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Luego del gran reto enfrentado en nuestro 70 aniversario en 2019, los cambios planteados para la Revista de la Facultad de Ciencias Agrarias representaron un desafío en la búsqueda constante de la excelencia académica y la divulgación de los distintos saberes de las ciencias agrarias multidisciplinarias.

Podemos decir que somos una revista digital Open Access en inglés indizada en bases de datos internacionales como Web of Science, Scoupes Embase, SciELO Argentina, entre otras, permitiéndonos posicionarnos más allá de las fronteras regionales y nacionales. Hoy, es notorio el reconocimiento de nuestros pares alrededor del mundo a nuestro compromiso, seriedad y profesionalidad en la difusión de las ciencias agrarias multidisciplinarias.

Somos una revista en el seno de una institución pública, que se enorgullece de su historia y su pertenencia, pero que al mismo tiempo se posiciona con visión al futuro en una realidad cambiante en tiempos extremadamente cortos.

Hoy celebramos nuestro 75 aniversario con la fuerte convicción en nuestra misión y visión, con la gran alegría de seguir contando con la participación desinteresada de excelentes profesionales que nos acompañan día a día en cada publicación junto a un equipo editorial profesional. Este aniversario se ve coronado con el aumento del impacto de nuestra revista en el ámbito nacional e internacional.

Todo estos logros y alcances nos dan la fuerza necesaria para seguir trabajando constantemente en mejorarnos y plantearnos nuevos desafíos. Muchas gracias a todos aquellos profesionales que siguen confiando en nuestra labor.

Dr. Ing. Agr. Rodrigo J. López Plantey
Director Científico

75º aniversario
Revista de la Facultad de Ciencias Agrarias
Universidad Nacional de Cuyo

Following the significant challenge encountered during our 70th anniversary in 2019, the proposed changes for the Revista de la Facultad de Ciencias Agrarias represented an ongoing pursuit of academic excellence and the dissemination of diverse knowledge within the agricultural sciences multidisciplinary.

We proudly state that we are an Open Access digital journal in English, indexed in international databases such as Web of Science, Scopus, Embase, SciELO Argentina, among others, enabling us to extend our reach beyond regional and national borders. Today, our peers globally recognize our commitment, seriousness, and professionalism in disseminating agricultural sciences multidisciplinary.

We are a journal within a public institution, proud of its history and heritage, yet forward-looking, adapting to a rapidly changing reality.

Today, we celebrate our 75th anniversary with a strong conviction in our mission and vision, and with the great joy of continuing to have the selfless participation of excellent professionals who accompany us in each publication alongside a professional editorial team. This anniversary is marked by the increased impact of our journal at both national and international levels.

These achievements and milestones provide us with the necessary strength to continuously improve and embrace new challenges. We extend our deepest gratitude to all the professionals who continue to trust in our work.

Dr. Ing. Agr. Rodrigo J. López Plantey
Scientific Director

75th anniversary
Revista de la Facultad de Ciencias Agrarias
Universidad Nacional de Cuyo

Revista de la Facultad de Ciencias Agrarias
Universidad Nacional de Cuyo

Tomo 56 (2) - Diciembre 2024

Índice

Green manuring and fertilization on rice (*Oryza sativa* L.): A peruvian Amazon study

Abonos verdes y fertilización en arroz (*Oryza sativa* L.): un estudio en la Amazonía peruana

Yuri Arévalo-Aranda [1, 2], Elmer Rodríguez Toribio [1, 2], Leodan Rosillo Cordova [2], Henry Díaz-Chuquizuta [1], Edson E. Torres Chávez [3], Juancarlos Cruz-Luis [4], Rita de Cássia Siqueira Bahia [1], Wendy E. Pérez [4*]

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Abstract

The study was conducted in Juan Guerra district, province and region of San Martin, Peru; it assessed two treatment sets: (1) nitrogen fertilizer dose (FN75, FN100); (2) green manure *Crotalaria juncea* (CroJ), *Canavalia ensiformis* (CanE), and without green manure. It was arranged in a split-plot design with four replications. During the experiment, we observed an important fluctuation in soil parameters. Notably, there was a decrease in soil carbon and nitrogen levels, likely attributed to microorganism metabolism. On the other hand, we observed that CanE significantly reduced the diseased tillers through "White Leaf Virus" (RHBV) by 2.82% compared to the control, and significant panicle fertility was achieved by CroJ (91.88%). No significant differences were obtained in yields during this first campaign; however, the highest reported yield was 8.36 t ha^{-1} with the CanE - FN100 treatment. Additionally, the nutritional quality of the rice was not affected by either green manuring or the application of chemical nitrogen fertilization. These findings allow deeper studies to consider strategic alternatives to reducing dependency on inorganic fertilizers among the poorest communities.

Keywords
split-plot • legumes • soil fertility • RHBV • regenerative agriculture

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Resumen

El estudio se realizó en el distrito de Juan Guerra, provincia y región de San Martín, Perú; se evaluaron dos conjuntos de tratamientos: (1) dosis de fertilizante nitrogenado (FN75, FN100); (2) abono verde *Crotalaria juncea* (CroJ), *Canavalia ensiformis* (CanE), y sin abono verde. Se dispuso en un diseño de parcela dividida con cuatro repeticiones. Durante el experimento observamos una fluctuación importante en los parámetros del suelo. Notablemente, hubo un decremento en los niveles de carbono y nitrógeno del suelo, comúnmente atribuidos al metabolismo microbiano. Por otra parte, observamos que CanE redujo significativamente los macollos enfermos por el "Virus de la Hoja Blanca" (RHBV) en un 2,82% en comparación con el control, y CroJ logró una fertilidad de panícula significativa (91,88%). No se obtuvieron diferencias significativas en los rendimientos durante esta primera campaña; sin embargo, el mayor rendimiento reportado fue 8,36 t ha^{-1} con el tratamiento CanE - FN100. Además, la calidad nutricional del arroz no se vio alterada por los abonos verdes o la fertilización química nitrogenada. Estos alcances permiten ahondar en los estudios para considerar alternativas estratégicas para disminuir la dependencia de los fertilizantes inorgánicos por las comunidades más pobres.

Palabras clave
parcela dividida • leguminosas • fertilidad del suelo • RHBV • agricultura regenerativa

Introduction

Rice (*Oryza sativa* L.) is cultivated in over 95 countries worldwide. Serving as a staple for over half of the world's population, rice plays an important role in various countries by significantly contributing to dietary needs, providing approximately 35-80% of consumed calories (9). Moreover, among prevalent cereal grains, it stands out for its exceptional characteristics, boasting the highest net protein utilization and digestible energy levels (64).

