c}	100 ^{c}

Different lowercase letters within the same column indicate significant differences among severity means and control according to Tukey's test ($p < 0.05$).

The • symbol indicates non-significant difference between disease severity and control (100%).

Gray highlight indicates 0% disease severity.

Diferentes letras minúsculas en la misma columna indican diferencias significativas entre las medias de severidad con respecto al control en relación al Test de Tukey ($p < 0,05$).

El símbolo • representa valores de severidad que no presentan diferencias significativas en relación con el control (100%)

Resaltado con gris indica 0% de severidad.

Figure 1. Grapes inoculated with *S. cerevisiae* BSc103- *B. cinerea* B15 (A) (0% severity) and inoculated with water- *B. cinerea* B15 (B) (100% severity), in preventive assays.

Figura 1. Uvas inoculadas con *S. cerevisiae* BSc103- *B. cinerea* B15 (A) (0% severidad) e inoculadas con agua- *B. cinerea* B15 (B) (100% severidad), en ensayos preventivos.

In curative *in vivo* experiments, 15 of the 18 preselected yeasts presented curative activity against two of the four *B. cinerea* strains assayed (9 *S. cerevisiae,* 1 *S. chevalieri*, 1 *C. sake*, 1 *H. vineae*, and 2 *T. delbrueckii*), reducing disease severity between 54.01 and 100%. Additionally, four isolates (3 *S. cerevisiae* and 1 *D. vanrijiae*) significantly reduced the rot halo caused by one *B. cinerea* strain with a severity between 0 and 39.88% (table 1, page 107).

S. cerevisiae BSc206 and BSc212 inhibited total growth of B11 during curative assays (table 1, page 107).

Possible mechanism of action

Eighteen yeast isolates that showed antifungal effectivity (table 1, page 107) were analyzed.

Dual culture assay

Five of the 18 yeast isolates did not inhibit any *B. cinerea* strain (B11, B14, B15, B24) in dual culture assays (table 2, page 109). Two yeasts (BSc27, BCs54) significantly inhibited three *B. cinerea* strains, whereas five isolates (BSc14, BSc16, BSc90, BSch26, BHv86) significantly reduced fungal development of two strains. Five yeast strains only inhibited one pathogenic strain (BSc96, BSc97, BSc103, BSc112, BSc140, BTd156). Table 2 (page 109), shows the highest inhibition percentages (50% or more) in *Saccharomyces* strains BSc27, BSch26, and BSc14 against B11, B15, and B24, respectively.

Yeast *'Killer'* activity

Thirteen yeast strains presented *'killer'* activity against at least one *B. cinerea* pathogenic strain**.** Seven yeasts (*5 S. cerevisiae, 1 T. delbrueckii, 1 D. vanrijiae*) showed *'killer'* activity against B11 strain, four *Saccharomyces* yeasts against B14, two yeasts against B15 and none yeast against B24 (table 3, page 109-110).

Antifungal activity of volatile organic compounds (VOCs)

From the 18 isolates, 13 yeast strains produced volatile compounds that significantly inhibited mycelial growth of at least one *Botrytis* strain (table 4, page 110). The most susceptible *B. cinerea* strain was *B. cinerea* B24 (7/18), followed by B11, B15 (6/18) and B14 (3/18).

Volatile compounds produced by *S. cerevisiae* BSc206 significantly inhibited mycelial growth of three *B. cinerea* strains, B14, B15, and B24, between 25.6 and 54.7% (table 4, page 110). Seven strains (4 *Saccharomyces* and 3 non- *Saccharomyces)* produced antifungal volatile compounds against 2 *B. cinerea strains* and 5 *Saccharomyces* yeasts against one *B. cinerea* strain. These 12 yeast strains inhibited mycelial growth between 21.4 and 76.9% (table 4, page 110).

Table 2. Mycelial growth inhibition (%) of *B. cinerea* strains after released dual culture assays with 18 yeast strains.

Tabla 2. Inhibición del crecimiento micelial (%) de cepas de *B. cinerea* luego de realizar ensayos de cultivos duales con 18 cepas de levaduras.

Treatments	Mycelial growth inhibition of *B. cinerea* strains (%)			
	B11	B14	B15	B24
S. cerevisiae BSc14	• [a]	• [a]	41.33 ± 2.52 [b]	52.33 ± 1.53 [c]
S. cerevisiae BSc16	• [a]	• [a]	40.00 ± 2.00 [b]	39.33 ± 1.53 [b,c]
S. cerevisiae BSc27	51.60 ± 1.5 [d]	24.0 ± 4.0 [a,b]	44.33 ± 1.53 [b]	• [a]
S. cerevisiae BSc90	37.60 ± 0.5 [b,c,d]	• [a]	• [a]	29.60 ± 1.5 [b,c]
S. cerevisiae BSc96	31.00 ± 1 [c,d]	• [a]	• [a]	• [a]
S. cerevisiae BSc97	• [a]	39.03 ± 3 [b]	• [a]	• [a]
S. cerevisiae BSc103	• [a]	• [a]	• [a]	31.30 ± 1.5 [b,c]
S. cerevisiae BSc112	35.30 ± 0.5 [b,c,d]	• [a]	• [a]	• [a]
S. cerevisiae BSc140	• [a]	• [a]	• [a]	29.30 ± 0.5 [b,c]
S. chevalieri BSch26	38.70 ± 2 [b,c,d]	• [a]	53.67 ± 0.58 [c]	• [a]
C. sake BCs54	47.30 ± 3.5 [c,d]	31.00 ± 1 [b]	• [a]	31.00 ± 1.51 [b,c]
H. vineae BHv86	47.00 ± 1 [c,d]	42.30 ± 3.5 [b]	• [a]	• [a]
T. delbrueckii BTd156	40.30 ± 2.5 [b,c,d]	• [a]	• [a]	• [a]
S. cerevisiae BSc60 *S. cerevisiae* BSc102 *S. cerevisiae* BSc206 *D. vanrijiae* BDv197 *T. delbrueckii* BTd165	• [a]	• [a]	• [a]	• [a]
Control	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]

Different lowercase letters within the same column indicate significant differences among means and SD of mycelial growth according to Tukey's test ($p \leq 0.05$).

The •symbol represents values not significantly different from control (0%).

Gray highlight indicates a mycelial growth inhibition percentage of 50% or more.

Diferentes letras minúsculas en la misma columna indican diferencias significativas entre las medias y el DS del crecimiento fúngico micelial con respecto al control ($p \leq 0,05$).

El símbolo • representa valores que no difieren significativamente con el control (0%).

Resaltado con gris indica porcentajes de inhibición del crecimiento micelial de 50% o superior.

Table 3. Yeast *"Killer"* activity against four *B. cinerea* isolates (B11, B14, B15, B24) at 25°C.

Tabla 3. Actividad "killer" de las cepas de levaduras frente a los cuatro aislamientos de *B. cinerea* (B11, B14, B15, B24), a 25°C.

Treatments	*B. cinerea* strains			
	B11	B14	B15	B24
***S. cerevisiae* BSc16**	+	-	-	-
***S. cerevisiae* BSc27**	-	+	-	-
***S. cerevisiae* BSc60**	-	+	-	-
***S. cerevisiae* BSc90**	+	-	-	-
***S. cerevisiae* BSc102**	+	-	-	-
***S. cerevisiae* BSc103**	+	-	-	-
***S. cerevisiae* BSc112**	+	-	-	-
***S. cerevisiae* BSc206**	-	-	+	-

Positive signs (+) indicate killer activity (presence of a clear zone around the yeast colony), and negative signs (-) indicate no "killer" activity.

Signos positivos (+) indican la presencia de zona transparente alrededor de las colonias de levadura, y los signos negativos (-) indican ausencia de actividad "killer".

***S. chevalieri* BSch26**	-	+	-	-
***T. delbrueckii* BTd156**	+	-	-	-
***T. delbrueckii* BTd165**	-	-	+	-
***D. vanrijiae*BDv197**	+	-	-	-
***S. cerevisiae* BSc14**	-	+	-	-
***S. cerevisiae* BSc97**	-	-	-	-
***S. cerevisiae* BSc140**	-	-	-	-
***C. sake* BCs54**	-	-	-	-
***H. vineae* BHv86**	-	-	-	-

Positive signs (+) indicate killer activity (presence of a clear zone around the yeast colony), and negative signs (-) indicate no "killer" activity.

Signos positivos (+) indican la presencia de zona transparente alrededor de las colonias de levadura, y los signos negativos (-) indican ausencia de actividad "killer".

Table 4. Effect of volatile compounds produced by 18 yeast isolates on mycelial growth of four *B. cinerea* strains (%).

Tabla 4. Efecto de los compuestos volátiles producidos por 18 levaduras sobre la inhibición del crecimiento micelial de las cuatro cepas de *B. cinerea* ensayadas (%).

Treatments	Inhibition of mycelial growth of *B. cinerea* strains (%)			
	B11	**B14**	**B15**	**B24**
S. cerevisiae BSc14	• ^{d}	62.60 ± 0.29 ^{b}	• ^{d}	64.20 ± 0.28 ^{a}
S. cerevisiae BSc16	• ^{d}	• ^{c}	66.80 ± 0.01 ^{c}	• ^{c}
S. cerevisiae BSc27	• ^{d}	• ^{c}	54.90 ± 0.06 ^{b}	69.70 ± 0.12 ^{a}
S. cerevisiae BSc60	• ^{d}	67.60 ± 0.1 ^{b}	• ^{d}	• ^{c}
S. cerevisiae BSc96	• ^{d}	• ^{c}	64.30 ± 0.02 ^{c}	• ^{c}
S. cerevisiae BSc97	77.60 ± 0.1 ^{c}	• ^{c}	• ^{d}	72.80 ± 0.06 ^{b}
S. cerevisiae BSc102	76.7 ± 0.05 ^{c}	• ^{c}	• ^{d}	• ^{c}
S. cerevisiae BSc112	78.60 ± 0.5 ^{c}	• ^{c}	• ^{d}	71.80 ± 0.49 ^{b}
S. cerevisiae BSc206	• ^{d}	50.20 ± 0.2 ^{a}	45.30 ± 0.26 ^{b}	74.40 ± 0.04 ^{b}
S. cerevisiae BSch26	• ^{d}	• ^{c}	• ^{d}	70.00 ± 0.32 ^{b}
C. sake BCs54	60.76 ± 0.5 ^{a}	• ^{c}	• ^{d}	65.80 ± 0.02 ^{a}
T. delbrueckii BTd165	72.70 ± 0.1 ^{b}	• ^{c}	23.10 ± 0.06 ^{a}	• ^{c}
D. vanrijiae BDv197	68.7 ± 0.05 ^{b}	• ^{c}	63.90 ± 0.1 ^{c}	• ^{c}
S. cerevisiae BSc90	• ^{d}	• ^{c}	• ^{d}	• ^{c}
S. cerevisiae BSc103				
S. cerevisiae BSc140				
H. vineae BHv86				
T. delbrueckii BTd156				
Control (H_2O)	**100** ^{d}	**100** ^{c}	**100** ^{d}	**100** ^{c}

Different lowercase letters within the same column indicate significant differences between means of mycelial growth according to Tukey's test ($p \leq 0.05$).

The •symbol represents mycelial growth not significantly different from the control (100%).

Letras distintas en de la misma columna indican diferencias significativas entre las medias para el crecimiento fúngico según la prueba de Tukey ($p \leq 0,05$).

El símbolo • representa valores de crecimiento micelial que no difieren significativamente con el del control (100%).

Yeast effect on conidial germination (CG) and germinal tube length (GTL) of *B. cinerea* in low-nutrient medium (diluted grape must)

In this assay, seven yeast strains (6 *Saccharomyces* and 1 non-*Saccharomyces*) significantly affected conidial germination (CG) and germinal tube length (GTL) of at least one *B. cinerea* strain. B24 was the most susceptible strain, inhibited by five yeasts (5/18), followed by B15 inhibited by 3 yeasts (3/18), B14 (2/18), and B11 inhibited by 1 yeast (1/18). Five *Saccharomyces* yeasts (*S. cerevisiae* BSc27, BSc102, BSc112, BSc206, and *S. chevalieri* BSch26) significantly inhibited conidial germination and reduced the germ tube length of one *B. cinerea* strain. *S. cerevisiae* Bsc60 significantly reduced two *B. cinerea* strains: B24 and B15 (table 5, page 111).

Table 5. Evaluation of conidial germination (CG; %) and germ tube length (GTL; µm) of *B. cinerea* strains in co-cultures with yeasts (excavated slides).

Tabla 5. Evaluación de la germinación de conidios (CG; %), y longitud del tubo germinal (GTL; µm) de cepas de *B. cinerea* en co-cultivos con levaduras (portaobjetos excavados).

Treatments	*B. cinerea* strains							
	B11		B14		B15		B24	
	CG	GTL	CG	GTL	CG	GTL	CG	GTL
S. cerevisiae BSc14	100 [b]	15.42 [b]	100 [b]	20.06 [b]	100 [b]	11.43 [b]	100 [b]	25.0 [b]
S. cerevisiae BSc16	100 [b]	15.67 [b]	100 [b]	21.85 [b]	100 [b]	11.40 [b]	100 [b]	25.02 [b]
S. cerevisiae BSc27	100 [b]	13.21 [b]	67.52 [a]	5.75 [a]	100 [b]	9.45 [b]	100 [b]	25.11 [b]
S. cerevisiae BSc60	100 [b]	14.55 [b]	100 [b]	22.23 [b]	71.49 [a]	5.87 [a]	61.22 [a]	3.56 [a]
S. cerevisiae BSc90	100 [b]	14.52 [b]	100 [b]	21.96 [b]	100 [b]	11.49 [b]	100 [b]	25.01 [b]
S. cerevisiae BSc96	100 [b]	17.23 [b]	100 [b]	21.54 [b]	100 [b]	11.42 [b]	100 [b]	25.02 [b]
S. cerevisiae BSc97	100 [b]	16.32 [b]	100 [b]	21.85 [b]	92.4 [b]	11.12[b]	100 [b]	25.07 [b]
S. cerevisiae BSc102	100 [b]	17. 54 [b]	100 [b]	21.15 [b]	67.44 [a]	5.62 [a]	100 [b]	25.71 [b]
S. cerevisiae BSc103	100 [b]	13.64 [b]	100 [b]	20.54 [b]	100 [b]	11.40 [b]	100 [b]	25.03 [b]
S. cerevisiae BSc112	100 [b]	13.56 [b]	100 [b]	19.55 [b]	100 [b]	11.42 [b]	63.87 [a]	3.78 [a]
S. cerevisiae BSc140	100 [b]	15.46 [b]	100 [b]	21.56 [b]	100 [b]	11.43 [b]	100 [b]	25.01 [b]
S. cerevisiae BSc206	100 [b]	16.87 [b]	100 [b]	22.78 [b]	98.76 [b]	10.13 [b]	71.55 [a]	3.56 [a]
S. chevalieri BSch26	100 [b]	17.84 [b]	100 [b]	21.87 [b]	100 [b]	11.45 [b]	67.54 [a]	2.78 [a]
C. sake BCs54	100 [b]	17.24 [b]	100 [b]	19.84 [b]	100 [b]	11.44 [b]	100 [b]	25.21 [b]
H. vineae BHv86	70.65 [a]	3.14 [a]	71.43 [a]	3.98 [a]	67.38 [a]	5.81[a]	69.11 [a]	3.24 [a]
T. delbrueckii BTd156	100 [b]	16.44 [b]	100 [b]	21.56 [b]	100 [b]	11.42 [b]	100 [b]	25.03 [b]
T. delbrueckii BTd165	100 [b]	15.38 [b]	100 [b]	20.59 [b]	100 [b]	11.47 [b]	100 [b]	25.02 [b]
D. vanrijiae BDv197	100 [b]	16.57 [b]	100 [b]	21.65 [b]	100 [b]	11.43 [b]	100 [b]	25.0 [b]
Control (H_20)	**100 [b]**	**17.96 [b]**	**100 [b]**	**21.97 [b]**	**100 [b]**	**11.4 [b]**	**100 [b]**	**25.77 [b]**

Different lowercase letters within the same column indicate significant differences between means of germinated conidia (CG) expressed in % and germinal tube length (GTL) in µm, according to Tukey's test ($p \leq 0.05$).

Gray highligh indicates values significantly differing from the control ($p \leq 0.05$).

Letras distintas en la misma columna indican diferencias significativas entre los valores de conidios germinados (CG) expresados en %, y longitud del tubo germinal (GTL) en µm, en relación al Test de Tukey ($p \leq 0,05$).

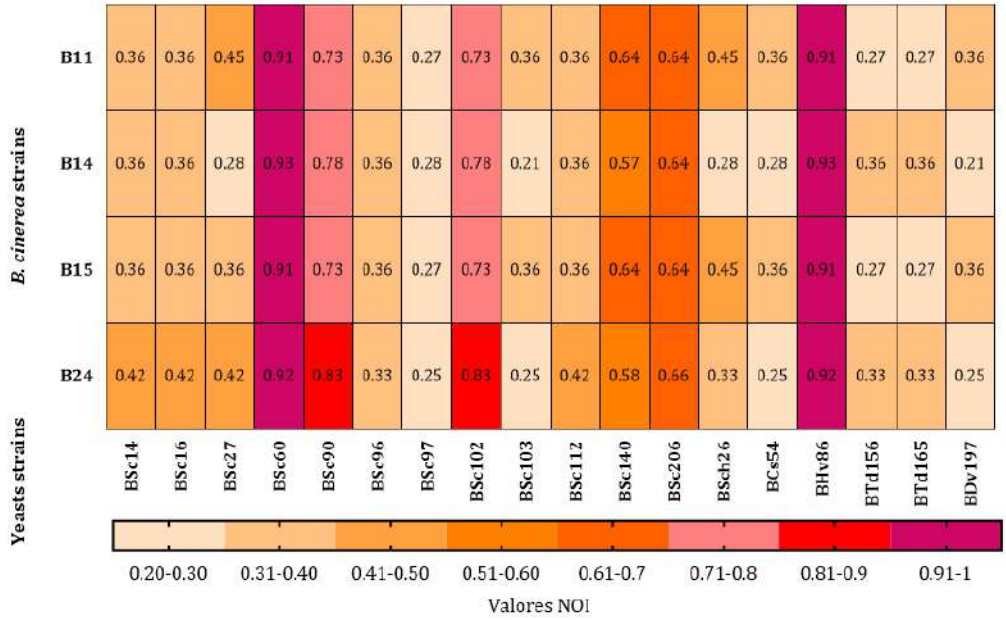
Resaltado con color gris indica valores que difieren significativamente del control ($p \leq 0,05$).

H. vineae BHv86 was the only isolate significantly inhibiting conidial germination and germinal tube length of all five *B. cinerea* B11, B14, B15, and B24. This yeast strain reduced conidial germination between 28.57 and 32.62%, and germ tube length between 12.57 and 50.96% (table 5).

Niche Overlap Index (NOI)

S. cerevisiae BSc60 and *H. vineae* BHv86 occupied the same niche as the 4 *B. cinerea* isolates. Both yeast strains competed with B11, B14, B15 and B24 for the nutritional sources. Four *S. cerevisiae*/four *H. vineae-B. cinerea* interactions presented NOI values between 0.91 and 0.93 (table 6, page 112). The remaining interacting pairs (64) presented NOI values between 0.21 and 0.83, indicating ecological coexistence (separate niches) (table 6, page 112).

Table 6. Niche overlap index (NOI) between 18 yeast and 4 *B. cinerea* strains.
Tabla 6. Índice de superposición de nichos (NOI) entre 18 cepas de levaduras y 4 cepas de *B. cinerea*.



Discussion

Yeast antifungal activity is strain-dependent, and therefore, screening numerous microorganisms becomes necessary for finding strains with broad inhibitory spectrums. Few reports have mentioned the potential use of yeasts of different species and genera with preventive and curative activity to control fungi on fruit tissues (5, 22). Our study first reports yeast species isolated from grape fermentation, like *S. cerevisiae*, *S. chevalieri*, *T. delbrueckii*, *D. vanrijiae*, *H. vineae* and *C. sake,* with preventive and curative antifungal activity against *B. cinerea* in table grapes and postharvest conditions. These enological yeasts demonstrated greater antifungal activity in preventive than in curative assays in table grape wounds (table 1, page 107). Pesce *et al.* (2018) determined that oenological yeasts have stronger antifungal activity than yeasts isolated from another habitat (olives), presenting competitive advantages against other microorganisms.

Commercially developing a product based on native microorganisms involves several steps, starting with isolation and selection of potential biocontrol strains exhibiting desirable characteristics, like different antifungal mechanisms of action (5). Our study examined 18 viticultural yeast strains promoting biocontrol of gray mold under preventive and curative conditions in white table grapes (table 2, page 109; table 3, page 109-110 and table 4, page 110; table 5, page 111 and table 6). We assessed biocontrol activity and modes of action of native yeasts against a pool of four native *B. cinerea* strains previously isolated from different vineyards, constituting a representative way to measure biocontrol activity. To the best of our knowledge, this is the first report on antifungal modes of action of *Saccharomyces, Torulaspora, Debaryomyces, and Candida* with preventive and curative effects against native *B. cinerea* strains isolated from Superior Seedless table grapes (table 1, page 107). The dual culture assay revealed that 72.2% (13/18) of the biocontrol yeasts inhibited mycelial growth of at least one *B. cinerea* strain in the PDA medium (table 2, page 109). Strains belonged to *S. cerevisiae*, *S. chevalieri*, *C. sake*, *H. vineae,* and *T. delbrueckii* species. Korres *et al.* (2011) state that yeasts synthesized and secreted suppressive antifungal substances like diffusible and volatile metabolites, and observed mycelial inhibition on

plates in dual cultures. "*Killer*" activity is a widespread characteristic among yeast species of different genera, including *Saccharomyces*, *Hansenula*, *Kluyveromyces*, and *Pichia*, conferring an ecological advantage over competitors (10). In this study, 13 yeasts belonging to *Saccharomyces*, *Torulaspora,* and *Debaryomyces* species showed positive *'killer'* activity against at least one *B. cinerea* strain at pH 4.5 (table 3, page 109-110). Fungal growth inhibition by volatile compounds avoids adverse environmental or toxicological effects (11). Parafati *et al.* (2015) found that the biocontrol activity of *S. cerevisiae* strains against *B. cinerea* on table grape berries was attributed to volatile organic compounds (VOCs) *in vitro* and *in vivo*. In our study, *Saccharomyces*, *Candida*, *Torulaspora,* and *Debaryomyces* isolates significantly inhibited mycelial diameter of different *B. cinerea* isolates through volatile compounds *in vitro* (table 4, page 110)**.** Primarily, biocontrol yeasts compete for nutrients and space (20). Co-cultures of *Saccharomyces* (BSc27, BSc60, BSc102, BSc112, BSc206, BSch26) and *Hanseniaspora* (BHv86) with *B. cinerea* conidia in liquid medium with low nutrients (excavated slides) showed that these yeasts significantly inhibited conidia germination and reduced germ tube length. In our study, *Hanseniaspora* yeast significantly reduced conidial germination (CG) and germinal tube length (GTL) of four *B. cinerea* strains (table 5, page 111). Qin *et al.* (2015) reported similar results in a co-culture of *H. uvarum* and *B. cinerea*, while Wilson and Lindow (1994) previously suggested that nutritional resources might mediate microorganism coexistence and competitive exclusion. To the best of our knowledge, our study first reports two yeast isolates, *S. cerevisiae* (BSc60) and *H. vineae* (BHv86) with NOI values of 0.91 and 0.93 when co-cultured with four *B. cinerea* strains (table 6, page 112).

S. cerevisiae (BSc27, BSc60, BSc112) and *S. chevalieri* (BSch26) presented the highest amount of possible antifungal mechanisms (four). Our results confirm multiple possible modes of action against pathogens like antibiosis (dual culture), *'killer'* activity, antifungal activity by volatile compounds, inhibition of conidial germination, reduction of germinal tube length (low nutrient medium) and competitive exclusion (NOI).

Conclusions

Under preventive and curative conditions, yeasts isolated from wine fermentation are key biocontrol agents against *B. cinerea* in white table grapes. Understanding yeast strategies against *B. cinerea* strains in preventive and curative assays is essential for selection and effective application. Our study highlights the importance of testing diverse mechanisms that native biocontrol yeasts apply against different *B. cinerea* isolates. In addition, considering that different isolates can exert more than one mechanism of action makes biocontrol an effective tool to prevent gray rot in white table grapes. Further studies should establish bio-antagonism effects on quality attributes of table grapes, the application of biofungicide yeast consortium and the effect of nonviable yeast cells against *B. cinerea*.

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Pesticide drift: comparing spraying systems under variable field climatic conditions

Deriva de pesticidas: un estudio comparativo entre sistemas de aspersión bajo condiciones climáticas variables de campo

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Abstract

Safe pesticide application must ensure efficacy in pest control while minimizing environmental and human health risks. This study investigated pesticide potential drift by comparing ground and aerial spraying systems under different climatic conditions. The research was conducted in Rio Verde, Goiás, Brazil, using a randomized block experimental design with 10 repetitions and a 2 x 2 split-plot scheme, considering spraying systems and climatic conditions as factors. *Favorable* and *Unfavorable* conditions were determined by relative air humidity, temperature, and wind speed. Aerial spraying was performed using a Cessna aircraft, while terrestrial spraying was done using a self-propelled Montana Parruda sprayer. Variables assessed included Volumetric Median Diameter (VMD), droplet density (DEN), and target coverage. Results revealed that aerial spraying has a higher drift potential, exceeding 180 m, compared to terrestrial spraying, limited to 90 m under unfavorable conditions. Although terrestrial spraying produces larger droplets, its shorter distance to the target and reduced speed minimize lateral movement, limiting drift potential. Droplet density and non-target area coverage were low for both systems, (0.1%). Under ideal conditions, aerial spraying is more efficient, but both methods require rigorous safety measures to prevent contamination risks. This study underlines the importance of considering droplet size and specific environmental conditions when choosing a spraying system, contributing to safer and more efficient agricultural practices.

Keywords
aerial application • terrestrial spraying • application technology

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Resumen

La aplicación segura de pesticidas en grandes cultivos es una preocupación crucial para garantizar la eficacia en el control de plagas y al mismo tiempo minimizar los riesgos ambientales y para la salud humana. En este contexto, este estudio investigó la posible deriva de pesticidas comparando sistemas de fumigación terrestre y aérea en diferentes condiciones climáticas. La investigación se realizó en Rio Verde, Goiás, Brasil, utilizando un diseño experimental de bloques al azar con 10 repeticiones. Se adoptó un esquema de parcelas divididas 2 x 2, considerando los factores de los sistemas de aspersión y las condiciones climáticas. Las condiciones favorables y desfavorables se determinaron mediante parámetros como la humedad relativa del aire, la temperatura y la velocidad del viento. La aspersión aérea se realizó mediante una aeronave Cessna, mientras que la aspersión terrestre se realizó mediante un aspersor autopropulsado Montana Parruda. Las variables evaluadas en este estudio incluyeron el diámetro medio volumétrico (VMD), la densidad de gotas (DEN) y la cobertura objetivo. Los resultados revelaron que la aspersión aérea tiene un mayor potencial de deriva, alcanzando distancias superiores a 180 m, en comparación con la aspersión terrestre limitada a 90 m en condiciones desfavorables. Aunque la pulverización terrestre produce gotas más grandes, su distancia más corta al objetivo y su velocidad reducida minimizan el movimiento lateral, lo que limita el potencial de deriva. La densidad de gotas y la cobertura del área no objetivo son bajas para ambos sistemas y se mantienen por debajo del 0,1%. En condiciones ideales, la fumigación aérea es más eficiente, pero ambos métodos requieren medidas de seguridad rigurosas para prevenir riesgos de contaminación. Este estudio enfatiza la importancia de considerar no solo el tamaño de las gotas sino también las condiciones ambientales específicas al elegir un sistema de aspersión, lo que contribuye a prácticas agrícolas más seguras y eficientes.

Palabras clave
aplicación aérea • fumigación terrestre • tecnología de aplicación

Introduction

Pesticides have been used in agriculture for centuries to protect crops against pests, diseases, and weeds (4). Despite studies demonstrating that their use can be reduced by combining other control methods, such as biological control (14), these products are still necessary for agriculture, especially considering large-scale cultivation and crop productive potential (16). This dependence on pesticides is evident in numbers. The European Union, Brazil, the United States, and China, worldwide major food producers, used approximately 827 million, 831 million, 1.2 billion, and 3.9 billion pounds of pesticides in 2016, respectively (5, 8, 25). This scenario remains for most food-producing countries (22). Therefore, adjusting the spraying system and minimizing pesticide impact on non-target organisms, is crucial.

Pesticide-safe application should consider four pillars: the formulated product, target, timing, and spraying system. The first three pillars directly affect system choice. Consequently, all 4 pillars should be analyzed jointly. Once these pillars are properly adjusted, efficient applications assure minimum non-target organism contamination (1). When these components are not well dimensioned, drift and evaporation, two main contamination pathways, are considerably increased. The adopted spraying system and the environmental conditions during application (timing) strongly influence risk potential (2, 3).

Pesticide drift is the unintentional transport of spray droplets away from the control target. Often, this transport leads to contamination of urban areas, forests, and rivers (3). Drift can be studied as primary and secondary drift. Primary drift results from the transport of an active ingredient away from the intended area, after passing through the spray nozzle, due to airflow during application (3). Secondary movement occurs after pesticide application due to chemical volatilization (15). Unlike secondary drift, many factors resulting in primary movement are largely under human control (3).

Studies on pesticide drift often focus on herbicide application risks given the possibility of intoxicating neighboring crops or native forests (3). Recently, this issue has gained attention given soybean cultivars resistant to dicamba and 2,4-D. These herbicides belong to the auxin mimics class, and the high sensitivity of dicotyledonous crops, including non-resistant soybeans, has increased crop damage in non-target areas. These reports are more frequent for dicamba (3). For example, in 2017, the USA reported 2708 cases of dicamba drift-induced injuries (21) while in Brazil, auxin herbicides stand as the main reported contamination in non-target areas. Between 2018 and 2021, 431 positive cases of auxin herbicide drift were recorded in the state of Rio Grande do Sul (9).

Although many studies address drift, associating this practice with contamination of neighboring crops, urban areas and native forests deserves particular investigation given human health and environmental safety. In Brazil, 2021 recorded 30 cases of pesticide drift in urban areas. Of these cases, 21 were caused by aerial applications of fungicides or insecticides (9). In Rio Verde, Goiás, 120 students were hospitalized due to drift caused by the aerial application of [thiamethoxam + lambda-cyhalothrin] (19).

Concerning human and environmental safety, Law N° 19423 of July 26, 2016, published in the Official Gazette on August 4, 2016, establishes restrictions on aerial spraying considering minimum distance from non-target locations: 500 m from urban perimeters and 250 m for public water reservoirs. For terrestrial sprayings, a minimum distance of 100 m is established from the urban perimeter, 200 m for public water reservoirs, and 50 m for isolated dwellings and animal clusters. Aerial application restrictions are stronger since droplet size and target distance may increase aerial drift compared to terrestrial spraying (2).

Despite restrictions, drift can reach greater distances. Even for primary drift, where the applicator can control some factors, drift still brings uncertainties during pesticide applications. Consequently, more studies should assess real drift, considering interactions between different spraying systems and environmental conditions. These studies are even more relevant in tropical conditions given higher frequency of unfavorable application conditions like high temperatures, lower relative humidity, and wind gusts (10). To facilitate drift deposit measurement processes, some researchers collect deposits on a drift test bench (11, 20) or in wind tunnels (6). Despite their advantages, these indirect methods cannot reproduce real aerial applications, and comprehensive field studies must be conducted (2). In this context, we studied the potential drift of ground and aerial spraying systems and the relationship between these systems and environmental conditions during field trials, identifying possible shortcomings in the current restrictions for pesticide spraying.

Materials and methods

The experiment was conducted in the municipality of Rio Verde (Goiás), Brazil (17°46'34.5" S 51°01'81.1" W). The region's climate is classified as B4 rB'4a' (humid; slight water deficiency; mesothermal; summer evapotranspiration less than 48% of the annual evapotranspiration), according to Thornthwaite (1948).

The experiment was conducted in a randomized complete block design with 10 replications. A 2 x 2 split-plot design was adopted to identify interactions between ground and aerial spray systems and climatic conditions during application. The climatic factor defined the main plots, while the spray system was defined in subplots. Two climatic conditions were considered, one *Favorable* and the other *Unfavorable*. Factor randomization in subplots was done by randomly selecting application moments for *Favorable* and *Unfavorable* classes. The parameters relative air humidity, instantaneous temperature, and wind speed determined *Favorable* and *Unfavorable* conditions (table 1, page 118). Climatic data were obtained using an INSTRUTHERM THAL-300 thermo-hygro-anemometer. A wind direction indicator (windsock) was installed in the experimental area to guide application direction.

Table 1. Climatic conditions during spraying with the different equipment.
Tabla 1. Condiciones climáticas durante la pulverización con los diferentes equipos.

Parameter	Favorable		Unfavorable	
	Aerial	Terrestrial	Aerial	Terrestrial
Application time (h)	10:10 a.m.	11:00 a.m.	12:05 p.m.	11:45 a.m.
Temperature (°C)	28.6	27.0	30.5	30.3
Relative humidity (%)	61.1	62.0	52.5	53.8
Wind speed (km h^{-1})	6.5	8.9	11.1	11.7

Aerial spraying was performed with a Cessna aircraft, Ag Truck model, with a capacity of 810 kg, equipped with Full Cone Hollow Core D6 Orifice 56 nozzles, set to provide a "Very Fine" droplet spectrum. Spray volume was 20 L ha^{-1}, at 26 Psi, with a travel speed of 187 km h^{-1} and flight height of 3 m. These parameters were determined by regional frequent use. Terrestrial spraying was performed using a self-propelled Montana Parruda sprayer, model MA2527, equipped with a Flat Fan Jet ST 03 nozzle, set to provide a "Large" droplet spectrum. Spray volume was 80 L ha^{-1}, and working pressure was 55 Psi, with travel speed of 20 km h^{-1} and a spray bar height of 0.50 m. These criteria were based on recommendations for each system for the lowest drift risk without compromising target coverage efficiency. Reservoirs of both spraying equipment contained only water. Regardless of the application system, applications were always perpendicular to wind direction.

Drift potential was estimated through hydro-sensitive papers attached to a wooden support at 45° angle relative to the wooden support. The 26 x 76 mm hydro-sensitive paper spray cards were purchased in TeeJet Technologies® (São Paulo, Brazil). The wooden supports were positioned equidistantly every 20 m, using the last external tip of the spraying bar as a reference, always perpendicularly to the application and in line with wind direction. Wooden supports positioned at the same distance from the spraying bar were placed every 10 m, totaling 100 meters (considering the 10 repetitions). Thus, the distance covered for each treatment was 100 m. Figure 1 illustrates the wooden supports distribution. Wooden supports were positioned at a maximum distance of 200 meters from the first wooden support.

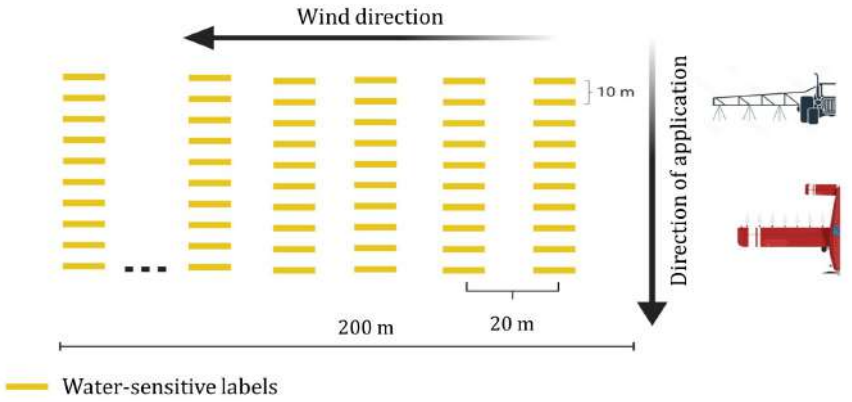


Figure 1. Scheme of the arrangement of water-sensitive papers in the experimental area.
Figura 1. Representación gráfica de la disposición de los papeles sensibles al agua en el área experimental.

After the spraying, the hydro-sensitive papers were removed and placed in a paper envelope for subsequent scanning using the CIR 1.5 software (13), at 600 dpi. After scanning, the parameters volumetric median diameter (VMD), droplet density (DEN) (drops cm^{-2}), and coverage percentage were obtained for each experimental unit.

Statistical analyses were performed using SISVAR software (7). After checking ANOVA assumptions, the F-test, was performed. When assumptions were not met, data were transformed using the Box-Cox criterion, followed by ANOVA and Tukey's test (p-value < 0.05).

Results

The ANOVA results for *Spray Systems* vs. *Environmental Conditions* are presented in Supplementary Material S1. The Volumetric Median Diameter (VMD) showed a significant interaction effect for either *Spray Systems* or *Environmental Conditions*, with distances exceeding 140 m.

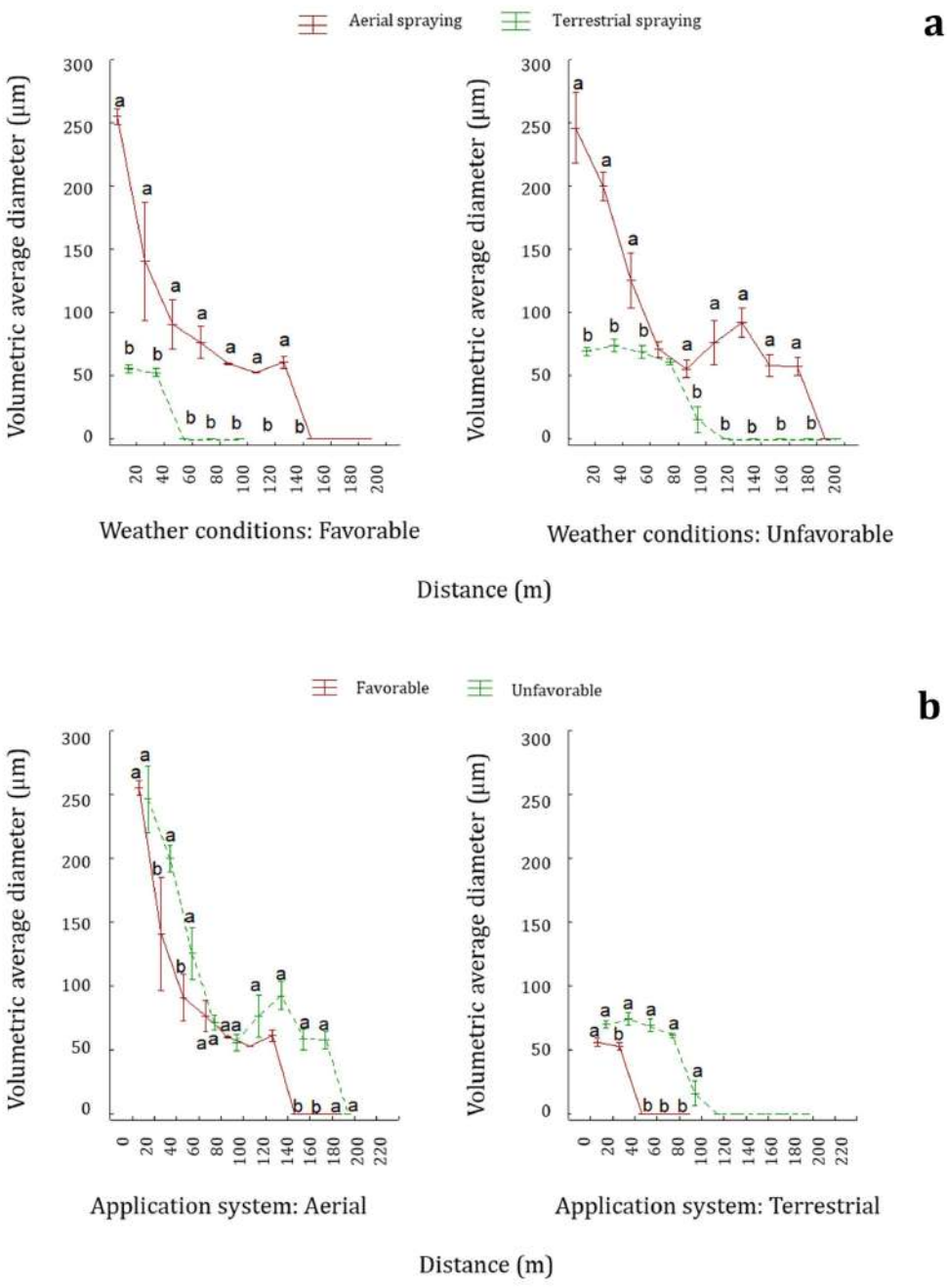
Figure 2 (page 120), shows differences in VMD between spraying systems under *Favorable* and *Unfavorable Conditions*. Under *Favorable* conditions, the aerial application system provided a higher VMD (ranging from 54 to 250 µm) compared to the ground-based system (ranging from 78 to 25 µm) for distances from 20 to 140 m. Beyond 140 m, no droplets were detected in either spraying system under *Favorable* conditions. Droplets were detected only up to 40 m for the ground-based spraying system. In aerial application, 74 µm VMD droplets were detected up to 140 m. Under *Unfavorable* conditions, the behavior between spraying systems for VMD was similar to *Favorable* conditions for most distances, with higher VMD values for the aerial system. Similarity in VMD between systems was only observed at 80 m.