In Perú, rice is one of the main crops, with a production of approximately 3,027.41 tons in 2022 (28). However, due to the increase in prices of chemical fertilizers, the cultivation area decreased by 47.2% during the February 2022 campaign (17), and for 2023 production exhibited a variation of -5.7%, experiencing a decline of 24.3% compared to 2021 and a 9.7% decrease compared to 2022 (28). Rice production in Peru is primarily located in the Coast and Amazon regions. Until 2016, over 55% of the national supply came from the Coast; however, since then, the majority has shifted to the Amazon. In 2022, rice production in the Amazon accounted for 50.5% of the national production, with approximately 87% coming from the regions of San Martín, Amazonas, Cajamarca, and Huánuco, with San Martín being the main producing region in Peru, yielding 877 thousand tons in 2022 (41). Nevertheless, farmers dedicated to this cultivation commonly face soil fertility issues, flooding, and phytopathological problems (55). In addition, the most common forms of land tenure are ownership and leased, in both situations the farmer must hire workers and this may minimize productivity. Besides, the leased have higher technical and allocative inefficiency costs (59). The advantages of green manures over other organic fertilizers include increased soil coverage, protection against erosion, reduced weed infestation, and decreased pests and diseases, ultimately enhancing crop quality and yield, reducing the use of pesticides and herbicides, preventing erosion, and improving soil fertility (40). Some studies demonstrated that green manures applied to rice-cultivated soils modified microbial abundance and composition, enzymatic activity, chlorophyll content, panicle number, yield, and crude protein content in rice cultivation (61). Others found improved physical soil characteristics (2), and increased dissolved organic matter content in soils (24). Moreover, depending on the nutrient and the fertilization dose, differences can be obtained in the edible part of the crop (21). Keeping the soil surface permanently covered by plants in the vegetative phase or as mulch is the most recommended management to protect and conserve the soil that directly influences the production of various crops (46). In the sense of increasingly using strategies more affordable, safer, and low-impact approach to crop growth, the organic amendments stand out as an alternative as part of a broader sustainable crop management strategy (23). However, it is necessary to extend knowledge of sustainable

techniques to farmers in the San Martin region as an alternative because it allows a reduction in production costs, nitrogen (N) fertilizers, and days, without altering performance. With the application of green manures, the profitability of the crop and agribusiness increases with the economic use of organic natural resources.

This study evaluated the effect of applying green manures prepared from Canavalia (*Canavalia ensiformis* L.) and Crotalaria (*Crotalaria juncea* L.) to improve soil fertility, partially reduce the use of N fertilizers, increase rice crop growth parameters, yield, and rice grain nutritional quality in plots located in Juan Guerra district, San Martín province.

Materials and methods

Study area

The experiment was conducted in the fields of the National Rice Program at the El Porvenir Agricultural Experimental Station - "Instituto Nacional de Innovación Agraria" (INIA) (S: 6°35'50", W: 76°19'30", altitude 219 masl) in Juan Guerra district, San Martín province and region, Peru (figure 1A), during the dry season from July 2022 to January 2023. The San Martin region experiences maximum temperatures ranging from 35.6 to 36°C and minimum from 12.1°C to 18°C, the estimated annual precipitation was approximately 1213 mm.

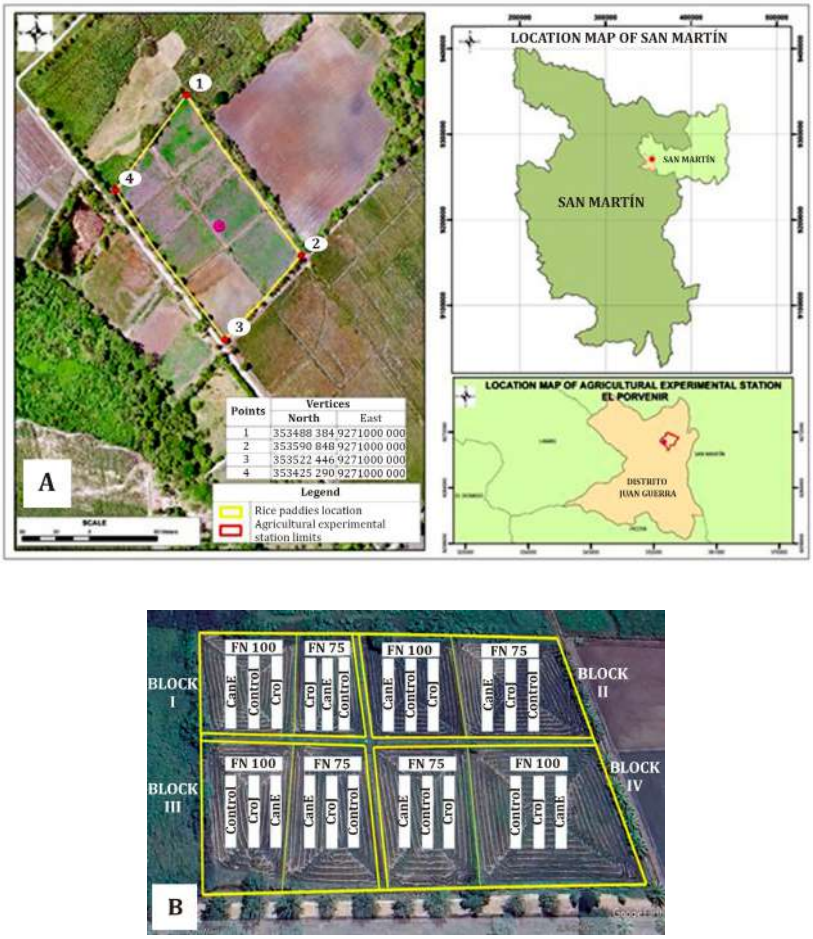


Figure 1. Geographical location of the rice paddies (A), and distribution of blocks, main plots, and subplots in the rice fields (B).

Figura 1. Ubicación geográfica de las parcelas de arroz (A), y distribución de los bloques, parcelas principales y subparcelas en el campo de arroz (B).

The initial sampling showed the following soil conditions: pH 7.11, electrical conductivity (EC) 0.14 ds m^{-1}, cation exchange capacity (CEC) 23 $cmol^{+}kg^{-1}$, organic matter (OM) 37.5g kg^{-1}, total N 2.0 g kg^{-1}, available P 17.56 mg kg^{-1}, and K 212.23 mg kg^{-1}, and a clayey texture, of a soil classified as a Vertisol.

Botanical material

Rice seed, INIA 507 "La Conquista'' variety, was acquired from the National Rice Program (INIA) at El Porvenir Agricultural Experimental Station. It corresponds to the PNA 2394-F2-EP4-6-6-AM-VC1 lineage obtained through individual pedigree selection for the resistance to *Burkholderia glumae*, the main causal agent of bacterial panicle blight (BPB) (36). Alternatively, *C. ensiformis* L. is widely cultivated in tropical and subtropical regions, and it is an annual or biannual herbaceous legume, very rustic, low, growth erect, and determined with a slow onset, reaching 1.2 m in height. It is resistant to variations in environmental conditions, insects, and microorganisms (56). *C. juncea* L. is a fast-growing legume, with high competition with weeds, and plant biomass production.

Field experiment

The experimental field, approximately 15,554 m^2 in size, was arranged in a split-plot design with 2 factors and 4 blocks. The blocks I (3,541 m^2), II (4,279 m^2), III (3,262 m^2), and IV (5,472 m^2) were divided into main plots by a partition wall. Additionally, the main plots Block1-FN100 (2,068 m^2), Block1-FN75 (1,473 m^2), Block2-FN100 (1,862 m^2), Block2-FN75 (2,417 m^2), Block3-FN100 (1,557 m^2), Block3-FN75 (1,705 m^2), Block4-FN100 (3,574 m^2), and Block4-FN75 (1,898 m^2) were subdivided into 3 subplots of approximately the same size (figure 1B, page 3). Therefore, the evaluated factors were: Factor 1, N fertilization dosage (main plot), with a reference dosage of 180 kg of N per hectare (391 kg of urea). The tested fertilization dosages included 100% (FN100) and 75% (FN75) of the reference dosage, this fertilization dosages were split into two applications of 50% of the dose at 40 and 55 days after sowing, during the tillering stage; and Factor 2, a type of green manure (subplot), which included *C. juncea* (CroJ), *C. ensiformis* (CanE), and without green manure (Control).