The VMD was higher for the *Unfavorable* condition and aerial spraying at 40, 60, 100, 120, 140, 160, and 180 m (figure 2b, page 120). The maximum distance at which droplets were detected for *Favorable* and *Unfavorable* aerial applications was 120 m (74 µm) and 180 m (77 µm), respectively. In the terrestrial spraying, the *Unfavorable* condition also resulted in a higher VMD, reaching a maximum distance of 100 m (25 µm) and 40 m (51 µm), respectively. These results demonstrate that environmental conditions at application will influence drift potential, with greater risk under *Unfavorable* weather conditions.

Under *Favorable* conditions, aerial application resulted in a higher droplet density on non-target areas, compared to terrestrial spraying. Droplet density values ranged from 2 to 23 drops cm^{-2} for aerial application and 1 to 9 drops cm^{-2} for terrestrial spraying (figure 3a, page 121). Under *Unfavorable* conditions, terrestrial spraying provided a higher droplet density than aerial spraying at 20, 40, and 80 m. Beyond 80 m, aerial spraying promoted higher droplet density, while terrestrial spraying had null density. Different droplet density between climatic conditions in aerial spraying was only observed at 80 m (figure 3b, page 121). Climatic conditions strongly impacted terrestrial spraying with higher droplet density under *Unfavorable* conditions compared to *Favorable* conditions and from 20 to 100 m.

Target coverage values were below 1% for all spraying systems and environmental conditions, (figure 4a and 4b, page 122). Aerial spraying provided higher coverage than terrestrial spraying, for *Favorable* and *Unfavorable* conditions (figure 4a, page 122). Beyond 80 m, aerial coverage was below 0.1%, regardless of climatic conditions. In terrestrial spraying, coverage below 0.1% occurred at 40 m from the target.

The maximum drift distance detected for ground application was 40 m and 90 m for *Favorable* and *Unfavorable* conditions, respectively (table 2, page 123). For aerial application, maximum drift values were 140 m and 180 m for *Favorable* and *Unfavorable* conditions, respectively.

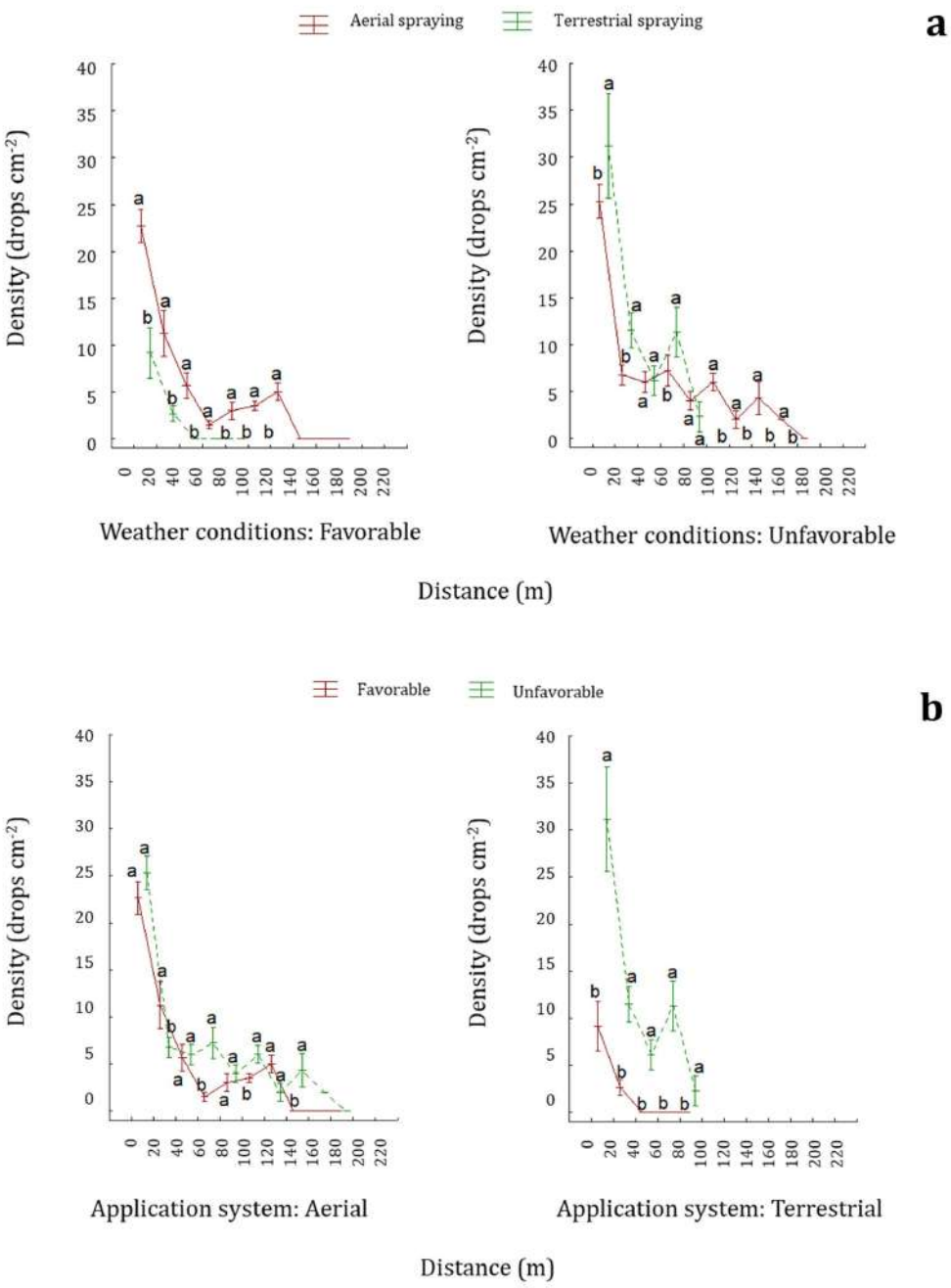


Lowercase letters show statistical differences.

Las letras minúsculas muestran diferencias estadísticas.

Figure 2. Volumetric mean diameter (µm) obtained by applications in **a**: two environmental conditions (favorable and unfavorable) from **b**: aerial and terrestrial application systems, over 200 meters considering perpendicular drift.

Figura 2. Diámetro volumétrico medio (µm) obtenido por aplicaciones en **a**: dos condiciones ambientales (favorable y desfavorable) y **b**: del sistema de aplicación aérea y terrestre en una distancia de 200 metros considerando un movimiento perpendicular de la deriva.

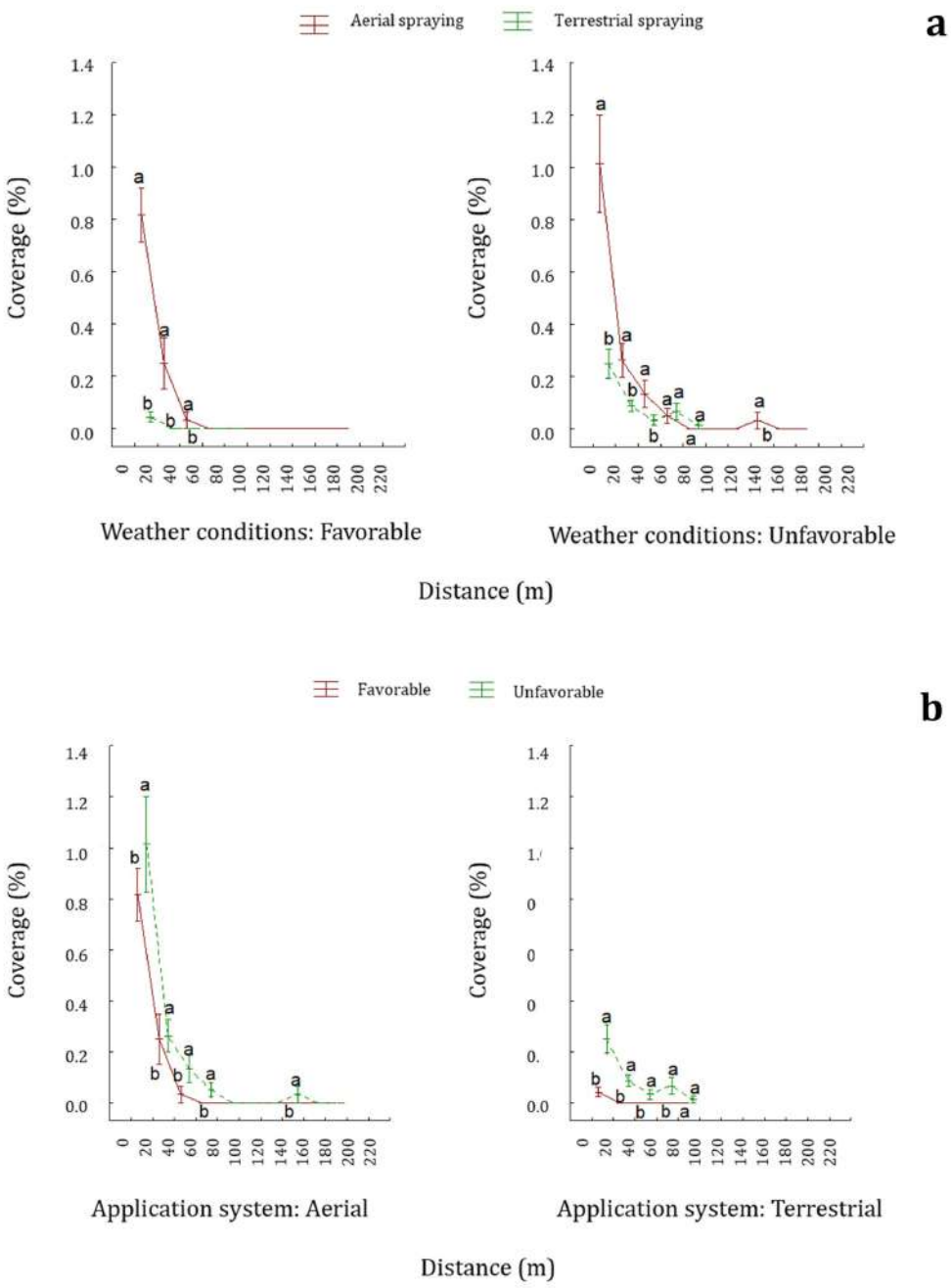


Lowercase letters in figure 3a differentiate application systems at each distance for each condition. Lowercase letters in figure 3b differentiate application conditions at each distance for both application system.

Las letras minúsculas en la figura 3a diferencian los sistemas de aplicación en cada distancia evaluados para cada condición de aplicación. Las letras minúsculas en la figura 3b diferencian las condiciones de aplicación en cada distancia evaluada para cada sistema de aplicación.

Figure 3. Density (drops cm^{-2}) in two environmental conditions (favorable and unfavorable) from aerial and terrestrial application systems over 200 meters considering perpendicular drift in relation to the application.

Figura 3. Densidad (gotas cm^{-2}) obtenida por aplicaciones en dos condiciones ambientales (*favorable* y *desfavorable*) del sistema de aplicación aérea y terrestre en una distancia de 200 metros considerando un movimiento perpendicular de la deriva con relación a la dirección de aplicación.



Lowercase letters in figure 4a differentiate the application systems at each distance evaluated for each application condition. Lowercase letters in figure 4b differentiate the application conditions at each distance evaluated for each application system.

Las letras minúsculas en la figura 4a diferencian los sistemas de aplicación en cada distancia evaluados para cada condición de aplicación. Las letras minúsculas en la figura 4b diferencian las condiciones de aplicación en cada distancia evaluada para cada sistema de aplicación.

Figure 4. Coverage (%) obtained by applications in two environmental conditions (favorable and unfavorable) from the aerial and terrestrial application system over a distance of 200 meters considering a perpendicular movement of the drift concerning the direction of application.

Figura 4. Cobertura (%) obtenida por aplicaciones en dos condiciones ambientales (favorable y desfavorable) del sistema de aplicación aérea y terrestre en una distancia de 200 metros considerando un movimiento perpendicular de la deriva con relación a la dirección de aplicación.

Table 2. Maximum drift distance and increase thereof depending on applications carried out in different modes and climatic conditions.

Tabla 2. Distancia máxima de deriva y aumento de la misma en función de aplicaciones realizadas en diferentes modos y condiciones climáticas.

Application system	Distance (m)		Increase [1/]	
	Favorable	Unfavorable	--- % ---	--- m ---
Aerial	140	180	28	40
Terrestrial	40	90	125	50
Application system	**Target coverage (%) [2/]**			
	Favorable		***Unfavorable***	
Aerial	25		18	
Terrestrial	31		23	

[1/] Increase in drift when comparing applications under ideal and adverse weather conditions. [2/] Target coverage provided on hydro-sensitive papers positioned across the application swath.

[1/] Aumento de la deriva al comparar aplicaciones en condiciones climáticas ideales y adversas. [2/] Cobertura objetivo proporcionada en papeles hidrosensibles colocados a lo largo de la franja de aplicación.

Discussion

Aerial spraying showed higher drift potential in both *Favorable* and *Unfavorable* conditions. Even terrestrial application had larger VMD (coarse droplets) than aerial spraying (fine droplets), it did not reach greater distances outside the target. The shorter distance to the target and the lower traveling speed reduced lateral movement of larger droplets, allowing only lateral movement of droplets with VMD under 50 µm in *Favorable* conditions and 75 µm for *Unfavorable* conditions. Droplets of this caliber are more susceptible to wind drag, even under ideal climatic conditions.

Even though aerial spraying produced a spectrum of finer droplets, the higher target distance, travel speed, and turbulence propelled larger droplets farther from the application point. Probably, the smaller droplets of the aerial system evaporated before reaching the hydro-sensitive papers. On the other hand, larger droplets have a longer lifetime (2, 21) and were transported by the wind to distances exceeding 120 m under *Favorable* conditions and 180 m under *Unfavorable* conditions. Droplets with VMD greater than 100 µm are less susceptible to wind transport. However, for aerial spraying, 125 µm drops deposition was up to 60 m from the target under *Unfavorable* conditions. Under *Favorable* conditions, drops with VMD greater than 100 µm were transported up to 40 m given wind speed. According to Baio *et al.* (2019), wind is the most influential factor in pesticide drift for aerial spraying.

The higher droplet density observed for terrestrial spraying under *Unfavorable* conditions at 20, 40, and 80 m was given by smaller droplets traveling longer distances. For up to 80 m from the target, the higher wind speed under *Unfavorable* conditions was the prime factor modulating droplet movement. However, the lower position of the application bar compared to aerial spraying minimized drift potential with drops recorded only up to 80 m. Several studies directly correlate target distance with drift potential (12, 17, 18). In addition, aerial spraying occurred at 3 m from the target (3 m) while terrestrial spraying was at 0.5 m, increasing average time for droplets to reach the target. Probably, these droplets evaporated under *Unfavorable* conditions, failing to reach water-sensitive papers after the target. Under *Favorable* conditions, aerial spraying showed smaller droplets reaching the water-sensitive papers with higher droplet density than terrestrial spraying.

Even though drift occurred at 90 and 180 m for terrestrial and aerial spraying, respectively, under *Unfavorable* conditions, the amount of active ingredient hypothetically reaching non-target areas, is low.

Coverage was less than 0.1% for both spraying systems under this condition, proportionally low when considering target average coverage of aerial and terrestrial spraying of 18% and 23%, respectively (table 2). The hypothetical dose reaching above 90 m would be 0.5% and 0.4% of the recommended dose for aerial and terrestrial spraying, respectively. However, some non-target organisms do not tolerate infinitesimally small doses of certain pesticides, such as dicotyledonous plants (3) or crayfish (24).

In general, higher temperature, lower relative humidity, and increased wind speed during both aerial and terrestrial spraying increased drift potential, with 28% and 125% for perpendicular and parallel distances to wind direction. Despite higher drift risk of aerial spraying, terrestrial spraying is strongly affected by environmental conditions. Under *Unfavorable* conditions, drift reached 90 m, exceeding the minimum 50 m distance established by Brazilian Law 19.423/2016 for areas with isolated dwellings and groups of animals. Considering other restrictions determined by Brazilian Law 19.423/2016, even under non-ideal conditions, terrestrial spraying proved safe. Under *Favorable conditions,* aerial spraying had a low drift risk, with maximum drift detected at 140 m, under the 250 m limit established by law.

Conclusions

The results indicate that pesticide drift in large crops is significantly influenced by spraying systems and environmental conditions. Aerial spraying shows a higher drift potential, reaching over 180 m, while terrestrial spraying under unfavorable conditions is limited to 90 m. System choice should consider droplet size and specific environmental conditions. Despite drift potential, coverage in non-target areas was under 0.1% for both systems. We highlight the importance of rigorous safety laws to minimize contamination, contributing to safer and more efficient agricultural practices.

Supplemmentary material

https://docs.google.com/document/d/1Clve2FAJgRYvPtRptjLEMh37Kj_OKtxs/edit?usp=sharing&ouid=111310786017351827239&rtpof=true&sd=true

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Feeding strategies for Holando Argentino steers aimed at different markets

Estrategias de alimentación de novillos Holando Argentino para diferentes destinos comerciales

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Abstract

The objective was to evaluate the performance and meat quality of Holando Argentino (HA) steers under different feeding strategies. One hundred twenty-eight HA steers (181.4 ± 25.5 kg of live weight [LW]) were allocated to four treatments: FL: feedlot finishing during 98 days; Gr1.25: grazing with 1.25% LW/day maize grain supplementation during 235 days; Gr0.70: grazing with 0.70% LW/day maize grain supplementation during 331 days; and GrFL: 287 days grazing background and 116 days feedlot finishing. Average daily gains (ADG) were 1.14, 1.02, 0.82, and 0.81 kg/day for FL, Gr1.25, Gr0.70, and GrFL, respectively ($p<0.01$). Adjusted productivity ranged between 710 and 741 kg LW/ha ($p>0.05$). GrFL and Gr0.70 presented the highest carcass weight (CW; 288.3 ± 5.0 and 267.8 ± 12.2 kg, respectively, $p<0.001$). Gr0.70 presented the lowest *longissimus thoracis* (LT) *L** ($p<0.01$) and the highest *a** ($p<0.05$). Intramuscular fat was the highest for GrFL (4.86 ± 0.93%, $p<0.05$). In all strategies, LT shear force presented values of tender meat (29.9 ± 3.4 N, $p=0.60$). HA steers have the flexibility to produce tender meat under different, high-productivity strategies.

Keywords

dairy breeds • grazing steers • supplementation • feedlot steers • shear force • intramuscular fat • meat color • fat color

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Resumen

El objetivo fue evaluar el desempeño y la calidad de la carne de novillos Holando Argentino (HA) alimentados bajo diferentes estrategias. Se utilizaron 128 terneros HA (181,4 ± 25,5 kg de peso vivo [PV]) que se asignaron a cuatro tratamientos: FL: terminación a corral durante 98 días; Gr1,25: invernada pastoril con suplementación con grano de maíz al 1,25% PV/día durante 235 días; Gr0,70: invernada pastoril con suplementación con grano de maíz al 0,70% PV/día durante 331 días; y GrFL: recría pastoril durante 287 días y terminación a corral durante 116 días. Los aumentos medios diarios fueron 1,14, 1,02, 0,82 y 0,81 kg PV/día para FL, Gr1,25, Gr0,70 y GrFL, respectivamente ($p<0,01$). La productividad ajustada varió entre 710 y 741 kg PV/ha ($p>0,05$). GrFL y Gr0,70 presentaron el mayor peso de res (288,3 ± 5,0 y 267,8 ± 12,2 kg, respectivamente, $p<0,001$). Gr0.70 presentó el menor L^* ($p<0,01$) y el mayor a^* ($p<0,05$) del *longissimus thoracis* (LT). El mayor contenido de grasa del LT fue producido por GrFL (4,86 ± 0,93%, $p<0,05$). En todas las estrategias, la resistencia al corte del LT presentó valores que corresponden a carnes tiernas (29,9 ± 3,4 N, $p=0,60$). Los novillos HA tienen la flexibilidad de producir carne tierna bajo diferentes estrategias de alta productividad.

Palabras clave

razas lecheras • novillos en pastoreo • suplementación en pastoreo • alimentación a corral • resistencia al corte • grasa intramuscular • color de la carne • color de la grasa

Introduction

The availability of Holando Argentino (HA) male steers in the Argentine pampas represents an opportunity for meat production, considering their high growth potential and favorable purchase-to-sale price ratio. Locally, different feeding strategies have been evaluated for HA steers. On one side, intensive grazing systems slaughter between 460 and 540 kg LW, aiming for export markets (19, 21). On the other hand, calf feeding systems with slaughter LW between 300 and 370 kg (20) satisfy local demands characterized by small cuts.

Dairy breeds present higher maintenance requirements and different fat deposition patterns than beef breeds, compromising early slaughter and market acceptability (3). One way to increase local acceptability and market allocation flexibility of meat from HA steers, fed under grazing systems, is to achieve high growth rates, reaching the finishing endpoint at moderate LW (*i.e.* <450 kg). In this sense, using energetic supplements can increase daily gain and fattening rate, reducing the growing and finishing periods (24, 37). Furthermore, new export market opportunities like tax-free meat quota for the European Union, called quota 481, could emerge (23). This quota applies to meat from steers fed with high-concentrate diets for a minimum of 100 days and less than 30 months of age at slaughter.

Concerning meat quality, previous research has shown that meat obtained from dairy breeds has similar overall quality to that obtained from British beef breeds (3). Accordingly, Latimori *et al.* (2008) found that HA steers fed under different strategies presented no differences in tenderness compared with British or crossbred steers, despite having lower marbling scores than meat from Angus steers.

Feeding strategies for finishing steers can vary from pasture-based to concentrated feeding, resulting in different LW gains, age at harvest, final LW, CW, and fatness degree, which can also impact meat quality and market destination (30). Therefore, strategies need to be evaluated taking all these aspects into account. The main objective of this study was to evaluate productivity, animal performance, and meat quality from HA steers fed under contrasting strategies, ranging from grazing with different supplementation levels to feedlot finishing. A second objective was to identify the main traits defining carcass and meat quality of HA steers across diverse feeding strategies and to quantify the relationships among these traits.

Materials and methods

Site and feeding strategies

The study was conducted at the Marcos Juárez Agricultural Experimental Station of the National Institute of Agricultural Technology (INTA). Grazing strategies were evaluated on mixed pastures of alfalfa (*Medicago sativa*) and tall fescue (*Lolium arundinaceum*), established on argiudoll soil with no limitations. The region's climate is temperate, with a mean temperature of 17.9°C and an annual rainfall of 887 mm (14). Live animal management was conducted according to the standards and conditions of the Animal Ethics Committee of INTA.

A total of 128 HA steers with 5 to 7 months of age (181.4 ± 25.5 kg LW) were purchased. Upon arrival at the Experimental Station, animals were treated against internal parasites and vaccinated against Clostridia and respiratory diseases. The animals were then allocated to four feeding strategies. Group FL: *ad libitum* feedlot system for 98 days, targeting the local market (30 steers, 202.9 ± 13.8 kg LW, 10 steers x 3 repetitions); group Gr1.25: grazing with 1.25 %LW/day dry cracked maize supplementation (DM basis) for 235 days, targeting both local and export markets (36 steers, 172.9 ± 19.6 kg LW, 9 steers x 4 repetitions); group Gr0.70: grazing with 0.70% LW/day dry cracked maize supplementation (DM basis) for 331 days, targeting export markets (32 steers, 195.0 ± 11.7 kg LW, 8 steers x 4 repetitions); and group GrFL: grazing background without supplementation for 287 days followed by *ad libitum* feedlot finishing for 116 days, targeting the export quota 481 (30 steers, 156.7 ± 23.8 kg LW, 10 steers x 3 repetitions).

Grazing management and supplementation

Pasture management and supplementation of the grazing strategies are summarized in table 1. Each experimental unit had pasture divided into 6 paddocks grazed rotationally, with independent water troughs (minimum of 16 cm/animal) and group feed bunks providing 0.67 m/animal. Steers grazed rotationally, with paddock occupation and resting periods ranging from 4 to 9 days and from 21 to 60 days, respectively, depending on pasture production. Gr1.25 and Gr0.70 included permanent supplementation with dry cracked maize grain and winter supplementation with alfalfa hay.

Table 1. Grazing management and supplementation.

Tabla 1. Manejo del pastoreo y suplementación.

Feeding strategy	N° EU	N° Steers (steers/EU)	Pasture surface (ha/EU)	Pasture stocking rate (steers/ha)	Pasture allowance (%LW/day)		Supplementation (%LW/day)	
					Autumn-Winter	Spring-Summer	Dry cracked maize	Alfalfa hay - Winter
Gr1.25	4	9	2.4	3.75	3.24 ± 0.14	4.54 ± 0.55	1.25*	0.45
Gr0.70	4	8	2.4	3.33	2.57 ± 0.18	4.78 ± 0.20	0.70	0.60
GrFL	3	10	3.5	2.83	4.59 ± 0.29	6.88 ± 0.05	--	--

Gr1.25: grazing finishing with high supplementation; Gr0.70: grazing finishing with low supplementation; GrFL: grazing background and feedlot finishing; EU: experimental unit; LW: live weight. Pasture allowance and supplements are expressed on a DM basis.* supplementation was delivered in two daily feedings.

Gr1.25: invernada pastoril con alta suplementación; Gr0.70: invernada pastoril con baja suplementación; GrFL: recría pastoril y terminación a corral. EU: unidad experimental; LW: peso vivo. La asignación de pastura y la suplementación están expresadas en materia seca. * suplementación dividida en dos entregas diarias.

Pre-grazing pasture biomass was estimated every two weeks (8 to 18 days depending on the season) by 10 sites of 0.25 m^2 cut at 3 cm height. A subsample (200-400 g) was dried at 60°C for 48 h to determine DM content and milled to 1 mm for subsequent analysis (table 2, page 129). Crude protein (CP) was determined according to Horneck and Miller (1988), while neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to Van Soest *et al.* (1991). Pasture metabolizable energy was estimated using the digestibility equation McLeod and Minson (1976).

Table 2. Pasture chemical composition.
Tabla 2. Composición química de las pasturas.

Autumn-Winter			
	Gr1.25	Gr0.70	GrFL
Crude protein (%)	21.4 ± 0.4	24.2 ± 1.4	14.5 ± 0.8
Neutral detergent fiber (%)	48.2 ± 0.8	40.6 ± 1.61	50.4 ± 0.9
Acid detergent fiber (%)	31.4 ± 0.4	31.5 ± 1.3	30.9 ± 0.5
Metabolizable energy* (Mcal/kg DM)	2.30 ± 0.1	2.29 ± 0.4	2.31 ± 0.2
Spring-Summer			
	Gr1.25	Gr0.70	GrFL
Crude protein (%)	16.9 ± 0.5	18.9 ± 1.0	12.9 ± 0.8
Neutral detergent fiber (%)	51.8 ± 1.3	44.7 ± 1.1	55.6 ± 0.5
Acid detergent fiber (%)	33.6 ± 0.6	32.8 ± 0.8	35.7 ± 0.3
Metabolizable energy* (Mcal/kg DM)	2.22 ± 0.2	2.25 ± 0.3	2.15 ± 0.1

Gr1.25: grazing finishing with high supplementation; Gr0.70: grazing finishing with low supplementation; GrFL: grazing background and feedlot finishing. * Calculated using the equation from McLeod and Minson (1976): Metabolizable energy (Mcal/kg DM) = 3.6 x (92.3 - 0.91 x %ADF) / 100.

Gr1.25: invernada pastoril con alta suplementación; Gr0.70: invernada pastoril con baja suplementación; GrFL: recría pastoril y terminación a corral. * Calculado usando la ecuación de McLeod y Minson (1976): Energía metabolizable (Mcal/kg MS) = 3,6 x (92,3 - 0,91 x %FDA) / 100.

Feedlot management

The FL and GrFL strategies used six outdoor pens of 250 m^2 for feedlot finishing. Animals assigned to the FL strategy had a 45-day pre-experimental period with *ad libitum* access to alfalfa hay, increasing mean LW from 177.6 to 202.9 kg before entering the feedlot finishing period. The analysis did not include this period. FL strategy used 30 HA steers allocated randomly in three pens for a finishing period of 98 days. Whereas for the finishing phase of the GrFL strategy, 30 HA steers were allocated in three pens for 116 days after the mentioned grazing background phase.

In both strategies, the proportion of grain in the diet was gradually increased during an adaptation period of 21 days. The final diet consisted of a typical finishing diet based on dry cracked maize grain, alfalfa hay, soybean meal, and a mineral supplement (table 3). It was delivered once daily between 8:00 and 9:00, adjusting the amount offered to attain 10% of feed refusal and ensure *ad libitum* access to feed. Each ingredient was sampled monthly, dried at 60°C for 48 h to determine DM content, and milled to 1 mm for the same analysis described for pasture quality. Mean diet metabolizable energy was estimated from metabolizable energy of each component reported by NRC (1996) and their respective proportion in the diet.

Table 3. Ingredients and chemical composition of feedlot diet.
Tabla 3. Ingredientes y composición química de las dietas de corral.

Diet ingredients (% in DM)	
Dry cracked maize	84.2
Alfalfa hay	10.1
Soybean expeller	5.3
Mineral supplement [A]	0.4
Chemical composition [B]	
Crude protein (% in diet DM)	12.7
Neutral detergent fiber (% in diet DM)	18
Acid detergent fiber (% in diet DM)	8.7
Metabolizable energy (Mcal/kg DM)	3.12

[A] supplement composition: Ca, 295 g/kg; mg/kg: Fe 15000, Mn 14889, Zn 13000, Cu 1600, Se 9, I 140, Co 250, monensin 8330; UI/kg: vit. A 3000000, vit. D 1200000, vit. E 100; [B] calculated from composition and energy concentration of individual ingredients (31).

[A] composición del suplemento: Ca, 295 g/kg; mg/kg: Fe 15000, Mn 14889, Zn 13000, Cu 1600, Se 9, I 140, Co 250, monensina 8330; UI/kg: vit. A 3000000, vit. D 1200000, vit. E 100; [B] calculado a partir de la composición y concentración energética de los ingredientes individuales (31).

Animal performance and feeding strategy productivity

Animals were individually weighed without fasting between 8:00 and 9:00, at the beginning, every 4-5 weeks, and at the end of each feeding period. LW was adjusted considering 5% of shrinkage. For feedlot systems (FL and GrFL), mean DM intake (DMI) was estimated per pen of 10 steers (experimental unit) as the difference between offered and refused feed over 5 days, calculated monthly. DMI was used to estimate feed conversion as the ratio of mean DMI to average LW gain.

Productivity, measured as LW production per pasture surface and adjusted surface, was estimated for the Gr1.25, Gr0.70, and GrFL feeding strategies. Surface adjustment considered maize grain equivalents used for Gr1.25 and Gr0.70 supplementation and GrFL pen feeding (16). The maize crop surface needed was calculated considering a mean yield of 12,000 kg/ha for the southeast of Córdoba, Argentina (15).

Carcass characteristics and meat quality

The timing of slaughter for each strategy was based on a visual evaluation of the necessary fatness degree for the aimed market, verified by local cattle buyers for both local and export markets. For GrFL, slaughter timing also required a minimum of 100 days on a high-concentrate diet to target the export quota 481. Three steers per experimental unit from Gr1.25 and Gr0.70, and four steers per experimental unit from FL and GrFL, were randomly selected for carcass and meat determinations, resulting in 12 carcasses per feeding strategy. The slaughter of steers from all feeding strategies was carried out at a commercial abattoir. At 48 h *postmortem*, CW was recorded, and a section containing the 10^{th}, 11^{th}, and 12^{th} ribs was removed from the left side of each carcass. Samples were kept at 4°C until 72 h *postmortem*. Then, ribs were deboned and separated into 2.5 cm thick steaks, vacuum-packed, and stored at -20°C until further analysis. When necessary, samples were thawed at 4°C for 24 h.

Fat thickness (FT) and ribeye area (REA) were measured at the 12^{th} rib using a gauge and digital planimeter, respectively. Intramuscular fat (IMF) content was determined in duplicate by the Soxhlet method (SOXTEC SYSTEM HT 1043 Extraction Unit) using an aliquot of 5 g per steak (10). The results are expressed as a percentage of fresh muscle tissue.

To determine the thawing loss, each steak was placed on a plastic mesh inside a sealed plastic container, preventing the sample from coming into contact with the released liquid, for 24 h at 4°C. The results were calculated as the difference between initial and final weights referring to initial weight and expressed as percentage (28).

Muscle and subcutaneous fat CIE colors parameters were obtained sixfold with a Minolta CR-400 (Konica Minolta, Japan). The colorimeter used illuminant D-65, 8 mm port size, 2° observer, and was calibrated on black and white plates. Measurements followed AMSA (2012) guidelines with 45 min of blooming. Also, pH was recorded on each steak (ThermoOrion 420Aplus; USA).

Water holding capacity (WHC) was determined following the filter paper press methodology described by Coria *et al.* (2020). The WHC was expressed as the percentage of free juice expelled (WHC = meat area / total liquid infiltrated area x 100). Cooking loss was determined by measuring the weight loss of samples after dry heat cooking (oven temperature: 170°C; sample thermal center temperature: 71°C) followed by 20 min of cooling at room temperature (5). The result was reported as a percentage of weight loss relative to the initial sample weight. Warner Bratzler shear force (WBSF) was assessed as described by Coria *et al.* (2020). Steak were cooked on a preheated electric grill (George Foreman, USA) to an internal temperature of 71°C. Eight cores (1.25 cm in diameter, 2.5 cm in height) per steak were removed parallel to the fibers, and WBSF was assessed with a TA-XT Plus® (Surrey, UK). The results were expressed in Newtons (N).

Data analysis

Linear models were adjusted considering feeding strategy as a fixed effect for productive, carcass, and meat quality traits. ANOVA was used to evaluate differences, and means were compared using the LSD test. For WBSF analysis, IMF content was initially included as a covariate. However, since no significant effect was found for the covariate ($p>0.05$), it was excluded from the model. Average daily gains were calculated through linear regression

models of LW as a function of days for each feeding strategy. Productivity per pasture surface and adjusted surface was estimated. Then, a linear model with the linear and quadratic components of the stocking rate was fitted to assess productivity as a function of stocking rate.

On the other hand, relationships between productive, carcass, and meat traits were evaluated using stepwise linear regression, including FT, REA, muscle lightness (L^*) and redness (a^*), IMF, and WBSF. The initial regressor variables were: CW, days on feed, total grain intake (TGI), and average daily gain (ADG) for FT and REA; CW, FT, ADG, and pH for muscle L^* and a^*; CW, days on feed, TGI, FT, and ADG for IMF content; and CW, pH, FT, IMF, WHC, and thawing losses for WBSF.

The models and analyses were carried out with the Infostat statistical program (6). All models used each group of steers as the experimental unit.

Results

Animal performance and feeding strategy productivity

The evolution of steers' LW under the different feeding strategies is shown in figure 1. Mean LW gains and final LW were different between treatments (table 4, page 132). FL strategy presented the highest LW gains, followed by Gr1.25, whereas the lowest gains were obtained with Gr0.70 and GrFL strategies. The final LW presented an inversed trend compared with LW gain, with 482.4, 464.9, 413.4, and 314.6 kg LW for GrFL, Gr0.70, Gr1.25, and FL, respectively.

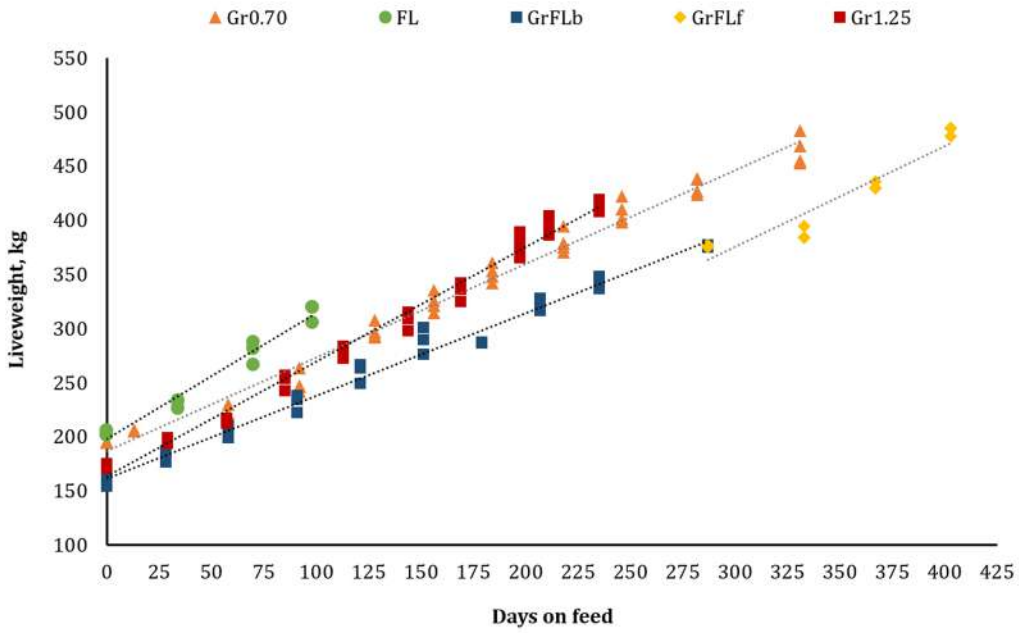


Figure 1. Live weight evolution of Holando Argentino steers under different feeding strategies.

Figura 1. Evolución del peso vivo de novillos Holando Argentino bajo diferentes estrategias de alimentación.

FL: Feedlot system ($y = 198.02 + 1.16\ x$, $R^2 = 0.97$, $p<0.001$); Gr1.25: Grazing finishing with 1.25 %LW/day of dry cracked maize supplementation ($y = 163.37 + 1.06\ x$, $R^2 = 0.99$, $p<0.001$); Gr0.70: grazing finishing with 0.70 %LW/day of dry cracked maize supplementation ($y = 186.42 + 0.87\ x$, $R^2 = 0.98$, $p<0.001$); GrFL: grazing background (GrFLb, $y = 161.26 + 0.76\ x$, $R^2 = 0.99$, $p<0.001$) and feedlot finishing (GrFLf, $y = 96.03 + 0.93\ x$, $R^2 = 0.89$, $p<0.001$). Values correspond to experimental unit means.

FL: terminación a corral ($y = 198{,}02 + 1{,}16\ x$, $R^2 = 0{,}97$, $p<0{,}001$); Gr1,25: invernada pastoril con suplementación al 1,25 %PV/día con grano de maíz partido seco ($y = 163{,}37 + 1{,}06\ x$, $R^2 = 0{,}99$, $p<0{,}001$); Gr0,70: invernada pastoril con suplementación al 0,70 %PV/día con grano de maíz partido seco ($y = 186{,}42 + 0{,}87\ x$, $R^2 = 0{,}98$, $p<0{,}001$); GrFL: recría pastoril (GrFLb, $y = 161{,}26 + 0{,}76\ x$, $R^2 = 0.99$, $p<0{,}001$) y terminación a corral (GrFLf, $y = 96.03 + 0{,}93\ x$, $R^2 = 0{,}89$, $p<0{,}001$). Los valores corresponden a las medias de cada unidad experimental.

Table 4. Animal performance and productivity.
Tabla 4. Desempeño animal y productividad.

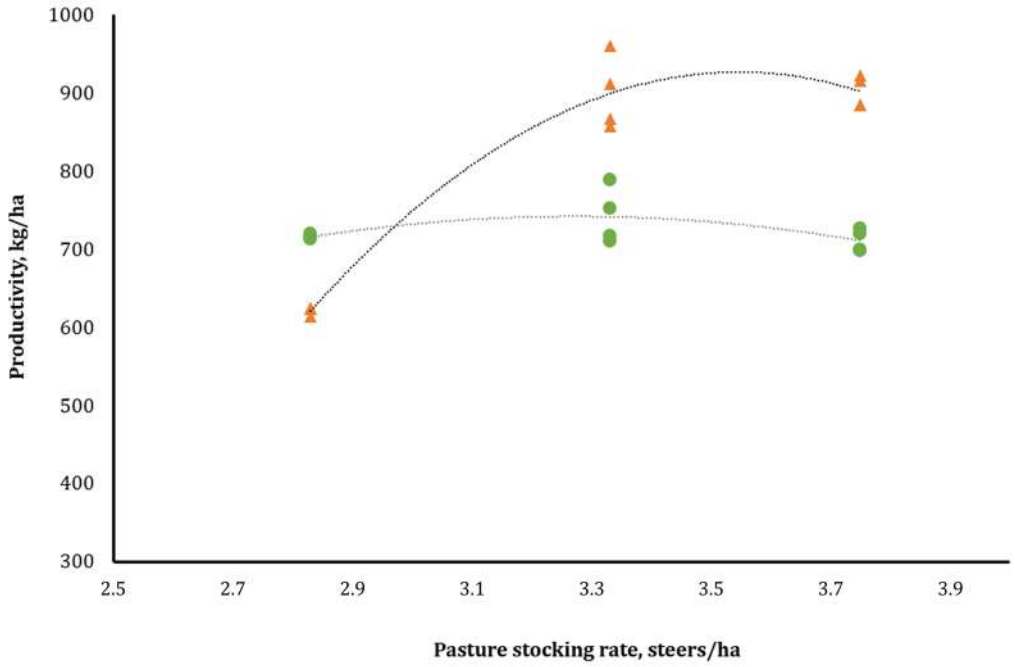
	FL	Gr1.25	Gr0.70	GrFL	*p-value*
Number of animals	30	36	32	30	-
Experimental units	3	4	4	3	-
Initial LW (kg)	202.9 ± 2.3 a	173.0 ± 1.9 c	195.0 ± 0.2 b	156.7 ± 3.2 d	< 0.001
Final LW (kg)	314.6 ± 8.3 d	413.4 ± 5.3 c	464.9 ± 14.0 b	482.4 ± 4.4 a	< 0.001
Days on feed	98	235	331	403	-
Grazing performance					
ADG (kg/day)	-	1.02 ± 0.02 a	0.82 ± 0.04 b	0.76 ± 0.01 c	< 0.001
Feedlot performance					
ADG (kg/day)	1.14 ± 0.07	-	-	0.92 ± 0.03	0.11
DMI (kg DM/day)	7.79 ± 0.35 b	-	-	12.54 ± 0.12 a	< 0.01
Feed conversion	6.85 ± 0.57 b	-	-	13.68 ± 0.33 a	< 0.01
Global performance					
ADG (kg/day)	1.14 ± 0.07 a	1.02 ± 0.02 b	0.82 ± 0.04 c	0.81 ± 0.01 c	< 0.001
Productivity (kg LW/ha pasture)	-	902 ± 20 a	899 ± 47 a	621 ± 6 b	< 0.001
Total grain intake (kg DM/animal)	643 ± 29 d	861 ± 9 b	764 ± 16 c	1224 ± 12 a	< 0.001
Productivity (kg LW/ha adjusted)	-	710 ± 15	741 ± 36	715 ± 4	0.24

Different letters indicate significant differences (p<0.05). FL: feedlot system; Gr1.25: grazing finishing with high supplementation; Gr0.70: grazing finishing with low supplementation; GrFL: grazing background and feedlot finishing; ADG: average daily gain, DMI: dry matter intake, LW: live weight.

Letras diferentes indican diferencias estadísticamente significativas (p<0,05). FL: terminación a corral; Gr1.25: invernada pastoril con alta suplementación; Gr0.70: invernada pastoril con baja suplementación; GrFL: recría pastoril y terminación a corral; ADG: aumento medio diario de peso vivo, DMI: consumo de materia seca, LW: peso vivo.

Grazing LW gain was highest for Gr1.25, followed by Gr0.70, while the grazing background phase of GrFL showed the lowest LW gains. Whereas feedlot finishing LW gain was not different between FL and GrFL (p=0.11). However, DMI was higher in GrFL than in FL (12.54 vs. 7.79 kg DM, p<0.01), as well as feed conversion (13.68 vs. 6.85, p<0.01).

Productivity per pasture surface was higher for supplemented grazing strategies (Gr1.25 and Gr0.70) than the background phase from GrFL. When productivity estimations included feedlot finishing of GrFL (both production and surface needed for feedlot diet ingredients) and the surface needed for Gr1.25 and Gr0.70 supplements supply, no significant differences were obtained (p=0.239). In all cases, adjusted productivity ranged between 710 and 741 kg LW/ha, presenting no response to the increase in stocking rates (figure 2, page 133).



Productivity per pasture surface (kg/ha, oranges triangles, y = -6570.60 + 4228.39 x - 596.22 x^2, R^2 = 0.96, *L*: p<0.001; *Q*: p<0.001) and adjusted productivity (kg/ha, green circles, y = -713.63 + 889.89 x - 136.04 x^2, R^2 = 0.32, *L*: p=0.097; *Q*: p=0.094) as a function of stocking rate. Surface adjustment was done considering maize grain equivalents used for supplementation in Gr1.25 and Gr0.70, and for pen feeding in GrFL. The maize crop surface needed to supply the grain equivalents used was calculated considering a mean yield of 12.000 kg/ha. *L*: significance of the linear component of the model; *Q*: significance of the quadratic component of the model. Values presented correspond to experimental unit means.

Productividad por superficie de pasturas (kg/ha, triángulos naranjas, y = -6570.60 + 4228.39 x - 596.22 x^2, R^2 = 0.96, *L*: p<0.001; *Q*: p<0.001) y productividad por superficie ajustada (kg/ha, círculos verdes, y = -713.63 + 889,89 x - 136,04 x^2, R^2 = 0,32, *L*: p=0,097; *Q*: p=0,094) en función de la carga animal. El ajuste de superficie se realizó considerando equivalentes de grano de maíz utilizados para suplementación en Gr1.25 y Gr0.70, y para la terminación a corral en GrFL. La superficie de cultivo de maíz necesaria para abastecer los equivalentes de grano utilizados se calculó considerando un rendimiento medio de 12.000 kg/ha. *L*: significancia de la componente lineal del modelo; *Q*: significancia del componente cuadrático del modelo. Los valores presentados corresponden a las medias de cada unidad experimental.

Figure 2. Productivity per pasture surface and adjusted productivity as a function of stocking rate.

Figura 2. Productividad por superficie de pasturas y por superficie ajustada en función de la carga animal.

Carcass and meat quality

Carcass characteristics and meat quality are shown in table 5 (page 134). GrFL and Gr0.70 feeding strategies presented the highest CW, followed by Gr1.25, while FL presented the lowest (p<0.001). FT did not differ between feeding strategies (p>0.05), whereas GrFL REA was larger than that of Gr1.25 and FL (p<0.05).

Regarding color parameters, meat from Gr0.70 was the only one to show differences, with lower *L** (p<0.01) and higher *a** than Gr1.25, GrFL, and FL (p<0.05). No differences were observed for the subcutaneous fat color parameters (p>0.05).

Meat hardness, estimated by *longissimus thoracis* WBSF, presented no differences between feeding strategies, nor for WHC nor losses due to thawing or cooking (p>0.05). IMF content of the *longissimus thoracis* was higher for GrFL than Gr1.25, Gr0.70, and FL (p<0.05).

Relationship between productive, carcass, and meat traits

FT was explained by TGI and ADG (table 6, page 134), whereas REA was explained by CW and days on feed. On the other hand, IMF content of the *longissimus thoracis* was explained by TGI as the only trait retained by the model (R^2 = 0.63).

In relation to muscle color, *L** was explained by FT and CW (R^2 = 0.68), while *a** was explained by ADG and FT (R^2 = 0.68). WBSF was explained by thawing losses, WHC, and FT as variables kept by the model (R^2 = 0.81).

Table 5. Carcass characteristics and meat quality.
Tabla 5. Características de res y calidad de carne.

	FL	Gr1.25	Gr0.70	GrFL	*p-value*
Number of carcasses	12	12	12	12	-
Experimental units	3	4	4	3	-
Carcass characteristics					
Carcass weight (kg)	197.9 ± 11.5 c	240.4 ± 9.6 b	267.8 ± 12.2 a	288.3 ± 5.0 a	<0.001
FT (mm)	8.3 ± 2.2	10.3 ± 2.6	6.6 ± 3.3	12.3 ± 3.7	0.16
REA (cm^2)	50.5 ± 1.2 b	51.7 ± 1.9 b	54.2 ± 4.0 ab	57.6 ± 2.7 a	0.04
Meat quality					
Thawing losses (%)	2.32 ± 0.55	1.48 ± 0.86	1.20 ± 0.77	1.28 ± 0.79	0.31
pH	5.64 ± 0.07	5.66 ± 0.06	5.66 ± 0.06	5.61 ± 0.03	0.59
Muscle color					
*L**	36.2 ± 0.2 a	36.0 ± 0.6 a	33.7 ± 0.7 b	36.2 ± 0.8 a	<0.01
*a**	18.9 ± 1.1 b	18.6 ± 1.3 b	22.5 ± 2.1 a	19.9 ± 0.3 b	0.02
*b**	11.1 ± 0.5	11.6 ± 0.6	12.3 ± 1.4	11.6 ± 0.6	0.39
Subcutaneous fat color					
*L**	72.6 ± 0.8	71.7 ± 2.1	73.4 ± 1.8	71.4 ± 2.6	0.52
*a**	4.6 ± 1.3	3.0 ± 0.7	5.9 ± 3.5	5.7 ± 2.1	0.33
*b**	11.4 ± 0.5	13.0 ± 1.4	12.1 ± 2.8	12.5 ± 0.3	0.66
Intramuscular fat content (%)	2.52 ± 0.55 b	3.10 ± 0.60 b	3.18 ± 0.66 b	4.86 ± 0.93 a	0.02
WHC (%)	30.9 ± 2.3	31.0 ± 2.7	28.4 ± 0.6	30.1 ± 1.9	0.29
Cooking losses (%)	22.8 ± 3.0	22.0 ± 2.8	22.8 ± 3.5	22.4 ± 2.5	0.97
WBSF (N)	29.1 ± 2.8	31.5 ± 4.2	29.5 ± 3.9	29.2 ± 3.3	0.60

Different letters indicate significant differences (p<0.05). FL: feedlot system; Gr1.25: grazing finishing with high supplementation; Gr0.70: grazing finishing with low supplementation; GrFL: grazing background and feedlot finishing, FT: 12th rib fat thickness, REA: ribeye area, *L**: lightness, from black (0) to white (100), *a**: redness, from green (negative values) to red (positive values), *b**: yellowness, from blue (negative values) to yellow (positive values), WBSF: Warner Bratzler shear force, WHC: water holding capacity.

Letras diferentes indican diferencias estadísticamente significativas (p<0,05). FL: terminación a corral; Gr1.25: invernada pastoril con alta suplementación; Gr0.70: invernada pastoril con baja suplementación; GrFL: recría pastoril y terminación a corral; FT: espesor de grasa dorsal en la 12° costilla, REA: área de ojo de bife, *L**: luminosidad, desde negro (0) a blanco (100), *a**: desde verde (valores negativos) a rojo (valores positivos), *b**: desde azul (valores negativos) a amarillo (valores positivos), WBSF: resistencia al corte de Warner Bratzler, WHC: capacidad de retención de agua.

Table 6. Relationship between productive, carcass, and meat traits.
Tabla 6. Relación entre parámetros productivos, de res y de calidad de carne.

Item	Intercept	Explanatory variables	Partial slopes	SE	*p-value*	R^2
FT (mm)	-18.69	Total grain intake, kg	0.01	4.1×10^{-3}	< 0.01	0.52
		ADG, kg/day	15.83	6.52	< 0.05	
REA (cm^2)	15.7	Carcass weight, kg	0.19	0.07	< 0.05	0.62
		Days on feed	-0.03	0.02	< 0.15	
IMF (%)	-3.4×10^{-3}	Total grain intake, kg	3.9×10^{-3}	8.7×10^{-4}	< 0.001	0.63
Muscle *L**	37.71	FT, mm	0.28	0.06	< 0.01	0.68
		Carcass weight, kg	-0.02	0.01	< 0.05	
Muscle *a**	32.92	ADG, kg/day	-10.35	2.75	< 0.01	0.68
		FT, mm	-0.26	0.11	< 0.05	
WBSF (N)	3.08	Thawing Losses, %	-3.02	0.6	< 0.001	0.81
		WHC, %	1.23	0.25	< 0.001	
		FT, mm	-0.59	0.15	< 0.01	

FT: 12th rib fat thickness, REA: ribeye area, *L**: lightness, from black (0) to white (100), *a**: redness, from green (negative values) to red (positive values), WHC: water holding capacity of the *longissimus thoracis*, IMF: intramuscular fat content of the *longissimus thoracis*, ADG: average daily gain, WBSF: Warner Bratzler shear force.

FT: espesor de grasa dorsal en la 12° costilla, REA: área de ojo de bife, *L**: luminosidad, desde negro (0) a blanco (100), *a**: desde verde (valores negativos) a rojo (valores positivos), WHC: capacidad de retención de agua del *longissimus thoracis*, IMF: contenido de grasa intramuscular del *longissimus thoracis*, ADG: aumento medio diario de peso vivo, WBSF: resistencia al corte de Warner Bratzler.

Discussion

Animal performance and feeding strategy productivity

Feeding strategies with higher energy supplementation levels resulted in greater LW gains during grazing. This was expected as pasture allocations were within the range of response to supplementation suggested by Beretta *et al.* (2006).

Comparing grazing strategies (Gr1.25 and Gr0.70), the higher LW gains of Gr1.25 led to faster fattening rates and earlier slaughters at lower LW than Gr0.70. Similarly, Manni *et al.* (2013) reported that increasing concentrate supplementation in 1 kg DM/day improved growth rates by 0.073 kg LW/day and 0.048 kg CW/day in growing dairy bulls. The authors also reported an increase in CW and a slight increase in carcass fatness, suggesting that concentrate supplementation improves growth and carcass fat deposition (25).

In the feedlot finishing phases, the higher DMI and the lower feed efficiency in GrFL compared with FL align with Lancaster *et al.* (2014), who compared calf-fed versus yearling systems. They suggested that lower feed efficiency of steers entering the feedlot older and heavier could result from higher maintenance requirements and higher fat composition in LW gain. This effect could be steeper in dairy breeds due to larger and more metabolically active organs than beef breeds (3). Moreover, GrFL steers were fed beyond the 8.0 mm subcutaneous FT endpoint (12.3 mm), which must have contributed to the decay in feed efficiency as reported by Zurbriggen *et al.* (2022).

In contrast to Lancaster *et al.* (2014), this study found no higher LW gains in backgrounded (GrFL) steers compared to FL steers. This may explain the lack of adjusted productivity advantages for GrFL relative to Gr0.70 and Gr1.25, since the increase in productivity through feedlot finishing relies on the high LW gains expected during this period.

Supplemented grazing strategies (Gr1.25 and Gr0.70) showed higher pasture productivity than not supplemented GrFL background phase, due to higher LW gains and stocking rates. However, pasture productivity was similar between Gr1.25 and Gr0.70, since the higher LW gain and stocking rate of Gr1.25 was offset by the shorter feeding period and the lower LW at slaughter.

When productivity was estimated, including the feedlot finishing period from the GrFL strategy and the adjustments for grain equivalents, no differences were found between GrFL, Gr1.25, and Gr0.70. All strategies achieved adjusted productivities between 700 and 750 kg LW/ha, corresponding with high productivity levels for intensified grazing systems. However, GrFL productivity was below the 1000 kg LW/ha previously reported for grazing background and feedlot finishing systems from the Argentine pampas (17, 22), which may compromise the strategy's viability.

Carcass characteristics and meat quality

Morales Gómez *et al.* (2022) compared feedlot and pasture systems with different LW gain targets (1.50 and 0.90 kg/day for feedlot and 0.90 and 0.60 kg/day for pasture) and found the highest FT in the steers from the feedlot system targeting high LW gains (1.50 kg/day). Furthermore, Morales Gómez *et al.* (2022) reported that pasture systems and feedlots targeting low LW gains (0.90 kg/day) presented FT at slaughter lower than 6 mm, which may have threatened meat quality (33). While grazing systems reported by these authors achieved LW gains above 0.60 kg/day, large variations in gain during the feeding period may have reduced fattening rate and resulted in leaner carcasses.

In the present study, the grazing strategies achieved higher mean LW gains (0.82 and 1.02 kg/day for Gr0.70 and Gr1.25, respectively) with low variations in LW gain through the feeding period (figure 1, page 131). These results explain the proper FT reached at slaughter. Consistent LW gains, supported by strategic supplementation and well-managed forage allowances, are key to achieving proper productivity and meat quality in grazing finishing systems. In this sense, even though FT did not differ between the feeding strategies, FT variations were explained by TGI and LW gain.

The increase in REA with higher CW was also found in previous research with beef steers (4, 8, 43). Gr0.70 and GrFL strategies achieved the highest REA due to the longer feeding periods together with moderate LW gains, which allowed the higher CW and muscle growth.

In this study, TGI mainly explained the IMF content of the *longissimus thoracis*, consistent with previous research showing increased marbling with the inclusion of high-starch diets. Testa (2017) found that marbling score was increased by including high-starch diets during the finishing of beef steers. Garcia *et al.* (2008) also indicated that diet was a determinant for IMF deposition in beef and dairy steers, with no differences between breeds. In addition, Manni *et al.* (2018) found that increasing energy intake and carcass fatness increased the IMF content of the *longissimus lumborum* in dairy bulls.

Despite using the same finishing diet, the difference in IMF between FL and GrFL was expected. Pethick *et al.* (2004) suggested that IMF deposits linearly between a CW of 200 and 400 kg. In the FL strategy, its short duration with no previous background led to lighter carcasses with a mean CW below 200 kg. In contrast, the pasture-based background of the GrFL strategy allowed a higher CW at feedlot entry. This could have allowed coupling the finishing period with high starch diets with the phase of linear increase in IMF proposed by Pethick *et al.* (2004).

In this sense, Lancaster *et al.* (2014) suggested that achieving moderate gains during long stocker phases could improve marbling at constant FT by reaching heavier placement weights at feedlot finishing. However, this could only apply to strategies including feedlot finishing. Whereas for grazing strategies, LW gains need to be high enough to ensure sufficient fat accretion and efficient feeding duration.

Meat color is one of the most important meat attributes since it defines consumers' purchase decisions (39). Subcutaneous FT influences carcass chilling rate and pH drop, and ultimately adequate meat pH, as major factors defining meat color (13). Page *et al.* (2001) proposed a 7.6 mm FT threshold to attain bright meat, which aligns with this study's results. The lower L^* obtained with the Gr0.70 strategy could be attributed to 6.6 mm FT reached, which was below this threshold.

Grass-finished Holando Argentino beef could have acceptable color if steers had enough fatness at slaughter. In this study, the increase in dry cracked maize supplementation from 0.70 to 1.25% LW/day resulted in higher L^* and lower a^*. The use of different feeding strategies can allow for targeting different fat endpoints and attaining the meat characteristics that consumers demand.

Despite all feeding strategies being contrasted in diet, weight, and age, muscle color parameters were within the light and medium meat color range (13). Meat a^* was above the 14.5 threshold for acceptability (11). In all cases, meat pH was within the normal range, suggesting that glycogen levels were enough in all strategies (13, 33).

Although the contrasting differences in feeding strategies, there were no differences in fat color. Fat yellowness (b^* value) is a major trait defining purchase decisions since it is undesirable for most consumers from markets of different countries (9). Fat b^* was between 11.4 and 13.0, lower than the 19.2 mean reported for grazing steers (27) and similar to the 14.1 mean reported for feedlot steers in Argentina (42).

Usually, pasture feeding increases b^* due to the higher carotene content in fresh pastures compared to concentrates. The lack of differences between strategies in this study may be due to the high LW gains of the grazing strategies. In this sense, maintaining high LW gains may have diluted carotenoids with subcutaneous fat accretion (9).

Shear force was explained by thawing losses, water holding capacity, and FT. However, the low change rate in shear force per mm FT was explained by all strategies achieving at least 6.6 mm of mean FT. This fat coverage was above the threshold proposed by Savell *et al.* (2005) and slightly below the 7.6 mm FT threshold proposed by Dolezal *et al.* (1982) to obtain tender meat.

Morales Gómez *et al.* (2022) found differences in meat WBSF between grazing and feedlot-finished steers. These differences were attributable to different muscle pH for feedlot and grazing animals (5.62 and 5.97, respectively) since final muscle pH of grass-fed animals could be associated with dark, firm, and dry meat. This evidences the low FT reached by this feeding system and differs from the FT obtained in the present study for grazing steers, which attained 6.6 mm of mean FT.

Previous research has proposed different WBSF threshold values for consumer unacceptability. Platter *et al.* (2003) suggested 43.12 N, while Miller *et al.* (2001) suggested 55.9 N. In the present study, meat from all strategies could be considered tender since WBSF values were below these thresholds.

The higher IMF content reached under the GrFL strategy did not affect *longissimus thoracis* shear force. According to previous research, IMF content explains only 17% of sensory panel tenderness variation (32). Moreover, Zurbriggen *et al.* (2022) reported that once 8.0 mm FT was reached, the increase in IMF from 2.7 to 7.3% only tended to reduce WBSF in British feedlot steers. However, the increase in marbling must not be belittled since it could improve juiciness and flavor (40) and may be needed to access some export markets.

Conclusion

Grazing finishing strategies for HA steers must achieve and maintain high LW gains to attain the fatness required to guarantee meat tenderness and reduce fat yellowness. Energetic supplementation can be used to achieve this but also to manipulate slaughter LW and IMF to meet different market demands.

Incorporating a grazing rearing phase before feedlot entry to increase the placement weight can increase IMF content, which is relevant for certain export markets. This strategy, however, presented the highest TGI and low feed efficiency, making its viability dependent on pricing conditions in the export market.

Overall, HA steers have the flexibility to produce high-quality meat under different feeding strategies. Production systems can strategically use maize grains as a supplement or in feedlot diets, managing stocking rates, LW gains, and finishing endpoints to achieve high productivity and also manipulating marbling and FT to obtain meat quality that different markets demand.

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Nutritional and morpho-anatomical characterization of *Phyllostachys aurea* (Poaceae, Bambusoideae, Bambuseae) foliage for Argentine livestock systems

Caracterización nutricional y morfo-anatómica del follaje de *Phyllostachys aurea* (Poaceae, Bambusoideae, Bambuseae) para sistemas ganaderos en Argentina

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Abstract

Bamboo cultivation in Argentina could represent a major economic activity if its various applications were revealed. This study characterized the anatomy and micromorphology of leaf blades by optical and scanning electron microscopes. Foliage leaves presented predominant parenchyma and scarce sclerenchyma. Foliage chemical and biological composition were analyzed in 3 populations of *P. aurea* sampled in two contrasting seasons of the year. The six samples evaluated showed 13% protein, adequate for ruminant feed. Neutral detergent fiber (aNDFom) was approximately 60% DM, a probable limiting factor for consumption. Significant differences in ADFom (acid detergent fiber) and ADLom (acid detergent lignin) favored spring results, with lower values than winter results. The presence of silica in different cell types could limit digestion. Fermentation kinetics indicated that dry matter digestibility is close to 50%, and higher in spring given lower amounts of indigestible components. In addition, all samples analyzed had a low content of immediately soluble material and a high content of potentially fermentable insoluble material. Anatomy and chemical-nutritional characterization allow *P. aurea* foliage to be considered in ruminant feeding.

Keywords
woody bamboo • nutritional value • leaf anatomy and morphology • ruminant feed

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Resumen

El cultivo de los bambúes en la Argentina no ha alcanzado la relevancia merecida, pudiendo representar un valor económico más importante si se hicieran conocer sus diversas aplicaciones. En el presente trabajo, se caracterizó la anatomía y micromorfología de las láminas mediante microscopio óptico y microscopio electrónico de barrido. Se analizó la composición química y biológica del follaje en 3 poblaciones de *P. aurea* muestreadas en dos estaciones contrastantes del año. Las hojas del follaje presentaron una predominancia de tejido parenquimático sobre el esclerénquima, el cual fue escaso. La presencia de sílice en diferentes tipos celulares podría limitar la digestión. El promedio de proteína bruta fue 13% para las seis muestras analizadas y resulta aceptable para alimentación de rumiantes en mantenimiento. A su vez, se observó un contenido de aFDNmo (Fibra en detergente neutro) del 60%bs, lo cual puede ser limitante para el consumo. Se identificó una diferencia significativa en FDAmo (Fibra en detergente ácido) y LDA mo (Lignina en detergente ácido) entre invierno y primavera, favorable a la primavera con menores valores de dichos analitos. La cinética de fermentación indicó que la digestibilidad de la materia seca es cercana al 50%, siendo mayor en primavera dada la menor cantidad de componentes indigestibles. Además, para todas las muestras analizadas, el contenido de material inmediatamente soluble fue bajo, mientras que el material insoluble potencialmente fermentable fue elevado. Los resultados del análisis anatómico, complementado con la caracterización químico nutricional, permiten considerar al follaje de *P. aurea* en la alimentación de rumiantes.

Palabras clave

bambú leñoso • valor nutricional • anatomía y morfología de las hojas • alimentación para rumiantes

Introduction

The millennial cultivation of woody bamboo in Southeast Asia has recently gained attention in some tropical and subtropical countries of America (*e.g.* Colombia, Ecuador, Bolivia, Brazil and Chile (Londoño, 2009). Parodi (1943) stated that bamboo cultivation in Argentina had not reached its productive and economic potential. Its development should be encouraged by revealing its various applications. Woody bamboo provides human feed (24), and materials for utensils, cosmetic products, and crafts (22, 28, 30). Other uses are related to forage, paper pulp, fibers, biochar and pyrolysis compounds (1, 2, 3, 10).

Woody bamboo has rapid vegetative growth from vigorous rhizomes (27). Clumps can be used after 3 to 4 years of implantation. In addition to high biomass production, wide adaptability and evergreen leaves make them potential candidates in forage production (40). After rational culm cutting, bamboo gives annual harvests for 30 to 120 years, depending on the species (17, 18). The vegetative growth phase of *Phyllostachys aurea* Carrière ex Rivière & C. Rivière lasts 15 to 30 years, followed by flowering (30, 37) with no clump death. In Argentina, this exotic species is cultivated for ornamental purposes and its culms are used in construction and crafts, while shoots are edible (39, 48). Bamboo foliage is an alternative forage for domestic cattle. However, information on its chemical-nutritional composition as feed for ruminants is scarce (29). Other countries have identified several genotypes, growing sites, and seasons for forage variability (6, 34, 40, 47). Biomass production occurs in the temperate and rainy season, with growth impeded in winter, under 10°C (19).

Panizzo *et al.* (2017) nutritionally evaluated the foliage of the native woody bamboo *Guadua chacoensis* (Rojas Acosta) Londoño & P. M. Peterson and suggested its use as grazing supplement. In southern Argentina, *Chusquea culeou* E. Desv. ("caña colihue") constitutes the main winter forage for bovine diet while other grasses remain covered in snow (25). We aimed to nutritionally and morpho-anatomically characterize *P. aurea* foliage for ruminant feed.

Materials and methods

Selection and collection of material

We used foliage leaves from 3 populations of the woody bamboo *P. aurea*: Lucien Hauman (S: 34°35'; E: 58°28'), Arturo Ragonese (S: 34°36'; E: 58°40') Botanical Gardens and wild bamboo from Buenos Aires Delta (S: 34°24'; E: 58°33'). We sampled in two contrasting production seasons, spring (October 2017) and winter (July 2018). Nine culms were harvested at each location (*i.e.* 3 culms × 3 bamboo sites per harvest). A pool of foliage leaves was used in nutritional characterization of each population. Additionally, segments of the middle portion of blades were selected for anatomical and micromorphological studies.

The climate was humid temperate (figure 1) (20). Monthly rainfall and mean temperature did not restrict clumps growth of *P. aurea*. However, summer in 2018 was drier with rains accumulated in May and June.

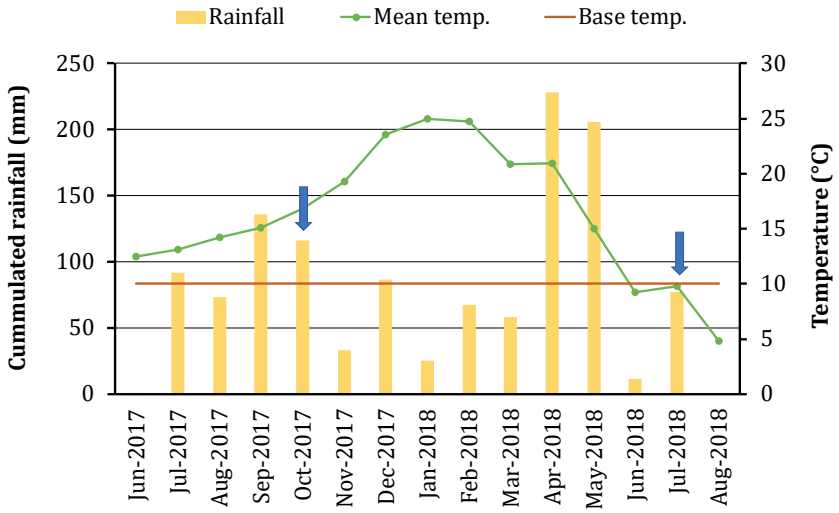


Figure 1. Average temperatures and cumulated rainfall during the months before *Phyllostachys aurea* foliage harvest (20); and growth base temperature of bamboo species according to Halvorson *et al.* (2010).

Figura 1. Temperaturas promedio y precipitaciones acumuladas durante los meses antes de la cosecha del follaje de *Phyllostachys aurea* (20) y temperatura base de crecimiento de especies de bambú según Halvorson *et al.* (2010).

Morphological and anatomical studies

The species' growth habit and leaf size, consistency, and indumentum followed McClure (1966) and Judziewicz *et al*. (1999) terminology. Foliage leaf blades collected in spring were dehydrated in alcohol series and embedded in paraffin following traditional anatomical techniques (12). Twenty µm-thick sections were cut with a rotary microtome and stained with safranine-Fast green. The following anatomical traits were considered: midrib and keel (position, vascular bundles, adaxial and abaxial sclerenchyma), abaxial and adaxial epidermis, bulliform cells, arm cells and intercellular spaces [sometimes referred to as "fusoid cells" (42), described in accordance to Ellis (1976, 1979) and Clark (2005). Observations were made with a Nikon® Microphot FXA optical microscope (Tochigi, Japan). Blade micromorphology was studied with a Scanning Electron Microscope (SEM, Phillips XL 30 Microscope (Phillips, The Netherlands). To describe abaxial and adaxial epidermal traits, small blade fragments were cleaned in xylene for 1.5 h with an ultrasonic cleaner (Cleanson, model CS 1106, Argentina). The material was air-dried, mounted, and coated

with a gold-palladium (40%-60%) alloy by a Thermo VGScientific, then observed using a Phillips XL 30 Scanning Electron Microscope (MACN-CONICET, Argentina). Papillae pattern in long cells follows (49). Foliar anatomy and micromorphology related content of sclerenchyma, silica cells, macro and micro hairs, prickle and hooks, with possible forage acceptability. To quantify phytoliths and describe their association in each period and locality, articulated (*i.e.*, more than two elements) and non-articulated elements (*i.e.*; a single element per morphotype), were considered (15). Morphotype description involved an *ad hoc* classification based on the proposals of Neumann *et al.* (2019).

Chemical-nutritional evaluation

Determinations included: dry matter (DM) by oven-drying at 105°C, ashes by incineration at 550°C (Cen; AOAC, 1990, Nº. 942.05), crude protein (CP; AOAC, 1990, Nº. 984.15), neutral and acid detergent fibers (42) using α-amylase and ash free (aNDFom and aADFom, respectively), and ash free acid detergent lignin (ADLom). Silica was determined by calcination technique (Si; Labouriau, 1983).

The *in vitro* evaluation of gas production (GP) followed the recommendations of Wawrzkiewicz *et al.* (2005). Measurements were made at regular intervals (*i.e.* 2, 4, 6, 8, 12, 16, 20, 24, 36, 48, 60, and 72 h). In addition, samples were incubated for up to 48 h to recover undigested residues in ANKOM® F57 filter bags determining DM digestibility (DMD_{iv}) and NDF ($NDFD_{iv}$). Cumulative net gas production (CNGP) and digestibility were corrected for blank (*i.e.* bottles without substrate) and incubated dry matter.

GP kinetics were adjusted to the equation $CNGP = a + b \times (1 - e^{-ct})$ (35). CNGP (ml/g DM) was determined at 12, 24, 48 and 72 h, parameters a, b and c, maximum hourly rate of GP (RGP_{Max}; ml/g DM.h) and the time at which it occurs (T_{RGPmax}; h) and average rate gas production (RGP_{Av}).

Statistical analysis

Comparisons were made between spring and winter considering the three populations as replicates. The data were analyzed with SAS® statistical program. Seasons were compared by a completely randomized one-way design, with no interactions. A Tukey test compared means at $p < 0.05$.

GP rates were compared via a model considering time (T) as a repeated measure and the interaction with season (S × T) with a completely randomized two-way design with interaction. A Tukey test compared means at $p < 0.05$.

Results

Morphological studies

The studied clumps were 2-9 m tall (figure 2A, page 144). Rhizome axillary buds formed edible shoots (figure 2B, page 144) and produced aerial culms. These were woody, hollow, 1-5 cm in diameter, with grooved internodes on the side of the bud, upper nodes spaced apart, and the lower ones asymmetric and proximate (figure 2C and figure 2D, page 144). Bamboos have culm leaves and foliage leaves. The formers have a protective function (figure 2B, page 144) and at maturity are deciduous, constituting abundant litter on the soil surface. Foliage leaves are photosynthetic and have perennial lanceolate leaf blades, 5-16 × 1-2 cm (figure 2D, page 144).

A: habit. B: young shoot. C: nodes and internodes of culm middle portion. D: basal nodes, internodes and foliage leaves. Scale bars: A: 2 m; B: 3 cm; C: 12 cm; D: 1.5 cm.

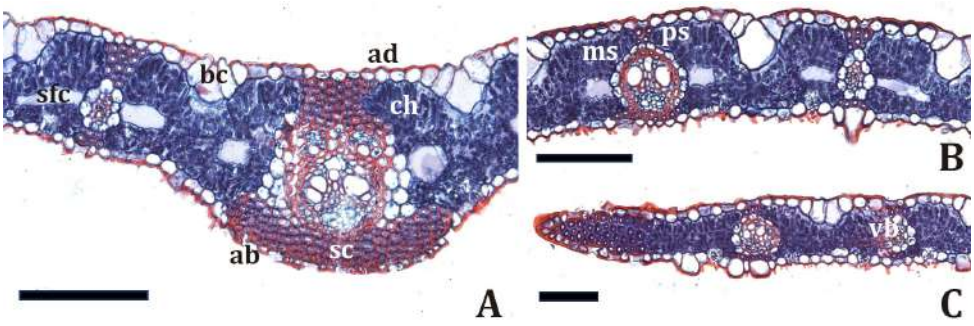
A: hábito. B: turión. C: nudos y entrenudos de la parte media de las cañas. D: nudos, entrenudos basales y hojas del follaje. Escalas: A: 2 m; B: 3 cm; C: 12 cm; D: 1,5 cm.

Figure 2 / Figura 2. *Phyllostachys aurea*.

Anatomical studies

In cross-section, foliage leaf blades present characteristic C_3 anatomy. Well-developed midrib, keel projecting abaxially, with a first-order vascular bundle surrounded by a double sheath consisting of an outer parenchyma sheath and inner mestome sheath, locked towards both surfaces (figure 3A). The adaxial epidermis exhibited a single layer of smooth-walled cells and groups of fan-shaped bulliform cells. The abaxial epidermis was papillose and formed by various cell types (*i.e.*, long cells, suberous cells, silica cells, and hooks, (figure 3A, 3B and 3C).

Chlorenchyma was diffuse and exhibited large intercellular spaces between fusoid cells, and on both sides of the vascular bundles in contact with parenchyma sheath. Arm cells are thin-walled and asymmetrically invaginated on the abaxial side. Scarce sclerenchyma. Girders associated with primary and secondary vascular bundles and strands at blade edges (figure 3C). In superficial view, leaf blades with SEM showed marginal hooks, smooth adaxial surface (figure 4A and 4B page 145) and abaxial epidermis formed by a diversity of cell types among which were long cells with short papillae, stomatal complex slightly sunken with elongated papillae of the long contiguous cells overarching the stomata (subtype IV), silica cells, suberous cells, bicellular microhairs, hooks and prickles (figure 4C and 4D page 145). The phytolytic association of all analyzed materials presented a greater abundance of morphotypes accompanying leaf epidermis: silica cells, suberous cells, microhairs, hooks, prickles, and fan-shaped bulliform cells (figure 5A, 5B and 5C, page 145). To a lesser extent, prismatic morphotypes with wavy edges derived from long cells and short cylindrical elements (figure 5A and 5B, page 145).

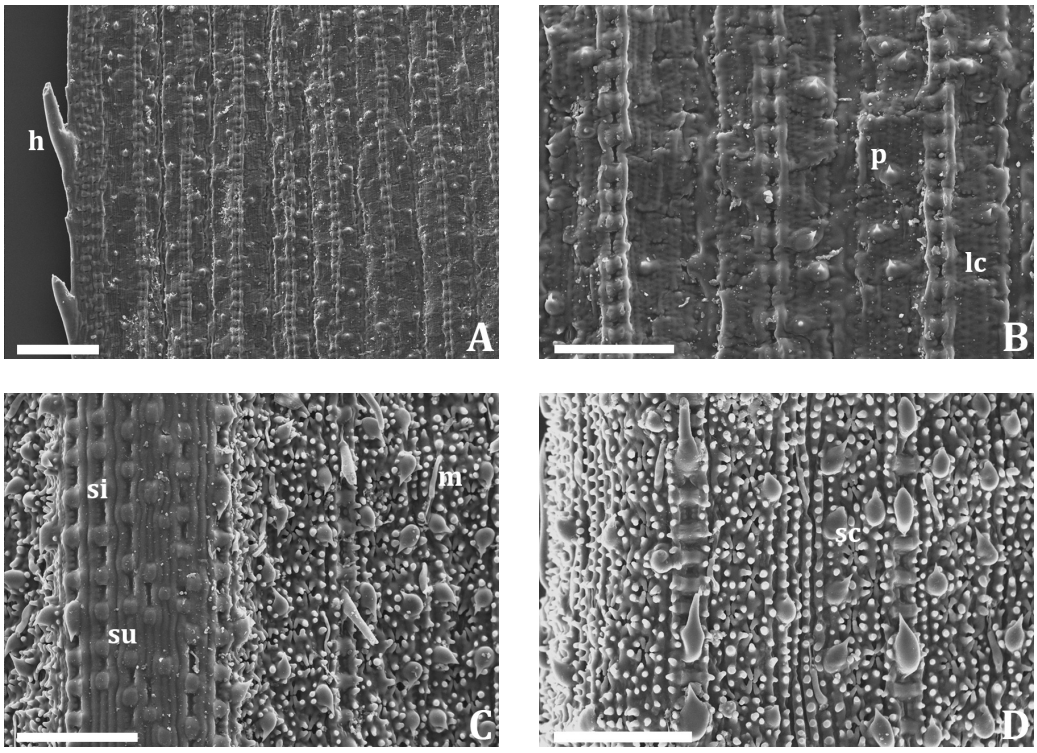


A: keel. B: arm. C: margin. ad: adaxial epidermis; ab: abaxial epidermis; bc: bulliform cells; ch: chlorenchyma; ms: mestome sheath; ps: parenchyma sheath; sc: sclerenchyma; sfc: spaces between fusoid cells; vb: vascular bundle. Scale bars: 100 µm.

A: costilla central. B: ala. C: margen. ad: epidermis adaxial; ab: epidermis abaxial; bc: células buliformes; ch: clorénquima; ms: vaina mestomática; ps: vaina parenquimática; sc: esclerénquima; sfc: espacios entre células fusoides; vb: haz vascular. Escalas: 100 µm.

Figure 3. Foliage leaf blade cross-section in *Phyllostachys aurea*.

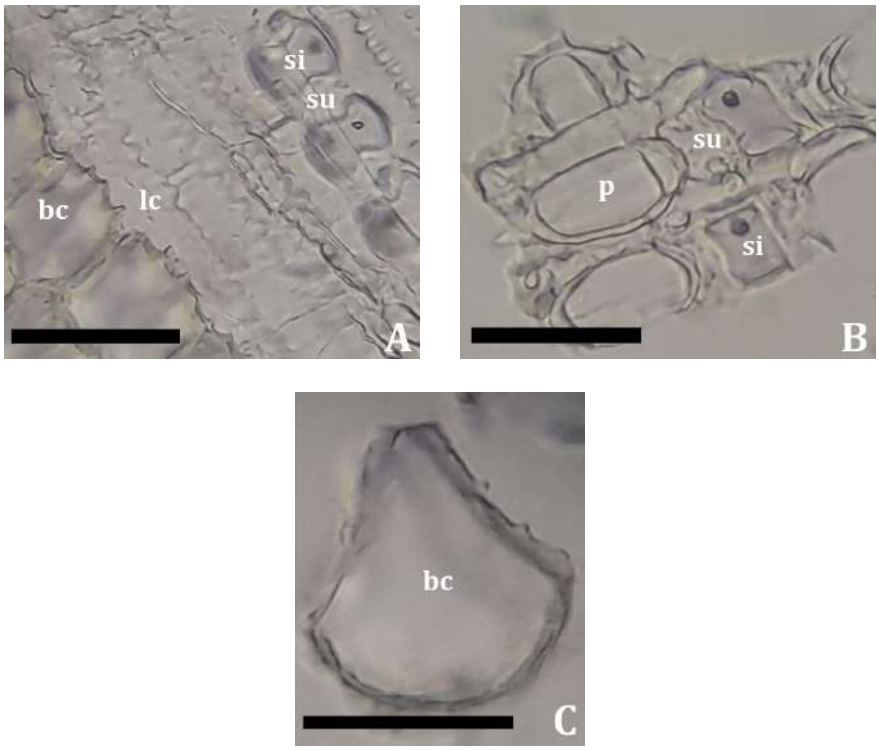
Figura 3. Lámina de *Phyllostachys aurea* en transcorte.



A-B: adaxial epidermis. C-D: abaxial epidermis. h: hook; lc: long cell; m: bicellular microhair; p: prickle; sc: stomatal complex; si: silica cell; su: suberous cell. Scale bars: A: 200 µm; B-D: 100 µm.

A-B, epidermis adaxial. C-D, epidermis abaxial. h, aguijón; lc, célula larga; m, micropelo bicelular; p, gancho; sc, aparato estomático; si, célula silícea; su, célula suberosa. Escalas: A, 200 µm; B-D, 100 µm.