Crops management

The sowing of CanE green manure was done with a spacing of 40 x 40 cm with 3 seeds per hole, while for CroJ a spacing of 30 x 40 cm, with 10 seeds per hole was used. During the pre-flowering stage, the plants were incorporated using a harrow, and the green manures were left to decompose for 98 days. Rice planting began in October and was conducted in two stages; first rice seedbeds were prepared, and then the seedlings were transplanted to the definitive field.

Preparing the seedbeds involved spreading pre-germinated rice seeds (120 kg) in an adjacent pond (300 m^2). Ten days after sowing, lambda-cyhalothrin and thiamethoxam (0.3 L ha^{-1}) were applied to control pests. The seedbeds were fertilized using urea (200 kg ha^{-1}) twelve days after sowing, and finally, fipronil (0.2 L ha^{-1}) was applied twenty days after sowing for pest control. Four seedlings were transplanted per hill at 25 x 25 cm. Fertilization used diammonium phosphate, potassium chloride, and magnesium sulfate as P, K, and Mg sources in doses of 150, 150, and 25 kg ha^{-1} respectively. Also, B was applied in a dose of 25 kg ha^{-1}.

Evaluation of the soil fertility

Composite soil samples were collected in three stages. The initial sampling occurred before the sowing of green manures; the second sampling fell out after green manure incorporation, and the final sampling was conducted post-rice harvest. For the soil analysis, the methods were: pH (EPA 9045D), Electrical conductivity (ISO 11265), N (ISO 11261), P (NOM-021-RECNAT-2000 AS-10), K (EPA 6020 B), Texture (NOM-021-RECNAT-2000 AS-09), Organic Matter (NOM-021-RECNAT-2000 AS-07), Cation exchange capacity (EPA 9081). The soil samples analysis was done at "Laboratorio de Suelos, Agua y Foliares" (LABSAF) at EEA El Porvenir (INIA).

Growth and yield parameters evaluation

For the rice evaluation, 10 random samples of 1 m^2 each were taken from the central part of each subplot. The assessed rice parameters included: white leaf virus infection percentage (RHBV), number of tillers per square meter (NTM), panicle length (PL), number of panicles per square meter (NPM), plant height (PH), panicle fertility percentage (PF), yield, and paddy grain (PG) or "unmilled rice" in kg ha^{-1}.

The harvest was conducted 140 days after planting. Evaluations were made following the Standard Evaluation System for Rice (29).

To determine the amount of dry matter (DM) and N incorporated by the green manures, plant samples were taken from the central part of each subplot, representing plants grown in a 1 m^2 area. Dry weight was determined by weighing oven-dried samples at 60°C after 72 hours and N content was determined by the Kjeldhal method. For rice grain nutritional quality analysis, a composite mixture was taken to "La Molina Calidad Total Laboratorios - UNALM" in Lima, standardized methods were followed (5, 30, 31, 32), and the digestible carbohydrates calculated by difference, *i.e.* 100 percent minus the sum in percent of fat, ash, fiber, and protein.

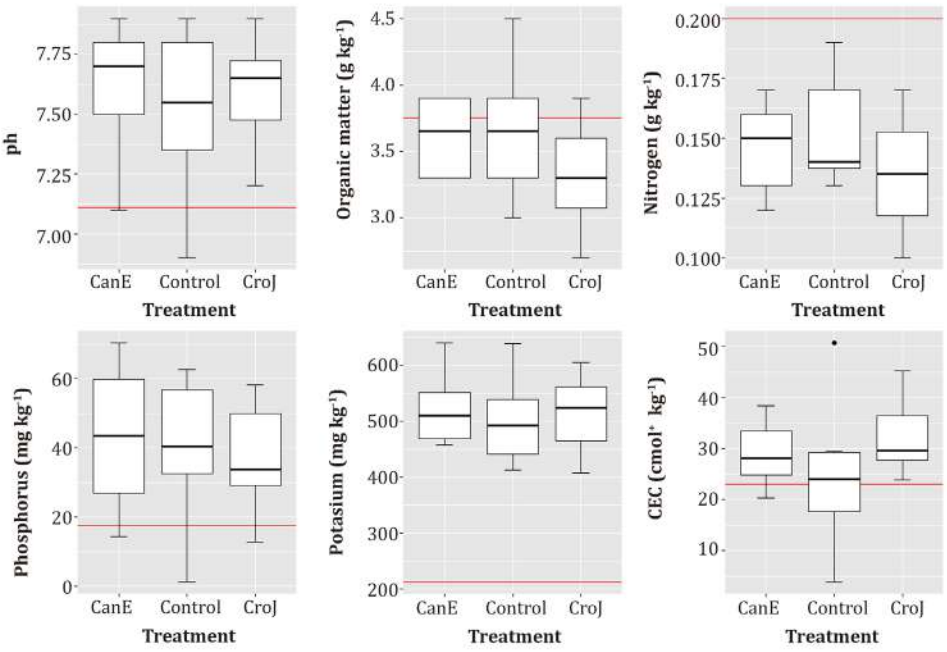
Statistical analysis

Data analysis was performed using the R statistical computing language and environment, version 4.2.1 (2023), along with the dplyr package (63), and agricolae package (19). The collected data were processed through a two-way ANOVA: green manure, N dose, and its interaction, and for mean comparison, the Fisher's LSD test was employed. In both tests, a significance level of $p < 0.05$ was considered.

Results

Soil physicochemical analysis

After green manure incorporation, the soil pH values were basic including the control, the organic matter content was medium as before. In the evaluation of macronutrients, the N content decreased, but the available P and K increased, and the CEC tended to show higher values with green manure. Nevertheless, the results were significant only for the N and K nutrients (figure 2).



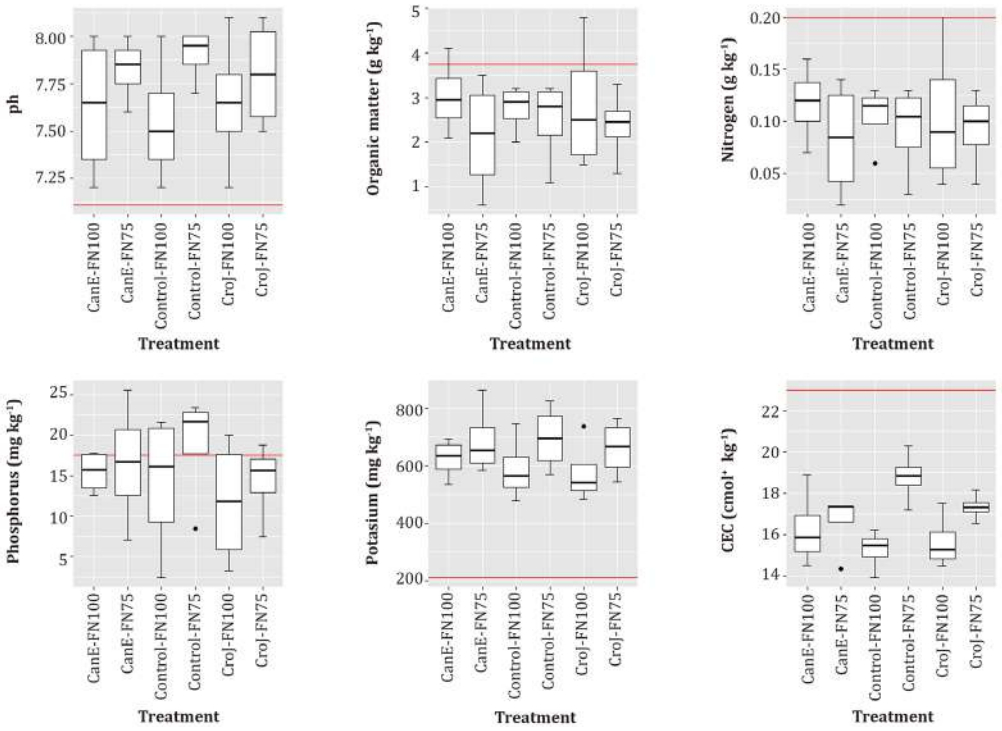
*The red line indicates the value at the initial soil sampling ($p < 0.05$).

*La línea roja indica el valor en el muestreo de suelo inicial ($p < 0,05$).

Figure 2. Soil parameters after green manure incorporation.

Figura 2. Parámetros del suelo después de la incorporación de abonos verdes.

The post-harvest physicochemical analysis showed that pH, N, K, and CEC values were statistically different compared to the initial sampling; it was found basic soil pH with significantly higher values, the OM percent was medium and exhibited a tendency to decrease (2.1 - 3.0 %), the N decreased in significant content, the available P showed similar values, the available K increased significantly, and the CEC showed meaningful lower values (figure 3).



*The red line indicates the value at the initial soil sampling ($p < 0.05$).

*La línea roja indica el valor en el muestreo de suelo inicial ($p < 0,05$).

Figure 3. Post-harvest soil parameters.

Figura 3. Parámetros del suelo poscosecha.

Green manure and rice growth/yield parameters

The green manure analysis showed that the average DM of CanE was 1.85 t ha^{-1} and the N content in vegetal tissue was 2.02% contributing to 37.41 kg of N ha^{-1}. Similarly, the average DM of CroJ incorporated into the soil was 3.59 t ha^{-1}, and the N content in plant tissue was 3.61% contributing to 129.35 kg of N ha^{-1}. Parameters evaluated in *O. sativa* showed that the tillers affected by RHBV did not reveal significant differences for the fertilizer effect or interaction ($p < 0.05$), however, concerning green manures, the treatment with the lowest RHBV incidence was CanE. The Analysis of Variance ($p < 0.05$) for the parameters NTM, PL, NPM, PH, Yield, and PG did not show significant differences for the fertilizer effect, green manure treatment, or interaction. However, it is important to mention that, despite the lack of significance, the FN75 treatment exhibited better results than the Control for NTM and NPM parameters, with 5.00% and 1.88% increments, respectively. Similarly, relating to the effect of green manures, it could be observed that NTM for CanE and CroJ treatments had 5.93% and 5.57% more tillers than the Control treatment. Likewise, for the NPM parameter, CanE and CroJ treatments increased the number of panicles by 4.15% and 2.50% compared to the Control. Similarly, the yield for CanE and CroJ treatments was 6.04% and 4.96% higher than the Control. In addition, CanE and CroJ treatments were 3.19% and 3.60% more than the Control for the PG parameter. We noted that the plot treated with CanE and 100% of the recommended N dosage attained the highest grain yield, reaching 8,355.75 kg ha^{-1}. Nevertheless, it was not statistically different (table 1, page 7).

Table 1. Agronomic parameters in *Oryza sativa*.
Tabla 1. Parámetros agronómicos en *Oryza sativa*.

Treatment		RHBV (%)	NTM (Tillers m^{-2})	PL (cm)	NPM (Panicles m^{-2})	Yield (kg ha^{-1})	PG (kg ha^{-1})	PH (cm)	PF (%)
Fertilizer Dose (FD)									
FN100		5.75 ± 2.67	245.71 ± 46.34	25.76 ± 0.85	256.42 ± 15.85	7926.00 ± 999.52	5056.70 ± 297.25	120 ± 0.07	90.5 ± 2.6
FN75		7.08 ± 2.71	258.00 ± 42.19	25.33 ± 0.36	261.25 ± 12.88	7720.78 ± 1169.52	4621.25 ± 810.65	116 ± 0.05	91.8 ± 1.8
Significance level		NS	NS	NS	NS	NS	NS	NS	NS
CV (%)		26.5	16.7	3.4	5.0	13.1	1.1	6.4	1.6
Green Manure (GM)									
CanE		5.25 ± 2.60	256.94 ± 28.50	25.62 ± 0.38	263.72 ± 10.79	8002.67 ± 692.86	4860.75 ± 481.83	120 ± 0.04	90.00 ± 3.2
Control		7.25 ± 2.43	242.56± 53.27	25.67 ± 1.04	253.22 ± 18.22	7546.60 ± 1336.10	4710.57 ± 781.00	118 ± 0.07	91.63 ± 1.6
CroJ		6.75 ± 3.01	256.06 ± 50.03	25.33± 0.72	259.56 ± 12.77	7920.90 ± 1164.04	4880.29 ± 780.05	117 ± 0.09	91.88 ± 1.6
Significance level		.	NS	NS	NS	NS	NS	NS	*
CV (%)		25.0	16.4	2.5	5.9	10.2	19.2	4.1	1.6
Fertilizer Dose x Green Manure (FD: GM)									
CanE	FN 100	5.50 ± 3.70	243.38 ± 25.23	25.75 ± 0.36	267.69 ± 11.64	8355.74 ± 599.93	5130.00 ± 379.93	122 ± 0.03	88.5 ± 3.7
	FN 75	5.00 ± 1.41	270.50 ± 27.71	25.49 ± 0.40	259.75 ± 9.71	7649.61 ± 654.06	4591.50 ± 451.70	117 ± 0.02	91.5 ± 1.9
Control	FN 100	6.50 ± 2.08	227.75 ± 47.02	25.99 ± 1.25	248.94 ± 11.08	7693.71 ± 1527.44	5047.33 ± 355.78	121 ± 0.04	91.5 ± 1.3
	FN 75	8.00 ± 2.83	257.38 ± 61.85	25.36 ± 0.83	257.50 ± 24.56	7399.49 ± 1332.15	4458.00 ± 968.02	115 ± 0.08	91.75 ± 2.1
CroJ	FN 100	5.25 ± 2.63	266.00 ± 63.57	25.57 ± 0.92	267.13 ± 6.84	7728.55 ± 774.57	4968.33 ± 178.67	117 ± 0.12	91.50 ± 1.3
	FN 75	8.25 ± 2.87	246.13 ± 39.19	25.13 ± 0.49	252.00 ± 13.47	8113.24 ± 1569.40	4814.25 ± 1087.25	117 ± 0.06	92.25 ± 2.1
Significance level		NS	NS	NS	NS	NS	NS	NS	NS
CV (%)		25.0	16.4	2.5	5.9	10.2	19.2	4.1	1.6

RHBV: White leaf virus, NTM: Number of tillers per square meter, PL: Panicle length, PG: Paddy grain, PF: Panicle fertility. The data in the table express the average and standard deviation ($\mu \pm \sigma$) of the evaluated parameters. Those values with different letters in the same column indicate significant differences between the treatments. ($p < 0.05$). "**" significant difference $p < 0.01$, "*" significant difference $p < 0.05$, "." significant difference $p < 0.1$, NS no significant difference.

RHBV: Virus de hoja blanca, NTM: Número de macollos por metro cuadrado, PL: Longitud de panícula, PG: Grano de arroz, PF: Fertilidad de panícula. Los datos de la tabla expresan el promedio y desviación estándar ($\mu \pm \sigma$) de los parámetros evaluados. Aquellos valores con letras diferentes en la misma columna indican diferencias significativas entre los tratamientos. ($p < 0,05$). "**" diferencia significativa $p < 0,01$, "*" diferencia significativa $p < 0,05$, "." diferencia significativa $p < 0,1$, NS no diferencia significativa.

Rice grain nutritional quality

The effect of factors of N fertilization dosage, the type of green manure, and the interaction did not present significant differences in the nutritional analysis of fat, ash, fiber, carbohydrates, and protein content (table 2, page 8).