Figure 4. Leaf blade of *Phyllostachys aurea* observed with a Scanning Electron Microscope.
Figura 4. Lámina de *Phyllostachys aurea* observada con Microscopio Electrónico de Barrido.



A: Fragment of leaf blade adaxial epidermis, general view. B: Prickles, silica and suberous cells, detail. C: Fan-shaped bulliform cell. bc, bulliform cell; lc, long cell; p, prickle; si, silica cell; su, suberous cell. Scale bars: 50 µm.

A: Fragmento de epidermis adaxial de la lámina foliar, vista general; B: Detalle de aguijones, células silíceas y suberosas. C: Célula buliforme con forma de abanico. bc, célula buliforme; lc, célula larga; p, gancho; si, célula silícea; su, célula suberosa. Escala: 50 µm.

Figure 5 / Figura 5. *Phyllostachys aurea.*

Chemical-nutritional evaluation

P. aurea did not present differences between seasons in DM, CP and aNDFom. Averages exceeded 500 g/kg HM and 133 and 623 g/kg DM of CP and aNDFom, respectively (table 1). The inorganic fraction reached 193 g/kg DM while 81% of leaf blades constituted organic matter. Structural carbohydrates predominated with a low concentration of water-soluble non-structural carbohydrates (*i.e.* 623 and 26 g/kg DM, aNDFom and WSC, respectively) and moderate CP contents (table 1). The high silica content (*i.e.* 82% of total ash) suggests the presence of indigestible material and lower organic matter for animal feed.

Table 1. Chemical composition of *P. aurea* leaf blades in winter and spring.
Tabla 1. Composición química de láminas de *P. aurea* en dos épocas del año.

	Winter	Spring	SE_{mean}	Significance
DM (g/kg MH)	490	458	3.5	0.003
Ashes	204	181	19.7	0.452
Silica	171	143	19.2	0.365
Crude protein	137	129	5.4	0.387
aNDFom	623	622	11.6	0.945
ADFom	298	281	4.2	0.044
ADLom	69	44	3.8	0.010
WSC	22	30	3.5	0.204

DM: dry matter; aNDFom: insoluble fiber in neutral detergent with α-amylase and free of ash; ADFom: ash-free acid detergent fiber insoluble fiber; ADLom: lignin in acid detergent in sulfuric acid and free of ashes; WSC: water-soluble carbohydrates; SE_{mean}: standard error of the mean.

Data are expressed in g/kg DM, unless indicated.

MS: materia seca; aFDNmo: fibra insoluble en detergente neutro con α-amilasa y libre de cenizas; FDAmo: fibra insoluble en fibra detergente ácida sin cenizas; LDAmo: lignina en detergente ácido en ácido sulfúrico y libre de cenizas; CS: carbohidratos solubles en agua; EE_{media}: error estándar de la media.

Los datos se encuentran expresados en g/kg MS, excepto que se indique lo contrario.

Phyllostachys aurea presented lower moisture content and higher concentration of ADFom and ADLom in winter than in spring, with increases of 7, 6 and 57% ($P < 0.05$), respectively (table 1). ADLom accumulation in the cell wall of winter leaf blades coincides with a 9% decrease in DMD_{in} in winter compared to summer harvest ($P < 0.05$; table 2, page 147). However, increases in ADF and ADL did not translate into statistically significant changes in IVNDF ($p = 0.16$), possibly given the greater SE_{mean} magnitude concerning DMD_{iv} (9.1 and 21.5 SE_{mean} for DMD_{iv} and $NDFD_{iv}$, respectively). Additionally, digestibility determined at 48 h, corresponding to potential values, could promote difference fading. *P. aurea* high cell wall content and greater winter lignification promoted digestibility not exceeding 54% and 36% for DM and aNDFom, respectively.

Leaf blade CP content was slightly below the threshold (13%DM) recommended for the ruminant diet. Moreover, since average soluble carbohydrates were 26 g/kg DM and aNDFom was over 600 g/kg DM, *P. aurea* foliage can be classified as medium-quality feed.

GP kinetics coincided with that described for ADFom, ADLom and digestibilities, being RGP_{Av} 1.8 times higher in spring and 1.7 in the PGAN at 24, 48 and 72 h, compared to winter (table 2, page 147; $P < 0.05$). This could be due to 36% less lignin of summer leaf blades, and 89% more organic matter not associated with the cell wall (*i.e.* 1000 - (Ash + CP + aNDFom) = 68 vs 36 g/kg DM; table 1). This hypothesis was supported by the greater DMD_{iv}, although no significant differences were detected for $DNFD_{iv}$.

Although the *c* rate was higher in spring leaf blades, the adjusted rate was 39% lower than in winter (*i.e.* 0.011 and 0.018 h^{-1}, respectively; table 2, page 147; $P = 0.047$). The model describing GP kinetics may overestimate *c* when the curve does not reach the plateau (figure 6, page 147). However, spring foliage showed GP net rates of 3.2 fold and twice higher at hour 1 and 18, respectively (*i.e.* E × T; $P = 0.034$; figure 6, page 147). In all cases, T_{RGPmax} was 3 hours after incubation. Higher GP rates at 1 and 3 hours could be due to WSC fermentation, while the later reduced rate is given by the lower availability of cell-wall carbon skeletons.

Table 2. *In vitro* nutritional evaluation of *P. aurea* leaf blades in winter and spring.
Tabla 2. Evaluación nutricional *in vitro* para dos estaciones del año de láminas de *P. aurea*.

	Winter	Spring	SE_{mean}	Significance
***In vitro* digestibility at 48 hours**				
DMD_{iv} (g/kg DM)	497	543	9.1	0.023
$NDFD_{iv}$ (g/kg NDF)	312	364	21.5	0.164
Cumulative gas production (ml/g DM) at different incubation times (h)				
24	33	59	3.4	0.006
48	51	85	4.2	0.004
72	73	114	5.3	0.006
Parameters of the kinetics of gas production				
RGP_{Max} (ml/g DM.h)	2.5	3.3	0.25	0.078
RGP_{Av} (ml/g DM.h)	1.2	2.1	0.09	0.003
a (ml/g DM)	2.0	2.0	1.20	0.736
b (ml/g DM)	154	138	17.0	0.539
a + b (ml/g DM)	140	156	16.5	0.514
c (h^{-1})	0.018	0.011	0.0018	0.047

SE_{mean}: mean standard error; DMD, dry matter digestibility; NDFD: insoluble fiber in neutral detergent digestibility; RGP_{Max}: maximum rate gas production; RGP_{Av}, average rate gas production; a: GP of soluble fraction; b: GP of potentially degradable fraction; c: *b* degradation rate (CNGP = a + b x (1 - e^{-ct}); Ørskov and McDonald, (1979).

Digestibility and kinetics of gas production.

EE_{media}: error estándar de la media; DMS: digestibilidad de la materia seca; DFDN: digestibilidad de la fibra insoluble en detergente neutro; TPG_{Max}: tasa máxima de producción de gas; TPG_{Prom}: tasa promedio de producción de gas; a: PG de fracción soluble; b: PG de fracción potencialmente degradable; c: tasa de degradación de b (PGNA = a + b x (1 - e-ct); Ørskov y McDonald, (1979).

Digestibilidad y cinética de producción de gas.

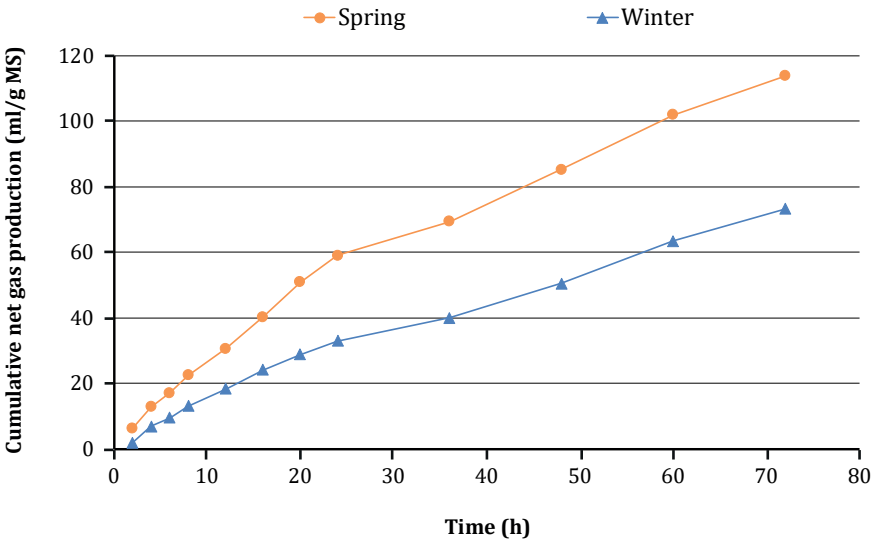


Figure 6. Cumulative net gas production (ml/g DM incubated) as a function of incubation hours of *P. aurea* leaf blades in spring and winter.

Figura 6. Producción de gas acumulada neta (ml/g MS incubada) en función de las horas de incubación de las láminas de *P. aurea* en dos estaciones del año.

Discussion

Morpho-anatomically, *P. aurea* foliage leaves presented predominant parenchyma and scarce sclerenchyma, desirable traits for forage use. As foliage matures, silica is deposited in bulliform cells, microhairs, hooks, and prickles, and to a lesser extent in suberous, long, and subsidiary cells in the stomatal complex (50). This study coincides with our anatomical and nutritional results showing that silica in different cell types could limit digestion. Silica in epidermis, trichomes, cell walls or lumen of grasses acts as a structural inhibitor of microbial digestion leading to lower acceptability and DMD (26). Thus, low DMDiv and NDFDiv could be explained by high epidermal Si contents preventing wall enzymatic degradation.

Chemical-nutritional composition of *P. aurea* leaf blades was statistically different between spring and winter harvests, as previously found (9, 19, 47). However, these differences did not modify productive implications in ruminant feeding, describing a forage of medium to low nutritional value (*i.e.* CP,133; aDFNom, 623; ADFom, 290; ADLom, 57; DMD_{iv}, 520; $NDFD_{iv}$, 338 g/kg DM). Other authors reported similar results (4, 40) and Bhardwaj *et al.* (2019) studying Jersey cows, characterized *P. aurea* with lower palatability and nutritional value than other bamboos like *Dendrocalamus hamiltonii* Nees & Arn. ex Munro, D. *asper* (Schult. & Schult. f.) Backer ex K. Heyne, *Melocanna baccifera* (Roxb.) Kurz, *Phyllostachys bambusoides* Siebold & Zucc. and *P. pubescens* (Pradelle) Mazel ex J. Houz.]. In contrast, Panizzo *et al.* (2017) described *Guadua chacoensis* leaf blades as better nutritional forage, with 220 g CP/kg DM, 541 g aNDFom/kg DM and 64% degradability at 48 hours.

Asaolu *et al.* (2010) and Halvorson *et al.* (2010) mentioned that bamboo is suitable for animals under maintenance conditions. The advantage of *P. aurea* is that leaf foliage blades are perennial and available when other forage species become scarce (4, 8, 9, 34). Mekuriaw *et al.* (2011), documented using bamboo foliage as feed for cattle, sheep, goats, and chickens in Ethiopia. In addition, our average aNDFmo content of 623 g/kg DM (table 1, page 146) could limit dry matter intake as for other forages (*i.e.*, aNDFom > 600 g/kg DM; Mertens, 1973). Compared to other forage species, *P. aurea* low GP and CNGP rates suggest lower rumen fermentation and a consequent negative effect on potential consumption (16, 45). However, its CP content over 120 g/kg DM would not limit rumen digestion or requirements of ruminant categories in maintenance (7).

P. aurea foliage presented lower nutritional value than most C_3 grasses and legumes grown in the temperate region of Argentina, both fresh and hayed or ensiled (*i.e.*, oats, barley, fescue, ryegrass, red clover and alfalfa; Jaurena and Danelón, 2006; Wawrzkiewicz *et al*., 2019). On the other hand, compared with C_4 (*i.e.*, rhodes grass, gatton panic, honey grass; Fernández Pepi *et al*., 2018) grasses, bamboo contributed more CP and a similar or lower cell wall content. For this reason, *P. aurea* would be an alternative to C_4 grasses, given its constant biomass production, nutritional quality and availability in winter or dry seasons.

Yayota *et al.* (2009) mention that low nutritional quality of bamboo cannot be completely explained by cell-wall quantity and composition. Factors like tannins or Si hinder microbiologic accessibility, slowing digestion (46).

Our results evidence foliar homogeneity of *P. aurea* throughout the year, for the temperate region of Argentina, and strong environmental adaptability while keeping stable chemical-nutritional characteristics. This allows considering *P. aurea* as an ingredient for ruminant supplementation in times of forage scarcity, increasing effective fiber intake. The use of foliage leaves, otherwise discarded after culms are used in construction and crafts, transforming a byproduct into an additional source of feed for ruminants.

Conclusions

Phyllostachys aurea presented morphological characteristics, growth habit, propagation form and vegetative cycle allowing high capacity for establishment and development in diverse environments. The C_3 leaf anatomy with abundant parenchyma and scarce sclerenchyma along with its chemical-nutritional composition suggest its value as feed for ruminants. Although cell wall content estimated as NDF limits potential consumption, bamboo could be used as supplement in unfavorable times and ensure fiber contribution. Finally, *P. aurea* presents homogeneous traits throughout seasons and sites (low phenotypic plasticity) constituting a food source throughout the year.

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Characterization of the pork sector in the productive core of Argentina: a look at small producers

Caracterización del sector porcino en el núcleo productivo de Argentina: una mirada hacia los pequeños productores

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Abstract

Pros and cons of pork production in Argentina underscore the need to have information to empower pork producers. This study characterizes three pork production strata (Small, Medium, and Large) in north Buenos Aires using surveys (n=40). We provide information on farms, management practices, infrastructure, technology and commercial activities. We found significant differences (p-value < 0.05) between strata in the use of artificial insemination and effluent treatment (mainly through lagoons and soil application) regarding infrastructure and technology. Additionally, there was a trend towards breeding in confined systems as the size of the production increased. Furthermore, despite 72.50% of surveyed producers having reported access to professional veterinary advice, we found a significant difference (p-value = 0.0167) in access between the Small (45.45%) and Large (100%) strata. Regarding commercialization, data indicated piglet sales as the predominant activity, with pig farming serving as a supplementary source of income for most producers. These findings show the need for professional intervention in smaller-scale pig farms to overcome structural barriers and access to the production chain.

Keywords
pig production • strata • Buenos Aires • infrastructure • health

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Resumen

Las fluctuaciones que presenta el sector porcino en Argentina resaltan la necesidad de disponer de información para potenciarlo. El objetivo de este estudio fue caracterizar tres estratos productivos porcinos (pequeño, mediano y grande) en el norte de la provincia de Buenos Aires mediante encuestas (n=40) proporcionando información sobre manejo, infraestructura, tecnología y comercialización. En términos de infraestructura y tecnificación, se identificaron diferencias significativas (p-value < 0,05) entre los estratos en el uso de la inseminación artificial y el tratamiento de efluentes (lagunas y aplicación al suelo), además de una tendencia hacia la cría en sistemas confinados a medida que el tamaño del estrato aumenta. Por otro lado, a pesar de que el 72,50% de los productores indicó contar con asesoramiento veterinario, se constató una diferencia significativa (p-value = 0,0167) entre el estrato pequeño y el grande en el acceso al servicio. En cuanto a comercialización, los datos evidenciaron que la venta de lechones es la actividad predominante, siendo la actividad porcina una fuente de ingresos económicos complementaria para la mayoría de los productores. Estos datos manifiestan la necesidad de intervención profesional en las explotaciones porcinas para superar barreras estructurales y aumentar el acceso a la cadena productiva.

Palabras clave
producción porcina • estratos • Buenos Aires • infraestructura • sanidad

Introduction

Global pork consumption ranks second only to poultry, with an average of 11.7 kg per capita annually. China is the leading producer, accounting for 41.3% of the total, followed by the European Union with 22.3% (23). In South America, Brazil is the largest producer, contributing 4.1% of global production, and ranking fourth in global exports. Argentina produces 0.7% of the world's pork, with 697 thousand tons destined mainly for domestic consumption, and to a lesser extent for export (22).

Pork production in Argentina has fluctuated over time, currently reaching 5 million heads, peaking at 8 million in the 1940s (10). This stock is concentrated in three provinces: Buenos Aires (23.7%), Córdoba (23.5%), and Santa Fe (14.1%), aligning with the agricultural core region (22). According to data from the Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA, 2022), there are 97,680 productive units (UP) in the country, with 90% having fewer than 50 mother sows. Only 3,313 UP reported slaughter activity in 2022 (26), highlighting the large number of small-scale producers not fully engaged in the production chain.

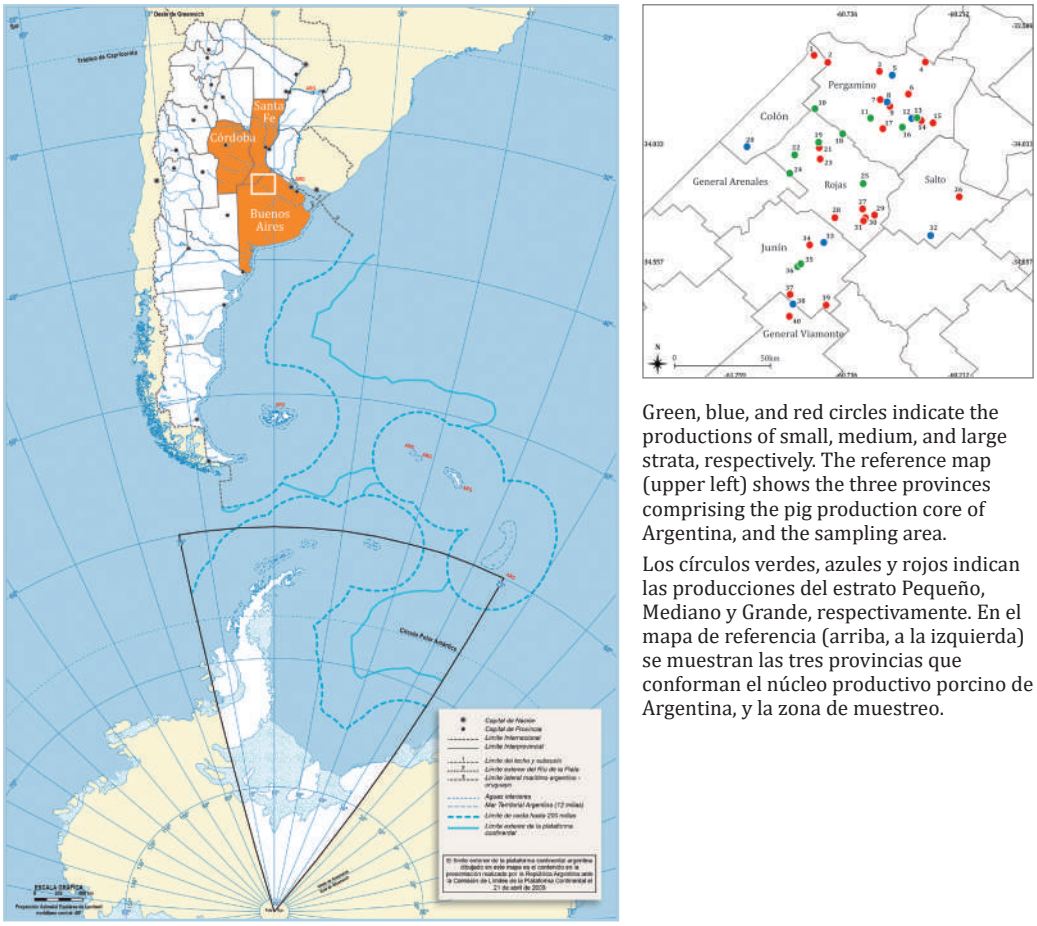
Currently, there is limited information on technical, health, and infrastructure development of small-scale producers. This sector faces several challenges, with most production relying on pasture, using traditional methods and low technological investment (11). Additionally, the prices of imported meat and fat from Brazil and Europe, place Argentina at a competitive disadvantage. Moreover, there is a lack of coordination between the production and processing sectors within the pork supply chain (9). However, the sector also presents opportunities, such as rising beef prices and the sharp decrease in China's pig stock due to African swine fever (26, 28).

In this context, understanding the current status of small-scale pork production is essential for increasing its involvement in the production chain. This study characterizes pork establishments in the north of the Buenos Aires province by providing information on herd composition, management, infrastructure, technology, and commercial activities, categorizing them by sow stock size.

Materials and methods

Study area

The study area is located in the Undulating Pampa region, from 33°42'43" to 34°47'75" S latitude and 61°52'30" to 60°20'38" W longitude. It is located in northwestern Buenos Aires province, the country's leading pork-producing region (figure 1) (30).



Source/Fuente: IGN (Instituto Geográfico Nacional)

Green, blue, and red circles indicate the productions of small, medium, and large strata, respectively. The reference map (upper left) shows the three provinces comprising the pig production core of Argentina, and the sampling area.

Los círculos verdes, azules y rojos indican las producciones del estrato Pequeño, Mediano y Grande, respectivamente. En el mapa de referencia (arriba, a la izquierda) se muestran las tres provincias que conforman el núcleo productivo porcino de Argentina, y la zona de muestreo.

Figure 1. Geographic location of the productions analyzed in this study.

Figura 1. Ubicación geográfica de las producciones analizadas en el estudio.

Data collection

Data were collected through semi-structured and face-to-face surveys with pork producers or establishment managers (n=40). The process followed the guidelines outlined by Albuquerque *et al.* (2014). Establishments were classified into three strata following the methodology of Argentina's Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA, 2022), grouping production units by the number of sow mothers (7): the 'Small' (S, n=11), 'Medium' (M, n=22), and 'Large' (L, n=7) strata included herds of 0-10, 11-100, and over 101 sow mothers, respectively. The survey had four sections: (a) Farm Size, (b) Technology and Personnel, (c) Health, and (d) Marketing.

The Farm Size section provided information about the number of females and males in the herd. Mean and standard deviation for each stratum were calculated using GraphPad Prism software version 6.01 (GraphPad Software, Boston, MA, USA).

The Technology and Personnel section collected data about the use of Artificial Insemination (AI) and effluent disposal and treatment processes to reduce contaminants (lagoons, watercourses, or irrigation) (24). Data was analyzed with a Pearson Chi-squared test (25). Information on the breeding system (outdoor, confined, or mixed systems) and workforce size (expressed as the number of individuals working either part-time or full-time) was also gathered (25). Paired differences between strata were calculated using an analysis of variance (ANOVA) with the Tukey test in R software (27).

The Health section included questions on veterinary advisory services received in the past year and the main health issues affecting the herd. Statistically significant differences were analyzed using the Pearson Chi-squared test in R software (27).

The Marketing section gathered information on the products sold during the survey, including piglets, market pigs over 100 kg, and processed products. The destination of these products included private buyers, slaughterhouses, aggregators, or personal consumption. Data on the level of cooperation with other producers (*e.g.* membership in producer groups or organizations) and the role of pig farming in family income was also collected. The term 'piglets' referred to animals up to 4/5 weeks old and under 15 kg, while "market pigs over 100 kg" were defined as castrated males and non-breeding females weighing more than 100 kg (12). 'Processed products' referred to the production of preserves, cured meats and salted products, including fresh, dried, or cooked sausages (7).

Results

Farm size

The study surveyed a total breeding stock of 2,759 individuals, averaging 68.98 ± 133.04 sow mothers and 2.66 ± 2.00 boars. The distribution of breeding stock across the Small, Medium and Large strata was 2.68%, 29.72%, and 67.60%, respectively (table 1).

Table 1. Mean, Standard deviation (±SD), 75% percentile (75%-per), maximum, and minimum number (Min/Max) of sow mothers and boars per stratum.

Tabla 1. Media, Desvío estándar (±SD), 75% percentil (75%-per), número máximo y mínimo (Min/Max) de cerdas madres y padrillos por estrato.

	Sow mothers				Boars			
Stratum	**n**	**Mean (±SD)**	**75%-per**	**Min/Max**	**n**	**Mean (±SD)**	**75%-per**	**Min/Max**
S	11	6.73 (±3.00)	9	2/10	11	1.09 (±0,70)	2	0/2
M	22	37.27 (±21.61)	51.25	12/80	21	3.286 (±2,03)	8	1/8
L	7	266.4 (±158.60)	400	140/550	6	3.33 (±2.16)	7	1/7
Total	**40**				**38**			

Technology and personnel

Producers using Artificial Insemination (AI) as a reproductive method accounted for 17.95%, while 82.05% relied on natural mating for breeding. A statistically significant difference was found between the Large stratum, where the majority of respondents used the technique, and the two smaller strata (figure 2, page 156).

N/D: no data available. References are indicated in the figure. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

N/D: sin datos. Las referencias se indican en la figura. * p-value < 0,05, ** p-value < 0,01, *** p-value < 0,001.

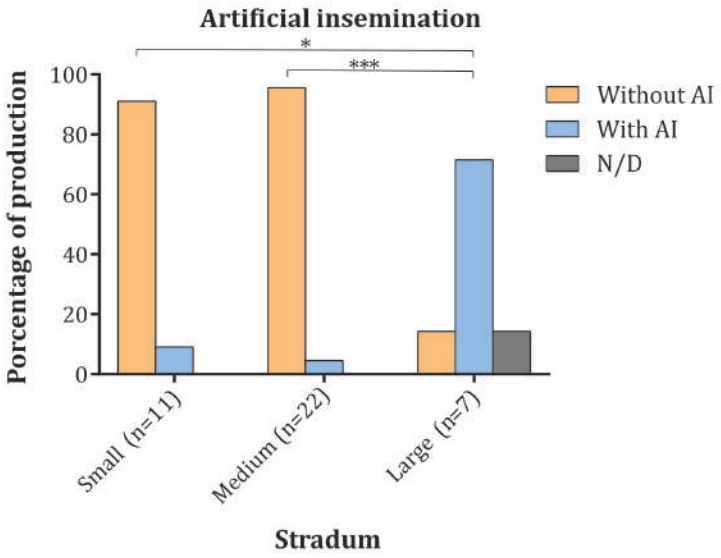


Figure 2. Grouped bar graph showing the percentage of productions using the Artificial Insemination (AI) technique across different strata.

Figura 2. Gráfico de barras agrupado donde se representa el número de criaderos que emplean la técnica de Inseminación artificial (IA) en los diferentes estratos.

The primary breeding method was the outdoor system (37.50%), followed by the mixed system (35%), and the confined system (27.50%). In the Small stratum, the outdoor breeding system was used to a greater extent (54.55%), followed by mixed systems (36.36%), and confined systems (9.09%). In the Medium stratum, 36.36% of the breeders used an outdoor system, 45.46% opted for mixed systems, and 18.18% employed a confined system. In the Large stratum, 85.71% used a confined rearing system, while 14.29% employed an outdoor approach.

Regarding effluent management, 52.50% of producers did not implement any treatment, 17.50% disposed of effluents directly onto the soil, 12.50% used settling lagoons, 10% combined both methods (lagoon and soil application), and 7.50% did not respond.

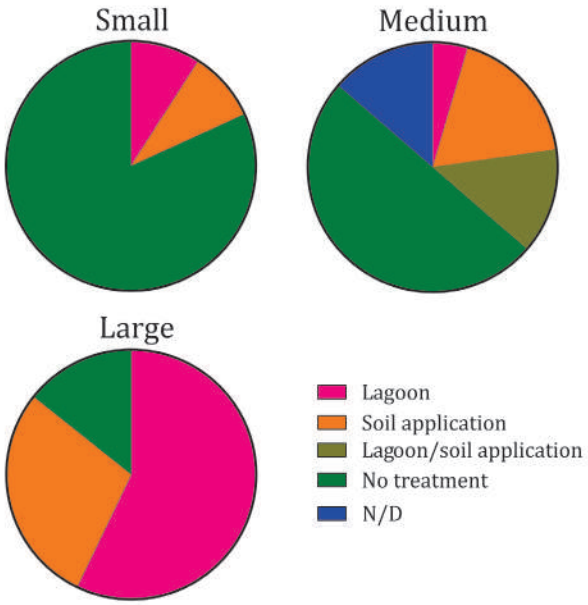
In the Small stratum, 81.82% of the producers did not treat effluents. In contrast, the Medium stratum decreased to 50%. These results differ from the Large stratum, where 85.71% of producers implemented some treatment.

Soil application and lagoons were the predominant treatments in the Medium and Large strata. In the Small stratum, two producers reported using treatment methods: one used lagoons, and the other disposed of waste through soil application (figure 3, page 157).

The average number of workers per breeding facility (n= 38) (full-time or part-time) was 2.05. In the Small stratum, the average was 1.36 ± 0.50 workers (with a maximum of 2), increasing to 2 ± 0.63 workers (with a maximum of 3) in the Medium stratum. The average was 3.50 ± 1.98 in the Large stratum (with a maximum of 7). The latter differed statistically from the other two strata (p-value < 0.05). The ratio of sow mothers per personnel (operators) per stratum was 4.95, 18.6, and 76.11 sow mothers per person in the Small, Medium, and Large strata, respectively.

Health

Of the total producers surveyed, 72.50% received professional veterinary advice. Small, Medium and Large strata, had 45.45%, 77.27% and 100%, respectively. The pairwise Chi-squared test showed a significant difference between the Small and Large strata (p-value = 0.0167).



N/D: no data.
N/D: sin datos.

Figure 3. Pie charts showing the proportion of producers by effluent treatment method across different strata.

Figura 3. Gráficos de tortas que representan la proporción de productores según el método de tratamiento de efluentes por estrato.

Regarding the most frequent diseases in the herds, 47.50% of the producers reported no frequent diseases. The remaining 52.50% identified pneumonia as a recurrent disease, with other issues such as parasites, diarrhea, and pre-weaning mortality being less common (figure 4, page 158). When disaggregated by stratum, the trend of pneumonia remained the primary concern across all groups, while a notable percentage of producers in the Small and Medium strata reported having no recurrent problems.

Marketing

Sixty-five percent (65%) of pig producers reported selling piglets. Additionally, 20% sold both piglets and market pigs over 100 kg, 12.50% sold piglets and processed products, and 2.50% sold only market pigs over 100 kg. Piglets were the predominant product across all three strata (table 2, page 158). These products were mainly marketed privately (60%). A smaller proportion of producers (22.50%) exclusively targeted slaughterhouses as their end customers, while 7.50% raised pigs for personal consumption. Five percent (5%) engaged in joint marketing with slaughterhouses and private sales, while the remaining 5% was split between bulk purchasers and private sales. Stratified data revealed that private sales were predominant in the two smaller strata, while the Large stratum was dominated by sales to meatpacking plants (table 3, page 158). Furthermore, 82.50% reported that pig production serves as a supplementary source of income, typically alongside grain production, while 17.50% considered pig production as their primary source of income.

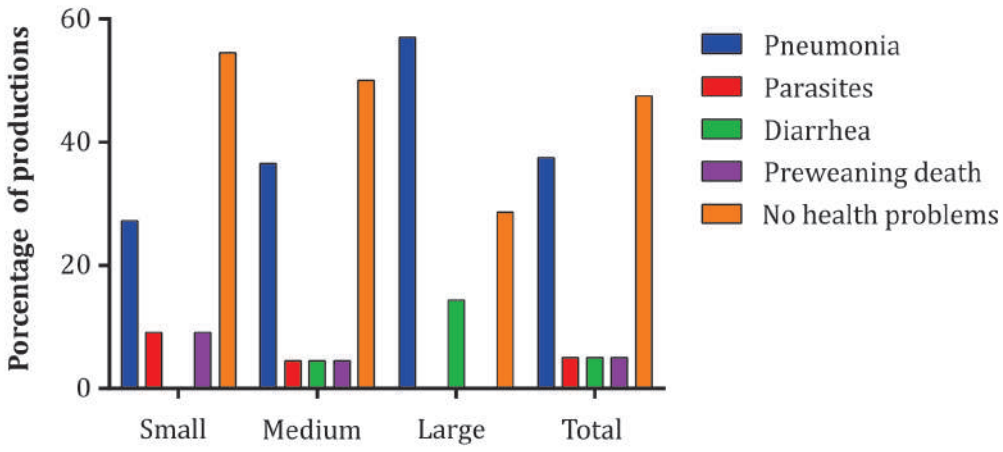


Figure 4. Frequency of recurrent diseases grouped by stratum.
Figura 4. Frecuencia de enfermedades recurrentes agrupadas por estrato.

Table 2. Proportion of final product marketed by stratum.
Tabla 2. Proporción del producto final comercializado por estrato.

	Product			
Stratum	**Piglets**	**Piglets/Manufacturing**	**Piglets/Market pigs**	**Market pigs**
S (n=11)	72.73%	18.18%	9.09%	-
M (n=22)	63.70%	13.60%	22.70%	-
L (n=7)	-	-	42.86%	57.14%

S= small stratum, M= medium stratum, L= large stratum.
S= estrato pequeño, M= estrato mediano, L= estrato grande.

Table 3. Destination of final products by stratum.
Tabla 3. Destino de los productos finales por estrato.

	Destination					
Stratum	**Private**	**Slaughterhouse**	**Private/ slaughterhouse**	**Private/ Collector**	**Collector**	**Own consumption**
S (n=11)	81.82%	9.09%	-	-	-	9.09%
M (n=22)	63.63%	18.18%	9.09%	4.55%	4.55%	-
L (n=7)	42.86%	57.14%	-	-	-	-

S= small stratum, M= medium stratum, L= large stratum.
S= estrato pequeño, M= estrato mediano, L= estrato grande.

In the Small, Medium and Large strata, pig farming represented supplementary income for 100%, 90.91% and 71.43% of respondents, respectively. Regarding the level of cooperativeness or association among producers, 7.50% (n=3) responded affirmatively, with all positive responses coming from breeders in the Medium stratum.

Discussion

The information presented in this study highlights the vulnerability and limited access to technology and infrastructure faced by small-scale producers, which makes it difficult for them to participate in the national pig production chain.

The animal stock recorded in this study confirms the trend of sow herd concentration in a few establishments at the national level (table 1, page 155). In Argentina, productions with fewer than 50 sows account for only 4% of the total sow stock (29). Despite representing the majority of productions, the two lower strata retain only 32.40% of the total sow stock. Within comparison, data from Buenos Aires province show that the proportion of producers unable to scale up between strata over the past decade has remained unchanged. Benéz and Cendon (2013) reported that farms with fewer than 50 sows, although the largest sector, retain only 41% of the stock.

To understand the cause of this disparity, it is essential to closely examine the production state. One approach in this study was quantifying artificial insemination (AI) as an indicator of technological adoption. The obtained data aligns with the 2018 agricultural census, where approximately 20% of commercially oriented productions use AI (18). These figures are discouraging, as AI usage in Argentina's core production regions does not exceed 18%. In contrast, leading pork-producing countries such as the United States and those in the European Union report AI usage in 60% to 90% of their productions (31, 32). Furthermore, the results vary among strata, with significantly greater access to AI in the Large stratum (figure 2, page 156). This stratum shows percentages similar to previous reports, which estimate that 85% of sows in intensive operations in Argentina are artificially inseminated (3). The technological gap between strata is clear. Although reproductive techniques like AI could become more accessible to family or small-scale producers in the near future. In many developing countries, this technique has been adopted despite significant infrastructure limitations (19, 20). In Brazil, for example, the use of AI in pigs increased by more than tenfold between the 1990s and 2000, reaching 70% today (13). The widespread adoption of IA in these countries was driven by research programs, education, and financial support provided by universities, governments, and commercial enterprises. These initiatives promoted the benefits of AI and made the technology more accessible to the community (15, 19).

In terms of breeding systems, there is a general trend toward outdoor pig farming, which decreases as the number of sows increases. This is partially explained by the capital requirements, as maintaining an extensive pig production system typically requires 40-70% less capital than a confined system (21).

Manure accumulation in pens is one of the most significant contributors to soil contamination (17). Although there is limited national-level data, methods such as stabilization lagoons, irrigation, and composting appear to be the most commonly used waste management approaches (5). In the study area, producers actively participate in effluent treatment. This contrasts with the province of Santa Fe, where, although 75% of producers have some form of organic waste storage, only 12.50% treat the effluents (16). In this province, the primary methods for waste disposal are ditches or pits, which contrasts with the use of lagoons and direct soil application reported in this study. As the stratum (sow stock) increases, producers' participation in effluent treatment becomes more common (figure 3, page 157). Effluent control requires substantial planning and investment, which may explain why many producers choose lagoons as a more cost-effective alternative (1, 21). These lagoons are less expensive and require less maintenance, while also allowing for the management of a large load and concentration of organic material (8). On the other hand, disposal through irrigation (direct soil application) is common because many pig producers largely engage in agriculture, and the investment in irrigation equipment or manure spreaders is relatively affordable.

Similarly to the lack of data on effluent treatment, there is no information available on workforce registration in the pig sector, making the data obtained in this study a first approximation. In many establishments, particularly in the Small stratum, the work is performed entirely by one person, which presents a disadvantage due to the occupational risks involved (*e.g.*, injuries, and zoonotic diseases) (6). The survey data allows us to calculate the relationship between the average number of sows and the number of personnel per stratum. It is observed that each worker in the Large stratum manages more animals than recommended for this type of activity. Typically, swine operations require 1.9 direct jobs for every 50 sow mothers in operations with 51 to 100 mothers and 1.7 jobs for every 50 sow mothers in operations with 101 to 500 mothers (14).

Animal health is an important factor impacting the economics and production performance, which accounts for 4% to 7% of the cost per kilogram of meat produced in a pig farm (32). Previous reports indicate that 93% of pig producers in Argentina do not have routine veterinary guidance or only consult a veterinarian sporadically (32). The results of this study differ from these previous findings, as 72.50% of producers reported receiving veterinary advice. These differences may be attributed to estimation scale and suggest a high level of access to professional consultation among regional producers. However, 27.50% of these producers lack veterinary advice, which is detrimental from a production standpoint and raises concerns for human health. According to Braun (2016), the production conditions in the small or subsistence stratum lead to health vulnerabilities for the overall pig population, due to the absence of a systematic approach and limited knowledge of good production practices. One recommended solution is for small-scale or family-owned production establishments to organize under the guidance of a single professional, which could help reduce costs (23). The importance of guidance lies in the ability to plan and manage a health program tailored to the specific circumstances of each establishment. In this context, all three strata identified pneumonia as a recurrent disease (figure 4, page 158). According to Bencomo (2010), pneumonia is present in 90% of pig farms and affects 80% of pigs globally, making it the most prevalent and economically impactful disease in pig production. Aside from the regular epidemiological surveillance conducted by government agencies, there are no formal records of recurrent pig diseases in the region.

The data on the commercialization of the pig farming enterprise not only provide insights into the current economic characteristics of each stratum but also serve as a basis for potential marketing strategies. In the Small stratum, 81.82% of producers sell their products, primarily piglets, through private sales. These figures highlight the limited access that producers in the Small and Medium strata have within the production chain. This is particularly relevant when considering that Buenos Aires is the province with the highest number of meat processing plants in the country (22). Furthermore, alternative markets, such as the production of processed products, can offer growth opportunities for smaller strata (4). As the results show, these strata have a higher percentage of manufacturing compared to the Large stratum, which can serve as an initial step towards expanding or developing their activities. The production of cured and salted meats accounts for 3.20% of the value of the food and beverage industry in the country (7).

The fragility is evident in the need for producers to rely on other rural activities for their livelihoods. The vast majority (82.50%) use pig production as a supplementary source of income alongside other agricultural activities. In this context, cooperatives or producer associations offer a viable way to achieve common goals, such as veterinary guidance, use of artificial insemination, acquisition of effluent treatment equipment, and the purchase of high-value genetic material, and to attain levels of competitiveness comparable to larger companies (21).

According to the data, over 90% of the surveyed producers are not part of any network or association. Cooperatives or associations are some of the powerful tools for overcoming individual limitations and achieving production levels comparable to those of large enterprises.

Conclusions

The results presented here reveal that small producers have limited access to technological resources (such as artificial insemination), operate with precarious infrastructure (with less use of intensive confinement systems and low investment in effluent treatment), and face challenges in sanitary control and access to the production chain or meatpacker sales.

It is crucial to counterbalance a concentrated pig production model, where a few producers dominate the entire stock, by promoting a diversified national production system that includes small producers in the production chain, allowing them to grow within the sector. Achieving this requires the involvement of competent authorities to develop national-level plans that provide financial assistance and training in proper health and herd management practices.

Supplemmentary material

https://docs.google.com/spreadsheets/d/1WR7VCWOBYpTW7pfn8aAMVMzE4aahvmHG/edit?usp=drive_link&ouid=111310786017351827239&rtpof=true&sd=true

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Nutritional characterization of *Larrea divaricata* Cav during winter and its potential as cattle and goats feed

Caracterización nutricional de *Larrea divaricata* Cav durante la temporada invernal y su potencial como alimento para bovinos y caprinos

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Abstract

The ever-increasing global demand for agricultural commodities and progressive climate change factors are displacing extensive beef cattle and goat ranching from temperate humid regions to peripheral regions with semi-arid characteristics. Extensive investigation is required on native desert plants to be safely incorporated into feed programs and to maintain the biodiversity and sustainability of these fragile ecosystems. *Larrea divaricata* is a native plant adapted to arid and semi-arid biomes of South and Western-South America. This research evaluates the nutritional composition of the browsing available canopy parts of *Larrea divaricata* during the winter season in a semi-arid region of Argentina. Its crude protein content resulted in 11.20% of dried matter and its soluble protein content resulted in nearly 80% of the crude protein. Acid detergent fiber fraction, ash-corrected neutral detergent fiber fraction, lignin, ash content, fat-like compounds, and non-fibrous carbohydrates resulted in 17.42, 35.51, 12.09, 9.96, 5.92 and 3.82% of dried matter, respectively. Essential bioelements Ca, Mg and K resulted within standard forage requirements. Total polyphenols and flavonoids resulted in 430 mg/g and 140 mg/g, respectively. These results demonstrate that *Larrea divaricata* can be an effective complement for winter-feeding beef cattle and goats in arid and semi-arid regions.

Keywords
native plant • shrub • jarilla • ruminant • desert • forage

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Resumen

La creciente demanda de productos agrícolas y el cambio climático, están desplazando la ganadería extensiva desde regiones templadas húmedas, hacia regiones periféricas semiáridas. Ello requiere de investigaciones exhaustivas respecto de plantas nativas, para incorporarlas eficientemente a los programas de alimentación y mantener la biodiversidad y la sostenibilidad de estos frágiles ecosistemas. La *Larrea divaricata*, es una planta nativa de las regiones áridas y semiáridas de Sudamérica. Este trabajo, evalúa la composición nutricional de las partes aéreas disponibles para el ramoneo de *Larrea divaricata* durante la temporada invernal. Su contenido de proteína cruda resultó 11,20% de la materia seca. La proteína soluble resultó cerca del 80% de la proteína cruda. La fracción de fibra detergente ácida, la fracción de fibra detergente neutra, la lignina, el contenido de cenizas, los compuestos grasos y los carbohidratos no fibrosos resultaron 17,42; 35,51; 12,09; 9,96, 5,92 y 3,82% de la materia seca, respectivamente. Bioelementos esenciales como Ca, Mg y K resultaron en niveles relevantes. Los polifenoles y flavonoides totales representaron 430 mg/g y 140 mg/g, respectivamente. Estos resultados, demuestran que *Larrea divaricata* puede ser un complemento eficaz para la alimentación invernal de ganado vacuno y caprino en regiones áridas y semiáridas.

Palabras claves
planta nativa • arbusto • jarilla • rumiante • desierto • forraje

Introduction

Larrea divaricata is a plant endemic to the southern and western territories of South America, including vast arid and semi-arid regions of Argentina, Bolivia, Chile and Peru (20, 30). *L. divaricata* is a perennial shrub, 0.9 to 1.8 meters tall, with resinous leaves that grow at the tips of the branches (7) and exhibit antifungal properties (4). This plant is closely related to *Larrea tridentata* (Sessé & Moc. ex DC.) Coville, which is endemic to western North America, including Mexico and the USA (32). Both species belong to the Zygophyllaceae family (32) and thrive only in their natural habitats (14, 27). Interestingly, countries where *L. divaricata* and *L. tridentata* are endemic account for 18% of world livestock inventory (31).

Extensive husbandry methods for beef cattle and goats are undergoing accelerated changes. The ever-increasing global demand for agricultural commodities, coupled with progressive climate change factors, is displacing extensive livestock ranching from temperate humid regions to peripheral areas with semi-arid characteristics (28). The situation in South America is no exception. Record prices of soybean, corn, wheat and sunflower, among other agricultural commodities (8), have driven extraordinary profitability of the cultivated land in the humid Pampa region of Argentina (3). As a result, extensive beef cattle and goat activities are being displaced from the humid Pampa region to the semi-arid Pampa region (34). The semi-arid Pampa region is located in central-south Argentina, between the humid Pampa region to the north and the Patagonia region to the south. The climate of the semi-arid Pampa region is dry with temperate summers and cold, harsh winters (43). Here, beef cattle and goat ranching are adapted to silvopastoral systems and incorporate native forests and wild pastures into the diet (6, 43). Figure 1 (page 165), shows the distribution of *L. divaricata* in Argentina, the direction of agricultural expansion and extensive livestock displacement toward semi-arid zones. This situation presents an opportunity for exploiting native vegetation as forage, without using agrochemicals, soil tillage, or artificial irrigation and with the advantageous approval of Carbon Neutral Meat Certifications.

Native forage species should be palatable, have adequate nitrogen content and exhibit high digestibility (15, 33, 39). However, extensive research is needed to safely incorporate native plants into livestock feeding programs, while maintaining the biodiversity and sustainability of the land (14, 15, 38, 40).

Figure 1. Distribution of *L. divaricata* in Argentine territory and displacement of extensive livestock as a consequence of agriculture.

Figura 1. Distribución de la *L. divaricata* en el territorio argentino y desplazamiento de la ganadería extensiva como consecuencia de la presión agrícola.

In the semi-arid Pampa region, beef cattle and goats browsing on *L. divaricata* are considered essential for nutrition during winter seasons. Here, pastures are dry, nutrient-poor, and scarce, either due to overgrazing, fires, or snow cover (13). However, there is little scientific information on the nutritional value of *L. divaricata* during the winter season (41).

This article studies the nutritional value of L. divaricata canopy leaves collected from specimens in the semi-arid Pampa region, during winter. It focuses on key nutritional parameters, including nutritional content, energy value, mineral composition, and digestibility. The analysis followed standard procedures recommended for assessing the nutritional requirements of beef cattle and goats. The discussion highlights the potential of *L. divaricata* as winter forage for beef cattle and goats.

Materials and Methods

Plant material

Samples of *L. divaricata* were obtained from two fields in the Province of La Pampa, Argentina. One field is located in the Department of Loventué, at an elevation of 308 meters above sea level, with coordinates 36°11’00” S latitude and 65°18’00” W longitude, while the other is in the Department of Chalileo, at 306 meters above sea level, with coordinates 36°13’32”S latitude and 66°56’25”W longitude. The samples were obtained from 30 randomly selected specimens located within a radius of 200 meters from the above-mentioned geographical references (15 specimens from Loventué and 15 specimens from Chalileo). All samples were taken during the winter season (late August). Only plant organs compatible

with browsing habits of cattle and goats were collected. All specimens appeared healthy and homogeneous. Leaf samples were taken without considering the size or age of the plant.

Sample conditioning

Samples of 100 g of leaves were collected from 30 randomly selected *L. divaricata* specimens until a total weight of 3 kg was reached. The obtained material was immediately placed in a dark dry place. The samples were mixed and dried under ambient conditions for 7 days until constant weight. Natural drying was preferred due to the presence of resin on the surface of the leaves. The dry matter (DM) and moisture percentages were determined gravimetrically. The dried material was ground in a blade mill, passed through a 2-mm mesh sieve, homogenized, and subdivided into several fractions for various types of analysis.

Other materials

A 95% ethanol aqueous solution (Purocol, Almirante Brown, Argentina) was used as solvent for polyphenol and flavonoid extraction. Distilled water was used as medium for extract resuspension. Folin-Ciocalteu reagent (Sigma Chemical Co.) and sodium carbonate (Alcalis de la Patagonia, San Antonio Oeste, Argentina) were used for polyphenols characterization. Reagent-grade sulfuric acid (Biopack, Buenos Aires, Argentina) was used for lignin determination. Analytical grade dichloromethane (CH_2Cl_2), methanol (MeOH) and N-hexane (Cicarrelli, San Lorenzo, Argentina) were used as extraction solvents for isolating *L. divaricata* resin.

Phytochemical determinations

Qualitative determinations of phytocompounds, including polyphenols and flavonoids, were conducted according to conventional spectrophotometry techniques (2, 5).

The total polyphenolic content was determined using the Folin-Ciocalteu method (37). Briefly, a sample of 1 g of dried material was extracted with 20 mL of 95% ethanol solution at room temperature for 24 h. Subsequently, the obtained extract was filtered and concentrated under vacuum to obtain 500 mg of dry extract. The dry extract was resuspended in 150 mL of distilled water and incubated in a water bath for 30 min at 90°C. A 2 mL aliquot of this resuspended extract was withdrawn and mixed with 1 mL of Folin-Ciocalteu reagent and 10 mL of distilled water. After vortexing, 12 mL of a sodium carbonate aqueous solution (290 g/L) were added to the mixture and left to react for 30 min in the dark. The absorbance at 760 nm of the blue-colored solution was recorded. Results were converted to total polyphenol content expressed in mg of gallic acid equivalents per 100 g of dry sample (mg GAE/100 g). Four random samples were taken from the dry and homogenized material for each polyphenol analysis. Then, three aliquots were taken from the stock solution obtained from each of these four samples. Finally, the absorbance readings of the aliquots were carried out twice.

The total flavonoid content is expressed as milligram quercetin equivalent per gram of extract (mg QE/g) and determined according to the Quercetin standard curve method (36). The aluminum chloride colorimetric method determined flavonoid content in *L. divaricata* extracts. Absorption readings at 425 nm were taken after 30 minutes of incubation in the dark. All analyses were performed in triplicate, and the results were averaged.

Evaluation of nutritional assets of *L. divaricata*

Core nutrients, minerals, and digestibility of *L. divaricata* were measured according to standard recommended techniques (46).

In cattle, forage crude protein (CP) indicates potential growth and productivity enhancement. CP is expressed as a percentage of DM, and determined by measuring nitrogen (N) content, using GAFTA and ISO methods (10, 21). It is important to note that the CP fraction includes non-protein nitrogenous substances such as amines, amides, urea, nitrates, peptides and isolated amino acids. These compounds are soluble, highly degradable and have less nutritional value than true proteins. A high level of CP does not always indicate a good protein level.

Soluble protein (SP) is reported as a percentage of the CP and indicates how much of the total CP is available for digestion. The acid detergent insoluble CP (ADICP) measures the

tightly bound protein not available for digestion. ADICP is a fraction of the acid detergent fiber fraction (ADF) bounded to the protein content. ADF measures the content of cellulose, lignin, Maillard compounds, silica and cutin in the DM. ADF is an indirect indicator of the degree of digestibility of the forage. The higher the ADF, the less digestible is the forage. It is expressed as a percentage of the DM, and determined according to ISO 13906 (25). Neutral detergent insoluble CP (NDICP) measures the amount of protein that is bound to the neutral detergent fiber fraction (NDF). It is expressed as a percentage of the DM, and determined according to ISO 16472 (26). NDF is a measure of the cell wall content in the DM and includes the content of hemicelluloses, cellulose and lignin. ADF is part of NDF. Both ADF and NDF vary with plant phenotype, age and season. The NDF corrected for ash content (aNDF) is expressed as a percentage of DM. High aNDF value generally occurs in plant species with low CP values (45).

Lignin is a polyphenol produced by the plant during its maturation. Lignin is responsible for plant rigidity and support. It is not digestible by ruminants and acts as a barrier to the ruminal microbial digestion of cellulose and hemicelluloses. The lignin content is expressed as a percentage of DM, and measured according to an adapted method based on ISO 13906 (25).

Non-fibrous carbohydrates (NFC) are sugar-related carbohydrates and starches. NFC are nearly 100% digestible. Starch is the most important fraction of the NFC. NFC are necessary for the growth of intestinal bacteria and the production of high-quality bacterial proteins (19). NFC is expressed as a percentage of DM. NFC is determined by measuring the total glucose content, after cleaving sugar-related carbohydrate and starch molecules into individual glucose molecules.

The above-mentioned parameters were determined using a Fibertec™ automated system (Gerber Instruments, Effretikon, Switzerland). The Fibertec™ automated system used programmed standard reference methods.

Ethereal extract (EE) is the lipid fraction of the DM. It is mainly composed of oils, fats and other high-energy nutrients. EE values greater than 14% can be toxic to rumen bacteria. Additionally, EE compounds are prone to becoming rancid during storage. It is expressed as a percentage of DM and determined according to an ether extraction method (9, 23).

The mineral profile in forage is an important factor for extensive livestock production. The ash fraction accounts for the macro and micro inorganic elements of the plant and those acquired from the environment. The ash fraction of common forages is usually less than 10% of DM. Ash contents higher than 10% are considered likely contaminated with soil materials. The ash content is expressed as a percentage of DM and measured gravimetrically, by comparing the weight before and after burning the sample (11, 22). Total mineral in ash was determined using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (NexION 2000 ICP Mass Spectrometer, Perking Elmer, Boston, USA). The elemental analysis was performed using an Atomic Absorption Spectrometer (AAS) (AANALIST 200, Perkin Elmer, USA), according to the standard methods (1, 12, 24). The following elements were determined: calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), phosphorous (P), manganese (Mn), sulfur (S), zinc (Zn), copper (Cu), and iron (Fe).

Evaluation of nutritional assets of *L. divaricata* resin

Subsamples of *L. divaricata* were withdrawn from the original dried sample and used to extract the resin. Fifty g of dried samples were immersed in 200 ml of CH_2Cl_2/MeOH (3:1) at 25°C for 24 h. The supernatant was recovered, filtered and the solvent was evaporated. Subsequently, the dried extract was immersed in N-hexane. The resin was dissolved in the upper N-hexane phase. The N-hexane phase was evaporated to isolate the resin material. The evaluation of the nutritional assets of the *L. divaricata* resin was carried out using the same procedures and methods described in the previous section.

Feedstuff *in vitro* rumen NDF digestion procedure and energy calculations

The total digestible nutrient content (TDN) is calculated as a fraction of the NDF content. Fiber digestibility (NDFD) is important to estimate how much fiber can be digested. NDFD is expressed as a percentage of NDF. The NDFD value is determined using the "traditional" Goering and Van Soest method (tNDFD) (1970). 30 h of digestion time was selected to define the extent of fiber digestion. The indigestible fiber content (uNDF) is expressed as a percentage of DM and defined as the part of the fiber that could not been digested after

30 h of digestion time. Fiber digestibility parameters were determined using a Fibertec™ automated system (Gerber Instruments, Effretikon, Switzerland).

The net energies for lactation (NEL), maintenance (NEM), and growth (NEG) account for the energies needed for milk production, physical activities such as breathing and walking, and muscle and bone formation, respectively. They are expressed in Mcal/kg and calculated according to standard procedures (35, 47).

The metabolizable energy (ME) is accurately determined through in vitro assays, following the traditional Goering and Van Soest method (1970) with the modifications proposed by Goeser (17, 18). This technique determines the feedstuff fiber digestibility in animal nutrition models. Feed samples were digested for 30 h using the standard procedure. The fiber digestion fraction was determined by the difference between intact and digested samples, according to Equations (1) and (2):

$$NDF = 100\frac{(bag + res) - (bag \cdot cf)}{(bag + S) - bag} \quad (1)$$

$$NDFD = 100\frac{1 - NDF_{res}}{NDF_0} \quad (2)$$

were:
S, *bag*, and *res* = the weight of the sample
bag = containing the sample and its residue, respectively
NDF_{res} = the NDF residue after digestion
NDF_0 = the initial NDF (*i.e.*, digestion time = 0 h).

In this test, each sample was analyzed in duplicate.

Statistical analysis

The total polyphenol content was calculated as the mean ± SD. Final mean concentration of polyphenols was calculated using a gallic acid standard curve. Comparison of mean values of measured parameters was performed by a one-way ANOVA (Infostat Software, version 2020) using Duncan's multiple range test, with a level of significance $p < 0.05$.

Results and Discussion

Phytocompounds in *L. divaricata* during winter

The total content of polyphenols and flavonoids in *L. divaricata* extracts resulted in 430.9 ± 2.27 mg GAE/g dry extract and 140.4 ± 2.81 mg GAE/g dry extract, respectively. Phytocompounds have beneficial effects on ruminants. They form tannin-protein complexes that protect proteins from microbial activity in the rumen. As a result, the proteins can reach the intestine and dissociate at the appropriate pH (36, 44).

Nutritional and mineral assets of *L. divaricata* during winter

Table 1 (page 169) summarizes the nutritional composition and mineral content of *L. divaricata*. It also compares *L. divaricata* values with typical nutritional and mineral assets of common cattle feeds (35). The water content resulted in 6.10 wt%. This value is nearly half of the humidity of Alfalfa-R, Corn-G, Sorghum-G, and Barley-G. This result suggests the potential for long-term silage of *L. divaricata* leaves. Low moisture content reduces the risk of fungal proliferation and helps preserve nutritional value of plant material for extended periods.

Table 1. Nutritional profile of browsing available canopy leaves of *L. divaricata* and other forages during winter.

Tabla 1. Activos nutricionales de las hojas de la copa disponibles para el ramoneo de *L. divaricata* durante el invierno y otros forrajes.

	L. divaricata	**Alfalfa-P**	**Alfalfa-R**	**Corn-G**	**Sorghum-G**	**Barley-G**
Humidity	**%WT**					
DM	93.9	21.0	88.9	88.0	86.1	88.0
H_2O	6.10	79.0	11.1	12.0	13.9	12.0
Assets	**%DM**					
CP	11.20	22.8	16.4	9.8	8.3	13.5
ADF	17.42	36.7	31.0	27.3	36.0	23.3
aNDF	35.51	40.0	43.0	12.0	54.9	21.0
EE	5.92	1.4	1.3	3.8	2.2	2.1
Ashes	9.96	11.6	10.0	1.3	1.7	2.3
Lignin	12.09					
NFC	3.82	18.4	22.0	65.0	34.5	62.7
Losses	4.08					
Protein	**%CP**					
SP	79.3-64.7	50	60	60	40	70
ADICP	20.71					
NDICP	35.27					
Minerals	% DM	% NRC	Mineral Content in Common Feed Supplements			
Ca	1.44	0.43-0.66	RGT, corn, sunflower, flax: 0.06–0.38			
K	0.78	0.90-1.00	barley, sunflower, sorghum, wheat: 0.76–1.08			
S	0.37	~0.20	RGT: 0.15, corn in plant: 0.20			
P	0.09	0.28-0.41	RGT: 0.26, corn: 0.22			
Mg	0.09	0.20-0.25	RGT: 0.16, corn in plant: 0.14			
Na	0.0447	~0.18	Corn: 0.06			
Fe	0.0229	0.005	Corn: 0.0048			
Mn	0.00563	0.004	Corn: 0.0010			
Zn	0.00312	0.004	Corn: 0.0046			
Cu	0.00232	0.001	Corn: 0.0013			

Alfalfa-P: alfalfa-based pastures in winter, Alfalfa-R: alfalfa rolls, Corn-G: corn in grains, Sorghum-G: sorghum in grains, Barley-G: barley in grains. NRC recommendations, RGT: RYE GRASS TAMA.

Alfalfa-P: pasturas de base alfalfa en invierno, Alfalfa-R: rollos de alfalfa, Maíz-G: maíz en grano, Sorghum-G: sorgo en grano, Barley-G: heno en granos. Recomendaciones de la NRC, RGT: RYE GRASS TAMA.

The composition of *L. divaricata* in terms of nutritional parameters resulted in 11.20% of CP, 17.42% of ADF, 35.51% of aNDF, 5.92% of EE, 9.96% of Ashes, 12.09% of lignin, and 3.82% of NFC. 4.08% of the material was lost during the analysis.

The CP of *L. divaricata* resulted higher than that of Corn-G and Sorghum-G, and lower than that of Alfalfa-R and Barley-G. In addition, the SP of *L. divaricata* resulted higher than that of Alfalfa-R, Corn-G and Sorghum-G, and lower than that of Barley-G. Interestingly, the SP ranged from 64.73% to 79.29% of the CP, confirming the potential of *L. divaricata* as protein source and nitrogen contributor to bacterial growth and microbial protein synthesis in the rumen (42). Insoluble fractions of CP accounted for 35.27% and 20.71% of CP for NDICP and ADICP, respectively. Thus, *L. divaricata* can be included in cattle diets without significant variations in digestibility and forage intake. *L. divaricata* meets the protein requirements for cattle growth and productivity. This is the first time these values have been reported in scientific literature.

The relatively low ADF and aNDF values further reinforce that *L. divaricata* is a good candidate for inclusion in cattle diets. Similar results have been reported for other forage trees, including *Larrea cuneifolia*, (aNDF = 18.30%) and *Prosopis torquata* (aNDF = 33%) (41). Moreover, the highly energetic EE and NFC fractions did not show excessive values that could disturb the bacterial balance in the rumen. However, the NFC content of *L. divaricata* resulted an order of magnitude lower than the reference forages, suggesting the need for starch-based feed complements.

The most abundant mineral elements in *L. divaricata* were Ca, K, S, P and Mg. In addition, it contained several essential and valuable microminerals, including Fe, Mn, Zn, and Cu. Concerning mineral content, *L. divaricata* meets the forage requirement for Ca, K, Fe, Mn, Zn, and Cu. But, it is deficient in P, Mg and Na.

Nutritional and mineral assets of *L. divaricata* resin during winter

The content of resinous secretions of *L. divaricata* ranged from 10% to 25% of the DM. The high resin content limits the use of *L. divaricata* as a large-scale forage plant (29). Table 2 presents the nutritional composition and mineral content of *L. divaricata* resin. The average values of ash and total EE resulted in 1.05% and 0.70%, respectively. The content of K, Na, Fe, Mg, Ca, Zn, Cu and Mn resulted in 65.00, 31.40, 23.30, 12.70, 5.75, 0.82, 0.67 and 0.14 mg% of resin, respectively. Interestingly, the Fe content in the resin and the leaves were almost identical. S and P were not detected. These results suggest that *L. divaricata* can be a good source of minerals, especially during winter.

Table 2. Chemical profile of *L. divaricata* resin during winter.
Tabla 2. Perfil químico de la resina de *L. divaricata* durante el invierno.

Resin Content	**10 - 25 %**
Components	% of resin
EE	0.70
Ash	1.05
Minerals	**mg% of resin**
K	65.00
Na	31.40
Fe	23.30
Mg	12.70
Ca	5.75
Zn	0.82
Cu	0.67
Mn	0.14
P	ND
S	ND

Note. N/D: Not Detected.
Nota. N/D: No Detectado.

Feedstuff *in vitro* rumen NDF digestion and energy values of *L. divaricata* during winter

The NFC and TDN content resulted in 41.37% and 60.2%, respectively. The NEL, ENG and ENM energy parameters are presented in table 3 (page 171). Concerning forage quality, *L. divaricata* shows values lower than the recommended standards. However, these findings are significant because *L. divaricata* provides a low-cost source of nutrients. This is particularly valuable for animal husbandry practiced in the semi-arid Pampa region during winter. Results are consistent with previously reported literature (41).

Table 3. Digestibility and energy calculations of *L. divaricata* during winter.
Tabla 3. Digestibilidad y cálculos de energía de *L. divaricata* durante el invierno.

DIGEST*	Values
NDFD 30, %FDN	12.8
uNDF 30	30.9
uNDF30om	27.4
NFC	41.3
NRC 2001 Energy calculations Dairy	
TDN 1X	60.2
ENL 3X Mcal/kg	1.35
ENG Mcal/kg	0.78
ENM Mcal/kg	1.36
EM 3X NRC2001 (Mcal/kg)	2.18
EM 1X NRC2001 (Mcal/kg)	2.23

Conclusions

The nutritional value of *L. divaricata* during winter was studied and characterized. The results of this study highlight the significant nutritional potential of *L. divaricata* as a supplementary feed for beef cattle and goats in the semi-arid Pampa region during winter. *L. divaricata* offers a balanced protein content, adequate EM, digestible NDF, and appropriate levels of EE, NFC, and minerals. This makes *L. divaricata* a suitable feed for beef cattle and goats during challenging periods and geographies where pastures are dry, poor in nutrients and scarce.

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Acceptability of bonbons made with camu-camu (*Myrciaria dubia* L)

Aceptabilidad de los bombones elaborados con camu-camu (*Myrciaria dubia* L)

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Abstract

The aim of this study was to formulate and evaluate different formulations of camu-camu bonbons, verifying which formulations obtained greater acceptability and maintenance of their nutraceutical potential. The bonbons were made of 2 chocolate-based toppings (white chocolate and milk chocolate), with three types of camu-camu-based fillings (candy, candy + jelly and candy + syrup). A sensory analysis was performed using a questionnaire and a hedonic scale ranging from 9 to 1 to evaluate appearance, colour, taste and texture. The hedonic scale used to assess purchase intentions ranged from 5 to 1. The physicochemical characteristics and bioactive compounds of the bonbons were evaluated. Bonbons had excellent acceptability rates, where consumers would definitely buy bonbons: white chocolate stuffed with camu-camu candy+camu-camu syrup (F3) and milk chocolate stuffed with camu-camu candy (F4). Consumers would 'probably buy' white chocolate stuffed with camu-camu candy (F1) based on its texture and high levels of vitamin C (VitC), antioxidant activity (FRAP) and (DPPH), phenolic compounds (Phen), and flavonoids (Flavon). Milk chocolate stuffed with camu-camu candy (F4), white chocolate stuffed with camu-camu candy+camu-camu syrup (F3), and white chocolate stuffed with camu-camu candy (F1) have excellent purchase percentages and levels of VitC, FRAP and DPPH, Phen, and Flavon, and especially titratable low acidity; these formulations are highlighted among consumers.

Keywords
by-products • functional properties • human health • industrial potential

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Resumen

El objetivo de este estudio fue formular y evaluar diferentes formulaciones de bombones de camu-camu, verificando cuáles obtuvieron mayor aceptabilidad y mantenimiento de su potencial nutracéutico. Los bombones se elaboraron con 2 coberturas a base de chocolate (chocolate blanco y chocolate con leche), con tres tipos de rellenos a base de camu-camu (dulce, dulce + gelatina y dulce + jarabe). Se realizó un análisis sensorial utilizando un cuestionario y una escala hedónica que iba del 9 al 1 para evaluar la apariencia, el color, el sabor y la textura. La escala hedónica utilizada para evaluar las intenciones de compra iba del 5 al 1. Se evaluaron las características fisicoquímicas y los compuestos bioactivos de los bombones. Los bombones tuvieron excelentes tasas de aceptabilidad, donde los consumidores definitivamente comprarían bombones: chocolate blanco relleno de dulce de camu-camu + jarabe de camu-camu (F3) y chocolate con leche relleno de dulce de camu-camu (F4). Los consumidores 'probablemente comprarían' chocolate blanco relleno de dulce de camu-camu (F1) basado en su textura y altos niveles de vitamina C (VitC), actividad antioxidante (FRAP) y (DPPH), compuestos fenólicos (Phen) y flavonoides (Flavon). El chocolate con leche relleno de dulce de camu-camu (F4), el chocolate blanco relleno de dulce de camu-camu + jarabe de camu-camu (F3) y el chocolate blanco relleno de dulce de camu-camu (F1) tienen excelentes porcentajes de aceptabilidad de compra y niveles de VitC, FRAP y DPPH, Phen y Flavon, y especialmente baja acidez titulable; estas formulaciones se destacan entre los consumidores.

Palabras clave
subproductos • propiedades funcionales • salud humana • potencial industrial

Introduction

Camu-camu (*Myrciaria dubia* (Kunth) Mc Vaugh), which belongs to the Mytaceae family, is among the native Amazonian species with great industrial potential. This potential for economic exploitation occurs due to the great benefits of the fruit, especially due to the high levels of vitamin C and bioactive compounds.

Among the chemical compounds present in camu-camu fruits, vitamin C is the most abundant, reaching up to 7,355 mg ascorbic acid (4). In addition, fruits contain other compounds, such as catechins and their derivatives, carotenoids, anthocyanins and flavonoids, which, together with vitamin C, provide high levels of antioxidant capacity (5).

Combined with the benefits of chemical compounds, fruits contain considerable amounts of micronutrients, such as mineral salts and fibre, which provide excellent nutritional and functional sources (16). However, due to several factors inherent to the culture, such as production concentrated at specific times of the year, the rapid loss of postharvest quality and the high acidity of the fruit when consumed in natura, the commercialization of the fruits is limited, and the fruits are little used by consumers.

One of the ways to explore the potential of camu-camu fruits is the development of industrial processing technology, a solution that seeks to reduce costs, add value and promote family farming (15). This technique has been used for the production of jellies (17), popsicles (15, 16) and yogurts (6) with high nutritional potential and food safety; therefore, this technique is a viable alternative to the use of camu-camu in byproducts (13). However, the use of bonbons as a byproduct of camu-camu fruit has not yet been tested.

Bonbons are some of the most consumed foods in the world and are one of the ways to market chocolate; they can contain several types of fillings that can be made with fruits, pieces of fruit, oil seeds, sugar, milk, butter, cocoa, liqueurs and other food substances covered with a layer of chocolate (8, 27). The insertion of products derived from fruits with excellent functional properties, whether artisanal or industrial, increases the possibility of consumer acceptance.

The commercialization of bonbons (of the most varied flavours) produced on an artisanal and/or industrial scale is growing and constant, moving the formal market (21). In the Amazon region, a large exploitation of products with fillings containing the most diverse fruits has been noted, such as Brazil nuts, cupuaçu and açai (19, 30, 32). Thus, due to the great functional and nutraceutical potential of the fruits, we emphasize the importance

of the production and characterization of byproducts based on camu-camu through the processing of parts of the fruit (seed, peel or pulp), in this way adding value to the product and maintaining its organoleptic and nutraceutical characteristics.

Thus, the objective of this study was to formulate and evaluate different formulations of camu-camu bonbons, verifying which formulations obtained greater acceptability and maintenance of their nutraceutical potential.

Materials and methods

Raw material

The fruits used were obtained from the Serra da Prata experimental farm belonging to Embrapa Roraima, located in the municipality of Mucajaí 62 km from Boa Vista and located at the geographical reference coordinates W 60°58'40" N 2°23'49". The fruits were manually harvested in the early hours of the day, considering the greenish-red bark colour, because at this stage, a longer shelf life is provided to the fruits, and the fruits retain their good quality attributes for a longer time (14, 18).

After harvesting, the fruits were packed in plastic bags, packed in coolers with ice and transported to the Post-Harvest, Agroindustry and Tissue Culture Laboratory, Embrapa - RR. Then, they were cleaned and sanitized with 0.02% sodium hypochlorite (NaClO) for 30 minutes, following the recommendations of the National Health Surveillance Agency (ANVISA). After cleaning, the fruits were manually pulped without water, and the pulp was separated from the peel and the seeds. A schematic representation of the steps for the production of byproducts is shown in figure 1 (page 177).

Experimental design

The experimental design was completely randomized in a 2 x 3 factorial scheme with three repetitions, each repetition consisting of 3 bonbons. The factors were the addition of 2 chocolate-based toppings (white chocolate and milk chocolate) with three types of camu-camu-based fillings (candy, candy + jelly and candy + syrup).

The mixtures of the formulations resulted in six different bonbons: F1-bonbon covered with white chocolate and stuffed with camu-camu candy (WCC); F2-bonbon covered with white chocolate and stuffed with camu-camu candy + camu-camu jelly (WCC+J); F3-bonbon covered with white chocolate and stuffed with camu-camu candy + camu-camu syrup (WCC+S); F4-bonbon covered with milk chocolate and stuffed with camu-camu candy (MCC); F5-bonbon covered with milk chocolate and stuffed with camu-camu candy + camu-camu jelly (MCC+J); and F6-bonbon covered with milk chocolate and stuffed with camu-camu candy + camu-camu syrup (MCC+S).

Industrial process

The camu-camu candy (the truffle type) used in all treatments was made using 48% white chocolate (Mavaleiro, São Paulo - Brazil), 24% can of cream (Bela Vista Dairy products, Goiás - Brazil), 4.8% tablespoons of condensed milk (Goias Minas industry, GOIAS - BRAZIL), 0.8% teaspoon of butter (INDUSTRY BRF, PARANA - BRAZIL) 20% cup of camu-camu pulp (Embrapa, Roraima - Brazil), and 2.4% dessert spoon of corn syrup (Industry Arco-íris, São Paulo - Brazil). The white chocolate was melted in a bain-marie model Magio MS-800F (Lactea Scientific, São Paulo - Brazil), and then the other ingredients were added and mixed until a homogeneous mass was formed. After this procedure, the homogeneous mass was refrigerated for cooling.

The jelly was obtained using the following ingredients in percentages: pure jelly (51% jelly, 49% sugar and 0.005% pectin) according to Grigio *et al.* (2021b). The ingredients were brought to medium heat until they were cooked and homogenized. Then, they were placed in glass jars to be added to the other treatments.

The seeds were removed manually and the camu-camu syrup was manufactured using the following ingredients at the percentages described: 45% fruit (peel+pulp) without seeds, 30% sugar and 25% water. The ingredients were placed over medium heat until the sugar had completely melted, forming a syrup next to the fruits. After this process, they were placed in glass jars to be added to the other treatments.

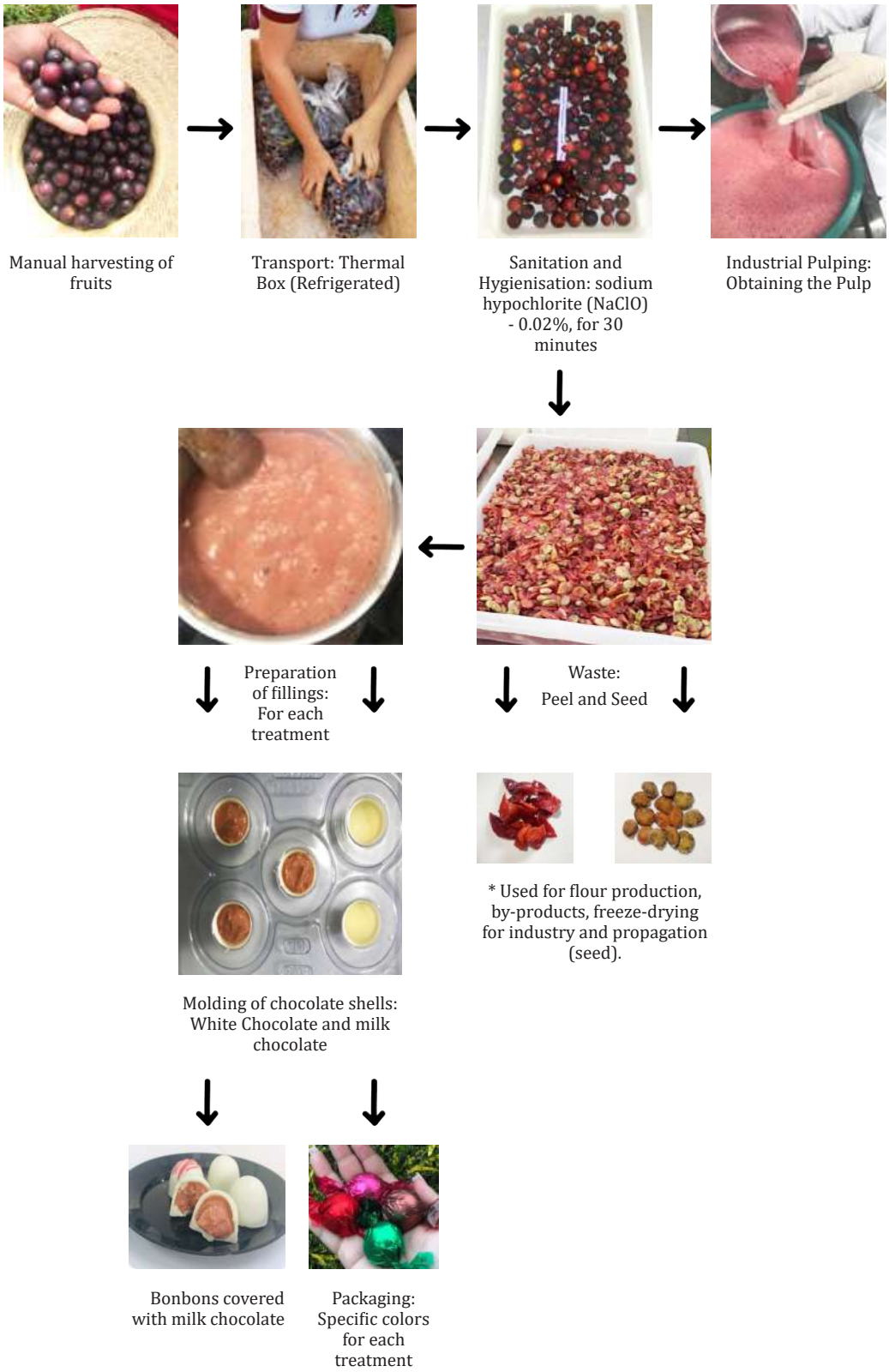


Figure 1. Schematic representation of the steps for the production of byproducts.
Figura 1. Representación esquemática de los pasos para la producción de subproductos.

The chocolates were melted in a bain-marie and moulded in round acetate moulds with a 5 cm cavity to cover the candy. After the chocolates were moulded, the fillings were distributed according to the treatments and then covered with a layer of chocolate to finish the moulding of the chocolates. Once finished, the chocolates were wrapped in aluminium foil with different colours that indicated the different treatments so that the sensory analysis of the products could be conducted.

Sensory analysis

This study was duly registered and approved by the Research Ethics Committee of the Federal University of Roraima, under number 41734015.0.0000.5302, and the sensory analyses were performed at Embrapa Roraima, with the participation of 40 untrained tasters. The samples were placed in disposable cups and coded with random numbers. Each appraiser received all six jelly formulations and one sheet containing a questionnaire and a hedonic scale ranging from 9 to 1 to evaluate appearance, colour, taste and texture (9-Liked it extremely, 8-Liked it a lot, 7-Liked it, 6-Somewhat liked it, 5-Indifferent, neither liked nor disliked, 4-Somewhat disliked, 3-Disliked, 2-Disliked moderately and 1-Disliked extremely), and another scale to gauge purchase intent, as previously reported (9), (1-Definitely would buy, 2-Probably would buy, 3-Maybe yes/maybe no, 4-Probably wouldn't buy, 5-Definitely would not buy). During the evaluations, the evaluators drank water to ensure no interference between the formulations analysed.

We calculated the product acceptability index using the expression IA (%) = A × 100/B, where A = the average grade obtained for the product and B = the maximum grade given to the product. Usually, an acceptability index ≥ 70% is considered to indicate good repercussion (11).

Physicochemical characteristics and bioactive compounds

pH (hydrogen potential): The pH was determined according to the methodology of AOAC (2012), by directly immersing a pH meter in the formulations. Soluble Solids (SS): SS were determined by refractometry with a portable refractometer (SOLOESTE brand, model RT-30ATC) with automatic temperature compensation (10 to 30°C), and the results are reported in °Brix (1). Titratable acidity (TA): Using the methodology described in AOAC (2012), 10 g of each formulation was diluted in 100 mL of distilled water. After the addition of the phenolphthalein indicator, the solution was titrated with a 0.1 M NaOH solution. The results are reported as mg of citric acid per 100 g^{-1} sample.

Ratio: The ratio between the quantities of SS and TA. Ascorbic acid (VitC) was extracted with 0.5% oxalic acid and titrated with 2,6-dichlorophenolindophenol (26).

Antioxidant activity (FRAP): The antioxidant capacity of each sample was estimated with the iron reduction method (FRAP) following a procedure adapted previously (29). Approximately 1 g of sample was mixed with 40 mL of 50% methanol, homogenized, and allowed to stand for 60 minutes at room temperature. After this period, the samples were centrifuged (25.406,55 g) for 15 minutes, and the supernatant was transferred to a 100 mL volumetric flask. Forty millilitres of 70% acetone was added to residue of the first extraction, and the residue was homogenized and allowed to stand for 60 minutes at room temperature. After one hour, the samples were centrifuged again (25.406,55 g) for 15 minutes, and the supernatant was transferred to a volumetric flask containing the first supernatant and the volume was completed with distilled water. The obtained extract, which was added to the FRAP reagent, was placed in a warm water bath at 37°C. The absorbance of the samples was measured at 595 nm, and the results are presented as mg of ferrous sulfate g^{-1} sample.

Antioxidant activity (DPPH): The antioxidant activity can be determined in terms of the oxidation inhibition potential using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as a reference (3). One gram of sample was weighed, after which 10 ml of ethyl alcohol was added, and the sample was homogenized and centrifuged for 50 minutes. After this period, the supernatant was removed with a pipette, and the solution was placed in a dark flask in an ice bath, to which 3 mL of ethanol was added. The absorbance of 500 µL of the sample extract supplemented with 300 µL of the DPPH solution was measured at 517 nm with a spectrophotometer. The results are expressed as µg of ascorbic acid equivalent g^{-1} sample.

The phenolic compound (Phen) content was determined according to the Folin-Ciocalteu spectrophotometric method described by Singleton *et al.*, 1999. An aliquot of 20 µL of sample was diluted to 1.58 mL with water, 100 mL of Folin-Ciocalteu reagent was added, and the sample was homogenized. Between 30 sec and 8 min, 300 µL of the sodium carbonate solution was added, and the mixture was homogenized again. The solutions were incubated at 20°C for 2 hours, and the absorbance of each solution was determined at 765 nm. The results are expressed as mg of g^{-1} gallic acid in the sample.

Total flavonoid (Flavon) content was determined with the aluminium chloride colorimetric assay (34) using quercetin as a standard. For extraction, a methanol solution and 5% aluminium chloride were added. After 30 minutes, the absorbance was measured at 441 nm with a spectrophotometer. For each sample, a blank was made containing the added methanol sample. The results are expressed in µg quercetin equivalents g^{-1} sample. The total anthocyanin content was determined according to the method described by Lees and Francis (1972), using cyanidin as a standard. Samples were added to an acidified methanol solution (HCl (85:15)), and after homogenization, the samples were stored in the dark. After storage for 24 h, the absorbance of the samples was measured at 520 nm using a spectrophotometer. The results are expressed as µg cyanidin equivalent g^{-1} sample.