Table 2. Rice grain nutritional quality.
Table 2. Calidad nutricional del grano de arroz.

Treatment		Fat	Ash	Fiber	Carbohydrate	Protein
Fertilizer dose (FD)						
FN75		2.05 ±0.3	4.94 ±0.6	9.36 ±0.5	75.15 ±3.8	7.22 ±0.3
FN100		2.15 ±0.2	4.98 ±0.3	9.48 ±0.5	73.87 ±0.4	7.35 ±0.3
Green Manure (GM)						
Control		2.13 ±0.3	5.05 ±0.4	9.22 ±0.5	74.02 ±0.4	7.13 ±0.2
Canavalia		2.05 ±0.1	4.89 ±0.5	9.38 ±0.5	75.43 ±4.5	7.38 ±0.3
Crotalaria		2.13 ±0.3	4.96 ±0.5	9.63 ±0.5	73.98 ±0.6	7.3 ±0.5
Fertilizer dose x Green Manure (FD:GM)						
FN 75	Control	2 ±0.4	5.3 ±0.4	9.03 ±0.3	73.93 ±0.6	7.07 ±0.3
	Canavalia	2.08 ±0.1	4.7 ±0.6	9.45 ±0.5	77.03 ±6.4	7.4 ±0.3
	Crotalaria	2.08 ±0.4	4.9 ±0.8	9.53 ±0.5	74.2 ±0.7	7.15 ±0.4
FN 100	Control	2.27 ±0.1	4.8 ±0.2	9.4 ±0.6	74.1 ±0.2	7.2 ±0
	Canavalia	2.03 ±0.2	5.08 ±0.4	9.3 ±0.5	73.83 ±0.6	7.35 ±0.3
	Crotalaria	2.18 ±0.3	5.03 ±0.2	9.73 ±0.6	73.75 ±0.5	7.45 ±0.5

The data in the table express the average and standard deviation (μ ± σ) of the evaluated parameters.

Los datos de la tabla expresan la media y la desviación estándar (μ ± σ) de los parámetros evaluados.

Discussion

Regarding the soil physicochemical analysis, in terms of pH, we can observe a significant increase in values, especially during the harvest phase, which might be attributed to organic anions in carboxylic acids commonly found in plant residues, resulting in a net alkalinization of soils (52). Also, urea transformation into ammonium carbonate potentially leads to a transient elevation in pH levels (50).

On the other hand, the soil C and N contents are expected to increase with the incorporation of green manure, because, the C: N ratios between 9.4 and 22.7 favor a mineralization process (15), and for CroJ and CanE are approximately 21.7±0.5 and 14±4, respectively (11, 22). However, we observed a statistically significant decrease in N after green manure incorporation and rice post-harvest. Some explanations could be that N losses rise when soil mineral N concentrations are high when supply surpasses crop demand. Excess mineral N from decomposed green manures can be lost through leaching as nitrate (NO^{3-}) and emitted as the greenhouse gas nitrous oxide (N_2O) (62). Labile fractions of C and N increase soil microbial activity, therefore, the reduction of available oxygen caused by this increase may stimulate denitrifying groups, leading to the subsequent loss of N in the form of N_2O (13). When the conditions for the mineralization of soil organic carbon are met this will lead to a high availability of easily available N and degradable C, these conditions provide hot moments for high N_2O fluxes. The management of vegetable crop residues and soil type significantly influences N_2O and NH_3 emissions, fine-textured soils, such as this research, tend to produce higher N_2O but lower NH_3 emissions than coarse-textured soils. Also, incorporating crop residues by plowing increases N_2O emissions (45). In addition, mineral N from the mineralization of soil organic matter (SOM) and plant residues in combination with periods of bare soil or sparse plant growth and precipitation surplus provide drivers for leaching (26). In other ways, the recalcitrant fraction of organic matter may bind nitrogen to aromatic carbon, reducing availability (53). Therefore, mainly mineral N released by legumes was susceptible to loss processes like soil denitrification and leaching, with the reduction in residual green manure N in the soil and the increase in cumulative N loss (37).

Organic matter is essential for stabilizing soil aggregates (35), in this sense, cover crops contribute to soil carbon stocks, v.g. they could increase their concentration by up to 12% (1.11 Mg C ha^{-1}) compared to a control treatment without cover crops (39). However, they are less effective in enhancing aggregate stability than farmyard manure and paddy straws due to their lower resistance to decomposition and stabilization (6). Also, non-conservationist cultivation practices can cause nutrient and C losses (25). In the present research, there was no significant difference in organic matter content, also in soils with high SOM, like this research, the existing organic matter already meets the nutrient requirements for grain crops, so additional organic matter does not significantly boost yields despite increasing SOM content. In addition, some authors reported that leguminous green manures did not increase grain crop yield significantly when SOM exceeded 3 g 100 g^{-1}, such as the value of initial sampling (38). Our results indicated that the green manures did not alter SOC, suggesting that it is not sensitive to short-term changes in soil quality. This finding highlights the need for a longer evaluation period to observe significant changes in SOM (14). Conversely, the increase in available K was statistically significant, this could be attributed to the high content of these and other nutrients in green manures, which are then released into the soil (1, 7, 16, 44, 57).

Sogata (*Tagosodes orizicolus*), is the main pest that affects rice production and transmits RHBV. A lower incidence of RHBV was shown with green manures because they control weeds, a strategy for plague management (51). Green manures have been employed by allelopathic effects, limiting the available space for weed growth and competing for essential resources such as water, light, oxygen, and nutrients, suppressing the potential for reinfestations (4, 49). Early studies showed that plants belonging to Crotalaria and Canavalia genera exhibited high predator diversity, and can create a more balanced ecosystem, promoting biodiversity and providing habitats for these beneficial predators (8, 18).

The PF (filled grains per panicle) was statistically significant for CroJ, which is an important factor for achieving good yields, and climatic conditions can be the reason for the formation of a higher number of grains (20).

The present research reached an 8.36 t ha^{-1} yield with the plot treated with CanE and 100% N dosage. However, it was not significant in the experiment, it is important to expose that in Peru, rice production in 2023 amounted to 8.2 t ha^{-1}, while in the province of San Martín and the district of Juan Guerra, the yield was approximately 7 t ha^{-1} (42). Besides, according to the reported yields using the INIA 507 variety, another study documented only a yield of 6.6 t ha^{-1} (27).

There were no notable distinctions in proximate analysis between treatments utilizing chemical N fertilization and those employing green fertilizers. Consequently, it can be established that the nutritional quality of rice remains unaffected by the substitution of chemical fertilization. The fat content was around 2.1 %, which is similar to Indian rice (2.463%) and Philippine rice (2.783%) (27). Concerning the protein content, it was 7.3% on average, close to the values of Mexican cultivars (6.8%) (3), the values observed in non-aromatic rice (6.97-7.17%) (60), and Brazilian variety with high amylose content and long grain (8.5%) (43). However, another rice variety from the San Martín region ("La Esperanza") exhibited an elevated protein concentration ranging between 9% and 9.48% (50). The established protein content range typically falls between 7% and 8% (34), consequently, the findings indicate a significantly higher protein content. The fiber had values of 9.4% on average, however, other studies report lower values like 2.4% (43). Regarding carbohydrates, values were presented at 74.5% on average, which is lower than other studies with non-aromatic rice (80.14-81.83%) (60), about this, N fertilizer rates can influence the concentration of non-structural carbohydrates at the filling stage (12).

The technology of green manures contributes to environmental benefits and their long-term application has proven to be economically advantageous (54). Other experiments that combined with N fertilization, showed a significant 9% increase in grain yield compared to using only chemical fertilization (33). However, establishment, management, and productivity of the subsequent cash crop influence the profitability of cover crops. It is also important to state aversion to risk, and characteristics specific to the producer and the farm can affect profit (10, 58).

Conclusions

Green manure biomass incorporation influenced soil physicochemical properties. Particularly, soil pH, P, K, and CEC increased, while N and OM declined. A lower incidence of RHBV was shown with green manures, and CroJ achieved a significant PF. Nevertheless, no significant differences were obtained in yields during this campaign, the superior outcomes were achieved through CanE, and the highest yield was 8.36 t ha^{-1} with the CanE - FN100 treatment. Concerning the proximal analysis, it can be concluded that the nutritional quality of rice remains unaffected by replacing chemical nitrogen fertilization with green manure fertilization.

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Gas exchange in yellow melon (*Cucumis melo*) crop under controlled water deficit (RDI) and application of a biostimulant

Intercambio gaseoso en el cultivo de melón amarillo (*Cucumis melo*) bajo déficit hídrico controlado (RDI) y aplicación de bioestimulantes

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Abstract

The São Francisco River Valley region in Brazil is a major producer of irrigated melons, facing stresses due to climate change. New strategies for crop management are essential to maintain sustainable cultivation. This study aims to evaluate the characteristics of melons under controlled irrigation deficit (RDI) and the use of a biostimulant. The experiment followed a completely randomized design with sub-subdivided plots. The main plots represented water levels: full irrigation (100% soil water availability - SWA) and deficit levels (80%, 60%, and 40% SWA). The subplots represented biostimulant application (with and without), and the sub-subplots represented collection periods: time I (17 to 26 days after planting - DAP), time II (27 to 36 DAP), and time III (37 to 46 DAP). The variable analyzed was gas exchange. Water restriction affects melons; however, some physiological characteristics show greater tolerance, demonstrating an adaptive response to moderate water deficit (80% SWA), regardless of the evaluation period. This allows for better water use efficiency. The biostimulant applied was not effective in promoting adjustments in the evaluated gas exchanges.

Keywords
Cucumis melo • physiology • irrigation management

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Resumen

En Brasil, el Valle del Río San Francisco es reconocido como un importante productor de melón bajo riego. Ante el estrés causado por el cambio climático, es crucial emplear nuevas estrategias de gestión del cultivo para garantizar su sostenibilidad. Por lo tanto, el objetivo de este estudio fue evaluar las características del melón bajo déficit de riego controlado (RDI) y la aplicación de bioestimulantes. El experimento se realizó en parcelas subsubdivididas. Las parcelas representaron diferentes niveles de agua: riego completo (100% de disponibilidad de agua del suelo - SWA) y niveles de déficit (80, 60 y 40% SWA). La subparcela consideró el uso de bioestimulante (con y sin), mientras que la subsubparcela abarcó los períodos de recolección: tiempo I (17 días después de la siembra - DDS hasta 26 DDS), tiempo II (27 a 36 DDS) y tiempo III (37 a 46 DDS). Las variables analizadas fueron el intercambio gaseoso. El melón mostró ser afectado por la restricción hídrica; no obstante, ciertas características fisiológicas mostraron ser más tolerantes, exhibiendo una respuesta adaptativa atenuada. Específicamente, la aplicación de déficit hídrico moderado (80% SWA), independientemente de la estación evaluada, permitió un mayor rendimiento en la eficiencia del uso del agua (UEU). Sin embargo, el bioestimulante no demostró ser eficiente para promover ajustes en el intercambio gaseoso.

Palabras clave
Cucumis melo • fisiología • gestión del riego

Introduction

Melon (*Cucumis melo* L.) is appreciated for its sweet flavor, functional and nutritional traits, and significant economic value. It thrives in diverse environments and management practices, particularly in the semi-arid Northeastern Brazil, where it exhibits year-round growth due to its exceptional productivity (5, 24). The climatic conditions of the São Francisco River Valley, characterized by high insolation and low rainfall, are conducive to melon production, fostering high photosynthetic rates and minimal disease incidence, thereby optimizing melon yields in the region (18).

The mid-region of the São Francisco River Valley, notably the Juazeiro-Petrolina area, is a prominent hub for melon production (14). In response to historically limited water resources from the main reservoirs of the São Francisco River, producers in these areas have increasingly adopted agronomic techniques to enhance water use efficiency.

Accurate water management is crucial to meet the crop's water requirements throughout its growth stages, ensuring optimal productivity. Physiological growth analysis serves as a valuable tool for understanding plant responses under varying environmental conditions, enabling comparisons across different cultivation systems (20).

Assessing drought tolerance requires a comprehensive evaluation of multiple physiological variables, such as water potential, stomatal conductance, temperature, and leaf transpiration, which collectively indicate plant performance under water stress (22). Parameters like transpiration, stomatal conductance, and photosynthesis directly influence crop growth, development, and yield, responding to soil water status and climatic variations (9).

Water use efficiency (WUE) metrics in agriculture facilitate the assessment of crop responses to varying water availability conditions. WUE is defined as the ratio of plant biomass production to the volume of irrigation water applied (11, 12).

Climate change exacerbates environmental challenges, particularly in semi-arid regions (8), potentially escalating drought vulnerabilities (7, 16) unless prompt interventions are implemented. Water scarcity in arid and semi-arid regions, such as the mid-region of the São Francisco River Valley, significantly impacts regional development.

Regulated deficit irrigation (RDI) has emerged as a key strategy in irrigation management, aiming to optimize water use efficiency by subjecting fruit trees, including melons, to controlled water stress during specific growth stages (1, 16). RDI entails applying reduced irrigation water at critical plant growth phases, enhancing WUE without compromising yield (3, 13, 14, 26, 27).

Various strategies for applying water deficit alter soil water availability, influencing leaf temperature variations and thereby affecting gas exchange and carbohydrate accumulation (31), ultimately influencing crop growth and productivity (28).

In addition to RDI, another management approach to mitigate regional climatic impacts involves biostimulant application. Biostimulants are formulations-comprising synthetic or natural substances-that promote plant growth and development by enhancing water and nutrient absorption. They influence vital plant processes, augmenting growth attributes like chlorophyll content, leaf area (4), carbohydrate levels, and fruit quality (32).

Therefore, this study aims to evaluate the physiological effects of RDI and biostimulant application on melons throughout their cultivation cycle in a controlled environment.

Material and methods

The research was conducted during November and December in a shaded environment (50% black mesh) at the experimental area of the Department of Technology and Social Sciences (DTCS), Campus III, State University of Bahia (UNEB), located in Juazeiro, BA, Brazil (9°25'09" S, 40°29'13" W, altitude approximately 368 m). The local climate is classified as Bswh, semi-arid, according to the Köppen classification, with an average annual rainfall of 540 mm. The experimental setup utilized 5-L containers filled with Fluvic Neosol soil sampled from the 0-20 cm layer. Chemical analysis of the soil was conducted at the UNEB Water, Soil and Limestone Laboratory (LASAC), with results presented in table 1.

Table 1. Results of the chemical analysis of the soil used in the research.

Tabla 1. Resultados del análisis químico del suelo utilizado en la investigación.

RESULTS											
EC	**pH**	**P**	**K**	**Na**	**Ca**	**Mg**	**Al**	**H+Al**	**BS**	**CEC**	**V**
$mS\ cm^{-1}$	-	$mg\ dm^{-3}$	$cmol_c\ dm^{-3}$ de TFSA								%
0.9	6.3	25	0.3	0.03	2.8	1.8	0.0	4.0	4.9	9.0	57.7

EC: Electric conductivity; pH: soil pH; P: phosphorus; K: potassium; Ca: calcium; Mg: magnesium; Al: aluminum; H+Al:potential acidity; BS: Bases sum; CEC: cation exchange capacity; V: percentage of base saturation.