Statistical analysis

The statistical analysis of the data is reported as the mean ± standard deviation. Analyses of variance (ANOVAs) were performed with F ($p<0.01$). Means were compared according to the test of minimum significant difference (LSD test) ($p<0.05$). The relationships between the parameters evaluated for the different bonbon formulations were estimated by calculating the Pearson correlation coefficient ($p<0.05$). The variables were subjected to a multivariate analysis. Multivariate data analysis was performed using principal component analysis (PC) to better show the distribution of different bonbon formulations and the effects of the intention to buy products on sensory characteristics, organoleptic quality and bioactive compounds. The analyses were performed with R software (Boston, USA) (25).

Results

Sensory characteristics

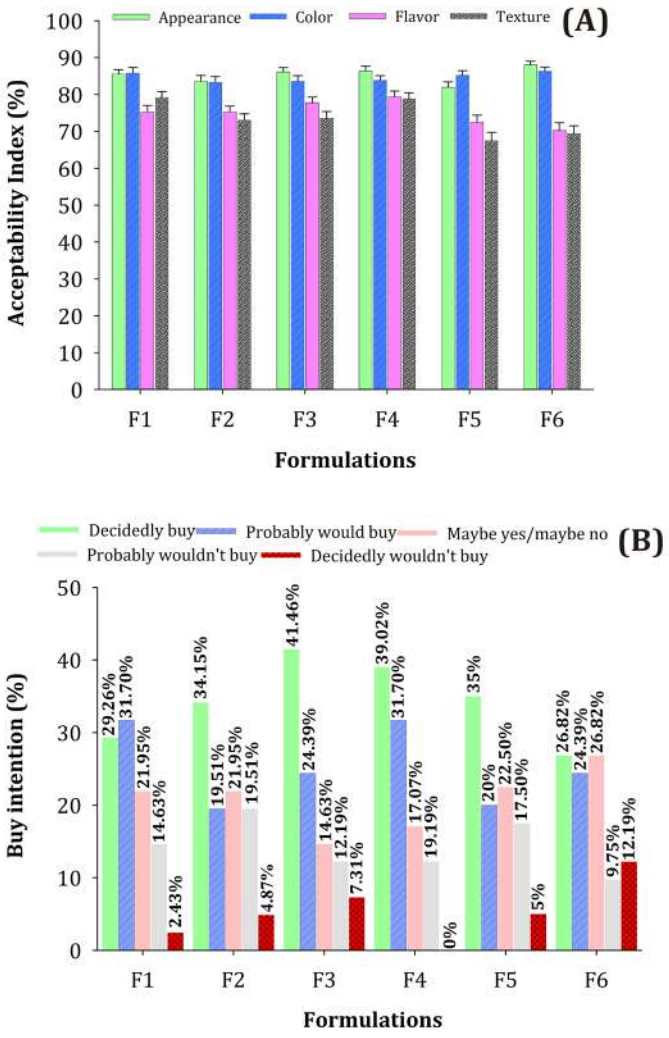
According to the sensory analysis (figure 2A, page 180), the bonbons covered with white chocolate filled with camu-camu candy (F1), camu-camu candy + camu-camu jelly (F2) and camu-camu candy+camu-camu syrup (F3) and those covered with milk chocolate with camu-camu sweet filling (F4) showed greater acceptability in terms of appearance, colour, flavour and texture, with indices greater than 73% (figure 2A, page 180).

The F4 and F3 bonbons had the most favourable flavours. The F1 and F4 bonbons, on the other hand, showed greater acceptance in terms of texture (figure 2A, page 180). Bonbons covered with milk chocolate and stuffed with camu-camu candy + camu-camu syrup (F6) and milk chocolate with camu-camu candy filling + camu-camu jelly (F5) provided poor consumer acceptance in terms of texture acceptability, with a low acceptability index (<70%) (figure 2A, page 180).

For purchase intent, consumers would definitely buy bonbons in the following order: F3 and F4 (figure 2B, page 180). Consumers would probably buy bonbons F1 and F4 (figure 2B, page 180). The lowest consumer uncertainty was recorded for the F3 and F4 bonbons, where participants reported maybe yes/maybe they would not buy (figure 2B, page 180). The greatest rejections, where consumers probably would not and decidedly would not buy the bonbons, were for the F5, F2, and F6 formulations, respectively (figure 2B, page 180).

Physicochemical and nutraceutical qualities

The results showed significant individual effects of coverage (white chocolate-WC and milk chocolate-MC) and fillings (candy, candy+jelly and candy+syrup) on the pH and soluble solid (SS) contents of the bonbons ($p<0.01$) (table 1, page 180).



Formulations: (F1) (white chocolate stuffed with camu-camu candy); (F2) (white chocolate stuffed with camu-camu candy + camu-camu jelly); (F3) (white chocolate stuffed with camu-camu candy + camu-camu syrup); (F4) (milk chocolate stuffed with camu-camu candy); (F5) (milk chocolate stuffed with camu-camu candy + camu-camu jelly); and (F6) (milk chocolate stuffed with camu-camu candy + camu-camu syrup). Means + standard deviations (*n*=40).

Formulaciones: (F1) (chocolate blanco y relleno de dulce de camu-camu); (F2) (chocolate blanco y relleno de dulce de camu-camu + gelatina de camu-camu); (F3) (chocolate blanco y relleno de dulce de camu-camu + camu-camu en almíbar); (F4) (chocolate con leche y relleno de dulce de camu-camu); (F5) (chocolate con leche y relleno de dulce de camu-camu + gelatina de camu-camu); y (F6) (chocolate con leche y relleno de dulce de camu-camu + camu-camu en almíbar). Medias + desviación estándar (n=40).

Figure 2. Acceptability index (A) and purchase intention (B) of different bonbon formulations.

Figura 2. Índice de aceptabilidad (A) e Intención de compra (B) de diferentes formulaciones de bombones.

Table 1. The average pH and soluble solid contents of bonbons covered with white and milk chocolate and stuffed with camu-camu candy, camu-camu candy + camu-camu jelly and camu-camu candy + camu-camu syrup.

Tabla 1. Valores promedio de pH y sólidos solubles de bombones cubiertos de chocolate blanco y con leche, rellenos de dulce de camu-camu, dulce de camu-camu + gelatina de camu camu y dulce de camu-camu + jarabe de camu-camu.

Covered	pH	Soluble Solids (°Brix)
White chocolate	4.87 a ± 0.27 a	52.55 ± 5.32 a
Milk chocolate	4.69 b ± 0.25 b	49.33 ± 5.83 b
Stuffed		
Camu-camu candy	5.06 ± 0.16 a	52.33 ± 1.63 b
Candy + camu-camu jelly	4.78 ± 0.07 b	56.5 ± 1.87 a
Candy + camu-camu in syrup	4.50 ± 0.17 c	44 ± 2.37 c
CV (%)	2.09	2.09

* Averages followed by the same letter in the column are not different from each other according to the least significant difference (LSD) test (p>0.05). ** Means ± standard deviations.

* Promedios seguidos de la misma letra en la columna no son diferentes entre sí, por la prueba de diferencia mínima significativa (LSD) (p>0.05). ** Media + desviación estándar.

The pH of the bonbons that were covered with WC was less acidic than that of the bonbons covered with MC ($p<0.05$). The pH of the bonbons that received the candy filling was less acidic than that of the bonbons that received candy+jelly or candy+syrup ($p<0.05$). The bonbons were also shown to have greater sweetness when receiving WC coverage than when receiving MC ($p<0.05$). The chocolates with the candy+jelly filling had the greatest sweetness, while those that received candy+syrup had the least sweetness, with low SS values ($p<0.05$) (table 1, page 180).

For the variables titratable acidity (TA), ratio (SSTA), ascorbic acid (VitC), anthocyanins (Antho), flavonoids (Flavon), phenolic compounds (Phen) and antioxidant activity (FRAP and DPPH), a significant effect of the interaction between the type of chocolate coating and filling with camu-camu was observed ($p<0.01$) (table 2).

Table 2. Average values of physicochemical characteristics, bioactive compounds and antioxidant activity of the bonbons covered with white and milk chocolate stuffed with camu-camu candy, camu-camu candy+camu-camu jelly and camu-camu candy+camu-camu syrup.

Table 2. Valores promedio de características fisicoquímicas, compuestos bioactivos y actividad antioxidante de los bombones cubiertos de chocolate blanco y con leche, rellenos de dulce de camu-camu, dulce de camu-camu+gelatina de camu-camu y dulce de camu-camu+jarabe de camu-camu.

Covered	Camu-camu candy	Candy+camu-camu jelly	Candy+camu-camu syrup
	Titratable acidity (mg citric acid 100 g sample^{-1})		
White Chocolate	2.63±0.17a A	3.23±0.10 a A	3.03±0.15 b A
Milk chocolate	2.90±0.32 a B	3.10±0.10 a B	4.66±0.57 a A
CV (%)	9.23		
	***Ratio* SSTA**		
White Chocolate	21.85±0.65 a A	17.97±0.50 a B	15.19±0.98 a C
Milk chocolate	17.64±1.41 b A	17.75±0.89 a A	9.10±1.22 b B
CV (%)	6		
	Ascorbic acid (mg 100 g sample^{-1})		
White Chocolate	828.79±6.15 a A	567.08±4.61 a B	307.34±4.61 a C
Milk chocolate	314.39±6.04 b B	359.69±7.99 b A	311.37±9.06 a B
CV (%)	1.48		
	Anthocyanins (µg cyanidin g sample^{-1})		
White Chocolate	44.55±0.67 a B	63.01±0.51 a A	34.15±0.51 a C
Milk chocolate	34.93±2.05 b B	39.97±0.88 b A	34.15±1.27 a B
CV (%)	2.69		
	Flavonoids (µg quercetin g sample^{-1})		
White Chocolate	913.42±13.42 a A	630.08±5.12 a B	341.49±5.12 a C
Milk chocolate	349.32±6.71 b B	399.66±8.87 b A	341.49±12.70 a B
CV (%)	1.87		
	Phenolic compounds (mg gallic acid g sample^{-1})		
White Chocolate	0.91±0.01 a A	0.63±0.01 a B	0.34±0.01 a C
Milk chocolate	0.34±0.00 b B	0.40±0.01 b A	0.34±0.01 a B
CV (%)	1.87		
	Antioxidant Activity (DPPH) (µg of ascorbic acid g sample^{-1})		
White Chocolate	91.34±1.34 a A	63±0.51 a B	34.14±0.51 a C
Milk chocolate	34.93±0.67 b B	39.96±0.88 b A	34.14±1.27 a B
CV (%)	1.87		

* Averages followed by the same lowercase letter in the column and uppercase letter in the row are not different according to the least significant difference (LSD) test ($p>0.05$). ** Means ± standard deviations.

* Promedios seguidos de la misma letra minúscula en la columna y mayúscula en la fila no son diferentes, por la prueba de diferencia mínima significativa (LSD) ($p>0,05$). ** Media + desviación estándar.

Covered	Camu-camu candy	Candy+camu-camu jelly	Candy+camu-camu syrup
	Antioxidant Activity (FRAP) (mg ferrous sulfate g sample^{-1})		
White Chocolate	920.13±6.71 a A	630.08±5.12 a B	341.49±5.12 a C
Milk chocolate	349.32±6.71 b B	399.66±8.87 b A	341.49±12.70 a B
CV (%)	1.61		

* Averages followed by the same lowercase letter in the column and uppercase letter in the row are not different according to the least significant difference (LSD) test (p>0.05). ** Means ± standard deviations.

* Promedios seguidos de la misma letra minúscula en la columna y mayúscula en la fila no son diferentes, por la prueba de diferencia mínima significativa (LSD) (p>0,05). ** Media + desviación estándar.

No significant differences in TA were observed between the bonbons with WC and MC coverage that received the fillings with candy and candy+jelly (p>0.05). The bonbons coated with MC and stuffed with candy+syrup were less acidic than the candies coated with WC filled with candy+syrup (p<0.05). WC bonbons with different fillings did not show significant differences regarding the TA (p>0.05). However, MC bonbons had higher TA values with candy+syrup fillings, differing statistically from those of bonbons filled with candy and candy+jelly (p<0.05) (table 2, page 181).

The bonbons with WC coatings filled with candy and candy+jelly had higher levels of ascorbate acid, anthocyanin, flavonoids, and phenolic compounds and greater antioxidant activity (DPPH and FRAP) than the bonbons with MC coatings (p<0.05). A significant difference was not observed between the bonbons coated with WC and those coated with MC that received candy+syrup fillings (p>0.05) (table 2, page 181).

The bonbons coated with WC and filled with candy had higher levels of ascorbic acid, flavonoids, phenolic compounds, DPPH and FRAP, differing statistically from the bonbons filled with candy+jelly and candy+syrup (p<0.05). The bonbons with the MC coating, which were filled with candy+jelly, presented the highest levels of ascorbic acid, flavonoids, phenolic compounds, DPPH and FRAP (p<0.05) (table 2, page 181). The values of anthocyanins were greater in bonbons filled with candy+jelly, both with WC and MC coatings (p<0.05) (table 2, page 181).

Multivariate analysis

The multivariate analysis of principal components (PCs) showed a cumulative variance of 89.06%. For PC1, the F1 and F6 bonbons contributed the most, with contributions of 51.60% and 27.34%, respectively. For PC2, the largest contributions were from bonbons F6 and F4, at 49.27% and 27.03%, respectively (figure 3, page 183).

The variables that showed the greatest correlations with PC1 were phenolic compounds (Phen), antioxidant activity (DPPH and FRAP), flavonoids, and ascorbic acid (AA) (r2= 0.95, p=0.03), pH (r2= 0.89, p=0.02) and the SSTA ratio (r2= 0.87, p =0.02). For PC2, only the purchase index of would definitely buy was significantly correlated (r2= -0.84, p=0.03) (figure 3, page 183).

The question of flavour acceptability (figure 2B, page 180) was what led most consumers to decide to buy the bonbons (r2= 0.78, p= 0.05), and colour negatively influenced this decision (r2=-0.82, p=0.01) (figure 3, page 183). The texture of the bonbons was the factor that influenced consumers to probably buy the bonbons (r2= 0.84, p=0.01); consumers who preferred the bonbons due to the texture also considered the greater flavour (r2= 0.75, p= 0.05) and lower acidity (>pH) (r2= 0.75, p=0.05) (figure 3, page 183). The flavour and colour acceptability indices of the bonbons contributed to greater uncertainty among consumers, as the lower the flavour (r2=-0.90, p=0.01) was, the greater the uncertainty of purchase, and the greater the colour intensity of the bonbons (r2= 0.74, p= 0.05) was, the greater the uncertainty of the purchase (figure 3, page 183).

The factors that influenced consumers to probably not buy bonbons were high levels of anthocyanins (Antho) (r2= 0.84, p=0.01) and soluble solids (SS) (r2= 0.93, p= 0.001) (figure 3, page 183). The majority of consumers who did not like the appearance probably would not buy bonbons (r2= -0.90, p= 0.001) (figure 3, page 183). For consumers who definitely would not buy bonbons, a high TA content was one of the indices that most contributed to this decision (r2= 0.86, p= 0.01), and F6 bonbons were the ones that most contributed to this decision. The greater the acidity of the sweets (pH) (r2 = -0.86, p= 0.01) and the smaller the SSTA ratio (r2= -0.85, p=0.01) were, the greater the percentage of consumers who decidedly would not buy the bonbons (figure 3, page 183).

Formulations: (F1) (white chocolate stuffed with camu-camu candy); (F2) (white chocolate stuffed with camu-camu candy + camu-camu jelly); (F3) (white chocolate stuffed with camu-camu candy + camu-camu syrup); (F4) (milk chocolate stuffed with camu-camu candy); (F5) (milk chocolate stuffed with camu-camu candy + camu-camu jelly); and (F6) (milk chocolate stuffed with camu-camu candy + camu-camu syrup).

Formulaciones: (F1) (chocolate blanco y relleno de dulce de camu-camu); (F2) (chocolate blanco y relleno de dulce de camu-camu + gelatina de camu-camu); (F3) (chocolate blanco y relleno de dulce de camu-camu + camu-camu en almíbar); (F4) (chocolate con leche y relleno de dulce de camu-camu); (F5) (chocolate con leche y relleno de dulce de camu-camu + gelatina de camu-camu); y (F6) (chocolate con leche y relleno de dulce de camu-camu + camu-camu en almíbar).

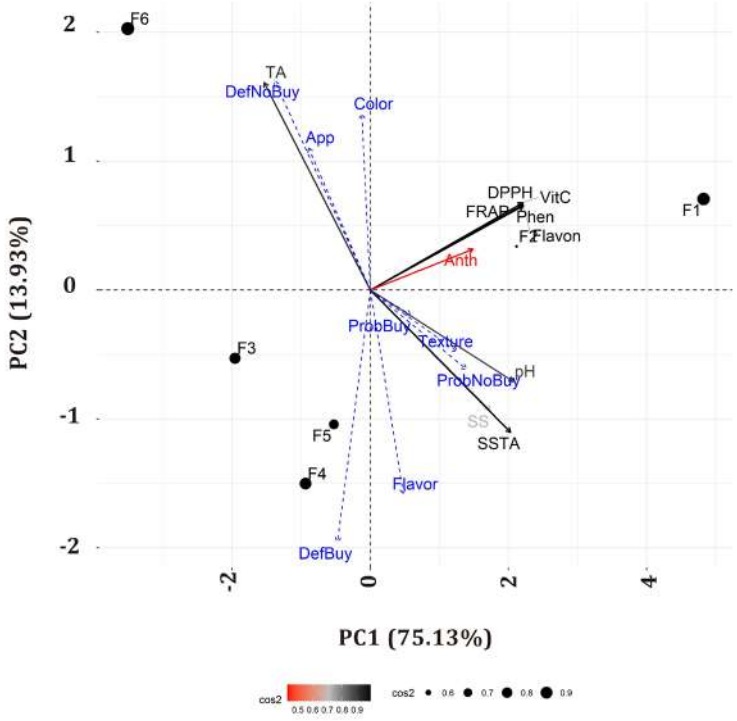


Figure 3. Principal component (PC) analysis of sensory characteristics, organoleptic quality and bioactive compounds was performed on different bonbon formulations with the intention of purchasing products (*n* = 114).

Figure 3. Análisis de componentes principales (PC) realizados en diferentes formulaciones de bombones y con la intención de adquirir productos sobre características sensoriales, calidad organoléptica y compuestos bioactivos (n = 114).

Confirming the results of the descriptive analysis (table 2, page 181-182), F1 bonbons showed the highest correlations with antioxidant activity (Pheno, Flav, AA, DPPH, FRAP and Antho) (figure 3). The lowest levels of antioxidant activity were found in the F4 and F3 bonbons, which consumers would definitely buy (figure 3).

Discussion

The highest taste acceptance index of bonbons occurs because products processed from camu-camu improve their acceptance (23), and when products are processed as jellies, improvements in acceptance and purchase intent occur (17, 33). The greater acceptance of products with stronger colours is because the first contact of consumers is through the visual aspect of the product (7, 22, 28). These flavour and colour parameters, along with the texture, provided bonbons with lower acidity and rigidity, resulting in higher levels of acceptability and purchase intent, which were also observed in jellies made from camu-camu (17).

The largest rejections of the bonbons were due to higher acidity, leading to low acceptance (<70%). The lowest acceptability rates (< 70%) due to acidity have already been observed in other studies, such as those involving popsicles (15, 16) and jelly (17); therefore, such formulations must be rejected. The use of a sensory analysis to better understand consumers is highly important because it provides valid and reliable product results (7). The evaluation of purchase intentions, on the other hand, provides us with important real parameters for the possible commercialization of products (17).

The pH values of the bonbons were higher than those observed in the literature for other products based on camu-camu (16, 17), and consequently, the products had higher acceptability rates. The lower acidity (>pH) of the candy filled with candy was caused by its composition containing a good percentage of white chocolate (48%), which increases the pH of the products (17, 27).

The bonbon covered with milk chocolate and filled with candy + camu-camu syrup (F6) showed a lower quality index SSTA ratio due to higher TA and lower SS (°Brix) contents and higher acidity (<pH) because the camu-camu syrup has camu-camu peel in its composition, and a second (14, 16, 24) had a greater amount of acids, as already observed in camu-camu fruits.

The higher levels of bioactive compounds in the F1 and F2 bonbons indicated a low purchase intention, especially regarding anthocyanin contents, where consumers probably would not buy the bonbons (figure 3, page 183). Formulations with higher levels of bioactive compounds cause astringency in the products, which has already been observed in other studies (16, 17). The presence of milk in the formulations negatively affects the amount of phenolic compounds, degrading an abundant portion of these compounds (2, 16), which was also observed in the present study, both for phenolic compounds and for other bioactive compounds that showed strong correlations with each other (figure 3, page 183).

The antioxidant activity has a direct correlation with the content of phenolic compounds (10, 12), which was also observed in the present study. High concentrations of ascorbic acid and other acids during cooking resulted in low consumer acceptance (17), which proves the low levels of these compounds in the formulations with candy + jelly and candy + camu-camu syrup fillings, together with the fact that milk chocolate negatively affects bioactive compounds, as already described.

Conclusions

Milk chocolate stuffed with camu-camu candy (F4), white chocolate stuffed with camu-camu candy+camu-camu syrup (F3), and white chocolate stuffed with camu-camu candy (F1) showed excellent purchase percentages and low rejection, with the highest acceptability evaluations of flavour and texture. In addition, due to the excellent levels of vitamin C, FRAP and DPPH antioxidant activity, phenolic compounds, and flavonoids, and especially the titratable low acidity and medium sweetness, these formulations are highlighted among consumers.

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Conflict of Interest

Declarations of interest: none'

Ethics approval statement

This study was duly registered and approved by the Research Ethics Committee of the Federal University of Roraima, under number 41734015.0.0000.5302

Antimicrobial and antioxidant properties of the woody endocarp of native and commercial walnuts from Argentina

Propiedades antimicrobianas y antioxidantes del endocarpio leñoso de nogales nativos y comerciales de Argentina

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Abstract

Juglans australis is a tree from the *Juglandaceae* family found in the southernmost region of America. Its small edible nuts are not commercialized, and their bioactive characteristics are unknown. This study first reports the antioxidant, antiradical, and antibacterial activity of extracts from this native walnut against phytopathogenic bacteria and compared with its commercial counterpart, *J. regia* L. Different extracts from the woody endocarp (shells) were obtained using methanol and ethyl acetate. Methanolic extracts significantly inhibited phytopathogenic growth at all concentrations tested (0.1, 1, and 10 mg/mL). The best activity was reported against *Xanthomonas.* Highest total phenolics and the most significant antioxidant activity were determined in methanolic extracts (TPC: 121 mg gallic acid equivalent (GAE)/g of dried peel, FRAP: 58.6 mmol Trolox/100 g of peel dried and 9.7 mM Trolox/100 g of dried peel). Extracts from both species demonstrated congruent patterns. Gallic acid was the most abundant compound in the methanolic extract. However, extracts demonstrated superior efficiency, suggesting a potential synergistic effect among their components. Antioxidant and antimicrobial activity of methanolic extracts against *Xanthomonas* make them potential control agents.

Keywords
Juglans australis • phytopathogens • polyphenols • bioactive compounds • sustainable agriculture • *Xanthomonas* sp.

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Resumen

Juglans australis es un árbol perteneciente a la familia *Juglandaceae* que se encuentra en la región más austral del continente americano. Aunque las nueces también son comestibles, son pequeñas y no se comercializan, sus características bioactivas son desconocidas. Este estudio constituye el primer informe sobre la actividad antioxidante, antirradical y antibacteriana de extractos de la nuez nativa frente a bacterias fitopatógenas, y su comparación con la especie comercial, *J. regia* L. Se obtuvieron diferentes extractos a partir del endocarpio leñoso (cáscaras) utilizando metanol y acetato de etilo. El extracto metanólico resultó ser la fracción más activa e inhibió significativamente el crecimiento de los fitopatógenos en todas las concentraciones analizadas (0,1, 1 y 10 mg/mL). La mejor actividad se registró para el género *Xanthomonas*. El mayor contenido de fenoles totales y la actividad antioxidante más significativa se determinó en el extracto metanólico (TPC: 121 mg de ácido gálico equivalente (GAE)/g de cáscara seca, FRAP: 58,6 mmol de Trolox/100 g de cáscara seca y 9,7 mM de Trolox/100 g de cáscara seca). Los extractos de ambas especies se comportaron de manera similar. Al analizar la composición química, el ácido gálico fue el compuesto más abundante en el extracto metanólico. Sin embargo, los extractos mostraron una eficiencia superior, lo que sugiere un posible efecto sinérgico entre sus componentes. La actividad antimicrobiana de los extractos metanólicos contra *Xanthomonas*, junto con su capacidad antioxidante, resalta su potencial aplicación como agentes de control de fitopatógenos.

Palabras claves

Juglans australis • fitopatógenos • polifenoles • compuestos bioactivos • agricultura sustentable • *Xanthomonas* sp.

Introduction

Phytopathogens cause significant economic loss in agriculture (35). Even considering synthetic pesticides imply detrimental environmental and human health consequences, several products are still being used. In the last decade, eco-friendly compounds have emerged as alternative pesticides (8, 19). These natural antimicrobial agents are safer and cheaper than chemical agents (48) contributing to the economy and environment (9, 19, 44).

Several plant bioactive compounds have shown antimicrobial activity (14). Phenolic-rich plant extracts have shown significant activity against phytopathogens (22, 42), including *Xanthomonas* spp., a major threat to crops like rice and citrus, which have developed resistance to chemicals and antibiotics (25, 29). Some studies report the effects of extracts as natural antimicrobials against *Xanthomonas* sp. (3, 23, 24, 30).

Other pathogens, like *Clavibacter michiganensis,* are responsible for significant losses in tomato (32), and the bacterium *Erwinia amylovora* mainly affects pome fruit trees like pear, apple, quince, and loquat (5, 28). Carnaval *et al*. (2022) observed the inhibition effect of seriguela (*Spondias purpurea* L.) extract on *Clavibacter michiganensis* pv *michiganensis* and *Xanthomonas phaseoli.*

The genus *Juglans* includes over 20 species, being *J. regia* L. majorly significant due to its extensively studied nutritional and functional properties (7, 15). Walnut by-products, including shells, are rich in bioactive phytochemicals with antimicrobial potential for medicine, food preservation, and agroindustry (1, 4, 26, 27). Although their potential as biopesticides is recognized, the antimicrobial activity of these compounds is still unexplored. Meanwhile, shell biomass is often undervalued despite being a cost-effective, renewable resource.

On the other hand, *Juglans australis* is a native walnut tree from the Juglandacea family inhabiting the most austral region of South America, and the Northwestern subtropical rainforest in Argentina, locally known as "Yungas" (11), in Jujuy, Salta, Tucumán, La Rioja, and Catamarca (figure 1, page 189). Its fruit is an indehiscent, subglobose drupe with a thick, adherent mesocarp and a rigid shell (endocarp) containing the embryo (39) (figure 1, page 189). Contrasting with the extensive knowledge about commercial walnuts, information on this native species remains scarce. To date, no research reports antimicrobial or antioxidant activity of *Juglans australis*; except for its activity against herpes virus (37).

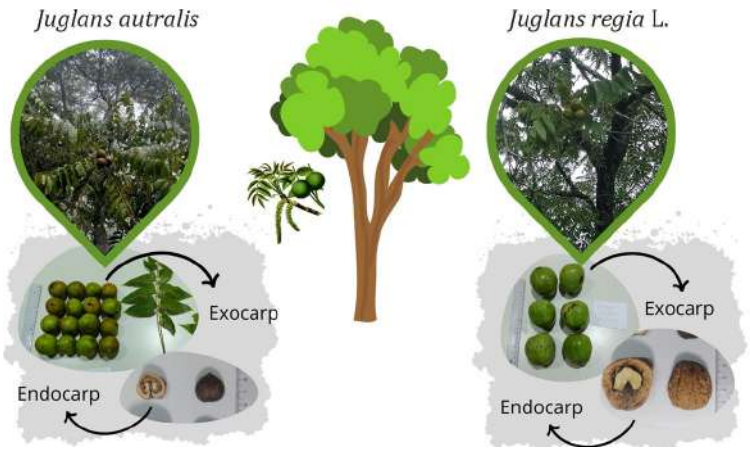


Figure 1. *Juglans australis* tree in Ancasti (Catamarca) city, Argentina (left) and *J. regia* (right). Morphological comparison of native *J. australis* and commercial *J. regia* L. fruits.

Figura 1 Árbol y frutos de la especie y *Juglans australis* (izquierda) y *Juglans regia* (derecha), correspondientes a la localidad de Ancasti, provincia de Catamarca, Argentina.

We aimed to analyze the antimicrobial and antioxidant properties of walnut shell extracts from the commercial *J. regia* and the native *J. australis* for future uses in agricultural management. These extracts would contribute to waste valorization while generating added value to the autochthonous species. Furthermore, extract chemical compositions allowed deeper comprehension of their bioactive activities.

Materials and methods

Sample collection

In 2021, samples of *J. regia* and *J. australis* walnut shells were collected in Ancasti, Catamarca, Argentina. Walnut shells were cleaned and dried under shaded conditions for a week. Selected samples were ground into small particles using a grinder.

Solvent extraction

Solvent extraction involved 50 g of the powdered walnut shells extracted with 250 mL of absolute methanol (MeOH) and ethyl acetate (AcOEt) for 45 min at room temperature and filtered through Whatman n° 4 (48). The solvents were evaporated under a vacuum in a Büchi R-210 rotavapor. The extracts obtained were redissolved in dimethyl sulfoxide (DMSO) to a final concentration of 0.1, 1, and 10 mg/mL and stored in the dark at 4°C for further use. All extractions were done in duplicate.

Determination of total phenolic content and antioxidant activities

Total polyphenol content (TPC) was determined colorimetrically using Folin-Ciocalteu's reagent at 765 nm. A standard curve was performed with gallic acid. The results were expressed as µg gallic acid/g dry weight (DW) (41). *In vitro* antioxidant activity was measured using the free radical elimination activity assay on 1,1, -diphenyl-2-picrylhydrazylradical (DPPH) (10), and ferric reduction capacity of plasma assay (FRAP) (6). Results are expressed in mmol Trolox equivalents/100 g dry weight (DW). All samples were analyzed in triplicate.

Identification of extract phenolic compounds by UHPLC-MS/MS

Phenolic compounds of extracts from walnut shells were identified by a UHPLC (Ultimate 3000 RSLC, Dionex - Thermo Scientific) equipped with a diode array detector and coupled to a TSQ Quantum ultra-triple-quadrupole mass spectrometer (TSQ Quantum Access Max, Thermo Scientific) and column Hypersil GOLD aQ (150 x 2.1 mm, 5 um)

(Thermo Scientific). The mobile phase was a binary mixture of solvents: mobile phase A corresponded to ultrapure water/formic acid solution (Merck, Darmstadt, Germany) (0.1% v/v), and mobile phase B corresponded to an acetonitrile / formic acid solution (Merck, Darmstadt, Germany) (0.1% v/v). Gradient conditions were as follows: 0-18 min, 97% A; 18-21 min, 90% B; 21-26 min, 97% A. Electrospray source of the MS was performed in negative mode. Eluate was monitored at 250 nm, flow rate was 0.3 mL min-1, injection volume was 15 µL, and the column was maintained at 35°C. Polyphenols were tentatively identified according to their retention times, UV/Vis spectra, high-resolution MS, and MS/MS spectra by comparison with pure compounds. We searched for gallic acid, cumaric acid, caffeic acid, ferulic acid, rutin, and eriocitrin (Sigma-Aldrich, St. Louis, MO, USA). The linearity of each calibration curve was confirmed by plotting the ratio of peak areas of phenolic compounds to the internal standard against compound concentration. Data were analyzed via LC-MS Xcalibur workstation software (Version 2.6, Thermo Fisher Scientific).

Antibacterial activity

Antibacterial activity of the different walnut shell extracts was evaluated against Gram-negative bacteria *Erwinia amilovora*, *Xanthomonas axonopodis* pv. *phaseolus,* and *X. campestris pv. campestris* 8004 and a Gram positive bacteria, *Clavibacter michiganensis*. Bacterial suspensions were prepared in Tryptic Soy Broth (TSB). By microdilution, microtiter plate wells were filled with bacterial suspension (10^7 CFU/mL) and the extract solution (final concentrations 0.1; 1 and 10 mg/ mL). Pure gallic acid, the main extract component, was incorporated in the antimicrobial assays at three different concentrations: 34 ppm (higher concentration detected in the extracts), 100 ppm, and 500 ppm. The extracts and gallic acid suspensions were prepared by diluting stock solutions in DMSO. Vehicle controls were prepared with DMSO and each bacterial culture. The 1% vehicle did not affect bacterial growth and was used as negative control. Streptomycin sulfate was also used as positive control. The microplates were incubated at 28°C for 24 h, and growth was detected using a microplate reader (Multiskan Go, Thermo) at 560 nm (45). Inhibition percentages were also calculated according to the Equation:

$$I\% = (Ac - As)/Ac) \times 100\%$$

where
Ac = absorbance of the control sample (phytopathogens without extract and antibiotic)
As = absorbance of phytopathogens + extract samples.

All assays were conducted three times and analyzed in triplicate.

Statistical analysis

ANOVA and Tukey test evaluated differences between treatments using INFOSTAT (Student version, 2020e).

Results and discussion

Total Phenolic compounds and Antioxidants activities

Table 1 (page 191), shows total phenolic content and antioxidant activity of organic extracts (ME: methanolic extract, EAE: ethyl-acetate extract) of *J. australis* and *J. regia*. Solvent extraction capacities varied significantly ($p < 0.05$). The highest phenolic content and reducing activity were observed in the methanolic extract corresponding to *J. australis* (121 mg GAE/g d.w. and 56.8 mmol Trolox/100 g d.w., respectively). However, the strongest antiradical activity was measured in the methanolic extract of *J. regia* ($p < 0.01$).

Tabla 1. Contenido fenólico total (TPC), actividades antirradicalias (DPPH) y reductoras (FRAP) de extractos metanólicos y de acetato de etilo obtenidos a partir del endocarpio de *J. australis* y *J. regia*.

Table 1. Total phenolic content (TPC), antiradical (DPPH) and reducing (FRAP) activities of methanolic and ethyl acetate of endocarp extracts from *J. australis* and *J. regia.*

Sample	Phenolic compounds (mg galic acid/g sample)	DPPH (mM Trolox/100 g DW)	FRAP (mM Trolox/100 g DW)
J. australis MeOH	121.1 ± 12.9 c	9.7 ± 0.6 b	56.8 ± 7.2 a
J. australis AcOEt	36.5 ± 5.4 a	2.06 ± 3 a	5.1 ± 0.2 b
J. regia MeOH	91.9 ± 13.4 b	11.5 ± 0.1 b	43.1± 0.6 a
J. regia AcOEt	39.9 ± 0.6 a	2.0 ± 0.1 a	13.8 ± 1.7 b

Value is expressed as mean ± standard error.
Different letters indicate statistically significant differences (p<0.05).
Los valores corresponden a la media ± error estándar.
Letras diferentes indican diferencias estadísticas (p<0,05).

Methanolic extracts had the highest total phenols content and the strongest antiradical and antioxidant activities. Extracts using polar solvents usually exhibit higher phenolic content and positively correlate with antioxidant potential (48). Among different factors in the extraction process, total phenolic compounds in walnut shell varies from 1 mg/g shell to 32.76 mg GAE mg/g shell (2, 26, 48). Here, we obtained 121 mg GAE mg/g shell in methanolic extracts of *J. australis*, significantly higher-more than two to ten times-than the reported (19).

When DPPH and FRAP activities were analyzed, methanolic extract of *J. australis* showed similar activity to *J. regia* and cited in the literature (42). Yang *et al.* (2014) analyzed the antioxidant and antiradical properties of walnut-shell extracts with different polarity solvents, demonstrating that methanol also shows the strongest antioxidant activity and reducing power. These established methods are reliable indicators of antioxidant potential in the native walnut, comparable with commercial *J. regia* activities, making it a potential source of bioactive compounds.

Solvent selection is crucial for antioxidant isolation by extraction methods. The chosen solvent significantly affects extract yield and its antioxidant activity due to the varying polarities of the extracted compounds (31). Methanolic and ethyl acetate extracts are commonly used in phytochemical studies. Methanol is a polar solvent that effectively extracts water-soluble compounds, including phenolics, flavonoids, and alkaloids. Ethyl acetate, on the other hand, is a less polar solvent that targets more lipophilic compounds, such as terpenes, steroids, and some fatty acids.

This difference in composition might depend on the genotype and environmental conditions during development, and maturity at harvest (2).

Identification and quantification of phenolic compounds

Phenolic compounds of walnut shell extract in *J. australis* and *J. regia* were determined using the UHPLC-MS/MS method. Results observed for *J. regia* extracts coincide with previous reports (2, 16, 18). The methanolic extract had the highest concentration of GLC (26.8 mg/L), followed by CMR (3.1 mg/L) and CFC (762 µg/L). Notably, rutin (RTN) was only detected in *J. regia* methanolic extract, albeit at a lower concentration (103 µg/L). In the ethyl acetate extract of *J. regia,* the most abundant were GLC (0.1 mg/L), followed by CMR (607 µg/L) and CFC (µg/L).

RTN in *J. regia* extracts suggests the potential unique properties of this species. Studies conducted on several parts of fruit and leaves consistently reveal that gallic acid is among the most abundant components in these extracts (2, 16, 18). Fernandez Argulló *et al.* (2021) recently reported that gallic, ellagic, and ferulic acids were the major phenolic compounds in walnut wood waste extracts. However, our study did not detect ferulic acid.

On the other hand, Gallic acid (GLC), caffeic acid (CFC), and cumaric acid (CMR) were identified in both *J. australis* extracts (methanolic and ethyl acetate) (table 2). The methanolic extract presents the highest GLC content (34000 µg/L), followed by CMR (672 µg/L) and CFC (351 µg/L). In ethyl acetate extracts, GLC was most abundant (9000 µg/L), followed by CMR (269 µg/L) and CFC (103 µg/L). Table 2 shows GLC content was significantly higher than CFC and CMR compounds for both extracts. Ours is the first characterization and quantification of phenolic compounds in this native walnut.

Table 2. Phenolic compounds in walnut shell extracts, and quantitative analysis of phenolic components in methanolic and ethyl acetate extracts of *J. regia* and *J. australis.*

Tabla 2. Compuestos fenólicos en extractos de cáscaras de nuez, y análisis cuantitativo del contenido de componentes fenólicos en extractos de acetato de etilo y metanólico presentes en *J. regia* y *J. australis*.

Extract	GLC (ppm)	CFC (ppb)	CMR (ppb)	RTN (ppb)
***J. australis* - MeOH**	34.2 ± 1.1	351 ± 46	672 ± 75	ND
***J. australis* - AcOEt**	9.2 ± 0.3	103 ± 10	269 ± 38	ND
***J. regia* - MeOH**	26.8 ± 0.6	762 ± 99	3.100 ± 0.6	103 ± 13
***J. regia* - AcOEt**	0.1 ± 0.0	26 ± 3	607 ± 45	ND

ppb = parts per billion = µg/L; ppm = parts per million = mg/L.

Note: AcOEt: ethyl-acetate; MetOH: methanolic; ppb = parts per billion = µg/L; ppm = parts per million = mg/L, GLC: gallic acid; CFC: caffeic acid; CMR: cumaric acid; RTN: rutin. ND: not detectable.

ppb = partes por billón = µg/L; ppm = partes por millón = mg/L.

Nota: AcOEt: acetato de etilo; MetOH: metanol; LOD: Límite de detección; ppb = partes por mil millones = µg/L; ppm = partes por millón = mg/L; GLC: ácido gálico; CFC: ácido cafeico; CMR: ácido cumárico; RTN: rutin. ND: no detectable.

Since studies on phenolic compounds are mainly conducted in *J. regia*, more information on *J. australis* extracts is needed. Considering differences between species, essential in-depth studies would allow understanding phytochemical profiles while identifying lost or gained compounds during crop domestication.

Antibacterial activity and gallic acid effects on bacterial growth

The effects of *J. regia* and *J. australis* extract and gallic acid (main compound in all extracts) were evaluated against phytopathogen growth (figure 2, page 193). All extracts tested showed the highest inhibition against *Xanthomonas* (figure 2 A and B, page 193). For *Xcc* 8004, methanolic extracts of *J. regia* (69.56%) and *J. australis* (72.31%) exhibited maximum inhibition at 10 mg/mL. Ethyl acetate extracts exhibited inhibitory activity against *Xcc* 8004, with *J. regia* demonstrating higher inhibition percentage (59.07%) than *J. australis* (41.61%).

All extracts from both *Juglans* sp. inhibited *Xanthomonas axonopodis* pv. *phaseoli*, exceeding 40% (figure 2B, page 193).