EC: conductividad eléctrica; pH: pH del suelo; P: fósforo; K: potasio; Ca: calcio; Mg: magnesio; Al: aluminio; H+Al: acidez potencial; BS: Suma de bases; CEC: capacidad de intercambio catiónico; V: porcentaje de saturación de bases.

The experimental design was completely randomized, consisting of four soil water availability (SWA) levels (40%, 60%, 80%, and 100% SWA) and three water stress application periods (time I: 17 to 26 days after planting (DAP); time II: 27 to 36 DAP; and time III: 37 to 46 DAP), arranged in subdivided plots. Sixteen SWA combinations were tested for each stress period: 100/100/100, 80/80/80, 60/60/60, 40/40/40, 100/80/80, 100/60/60, 100/40/40, 100/100/80, 100/100/60, 100/100/40, 100/80/100, 100/60/100, 100/40/100, 80/100/100, 60/100/100, and 40/100/100% SWA. During non-stress periods, irrigation was adjusted to maintain soil water at field capacity (100% SWA). The subplot factor included the absence or presence of a biostimulant.

The biostimulant (300 mL ha^{-1} concentration), obtained through biological fermentation of organic compounds, was applied twice: six days after transplant and pre-flowering. Each experimental unit provided data, and irrigation depth was calculated for each treatment using graduated cylinders, applied at two-day intervals.

Seedlings of the 'Gold Mine' cultivar, a yellow-type melon belonging to the *inodorus* group, were grown in a greenhouse using commercial substrate in polystyrene trays with 128 cells for germination. At 12 DAP, seedlings were transplanted into 5-liter pots filled with gravel for improved drainage, covered with a fine mesh to prevent soil loss, and filled with soil.

Following a five-day acclimatization period, water deficit treatments commenced at 17 DAP. Biostimulant application was individually administered using a 20-mL syringe in the specified periods. Fertilization was conducted via fertigation three times per week, tailored for the estimated plant population based on a 0.3 x 2.0 m spacing.

During each water stress period, gas exchange parameters including net photosynthesis, leaf transpiration, stomatal conductance, and leaf temperature were analyzed using a portable infrared CO_2 analyzer (IRGA - LiCOR 6400XT) on fully expanded leaves, between 10:00 am and 12:00 pm on sunny days. Water use efficiency (WUE) was calculated as the ratio of photosynthetic rate to transpiration.

Data were subjected to analysis of variance (ANOVA) and significant differences were determined using the Tukey test ($p < 0.05$). Statistical analyses were performed using SISVAR 5.6 software (9).

Results and discussion

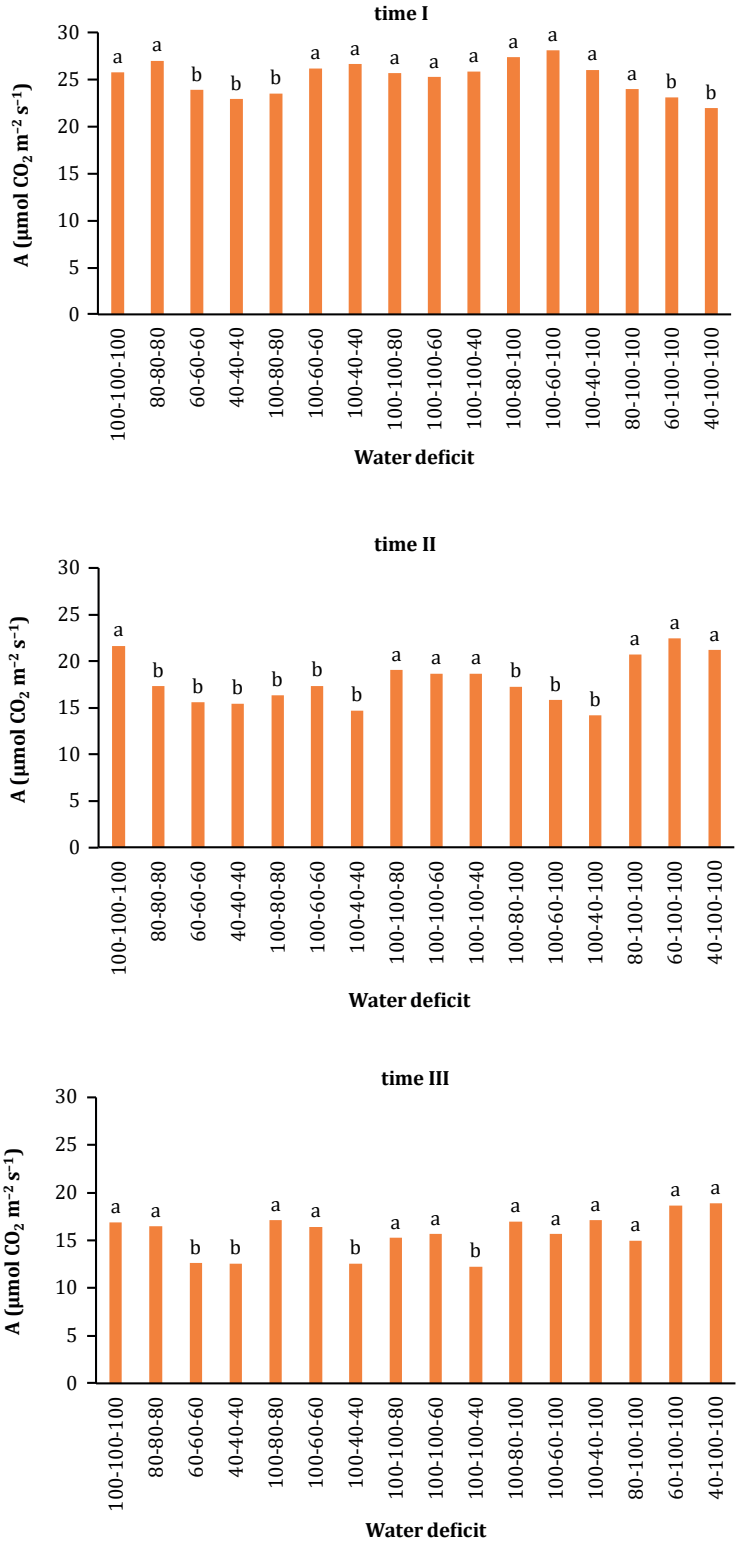
Despite previous studies (4, 29) demonstrating that biostimulants enhance plant metabolism and structure by improving water and nutrient absorption and tolerance to water stress, the analysis of variance in this study revealed no significant interaction between treatments or exclusive differences in evaluated physiological variables compared to RDI. The lack of biostimulant effects on physiological variables may be attributed to the cultivation period, which coincided with the hottest time of the year, likely impacting plant physiological and biochemical characteristics. Nonetheless, there is limited literature discussing the biostimulant's influence on gas exchange parameters.

The analysis of photosynthesis data indicated statistical significance ($p < 0.01$) and a significant interaction between time and water deficit levels. Figure 1 (page 18), illustrates the impact of water deficit on photosynthesis levels, represented by *A - net assimilation rate* of CO_2 *(µmol* CO_2 m^{-2} s^{-1}*)*. Plants irrigated at 100% SWA during times I, II, and III exhibited minimal reductions in photosynthetic activity. Moderate and severe water deficits (60% and 40% SWA) at any time resulted in significantly lower photosynthetic activity. As noted by Vendruscolo *et al.* (2017), adequate irrigation enhances internal CO_2 concentration in plants; however, water availability directly limits photosynthesis, with high CO_2 concentrations correlating with increased stomatal conductance (gs). Thus, stomatal closure primarily restricts photosynthesis, as reduced stomatal apertures hinder CO_2 diffusion, a phenomenon observed in this study.