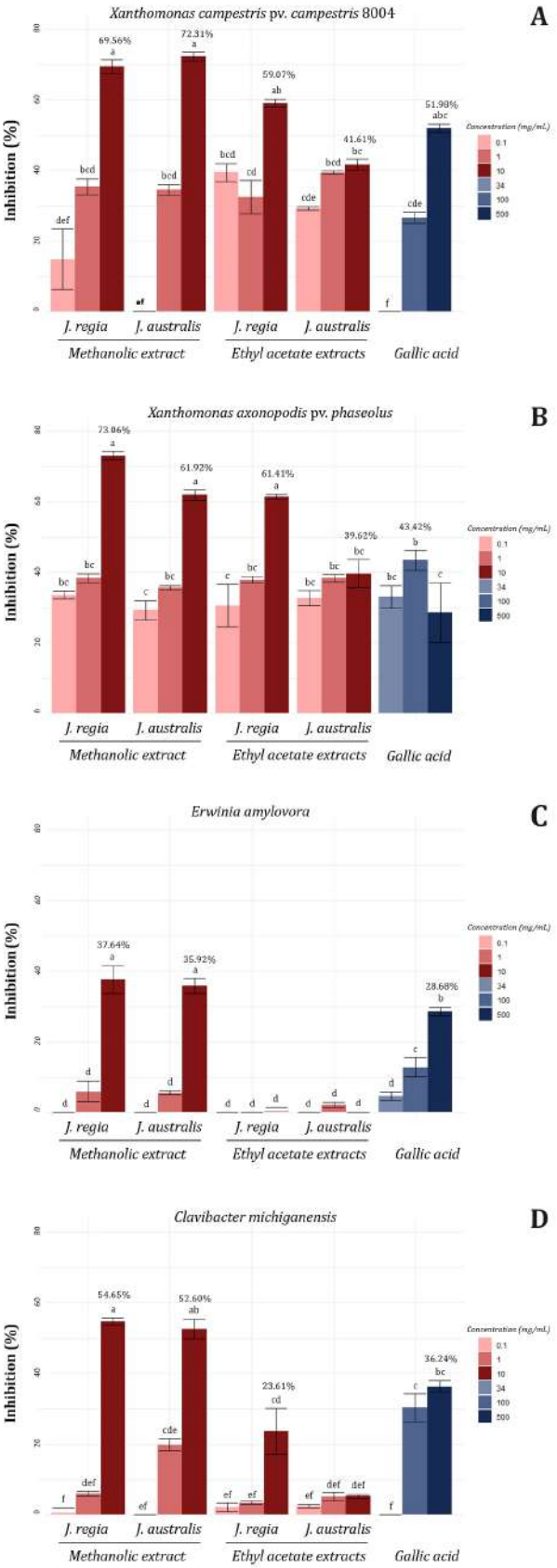
Considering *Erwinia amylovora*, methanolic extracts from both species diminished bacterial development in 37.64% (*J. regia*) and 35.92% (*J. australis*) at the highest tested concentration (figure 2C, page 193).

Once more, methanolic extracts at 10 mg/mL reached 54.65% and 52.60% inhibition against *Clavibacter michiganensis*, for *J. regia* and *J. australis*, respectively (figure 2D, page 193).

Gallic acid did not show substantial inhibition on the phytopathogens evaluated. At the highest concentration, it inhibited *X. axonopodis* (51.95%) (figure 2, page 193). Other studies mention antimicrobial activity of different parts of *J. regia* principally against pathogens of importance in human health (38), but none on antimicrobial effect against phytopathogens. Here, we observe that the methanolic extract from walnut shells had the best antimicrobial activity against the phytopathogens assayed even at the minimum concentration (0.1 mg/mL).

Shell extracts have inhibitory effects against *Xanthomonas* sp. and the highest amount of gallic acid (table 2), probably involved in antibacterial activities. Gallic acid is extensively studied, and its mechanism of action as an effective antimicrobial is well known (13, 21, 40).

Vu *et al.* (2017) report that gallic acid in walnuts can be found either in its free form or as part of hydrolyzable tannins. Nevertheless, gallic acid was less effective at inhibiting the phytopathogens assayed, suggesting a synergistic effect of the minor components.



Each value is expressed as mean ± standard error. Different letters indicate statistically significant differences ($p<0.01$).

Cada valor se expresa como media ± error estándar. Las diferencias estadísticamente significativas se indican con letras diferentes ($p < 0,01$).

Figure 2. Antibacterial activity of shell extracts from walnuts.

Figura 2 Actividades antibacterianas contra bacterias fitopatógenos de los diferentes extractos de cáscara de nuez.

Few studies have examined walnut shell extracts' antimicrobial effects, typically requiring higher concentrations (1-100 mg/mL) (31, 32, 44). Several reports used minimum bactericidal concentrations above 20 mg/mL for *J. regia* extracts (43) with notable activity against gram-negative bacteria like *E. coli* and *P. aeruginosa* (36). In our study, methanolic extracts effectively inhibited Gram-positive and Gram-negative phytopathogens at 10 mg/mL, particularly *Xanthomonas spp,* as previously reported with extracts from six walnut cultivars against Gram-positive (*Bacillus cereus, Bacillus subtilis*, *Staphylococcus aureus*) and Gram-negative bacteria (*P. aeruginosa, E. coli, Klebsiella pneumoniae*) (34).

Recently, the search for new natural compounds has gained interest given antioxidant and antimicrobial properties. Native plants are valued for economic and ecological benefits, with preservation playing a vital role (22).

The scarce information about using *J. australis* phytochemicals evaluates the effect of leaves and stem extracts on Herpes simplex virus (37). Here, we could demonstrate the efficacy of the native extracts over various phytopathogens.

Conclusion

In this study, we first report antibacterial activity of extracts from *J. australis* against phytopathogenic bacteria, and first findings on their particular antioxidant and antiradical activities. This contributions could enhance regional value. This research first characterizes walnut shell extracts from *J. australis*. Our findings demonstrate that methanolic extracts exhibit significant antimicrobial activity against *Xanthomonas* sp., suggesting natural biocontrol alternatives to copper-based formulations.

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Obtaining a lipid extract from peach palm (*Bactris gasipaes* Kunth) epicarp. Quantification of carotenoid content and application as a food additive

Obtención de un extracto lipídico a partir del epicarpio de chontaduro (*Bactris gasipaes* Kunth): Cuantificación del contenido de carotenoides y aplicación como aditivo alimentario

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Abstract

The agro-industrial assessment of fruit by-products as food additives would allow compliance with Sustainable Development Goals. This research aimed at the homogenizer-assisted extraction of total carotenoids from peach palm (*Bactris gasipaes*) peel (epicarp) with sunflower oil. We also studied its application as a natural additive in white corn flour food. The response surface methodology and the rotational composite central design quantified the extraction process. The studied factors were extraction speed, temperature, time, and liquid-solid ratio. Total carotenoid content in the extract (336.06 µg/g dried epicarp) was optimized at 50°C, with 76 seconds, extraction speed of 19200 rpm, and liquid-solid ratio of 48.75 mL/g. The green extract obtained from homogenizer-assisted extraction constitutes a natural additive with agro-industrial potential for use in roasted corn cake, increasing carotenoid (30.60 µg/g of *β*-carotene), provitamin A (4.14 µg/g) and antioxidant activity (11.57 % DPPH).

Keywords
Bactris gasipaes • *β*-carotene • corn agroindustry • natural dye • homogenizer -assisted extraction

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Resumen

El uso agroindustrial de los subproductos de las frutas, como aditivos alimentarios, podría ser una alternativa para el cumplimiento de los Objetivos de Desarrollo Sostenible. Por lo tanto, el objetivo de esta investigación fue la extracción asistida por homogeneización de carotenoides totales de la cáscara (epicarpio) de chontaduro (*Bactris gasipaes*) con aceite de girasol y su aplicación como aditivo natural en un producto alimenticio elaborado con harina de maíz blanco. Se aplicó la metodología de superficie de respuesta junto con el diseño central compuesto rotacional para cuantificar el proceso de extracción. Los factores de estudio fueron la velocidad de extracción, temperatura, tiempo y relación líquido-sólido. Los carotenoides totales en el extracto obtenido (336,06 µg/g de epicarpio seco) se optimizaron a una temperatura de 50°C, con un tiempo de 76 s, velocidad de extracción de 19200 rpm y relación líquido-sólido de 48.75 mL/g. El extracto verde obtenido de la extracción asistida por homogeneización es un aditivo natural con potencial agroindustrial para uso en alimentos como la arepa de maíz, debido al incremento en los valores de carotenoides (30,60 µg/g de *β*-caroteno), provitamina A (4,14 µg/g) y actividad antioxidante (11,57 % DPPH).

Palabras clave

Bactris gasipaes • *β*-caroteno • agroindustria de maíz • colorante natural • extracción asistida por homogeneización

Introduction

The peach palm (*Bactris gasipaes*) is cultivated in Nicaragua, Honduras, Costa Rica, Panama, Colombia, Venezuela, French Guiana, Brazil, Bolivia, Hawaii, Indonesia, Malaysia and Reunion Island (8). The common name of this fruit changes among countries. It is called pupunha (Brazil), pejibaye (Costa Rica and Nicaragua), pijuayo (Peru), person (Guyana), chontaduro (Colombia and Ecuador), and peach palm (English-speaking countries) (8). The fruit's pulp is consumed mainly cooked with salt, or processed into flour for bakery or animal feed (8, 13). Additionally, indigenous communities in Peru, Bolivia and Brazil obtain fermented beverages (8). Even though peels constitute an important source of carotenoids (330 µg/g) (12), and represent 10-12 % of total fruit weight, they are eliminated during consumption and processing (11, 15).

Carotenoids like *β*-carotene, *α*-carotene, *β*-cryptoxanthin, zeaxanthin, and lycopene, provide fruits and vegetables with characteristic yellow, red, and orange colors (9). At a functional level, they have antioxidant activity, and some are a source of provitamin A, with potential applications for health and nutrition (14). Furthermore, carotenoids in processed foods highlight color and encourage consumption (5, 22). The agro-industrial use of peels as by-product would significantly contribute to the food, pharmaceutical and cosmetic industries (17) while reducing waste generation, avoiding economic losses (25, 27) and supporting the reduction of greenhouse gases (27).

The green extraction methodology can obtain molecules of interest in different plant matrices (1, 10, 16, 17, 18, 24). The interaction of emerging technologies with biodegradable solvents makes these extraction processes environmentally friendly while reducing health risks. Additionally, these processes become more efficient when fewer solvents, less extraction time, and less energy are used (16, 18). Despite extraction efficiency, green carotenoid extraction studies using homogenizer-assisted extraction (HAE) are still scarce (1, 3). HAE, also known as high-shear homogenization, is a mechanical method based on high-speed homogenization, generating a shear effect between analyte and solvent, causing cell wall rupture and releasing the active compound of interest (24).

Therefore, new research is needed on food enriched with bioactive compounds, such as carotenoid pigments (7). This research aimed to obtain a carotenoid-rich extract using the homogenizer-assisted extraction from peach palm epicarp with sunflower oil and study its application as a natural additive.

Materials and methods

Sample collection and preparation

Red peach palm fruits with commercial maturity were acquired in the local market of Palmira, Department of Valle del Cauca, Colombia. Whole, healthy fruits were washed with water and disinfected with sodium hypochlorite at 150 ppm. The fruits were conventionally cooked in water for 60 min at boiling temperature (kg fruit/2 L water). Then, peels (epicarp) were removed using a disinfected, manual, stainless steel fruit peeler. Epicarp flour was produced according to previous studies (11). The epicarp was dehydrated in a convection oven (Binder ED 53 UL, Germany) at 60 ± 2°C until 10-11 % moisture. Dehydrated samples were crushed in an electric mill to particle size ≤ 0.25 mm. This flour was refrigerated in a sterile amber glass bottle at 4°C for later use.

Homogenizer-assisted extraction of epicarp carotenoids

The homogenizer-assisted extraction (HAE) was carried out in an ultra-turrax (T 18 digital, IKA, Janke & Kunkel, Germany) using sunflower oil as extraction solvent. Treatments were processed according to the established extraction parameters shown in table 1.

Table 1. Central composite rotatable design with independent variables and coded levels.
Tabla 1. Diseño central compuesto rotacional con variables independientes y niveles codificados.

Independent variables	Coded levels				
	-α (-2)	-1	0	+1	+ α (+2)
	Experimental levels				
Temperature (°C)	30	40	50	60	70
Time (s)	60	70	80	90	100
Speed (rpm)	16000	18000	20000	22000	24000
Liquid-solid ratio (mL/g)	30	40	50	66	90

Total carotenoids (µg/g dried epicarp) were determined according to the spectrophotometric method (15), using a molar extinction coefficient of 7.10×10^4 $M^{-1}cm^{-1}$ and sunflower oil as blank (15). The HAE was optimized via the response surface methodology combined with the rotational composite central design (RCCD). Table 1 shows coded factors, central points, and extreme values. Preliminary experiments identified central points, confirming that the liquid-solid ratio, temperature, time, and extraction speed significantly affected extraction.

Extract application in corn griddle cake

The optimized extract was used as a natural additive in corn griddle cake. Two treatments were elaborated: a control with precooked white corn flour (WCF), and another with white corn to which 50 mL of the lipid extract was added as a natural additive. In all cases, 100 g of flour were mixed with 2 g of salt and 145 g of water. Kneading time was 5 minutes, and standing time was 3 minutes. All samples were 4 cm diameter and 1 cm thick. They were cooked on a preheated plate at 180 °C for 10 minutes (5 minutes on each side) obtaining the brownish and crunchy texture of traditional corn griddle cake.

Concentration of carotenoid and provitamin A in corn griddle cake

Carotenoids (µg /g of corn grilled cake) were determined by spectrophotometry (17). Absorbance of the organic phase was measured at 444, 450, and 451 nm and compared to hexane with a spectrophotometer (Genesys 20 UV-Vis, Thermo Electron Scientific Instruments LLC, Madison, WI, USA).

Carotenoid concentration (µg/g of sample) was calculated using extinction coefficients (E% 1 cm) in hexane: 2460, 2480, 2560, and 2800 for *β*-cryptoxanthin, zeaxanthin, *β*-carotene and *α*-carotene, respectively. The provitamin A, expressed as retinol activity equivalents (RAE, µg/g of corn grilled cake), was calculated using a conversion factor of 12 for *β*-carotene and 24 for the other provitamins according to equation 1, as reported by the standard method (20).

$$\text{RAE} = \frac{\mu g\ (\beta - \text{carotene})}{12} + \frac{\mu g\ (\beta - \text{cryptoxanthin}) + \mu g\ (\alpha - \text{carotene})}{24} \quad (1)$$

Determination of antioxidant activity in corn griddle cake

Antioxidant activity AA (%) was determined as inhibition percentage of the radical DPPH (2,2-diphenyl-1-picrylhydrazyl) according to the colorimetric method (26).

Color parameters in corn griddle cake

Sample surface color was evaluated using the CIEL*a* b* coordinates, measured with a CR-400 Colorimeter, Konica Minolta Tokyo, Japan, with 2° observer settings and D_{65} deuterium lamp. The equipment was calibrated using a standard measurement plate: Y = 89.50, x = 0.3176, y = 0.3347. In addition, the Chroma (C *), hue angle (h °), and total color difference, TCD, were calculated with equations 2-4:

$$C^* = (a^{*2} + b^{*2})^{1/2} \quad (2)$$

$$h° = \tan^{-1}(b^*/a^*) \quad (3)$$

$$\text{TCD} = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2} \quad (4)$$

Experimental design and statistical analysis

The HAE was optimized with the response surface methodology combined with rotational central design of 29 experiments, where 16 were factorial points, 8 were axial points, and 5 were central points. The study factors were the liquid-solid ratio, temperature, time, and extraction speed. Factor effects and their interaction were evaluated with a second-order polynomial model to estimate the variables. Factor effects were identified with ANOVA ($p < 0.05$), and model reliability was evaluated with the coefficient of determination, R^2, lack of Fit, and coefficient of variation. The statistical software Design Expert (Version 11, Stat-Easy, Godward, MN, USA) was used in optimization design. The t-Student test validated the optimization model, and evaluated the two grilled corn cake formulations. The statistical analysis was run in the Minitab version 18 statistical package for Windows.

Results and discussion

Response surface optimization and contour plots

Table 2 (page 201), shows total carotenoids in each treatment evaluated during the HAE. Results ranged from 140.00 to 341.56 µg/g dried epicarp. These values are lower than the 440-670 µg/g obtained in peach palm epicarp (13).

The ANOVA model presented a $p < 0.0001$, monitoring HAE optimization of the response variable of interest (total carotenoids). Factors, interactions (temperature*time, temperature*ratio, time*speed, and time*ratio) and quadratic effect on the independent variables significantly affected carotenoid extraction (table 3, page 202). Lack of fit was not significant ($p > 0.05$). The $R^2 = 0.9994$, R^2 adj = 0.9987, R^2 pred = 0.9966 and CV% = 0.844 (table 3, page 202) indicated good regression fit according to the following equation:

$$\mathbf{Y = 340.115 - 9.136X_1 + 1.247X_2 - 2.866X_3 + 7.117X_4 + 1.539X_1 * X_2 - 0.629X_1 * X_3 + 10.182X_1 * X_4 + 1.325\ X_2 * X_3 + 15.919\ X_2 * X_4 - 0.802X_3 * X_4 - 45.374X1^2 - 12.116X2^2 - 15.498X3^2 - 42.769X4^2}$$

Table 2. Central composite rotatable design with experimental total carotenoids.
Tabla 2. Diseño central compuesto rotacional con resultados experimentales de carotenoides totales.

Run	Temperature X_1	Time X_2	Speed X_3	Liquid-solid ratio X_4	Analytical results total carotenoids (µg/g dried epicarp)
1	1	1	1	1	251.03
2	0	0	0	2	184.00
3	1	-1	-1	1	218.67
4	-1	1	-1	1	247.84
5	-2	0	0	0	176.00
6	0	0	0	0	341.56
7	1	1	1	-1	182.84
8	-1	-1	1	1	212.00
9	0	0	-2	0	285.69
10	0	0	0	0	340.76
11	-1	-1	-1	1	219.49
12	0	0	0	0	339.52
13	2	0	0	0	140.00
14	-1	1	-1	-1	220.40
15	0	0	0	0	339.75
16	0	-2	0	0	287.49
17	1	-1	1	1	208.00
18	-1	1	1	-1	222.49
19	1	1	-1	1	252.00
20	1	1	-1	-1	186.08
21	-1	1	1	1	242.54
22	0	0	2	0	269.32
23	1	-1	1	-1	208.45
24	-1	-1	1	-1	251.96
25	-1	-1	-1	-1	254.25
26	0	0	0	0	338.99
27	0	0	0	-2	152.84
28	0	2	0	0	294.58
29	1	-1	-1	-1	216.62

Table 3. ANOVA for the fitted quadratic polynomial model estimated for total carotenoid content of peach palm epicarp.

Tabla 3. Análisis de varianza del modelo polinomial cuadrático estimado para el contenido total de carotenoides a partir del epicarpio de chontaduro.

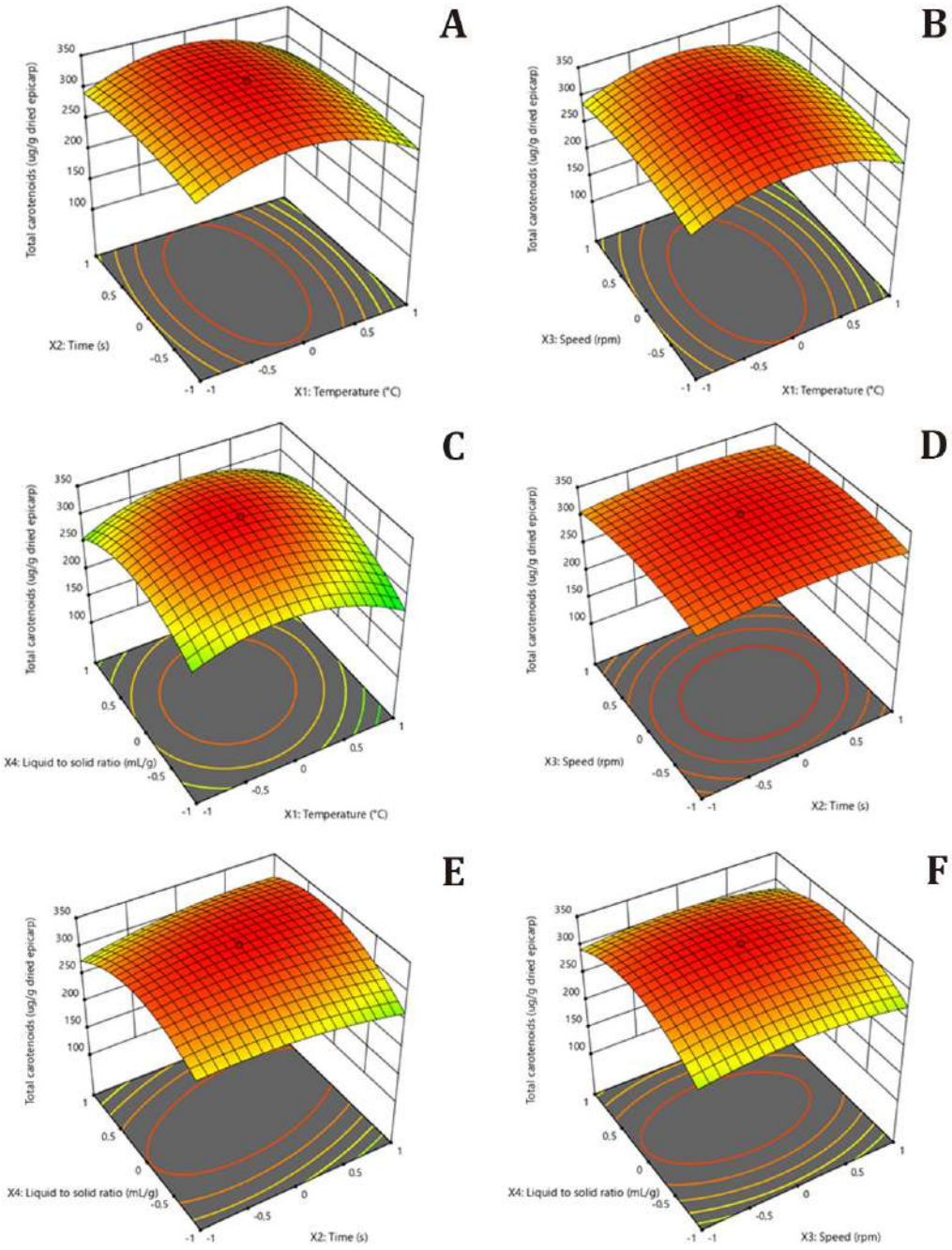
Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	94301.14	14	6735.80	1584.26	< 0.0001	Significant
X_1-Temperature	2003.51	1	2003.51	471.22	< 0.0001	
X_2-Time	37.37	1	37.37	8.79	0.0102	
X_3-Speed	197.14	1	197.14	46.37	< 0.0001	
X_4-Ratio	1215.74	1	1215.74	285.94	< 0.0001	
X_1*X_2	37.93	1	37.93	8.92	0.0098	
X_1*X_3	6.34	1	6.34	1.49	0.2424	
X_1*X_4	1659.04	1	1659.04	390.21	< 0.0001	
X_2*X_3	28.11	1	28.11	6.61	0.0222	
X_2*X_4	4055.07	1	4055.07	953.75	< 0.0001	
X_3*X_4	10.29	1	10.29	2.42	0.1421	
X_1^2	53418.47	1	53418.47	12564.02	< 0.0001	
X_2^2	3808.82	1	3808.82	895.83	< 0.0001	
X_3^2	6232.16	1	6232.16	1465.80	< 0.0001	
X_4^2	47461.37	1	47461.37	11162.91	< 0.0001	
Residual	59.52	14	4.25			
Lack of Fit	55.25	10	5.52	5.17	0.0638	Not significant
Pure Error	4.28	4	1.07			
Cor Total	94360.66	28				

R^2 = 0.9994, R^2 adj = 0.9987, R^2 pred = 0.9966, and CV% = 0.844

Figure 1 A-F (page 203), shows response surfaces of interaction and quadratic effects in carotenoid extraction.

Figures 1A and 1B (page 203), show response surfaces generated by significant effects of time and temperature, and speed and temperature. Augmented extractions were observed with increasing time, speed, and temperature, while at over 50°C, extraction was reduced. This was previously observed on HAE in oligosaacharides from banana pulp (2, 18). However, these authors did not observe a significant impact on extraction speed of phenolic compounds and chlorophylls during this process (2, 16).

Carotenoids increase during HAE due to a higher mass transfer coefficient between carotenoid pigments and sunflower oil. This generates a mechanical breakdown of the biological matrix through shearing during HAE, reducing viscosity and accelerating diffusion and breakdown of protein-carotenoid bonds in the plant matrix (16, 21). Carotenoid reduction with increasing temperature during HAE may be associated with isomerization and oxidative degradation (21).



Time *vs* temperature (**A**), speed *vs.* temperature (**B**), liquid-solid ratio *vs.* temperature (**C**), speed *vs.* time (**D**), liquid-solid ratio *vs.* time (**E**) and liquid-solid ratio *vs.* speed (**F**).

Tiempo vs temperatura (**A**), velocidad vs temperatura (**B**), relación líquido-sólido *vs.* temperatura (**C**), velocidad vs tiempo (**D**), relación líquido-sólido vs tiempo (**E**) y relación líquido-sólido *vs.* velocidad (**F**).

Figure 1. 3D surface plots of the effect of temperature, time, speed and liquid-solid ratio in the homogenizer-assisted extraction (HAE) of total carotenoids from *Bactris gasipaes* epicarp.

Figura 1. Gráficos de superficie 3D del efecto de la temperatura, el tiempo, la velocidad y la relación líquido-sólido en la extracción asistida por homogeneizador (HAE) de carotenoides totales del epicarpio de *Bactris gasipaes*.

Figure 1C (page 203), shows the response surface generated by temperature and liquid-solid ratio effects on the total carotenoid extraction. A quadratic effect was evidenced, at the beginning by a significant increase in extraction after an increase in liquid-solid ratio and temperature. Later, extraction levels were reduced when the liquid-solid ratio surpassed 50 mL/g, and temperature exceeded 50°C. Liquid-solid ratio effects in HAE of bioactive compounds in plant by-products were reported by Eyiz *et al*. (2020) in red grape pomace. The results presented here are consistent with the principles of mass transfer exposed by Wong *et al*. (2015), who stated that the concentration gradient between liquid and solid constitutes the driving force, which is greater for a higher liquid-solid ratio. On the other hand, extraction reduction of total carotenoids for ratios above 50 mL/g could prolong solvent diffusion distance into the matrix (23, 29).

Figure 1D (page 203), shows the response surface generated by speed and time effects on total extraction. Factors interaction positively affected extraction. Increased concentrations may have resulted from shearing and mechanical damage, transferring pigments to the solvent (16). Figures 1E and 1F (page 203), validate liquid-solid ratios, time, and speed effects in extracting total carotenoids from peach palm epicarp. A quadratic and interaction effect was observed on the response variable. The optimal HAE point of total carotenoids was 336.06 µg/g dried epicarp, with 50°C, 76 s time, 19200 rpm and a liquid-solid ratio of 48.75 mL/g. When experimentally validating the process factors established in HAE optimization, a carotenoid content of 334.97 ± 1.06 µg/g dried epicarp was obtained, not significantly different ($p > 0.05$, $n = 4$) from the theoretical one. Therefore, experimental values were adjusted to the quadratic model. When comparing optimized values with the maceration method (sunflower oil for 24 h), HAE exceeds the concentration of the conventional method (113. 94 µg/g dried epicarp) by 2.95 times. Extraction efficiency of bioactive compounds with HAE in plant matrices was previously described (16). These authors achieved high extraction rates with shearing, rupturing the plant matrix in a few seconds, increasing mass transfer coefficient (16). In addition, this method uses agitation, accelerating extraction and increasing mass transfer from the plant matrix to the solvent with diffusion and osmotic processes.

Application of the optimized extract in corn griddle cake

All response variables were significantly affected ($p < 0.05$, table 4, page 205). The enriched corn griddle cake (ECC) presented statistically higher carotenoids, provitamin A, and antioxidant activity than white corn flour (WCF). These differences are mainly due to the incorporation of the lipid extract in the ECC formulation. Other studies have used peach palm lipid extract in food matrices as bakery products, emulsions, and Frankfurt sausages (5, 17, 19). For example, de Souza Mesquita *et al*. (2020) reported increasing carotenoid pigments and provitamin A in mayonnaise made with this lipid extract. Bioactive compounds can influence antioxidant capacity in food matrices, and the addition of carotenoid pigments in the samples may increase antioxidant capacity, as stated in guava pulp after homogenization treatment (4).

Color attributes evaluated in crumb and crust showed L^* and $h°$ were statistically reduced in ECC, and C^* significantly increased compared to WCF. The TCD had a greater difference between ECC and WCF (table 4, page 205). These results are explained by the higher concentration of carotenoid pigments in ECC.

These pigments absorb part of the visible spectrum, favoring the yellow color in ECC. Meanwhile, low carotenoid concentration in WCF resulted in greater reflection of the visible spectrum, generating a white color in the samples. Suo *et al.* (2023) confirm changes in the white color of French fries to reddish tones when fried in corn oil enriched with carotenoid.

Table 4. Carotenoids, provitamin A, antioxidant activity and color attributes in two corn griddle cake formulations.

Tabla 4. Carotenoides, provitamina A, actividad antioxidante y atributos de color en dos formulaciones de arepas de maíz.

Parameter	Formulation type	
	White corn flour (WCF)	Enriched corn griddle cake (ECC)
β-Carotene[1]	0.80 ± 0.01[b]	30.60 ± 0.02[a]
α-Carotene[1]	0.40 ± 0.01[b]	28.50 ± 0.03[a]
β-Criptoxantin[1]	4.30 ± 0.02[b]	9.80 ± 0.03[a]
Zeaxantin[1]	9.20 ± 0.05[b]	30.30 ± 0.06[a]
Provitamin A[2]	0.26 ± 0.01[b]	4.14 ± 0.03[a]
DPPH (%)	4.61 ± 0.57[b]	11.57 ± 1.64[a]
L^*_{crumb}	81.93 ± 0.24[a]	72.05 ± 0.06[b]
C^*_{crumb}	14.40 ± 0.35[b]	43.09 ± 0.60[a]
h_{crumb}	93.50 ± 1.75[a]	87.81 ± 0.04[b]
TCD_{crumb}		30.45 ± 2.76
L^*_{crust}	80.65 ± 0.48[a]	72.29 ± 0.02[b]
C^*_{crust}	14.27 ± 0.21[b]	45.97 ± 0.57[a]
h_{crust}	95.48 ± 1.34[a]	86.16 ± 0.05[b]
TCD_{crust}		33.05 ± 1.45

[1] µg of compound/g of corn grilled cake, [2] RAE µg/g of corn grilled cake, averages on the same column followed by different letters vary significantly from each other ($p < 0.01$) according to t-Student test.

[1] µg de compuesto/g de arepa de maíz, [2] RAE µg/g de arepa de maíz, los valores promedios en la misma columna seguidos de letras diferentes varían significativamente entre sí ($p < 0,01$) según la prueba t-Student.

Conclusion

HAE was adequate for carotenoid extraction in peach palm epicarp. Maximum extraction of total carotenoids was reached when processing the samples at 50°C, 76 s, 19200 rpm, and liquid-solid ratio of 48.75 mL/g. In addition, the HAE method presented the best extraction performance for total carotenoids compared to extraction with maceration. The green extract obtained from homogenizer-assisted extraction is a natural additive with agro-industrial potential for use in roasted corn cake, increasing carotenoids (30.60 µg/g of β-carotene), provitamin A (4.14 µg/g) and antioxidant activity (11.57 % DPPH).

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Arachis genetic resources: evaluation of peanut smut resistance in wild species

Recursos genéticos de *Arachis*: evaluación de resistencia al carbón de maní en especies silvestres

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Abstract

Genetic resources are essential for crop improvement. Particularly, wild species related to peanuts are an important source of resistance to various factors. *Thecaphora frezii*, a pathogen causing peanut smut, leads to yield losses in Argentina's peanut sector up to 35%. This study evaluated the response of 11 diploid species with A, B, F and K genomes, *A. monticola* (AABB), and diploid interspecific hybrids (BB), to *T. frezii* over two cropping seasons. Plants were grown in 20L pots (three replicates each) under field conditions and inoculated with teliospores of the pathogen (20,000 tel./g of soil). The disease was quantified through incidence (% of diseased pods) and severity (scale from 0 to 4). Among A genome species, *A. duranensis* exhibited the highest incidence at 15.27%; for K genome species, *A. batizocoi* reached 13.18%. Resistance to *T. frezii* was observed in the wild species *A. diogoi* and *A. stenosperma* (A genome), *A. williamsii* (B genome), A. *trinitensis* (F genome), *A. cruziana* (K genome), and the intragenomic hybrids, constituting new records. Our findings expand the peanut gene pool information for breeders and identify resistant genotypes, supporting the need to preserve wild peanut germplasm to ensure its availability.

Keywords
Thecaphora frezii • *Arachis hypogaea* • wild peanut • resistance • genomes

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Resumen

Los recursos genéticos son fundamentales para el mejoramiento de los cultivos. Particularmente, las especies silvestres afines al maní cultivado constituyen una valiosa fuente de resistencias. *Thecaphora frezii* Carranza & Lindquist ocasiona pérdidas en el sector manisero argentino de aproximadamente el 35% del rendimiento. Se evaluó, durante dos campañas, el comportamiento frente a *T. frezii* de 11 especies diploides con genomas AA, BB, FF y KK; *A. monticola* AABB, y un híbrido interespecífico diploide BB. Los materiales se sembraron en macetas de 20L (tres repeticiones por c/u), en condiciones de campo, inoculándose con teliosporas del patógeno (20000 tel./g de suelo). Se cuantificó la enfermedad mediante la incidencia (% de vainas enfermas) y la severidad (escala de 0 a 4). Entre las especies con genoma A, *A. duranensis* presentó la mayor incidencia, 15,27%; y en las de genoma K, *A. batizocoi*, 13,18%. La resistencia a *T. frezii* hallada en las especies silvestres *A. diogoi* y *A. stenosperma* (genoma A), *A. williamsii* (genoma B), *A. trinitensis* (genoma F), *A. cruziana* (genoma K) y en el híbrido intragenómico BB constituyen nuevos registros. Nuestros resultados permiten ampliar el acervo genético del maní y generar genotipos resistentes; ratificando que el germoplasma de maní silvestre debe preservarse cuidadosamente para asegurar su disponibilidad.

Palabras clave
Thecaphora frezii • *Arachis hypogaea* • maníes silvestres • resistencia • genomas

Introduction

Peanut (*A. hypogaea* L.) is an allotetraploid species (2n=4x=40, AABB) originated in South America. It is cultivated in warm regions worldwide, with an annual production of 45.5 million tons (32). Given the small intern market, Argentina exports approximately 80% of its production. Córdoba province accounts for nearly 90% of Argentina's peanut industry (28).

The fungus *Thecaphora frezii* Carranza & Lindquist causes peanut smut, an endemic disease in Argentina (23) first detected in commercial crops in north-central Córdoba (18). Symptoms include pod malformation and replacement of seeds by dark-brown teliospores (figure 1, page 211). Literature reports almost 50% incidence (21) and yield losses up to 35% (20, 23).

Current management practices, such as tillage, crop rotation, cultivar selection, fungicide and fertilizer applications or soil amendments, have had limited success in reducing yield losses (1, 11, 22). However, the development of recombinant inbred lines (RILs) has enabled breeding strategies, generating resistant genotypes (8). These RILs originated from crosses involving one synthetic amphidiploid parent, obtained from a triple cross among wild species [(*A. correntina* x *A. cardenasii*) x *A. batizocoi*)]^{4x}, and an experimental line of *A. hypogaea*. These findings highlight the importance of wild species as biotic resistance sources in breeding strategies, targeting novel genetic resources with stable resistance to peanut smut.

Wild *Arachis* diploid species with A, B, D, F y K genomes (24, 25, 29, 30), are phylogenetically close to cultivated peanuts, constituting the genetic secondary pool PG-2 (14), defined as those species that can be crossed with local cultivars/elite germplasm, to produce fertile F1. Some of these species have been previously evaluated for smut resistance (3). Most of them are preserved in the "Banco de Germoplasma BGCTES" (IBONE, FCA-UNNE) germplasm bank. Although these genetic resources constitute fundamental breeding resources (31), they have not yet been fully exploited.

This study evaluated wild *Arachis* species with A, B, F y K genomes (24, 25) and intragenomic reciprocal hybrids (10), conserved in the BGCTES germplasm bank, aiming to identify wild sources of resistance against peanut smut.

Materials and Methods

Eleven diploid species with AA, BB, FF, and KK genomes, a tetraploid *A. monticola* AABB, and two intragenomic diploid hybrids (table 1) were evaluated over two cropping seasons (2019/2020 and 2020/2021), under field conditions at Criadero El Carmen in General Cabrera, Córdoba, Argentina (32°49'39.49" S - 63°51'55.57" O). The species were sown in 20L pots during the first week of December and maintained under field conditions in a completely randomized design with three replicates. Susceptible *A. hypogaea* var. Granoleico was included as a control treatment. Teliospores obtained from infected pods were used to inoculate the pots at a concentration of 20000tel/g soil. During harvest in April, pods were manually opened to quantify disease incidence (percentage of infected pods) and severity, following the 0 to 4 scale proposed by Astiz Gassó *et al*. (2008), where 0=healthy pod, 1 = normal pod with initial seed infection, 2 = normal pod with 50% seed infection, 3 = normal pod with 75% seed infection, and 4 = deformed pod with 100% seed infection. Severity was calculated using equation 1.

$$\textbf{Severity} = (0.x_0 + 1.x_1 + 2.x_2 + 3.x_3 + 4.x_4) \,/\, \textbf{total fruit number} \quad (1)$$

where
(x_0-x_4) = the number of fruits in each classification
(0-4) = the classification number.

Data were analyzed using ANAVA and Duncan´s test ($p \leq 0.05$) with InfoStat software 2020 (9).

Table 1. Analyzed material (species/hybrids, collection data and genome) and disease evaluation results.
Tabla 1. Material analizado (especies/híbridos, datos de colección y genoma) y resultados obtenidos de la evaluación de la enfermedad.

Species	Collection data*	Genome	Inc.		Sev.	
A. cardenasii	K 36021. Bolivia, Santa Cruz, Prov. Chiquitos	**AA**	0	a	0	a
A. cardenasii	K 36015. Bolivia, Santa Cruz, Prov. Chiquitos	**AA**	0	a	0	a
A. diogoi	G 10602. Paraguay, Alto Paraná, Pto. Casado	**AA**	0	a	0	a
A. duranensis	V 14167. Argentina, Salta, Capital	**AA**	6.31	a	0.24	ab
A. duranensis	K 7988. Argentina, Salta, Campo Durán	**AA**	15.27	b	0.57	c
A. kuhlmannii	K 30017. Brasil, MS, Aquidauana	**AA**	0	a	0	a
A. kuhlmannii	V 7639. Brasil, MS, Miranda	**AA**	0.78	a	0.01	a
A. stenosperma	V 10309. Brasil, MG, Rondonopolis	**AA**	0	a	0	a
A. ipaënsis	K 30076. Bolivia, Tarija, Gran Chaco, Ipa	**BB**	0	a	0	a
A. magna	K 30097. Bolivia, Sta. Cruz, Velazco, San Ignacio	**BB**	0	a	0	a
A. williamsii	W 1118. Bolivia, Beni, Trinidad	**BB**	0	a	0	a
[A. williamsii x A. ipaënsis]^{x2}	W 1118 x K 30076. Argentina, Corrientes, IBONE	**BB**	0	a	0	a
[A. ipaënsis x A. williamsii]^{x2}	K 30076 x W 1118. Argentina, Corrientes, IBONE	**BB**	0	a	0	a
A. trinitensis	W 1117. Bolivia, Beni, Trinidad	**FF**	0	a	0	a
A. batizocoi	K 9484. Bolivia, Sta. Cruz, Parapetí	**KK**	13.18	b	0.41	bc
A. cruziana	K 36024. Bolivia, Sta. Cruz, Chiquitos	**KK**	0	a	0	a
A. monticola	K 30061. Argentina, Jujuy, Lozano	**AABB**	0	a	0	a
A. monticola	K 30062. Argentina, Jujuy, Yala	**AABB**	0	a	0	a
A. hypogaea variedad Granoleico	Argentina, Córdoba, Gral. Cabrera	**AABB**	50.65	c	1.45	d

Except for the control, the analyzed materials are stored at the BGCTES (Corrientes, Argentina).

* G, W.C. Gregory; K, A. Krapovickas; V, J.F.M Valls; W, D.E. Williams; Inc. incidence (%), Sev. Severity (0-4). Different letters in each column indicate statistically significant differences ($p \leq 0.05$).

Los materiales analizados se mantienen en el BGCTES (Corrientes, Argentina), excepto el control.

* G, W.C. Gregory; K, A. Krapovickas; V, J.F.M Valls; W, D.E. Williams. ; V, J.F.M Valls; W, D.E. Williams. Inc. Incidencia (%), Sev. Severidad (0-4). Letras diferentes indican diferencias estadísticamente significativas ($p \leq 0,05$).

Results

Only species with AA and KK genomes exhibited symptoms of fungal infection. Among AA species, both entries of *A. duranensis* and one of *A. kuhlmannii* showed affected pods, with *A. duranensis* K 7988 presenting the highest average incidence of 15.27% (figure 1). Among KK genome species, *A. batizocoi* showed an average incidence of 13.18%, while *A. cruziana* showed no affected pods. Conversely, no affected pods were observed in species with BB and FF genomes, in the tetraploid *A. monticola* AABB, or BB hybrids [*A. ipaënsis* x *A. williamsii*]^{2x} and its reciprocal cross. The control, *A. hypogaea* var. Granoleico, displayed an average incidence of 50.65%. This value, along with those obtained for the wild species *A. duranensis* and *A. batizocoi*, showed statistically significant differences with the other wild species and the interspecific hybrid results (table 1, page 210).

Powder inside the pods evidences *Thecaphora frezii* teliospores. Scale bar = 1 cm.

El polvillo que se observa en el interior son las teliosporas de *Thecaphora frezii*. Escala de barra = 1 cm.

Figure 1. *Arachis duranensis* pods (K 7988) with smut (Severity Scale: 4).
Figura 1. Vainas de *A. duranensis* (K 7988) afectadas con carbón (Grado de Severidad: 4).

Regarding severity analysis in wild species, *A. duranensis* K 7988 achieved the highest value at 0.57, followed by *A. batizocoi*, with an average of 0.41. The control species, *A. hypogaea* var. Granoleico, had an incidence value of 1.45. Statistical analysis revealed significant differences ($p \leq 0.05$) between these values and those obtained for the rest of the species evaluated.

Discussion

The *Arachis* genus includes nine infrageneric sections according to cross-compatibility and exomorphic traits (17). Among these, the Arachis section is notable for comprising the largest number of wild species (32 spp.), and for its economic importance, as it includes the cultivated peanut *A. hypogaea.* In this section, 15 species possess A genome, six have B genome, K and G genomes are represented by three species each, F genome is represented by 2 species and only one has D genome (24, 25, 26, 29, 30). According to Harlan and Wet (1971), all 32 species integrate *A. hypogaea* secondary gene pool (PG-2).

Wild diploid *Arachis* species constitute valuable gene-transfer resources for cultivated peanuts, providing resistance to biotic and abiotic factors. Several techniques and methodologies have been developed for gene introgression from wild to cultivated genotypes (7, 31). In Argentina, the introgression of resistance to peanut smut from wild species has allowed important breeding advancements, such as the development of EC - 191 RC (AO) and EC - 394 RC (AO); (19).

Our results revealed a close relation between genome types and resistance to *T. frezii.* Accessions with A genome responded as previously observed by De Blas *et al.* (2019), except *A. duranensis*. Previous evaluations indicated susceptibility in *A. duranensis* (3), which aligns with our results, thus constituting the first susceptible A genome species identified. In our tests involving K genome species, *A. cruziana* was non-susceptible, while *A. batizocoi* exhibited susceptibility, which contrasts with prior results (8). Despite that, values were not markedly higher than those reported in this work. Species with B genome, alongside with [*A. ipaënsis* x *A. williamsii*]^{x2} and the reciprocal hybrid, showed non-infected pods, suggesting that B genome would be resistant to *T. frezii*, as previously reported (8).

Resistance to *Thecaphora frezii* in the wild species *A. diogoi*, *A. stenosperma* (A genome), *A. williamsii* (B genome), *A. trinitensis* (F genome), *A. cruziana* (K genome), and the diploid intragenomic hybrids constitute new records for *Arachis* genus.

The identification of resistant resources offers new breeding opportunities for peanut improvement. The literature documents successful incorporations of wild *Arachis* species in breeding programs. For instance, the A genome species *A. cardenasii* and *A. stenosperma*, which exhibit resistance to *T. frezii,* have also shown resistance to nematodes, rust and leaf spots (31). NemaTAM, a nematode-resistant genotype, was developed from wild *A. cardenasii* (27). Additionally, resistance to *Meloidogyne arenaria* was successfully transferred from *A. stenosperma* to tetraploid peanut (4).

Considering tetraploids, *A. hypogaea* is a segmentary allotetraploid with 2n=40 chromosomes (6, 13, 15, 16). The AABB cultigen arose through interspecific hybridization of two diploid AA and BB species (*A. duranensis* and *A. ipaënsis*, respectively), followed by chromosomal duplication (5, 6, 12). This event originated the wild tetraploid ancestor *A. monticola,* which subsequently underwent domestication, resulting in the cultigen *A. hypogaea.* In this study, our control species *A. hypogaea* exhibited the highest value of disease incidence and severity, whereas *A. monticola* showed no susceptibility. Given the susceptibility of *A. duranensis* to *T. frezii*, we hypothesize that *A. monticola´s* resistance may be derived from the B genome in *A. ipaënsis,* a hypothesis that could be further explored.

Conclusions

Our results provide valuable insights into wild *Arachis* species as sources of resistance to peanut smut disease, enabling breeders to expand peanut genetic pool and develop resistant genotypes. This underscores the importance of carefully preserving wild peanut germplasm collections to ensure their availability for future breeding efforts.

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REVIEW

Overview of garlic waste management, circular economy and upcycling

Perspectivas de la gestión de residuos del ajo, economía circular y transformación

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Abstract

In the last three years, 2 billion tonnes of untreated and dismissed agricultural wastes have been accumulated without adequate management of reuse or final disposal, resulting in dumping or burning. The circular economy concept has gained increasing global recognition for addressing environmental and economic challenges. Garlic, the second most common bulb vegetable cultivated worldwide, generates significant waste during its industrial processing, including husks, stalks, straws, and leaves. These wastes, representing 3.0 to 3.7 million tonnes of residual biomass per year, are currently underutilised, with the usual treatment involving dumping in landfills or direct burning, leading to increased soil and air pollution. In this review, we aim to encourage innovation by presenting a search for state-of-the-art garlic waste management. We identified studies about garlic residual biomass valorisation as raw material for obtaining different extracts and polymers, even energy or biofuels. Finally, following circular economy principles, we propose potential uses for garlic by-products to be repurposed or upcycled as materials within agricultural or other production chains. The information above reveals an increasing demand and interest in garlic waste valorisation. Future studies are needed to exploit garlic by-products as important sources of biopolymers and phytochemicals.

Keywords

bioactive compounds • biopolymers • circular economy • garlic waste • organosulfur compounds • pectin • valorisation

Resumen

En los últimos tres años, se han acumulado 2 mil millones de toneladas de residuos agrícolas no tratados y desechados sin una adecuada gestión o disposición final para su reutilización, resultando en vertederos de basura o siendo quemados. El concepto de economía circular cada vez ha ganado mayor reconocimiento mundial para abordar desafíos ambientales y económicos. El ajo, siendo la segunda hortaliza más cultivada en el mundo, durante su procesamiento industrial genera cantidades significativas de catáfilas (piel), tallos, paja y hojas. Dichos residuos, que representan de 3,0 a 3,7 millones de toneladas de biomasa residual por año, actualmente son subutilizados. El tratamiento convencional es disponerlos en vertederos o quemarlos directamente, aumentando la contaminación del aire y el suelo. En este trabajo nuestro objetivo es fomentar la innovación presentado una búsqueda del estado del arte en la gestión de residuos del ajo. Identificamos estudios sobre la valorización de biomasa residual de ajo como materia prima para obtener diferentes extractos y polímeros, incluso para la obtención de energía. Finalmente, de acuerdo con los principios de economía circular, proponemos posibles usos de los subproductos del ajo para su reutilización o transformación como materias agrícolas o en otras cadenas productivas. La información anterior revela una demanda y un interés creciente en la valorización de los residuos de ajo. Se necesitan estudios futuros para investigar y explotar aún más los subproductos del ajo como fuentes valiosas de biopolímeros y fitoquímicos.

Palabras clave

compuestos bioactivos • biopolímeros • economía circular • residuos del ajo • compuestos organosulfurados • pectina • valorización

Introduction

Nowadays, there is a global awareness of the problems derived from accumulating residues from agricultural production and their environmental impact. For example, in only three years, 3.9 billion tons of biomass can be produced (5, 65), accumulating 2 billion tons of untreated residues from the agriculture industry (55, 79). Given the world population growth and the expansion of new cultivation areas, these numbers are expected to increase in the following years. With 10 billion people by 2050, global waste growth will rise to 70%,

increasing the impact on the food system from 50% to 90% (39). For this reason, the study of agro-industrial waste worldwide production, valorisation and, employment for other purposes become important topics of study.

Among the wastes of agricultural production, we are focusing on the by-products derived from the cultivation and industrialisation of garlic (*Allium sativum* L.). The global garlic commerce is reflected by its high consumption per capita (7.6 kg by 2018) and a significant cultivated area (1.546.741 ha), harvesting 28.49 million tonnes with a world average productivity of 18.4 t/ha (19). China, Spain, and Argentina are the leading garlic producers and exporters, representing almost 90.8% of the global sales market (48). The commercial context estimates that garlic cultivation will increase in the coming years, reaching 31.1 million tonnes per year by 2025 (28), with an annual average growth of 4.72% (22). This vegetable is sold as fresh or industrialised products such as paste, dehydrated, powder, essential oil, oil macerated, and aged extract. However, the industrial process dismisses 25-30% of solid waste (16, 38). Garlic harvesting has implicit waste generation in cleaning, cutting, drying and processing, to obtain phytochemicals and dietary supplements. These facts increase residual biomass tons with no responsible disposal, generating over 3.7 million tonnes of by-products annually (80).

Beyond garlic's culinary use, it is known for its relevant biological and medicinal properties, contributing to its increasing demand, reaching 28 million tons in 2020 (80). Garlic properties and biological activities have been widely studied, demonstrating its preventive effect against chronic diseases such as cancer, antithrombotic (25), antibacterial, antifungal, and antiviral (40, 78, 83). These properties have been attributed to their composition in organosulfur and phenolic compounds. This factor has significantly bolstered the surge in demand to generate medicinal and functional products.

The emergence of the circular economy concept gained traction in the 1980s, advocating for an economic model that prioritizes resource optimisation, waste reduction, environmental preservation, and fosters innovation (73). At its core lies the principles of waste reduction, reuse, recycling, and resource recovery. Upcycling, as a part of the circular economy, promotes waste reduction by reusing existing materials, encouraging creativity and innovation to generate higher-value products without relying on new resources (77). The potential of garlic waste to generate higher-value products offers a promising outlook for waste management. These principles aim to minimize waste and maximize value from products, materials, and resources, inspiring strategies to mitigate ecological issues.

Thus, in this review, we summarize some effective strategies for waste management and explore the potential use of garlic waste as value-added by-products, intending to hopefully define future research direction. The data collection was conducted using academic databases such as Scopus and Google Scholar; the information was organised and summarised to identify research gaps and facilitate the approach to this problem. In agreement with Elsevier's abstract and citation database Scopus, a search was done over the last 10 years using the keywords "garlic" and "wastes". We then selected studies focusing on garlic husks, straws, or both. The previous Scopus search was complemented by another in Google Scholar, which used keywords such as 'garlic wastes,' 'garlic husks and straws,' and 'agro-industrial garlic wastes. Other investigations consulted in Google Academics were also considered to discuss garlic waste treatment for the extraction of different polysaccharides and polymers.

Garlic wastes

Waste generation begins before the garlic harvesting process, starting with removing garlic scapes to enhance bulb growth and development. Figure 1 (page 218), details garlic residual biomass production. The matured garlic bulbs are harvested, undergoing a curing process (35°C, 2 weeks), and finally stored in sheds under ambient conditions for 2 months. Garlic packing sheds are facilities dedicated to the processing and preparation of garlic before distribution and sale. Some common tasks performed in the sheds include sorting, selection, cleaning, and packaging. Consequently, a large amount of waste is generated, mainly from stems and roots are used for energy, animal feedstock, and bio-compost (12). Unfortunately, a relevant part of garlic waste is disposed of in landfills (38). Meanwhile, dry garlic is used for direct consumption and processed spice products, an important commodity with relevant economic value (84).

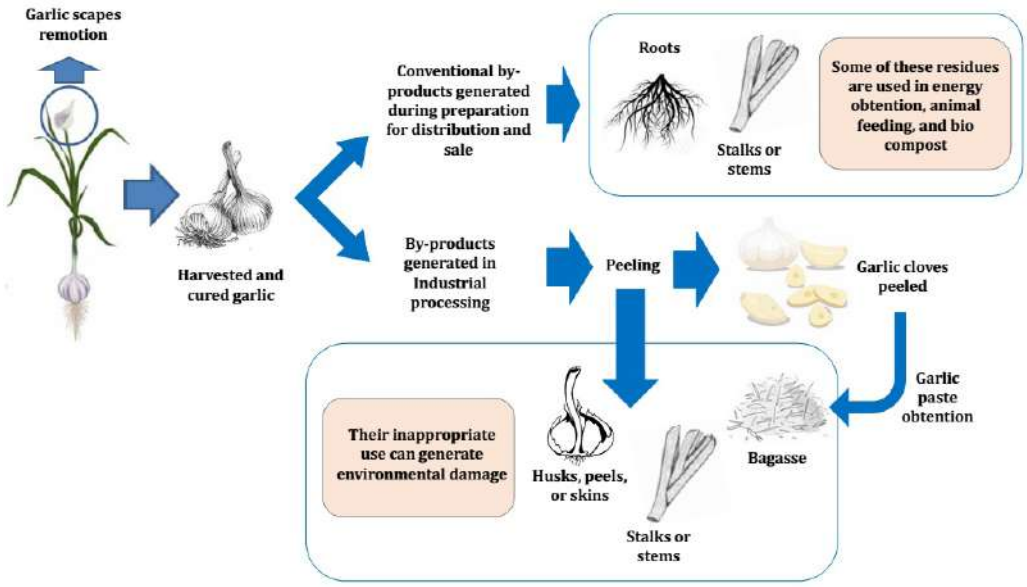


Figure 1. Scheme of wastes produced during garlic harvesting and processing
Figura 1. Esquema de los residuos generados durante la cosecha y procesamiento del ajo.

Figure 1 also shows that industrial processing starts with crushing garlic bulbs and peeling them to obtain residual husks and straws (52). After the peeling, 25 to 30% of inedible parts are considered solid wastes, consisting mainly of dried stems (stalks) and peels (outer and inner husks) (16, 21). Garlic stalks represent 10%, and husks reach 25% of the total garlic weight in these solid wastes (14, 69). In the garlic paste industry, a dried pulpy residue called bagasse is obtained; however, there is currently no information about the residues generated in this process. In this way, low reuse and valorisation of garlic wastes negatively impact the agricultural ecosystem, e.g., untreated garlic residues are directly burned increasing the environmental impacts on soils and air (76).

Fortunately, in the last decade, different investigations have focused on garlic by-product valorisation for obtaining diverse bio-compounds and finding other ways to process them to mitigate their environmental impacts. Additionally, circular economy, as a current strategy, focusing on agro-industrial waste reduction by using them as raw materials in other value chains. As a result, bio-products and bio-energy are now being produced from wastes, improving the environmental quality (6). For example, garlic residual biomass has been used in biorefinery for energy generation and bioethanol production due to its chemical composition.

A circular economy, in concordance with Sustainable Development Goals (SDGs), is more specifically on sustainable consumption and production and climate change, which has a crucial role in waste management, changing the way from a linear economy to a circular one (92). In this sense, figure 2 (page 219) compares the traditional and modern ways of conceiving garlic waste treatment. One of these is based on linear economy or by-products conventional disposal derived from garlic, resulting in dumping, burning, and even animal feeding. The second strategy for solving the inappropriate use of garlic by-products is to consider a circular economy, obtaining bio-products and bioenergy from them.

Our search covered the last 10 years of publications with the keywords “garlic” and “wastes” on both Scopus and Google Academic databases. The data showed that garlic husks, peels, and skins (63%) are the most studied by-products, followed by garlic stems or stalks (23%). Finally, other garlic wastes are either not specified or are a mixture (figure 3, page 219).

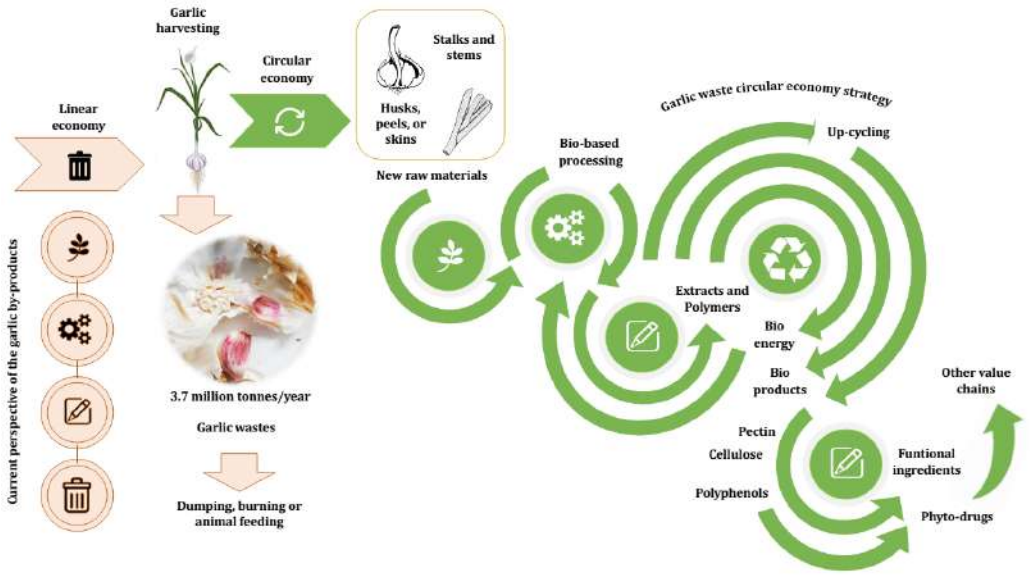


Figure 2. Comparison between the current disposition of by-products *vs* circular economy strategy.

Figura 2. Comparación de la disposición actual de subproductos contra una estrategia de economía circular.

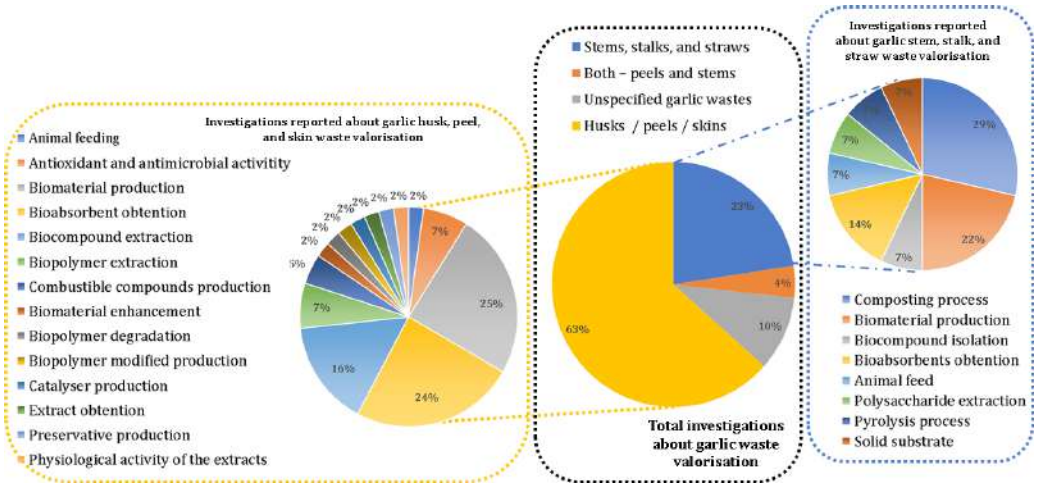


Figure 3. Results of the bibliography search regarding the kind of garlic waste and the objective of valorisation studied.

Figura 3. Resultados de la búsqueda bibliográfica mostrando el tipo de residuo de ajo y el objetivo de valorización estudiado.

Accordingly, in recent years the main uses of garlic waste have been related to second-generation products, minimising the negative impact on the food supply by harnessing resources that would otherwise be considered waste. These untreated materials are advantageously used as biomaterial and bio-adsorbent to remediate environmental pollution. Similarly, some approaches align with the circular economy using garlic by-products as solid substrates and in composting processes. In this context, some studies, improving extraction techniques have obtained bioactive compounds and

bio-polymers showing innovative potential as bio-products, given their functional and biological activity. Cellulose is the most common bio-polymer investigated and extracted from garlic straw wastes. However, other understudied bio-polymers are polysaccharides and dietary fibre such as pectin.

Research on garlic husks and straws for waste valorisation began several years ago. Research aimed to provide potential uses for these by-products, transforming them into value-added products, highlighting garlic biomass as a low-cost alternative.

Garlic by-products' main uses

Use as biomaterial and bio-adsorbent production

According to the references detailed in table 1, garlic waste is mainly used as biomaterial or bio-adsorbent. Husk, peel, and skin represent 31% of raw material used for this purpose and 36% is represented by stem, stalk, and straw. For example, biomaterials, such as carbon particles and biochar, which also serve as bio-adsorbents, have been obtained from garlic waste. different biomaterials, including an aerogel with cellulose nanoparticles (CNP), are proposed from husks, peels, and skins (26). Garlic residual biomass has also been reported as a precursor carbon electro-catalyst material (2). Additionally, using garlic skins, a film's microstructural properties have been improved for environmentally friendly dish creation (27), and cellulose nanofibre and nanoparticles have also been developed (56). Garlic waste has also been used to obtain a novel shape-stable phase-change material with garlic peels (47) and to prepare a cobalt-garlic peel nanocomposite (88). A study focused on generating eco-friendly biochar microparticles from garlic stalks and stems (table 1). Furthermore, garlic stems have produced low-cost and eco-friendly biochar microparticles (86).

Table 1. Garlic waste valorisation as a biomaterial and bio-adsorbent.
Tabla 1. Valorización de los residuos del ajo como biomaterial y bio-absorbente.

		Main use (Reference)
Husk, peel, and skin 	Bio-adsorbent	**To remediate**
		Dyes (7, 49, 51, 57)
		Heavy metals, dyes, and antibiotics (76)
		Radioactive compounds (54)
		Antibiotics (89)
		Heavy metals (91)
		No specified (62)
	Bio-material	**To produce or synthesise**
		Aerogels (26)
		Films (27)
		Carbon particles or biochar (2, 18, 29, 43, 45, 46)
		Composites (47, 88)
		Nanoparticles (56)
		Nanofibre (87)
Stalk and stem 	Bio-adsorbent	**To remediate**
		Heavy metals (61)
		Dyes (35)
	Bio-material	**To produce or synthesize**
		Carbon particles or biochar (50, 63, 86)

Table 1 (page 220) illustrates that bio-adsorbent production from garlic waste is the second most investigated value-added product. Garlic husks, peels, and skins have been reused mainly as bio-adsorbents of the dyes cationic blue 41, methylene blue, and Direct Red 12B (7, 49, 51). Additionally, garlic stalks or stems have been revalorised as bio-adsorbents of methylene blue dye (37).

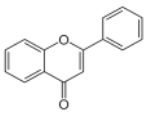
Other applications of garlic husks and stems are related to removing heavy metals and even pollutants as radioactive compounds from soil and water (54, 61, 62, 91). This last use has not been studied as extensively as dye remediation. Additionally, they have been used to absorb antibiotics such as Rhodamine B (76, 89). The effectiveness of garlic by-products bio-adsorbents and contaminants removers is due to garlic residual biomass high content of cellulose hemicellulose content, and some pectic components, which hydroxyl and carboxyl groups bound to pollutant molecules to remove them (57).

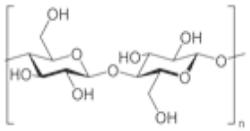
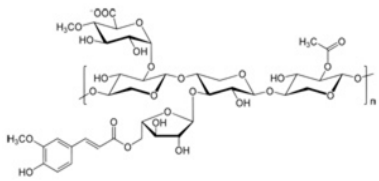
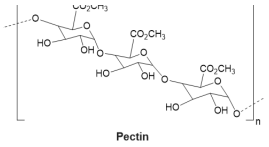
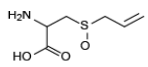
As mentioned in the previous lines, garlic residual biomass has been commonly used in the last years as a precursor to produce carbon materials or biochar for different applications (18, 29, 43, 45, 46, 50, 63). Biochar results from burning agricultural biomass organic material as an adsorbent, insulant, and carbon electrode to store energy, packaging, and pollutant treatment in air and water remediation (72). That means garlic husks and stems have suitable properties for reinforcement ingredients given their lignocellulosic composition, which is also natural, renewable, and biodegradable (68).

Bioactive compounds and biopolymers extraction

From ancient years garlic has been known for its medicinal properties. These properties are attributed to phytochemicals such as organosulfur compounds, phenolic compounds, and polymers (inulin and pectin), among others (13, 59, 70, 85). Figure 3 (page 219), depicts that 7% of related studies are focused on the valorisation of bioactive compounds found in wastes. These works are about different extraction processes, phytochemical activities evaluation, and food additive production from bioactive compounds. Table 2 summarizes articles about each chemical bioactive group mentioned.

Table 2. Valorisation of garlic wastes to obtain bio-compounds and biopolymers.
Tabla 2. Valorización de residuos del ajo para la obtención de biocompuestos y biopolímeros.

Compounds of interest	Study [Reference]
Total phenolic compounds (TPC) and polyphenols  **Phenolic compounds (PC)**	Investigation of antioxidant and antimicrobial properties of garlic peel extract (*Allium sativum*) and its use as a natural food additive in cooked beef (32).
	Garlic (*Allium sativum* L.) husk waste as a potential source of phenolic compounds: Influence of extracting solvents on its antimicrobial and antioxidant properties (34).
	Extraction of phenolic compounds and antioxidant activity from garlic husk using carbon dioxide expanded ethanol (14).
	Evaluation of phytochemical activities of aqueous and ethanolic garlic peel extract (24).
	Comparison of the antioxidant activity of garlic cloves with garlic husk and stem: determination of utilisation potential of garlic agricultural wastes (81).
	Garlic (*Allium sativum* L.) peel extracts: From industrial by-product to food additive (20).

Compounds of interest	Study [Reference]
Polymers 	Starch-based bio-composite films reinforced with cellulose nanocrystals from garlic stalks (1).
	Isolation and characterization of cellulose nanocrystals from garlic skin (66).
	Polysaccharide from garlic straw: Extraction, structural data, biological properties and application to beef meat preservation (35).
	Isolation and structural characterization of cellulose nanocrystals extracted from garlic straw residues (36).
	Extraction and Characterization of Cellulose Microfibres from Agricultural Wastes of Onion and Garlic (67).
	Structure and morphology of cellulose fibres in garlic skin (64).
Dietary fibre 	Modification of insoluble dietary fibre from garlic straw with ultrasonic treatment (30).
	Antioxidant and physicochemical properties of soluble dietary fibre from garlic straw as treated by energy-gathered ultrasound (31).
	Determination of nutritional and mineral composition of wasted peels from garlic, onion, and potato (90).
Pectin  Pectin	Garlic peel derived high-capacity hierarchical N-doped porous carbon anode for sodium/lithium-ion cell (74).
	Effect of garlic peel on haematological, biochemical, and digestive enzyme activity in beluga juvenile (*Huso huso*) (15).
	Pectin extraction from garlic waste under dual acid condition (75).
	Synthesis and characterisation of edible films from garlic (*Allium sativum*) husk components (42).
Organosulfur compounds (OSC)  S-allyl-L-cysteine sulfoxide **(alliin)**	Comparison of two extraction methods (high-pressure extraction vs. maceration) for the total and relative amount of hydrophilic and lipophilic organosulfur compounds in garlic cloves and stems. An application to the Italian ecotype "Aglio Rosso di Sulmona" (23).
	Nematocidal effects of extracts of garlic, grape pomace and olive mill waste, on *Meloidogyne incognita*, on grapevine cv Chardonnay (53).

As a result of the bibliographic search, the words aimed at biopolymers reached 7% for husk, peel, skin waste, stem, stalk, and straw waste. Garlic macro components are water, carbohydrates, proteins, fibres, some fats and polysaccharides (3), including biopolymers which have been poorly analysed. These components also contribute to the specific organoleptic characteristics, as well as the medicinal and nutritional properties of garlic (33). All these characteristics make garlic and its by-products a special raw material to use as a functional ingredient (4). Figure 4, schematizes the bio-compounds and highlights the main obtention source from garlic residual biomass.

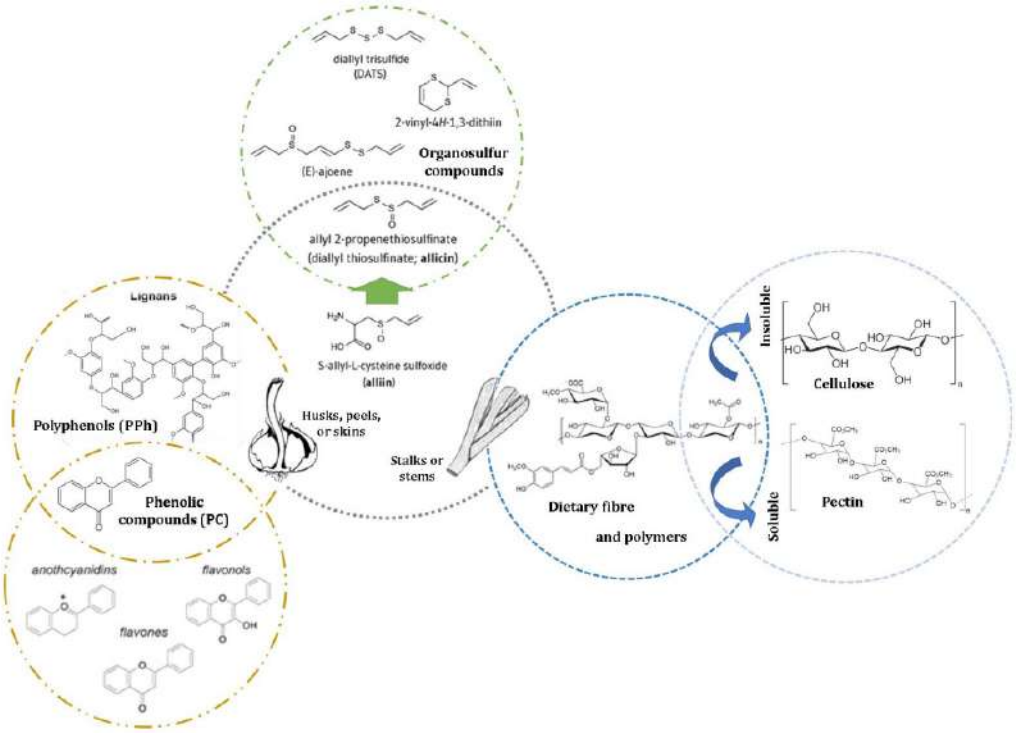


Figure 4. Bioactive compounds and biopolymers extracted from garlic husks and stalks wastes.

Figura 4. Compuestos bioactivos y biopolímeros extraídos catáfilas (piel) y tallos residuales del ajo.

Other studies have addressed different *Alliaceae* wastes in terms of their extract's bioactivity and composition (41, 58, 60). These studies provide critical information to support the upcycling of products like phytotherapeutics and food supplements. They also show that garlic stems/straws or husks/peels by-products have potential uses given their composition in bio-compounds and underline their diverse bioactivities.

Bioactive compounds

Phenolic compounds

Several garlic by-product extracts have been designed and obtained due to their phenolic compounds' antimicrobial, anti-inflammatory, and antioxidant activities (table 2, page 221-222). Likewise, in 2014, a study analysed polyphenols extraction from garlic husks using five different hydroalcoholic solvents, demonstrating their antioxidant and antimicrobial activity (34). Ifesan *et al.* (2014) evaluated garlic peel crude ethanolic extract, measuring its TPC content and antioxidant activity. The authors showed the effects on lipid peroxidation and microbial growth, proving garlic peel's potential use as a natural food additive.

Recent studies have focused on improving the extraction techniques to obtain phenolic compounds. For this purpose, Chhouk *et al.* (2017) tested a new extraction method using supercritical fluids with carbon dioxide-expanded ethanol, measuring these extracts' antibiotic and antioxidant properties. Fortunata *et al.* (2019) tried to obtain diverse bio-compounds from garlic peels through aqueous and alcoholic extractions by comparing the phytochemical profiles obtained. Besides, Tahmas Kahyaoğlu (2021) evaluated the ethanol extract from garlic cloves, husk, and stem using an ultrasonic bath, evaluating their TPC content, total flavonoid, and antioxidant activity. Finally, dos Santos *et al.* (2022) used garlic peel as a co-product to extract bio-compounds by maceration and turbolisation. In the study, the crushed biomass was incorporated into the solvent (water and hydroalcoholic solutions) using a blender, evaluating the bioactive compounds and their bio-activities.

The previous reports illustrate garlic waste hydro-alcoholic extracts with applications in nutraceutical, medicinal, and pharmacological fields. According to the extractant, these investigations exhibit different alternative methods to obtain a wide range of polyphenols. They also show potential uses as antioxidants and antimicrobial activities against diverse pathogenic diseases as an alternative response to the current bacteria drug multi-resistant problem.

Organosulfur Compounds (OSC)

Despite the increasing interest in evaluating and finding new uses for garlic wastes to obtain bio-compounds such as OSCs, few investigations have addressed this aim. Of the total literature consulted, only one reported an extraction method to isolate OSCs from garlic straws. However, a similar task using garlic peels or husks was not reported (table 2, page 221-222). It is crucial to underline the need for further research on unexplored biopolymers and the possible novel extraction techniques and the new biological activity assay alternatives derived from this, mainly highlighting biocidal properties. In this way, Ferioli *et al.* (2020) evaluated two extraction methods, using different solvents to identify the total OSCs and their relative amounts in garlic clove and stem extracts. They detected the highest OSCs amounts in garlic cloves. Another study by Martinotti *et al.* (2016) evaluated the nematicide effect against *Meloidogyne incognita*, employing aqueous extracts from garlic bulbils with promising results.

Biopolymers

Cellulose extraction

The most common polymer extracted from garlic husks and straws is cellulose. Agustin *et al.* (2013) obtained cellulose from garlic stalks through chemical hydrolysis and sonication, obtaining nanocrystals, which were applied to produce a bio-composite film with starch. Another investigation obtained microfibre and nanocrystal celluloses from garlic skins by chemical hydrolysis, characterising the polymers by FT-IR, TGA, SEM, XRD, and SEM physical methods (66). A similar and recent study focused on the obtention of nanocellulose crystal, but in this case from garlic straw residues. The polymer was also extracted by chemical hydrolysis and characterised by the same analytical techniques (36). Likewise, garlic stalks and skins were treated to determine their chemical composition and obtain micro-cellulose fibres by alkaline hydrolysis. After a chlorite blanched process, the micro-cellulose fibres were characterised by FT-IR, TGA, and SEM physical assays (67). Similarly, Raimo (2020) studied a garlic skin extract's cellulose structure and morphology without any use or valorisation, evaluating it by microscopic and optical assays. Finally, a similar and recent study focused on the obtention of nanofibre cellulose, but in this case by a hydrothermal extraction pre-treatment (87).

In some studies, garlic husks and stalks were mainly evaluated to obtain different kinds of cellulose given the amount of this polymer in the residual material, which shows a novel source of cellulose fibres. For example, garlic straw contains 41% cellulose (36), garlic husk cellulose content reaches 19% (8). The biopolymer extracted/modified depicted important value-added characteristics, like tensile strength, moisture resistance, and thermal stability (68). Garlic husks and stalks are a potential bioresource with important technological applications in polymer matrices. They can be used mainly as a biodegradable film, to obtain bio-composites, or in food packaging production, being an eco-friendly material. This potential for circular economy practices is inspiring for the future of agricultural waste management.

Garlic wastes dietary fibre

Dietary fibre refers to non-digestible carbohydrates, including "resistant starch" and non-starch polysaccharides, cellulose, and pectin, among others. Generally, it is extracted by different hydrolysis methods using both acid and alkaline solutions. These fibres are usually used to produce dietary supplements due to their health benefits such as improving gut mobility, microbiota profile, facilitating weight loss, and reducing insulin sensitivity (9). Recently, few works have reported the soluble and insoluble dietary fibre content in garlic husks and straws (table 2, page 221-222). Of these, just one referenced garlic peel waste composition, in which dietary fibre was the highest part of the total residual biomass weight representing almost 62% (90). Huang *et al.* (2018) achieved to modify the functional, physicochemical, and structural characteristics of insoluble dietary fibre from garlic straw. Then, the same authors published that is possible to alter soluble dietary fibre from garlic straw with an energy-gathered ultrasound treatment (31). They provided information on the physicochemical characteristics and antioxidant capacity of the modified garlic straw dietary fibre. The most common research applied to garlic wastes (husks and straws) considers polymer obtention, mainly cellulose. Herein, Kallel *et al.* (2015) studied the isolation from garlic straws of a new polysaccharide mainly composed of glucose, mannose, galactose, and xylose. The polysaccharide was extracted in hot water, and its antimicrobial and antioxidant activities and structural conformation were assessed. According to the previous information, focus should consider these by-products reusing, given the fibre dietary content accessible and easily modified by conventional methods.

Pectin

It is interesting to explore the extraction of pectin from garlic by-products. It has been demonstrated that pectin extraction can allow differences in the esterification and methylation degrees, even in galacturonic acid content (75). These parameters influence the jell formation capacity of the extracted pectin (10).

Some previous pectin investigations from garlic peels have been retaken reporting it as a component of garlic peels constituting 27% of its biomass (15, 74). Similar data was described in a recent investigation that evaluated the pectin composition from garlic waste (peel, stem, and straw), reporting a pectin content of 22.4% (75). Thus, Kumar *et al.* (2022) synthesized and characterized different edible films from pectin garlic husk, demonstrating the potential of this biomass for making food packaging. The investigations in animal feeding have evaluated haematological and biochemistry parameters as digestive enzymes in fishes. The research demonstrated health benefits when the fish diet was supplemented with garlic peels, given to pectin content (15).

Despite these previous investigations, few findings have demonstrated that garlic peels are an important source of biopolymers focusing on its pectin content and functional potential. One of these emphasised the functional properties of the dietary fibre pectin in animal feed (table 2, page 221-222). It is important to further investigate other specific characteristics of the extracted pectin. Likewise, to know pectin-specific rheological properties and establish its potential uses as a nutraceutical assigning it a technological application in the food industry.

Current advancements and future trends

Though the circular economy has several practices for reducing environmental damage and the carbon dioxide footprint, residual biomass re-utilisation is the most extended practice. Thus, agro-industrial reuse of wastes or by-products involves reintegrating them into the productive system as raw material and removing value-added products useful for other production chains. Therefore, the productive systems must integrate into other practices if they want a circular economy-producing approach. These practices include repairing, reassembling, renovation, and upcycling proposed to incorporate by-products in new productive chains. Besides, a circular economy strategy helps to close the productive chain by obtaining bio-products and bio-energy from agro-industrial by-products. Some projects have been planned to produce garlic sustainability in Argentina. Burba *et al.* (2021) established a cleaner garlic production process by adopting some strategies. These include

transforming tonnes of residual biomass into fuel pellets for heat locations and reusing wood pallets, cardboard, and residual plastics such as raw materials to produce new packaging. These strategies should be integrated into other industrial and territorial organisation approaches and the garlic sector's Good Farming Practices (GFP). Nevertheless, the planned project does not consider bio-products from this garlic residual biomass.

Several studies support the idea that waste valorisation is a current point of discussion and a circular economy strategy using conventional techniques such as composting, digestion, or biotransformation is a possible way to do it. Upcycling is a recent innovative technique to obtain biofuel and bio-compounds and produce microbial growth media (17, 44, 71, 82). In this way, the revision of literature concerning garlic by-products shows the potential of obtaining bio-polymers and bio-compounds for bio-energy and bio-products generation. The final purpose of this review is to examine the links in the garlic production chain as sources of inputs from which new chains could be integrated to obtain additional by-products, thereby enhancing added value.

Conclusions

In summary, the present work analyses the state-of-the-art of garlic by-product valorisation. We identified references regarding garlic husk and straw valorisation as raw materials for obtaining different extracts and polymers, even energy or biofuels. Nevertheless, some current disposal practices of this biomass contribute to environmental damage. As an upcycling strategy, this raw material could be reused in production systems to exploit garlic by-products' physicochemical characteristics. Their reuse can diversify the number of bio-products obtained from them. Knowing that garlic is the second bulb worldwide distributed, and its consumption and processing have increased by-product generation. There is a need to deepen studies that contemplate waste treatment in considering circular economy. Fortunately, some investigations have focused on garlic by-product valorisation in recent years. Either extracting different bioactive compounds or seeking other ways to process it as a strategy for agro-industrial waste reduction.

This review evidences that the valorisation of garlic by-products can be divided into two clusters: the production of biomaterials and the extraction of bioactive compounds. These works highlight the advantages of reusing a discarded material such as a low-cost natural source of easy accessibility to be applied at an industry level. Since garlic husks and straws represent almost 10% of the total weight of garlic, reducing their amount of worldwide solid waste could be a trending topic. Nevertheless, using this raw material in other value chains for obtaining bio-products and bio-energy, still represents a major underexplored topic. Bio-compound isolation is another relevant aim in residual garlic biomass revalorisation, and it demonstrates its potential applications in the nutraceutical, medicinal, and pharmacological fields. Though there is increasing interest in evaluating and finding new uses and valorisation of garlic wastes, their upcycling is scarce. More deep studies focused on garlic peels and straws, as an important source of other unexplored biopolymers and phytochemicals in this complex matrix, should be addressed. Therefore, investigating new substances from garlic peels and straws will improve the extractive techniques as the structural characterisation and the biological screening assays. The discovery of new evidence to revalorise this raw discarded material in diverse technological applications must consider the SDGs and circular economy approaches.

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