At times I and III, using 80% SWA showed statistical similarity ($p < 0.05$) to the control, indicating no decrease in carbon assimilation or photosynthetic capacity under moderate water deficit. This finding aligns with previous research (23), which evaluated gas exchange in melon plants under different irrigation frequencies, and Ferrerira (2011), which examined water stress in sesame plants, supporting our results by reporting reduced photosynthetic activity with decreased irrigation frequency.

Stomatal conductance (gs) data (figure 2, page 19), analyzed via ANOVA, revealed a significant interaction between time and water deficit levels. Examination across time periods indicated a decline in leaf surface water vapor conductance over time (I, II, and III), correlating with the photosynthesis data (figure 1, page 18), where photosynthetic levels decreased correspondingly. During time I, treatments maintaining 100% SWA exhibited the highest averages. The treatment with 80% SWA statistically mirrored ($p < 0.05$) the control, allowing higher water vapor conductance and photosynthesis levels (27.01 µmol.m^2.s^{-1}). Maximum and minimum gs values per time period were as follows: 100% SWA (0.544 mol.m^2.s^{-1}) and 40% SWA (0.264 mol.m^2.s^{-1}).

Time II showed a similar pattern to time I, with highest conductance values in non-stressed plants and lowest in stressed plants: 100% SWA (0.381 mol.m^2.s^{-1}) and 40% SWA (0.162 mol.m^2.s^{-1}), respectively. This aligns with findings by Vendruscolo *et al.* (2017), who reported increased gs with higher water availability in eggplants. However, during time III, no significant differences in water deficit levels were observed. As noted by Melo *et al.* (2014), studying gas exchanges aids in understanding melon responses to soil water deficits and quantifying species' acclimation to adverse conditions. Reductions in gs throughout the phenological cycle reflect photosynthetic adjustments, as melon development (15 DAP: vegetative, 30 DAP: flowering, 45 DAP: fruiting) increases leaf area and stomatal density, enhancing gas exchange efficiency. Without this efficiency, both melon types risk excessive water loss, potentially leading to dehydration and hindered growth.

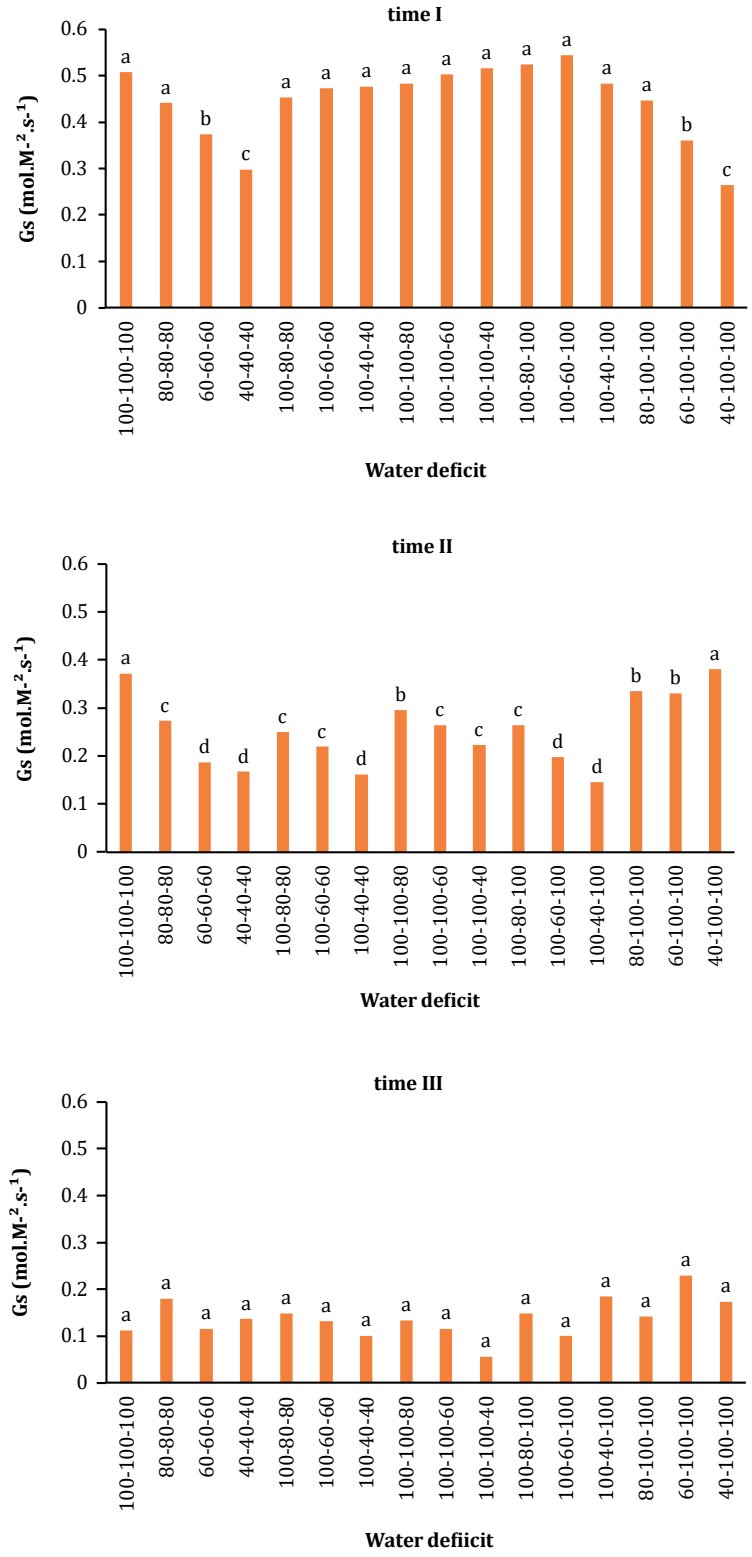


Means followed by the same letters do not differ at 5% probability level by Tukey's test.

Medias seguidas de letras iguales no difieren al nivel de probabilidad del 5% por la prueba de Tukey.

Figure 1. Changes in A - net assimilation rate CO_2 (µmol CO_2 m^{-2} s^{-1}) observed at different times (time I: 17 to 26 DAP; time II: 27 to 36 DAP; and time III: 37 to 46 DAP) in relation to the interaction with water deficit levels.

Figura 1. Cambios en A - tasa de asimilación neta de CO_2 (µmol CO2 m^{-2} s^{-1}) observados en diferentes momentos (Tiempo I: 17 a 26 días después de la siembra - DAP; Tiempo II: 27 a 36 DAP y Tiempo III: 37 hasta 46 DAP) en relación con la interacción con los niveles de agua.



Means followed by the same letters do not differ at 5% probability level by Tukey's test.

Medias seguidas por letras iguales no difieren al nivel de probabilidad del 5% por la prueba de Tukey.

Figure 2. Changes in stomatal conductance - (gs) (mol m^{-2} s^{-1}) at different times (time I: 17 to 26 DAP; time II: 27 to 36 DAP; and time III: 37 to 46 DAP) in relation to the interaction with water deficit levels.

Figura 2. Cambios en (gs) - conductancia estomática (mol m^{-2} s^{-1}) en diferentes tiempos (Tiempo I: 17 a 26 días después de la siembra - DAP; Tiempo II: 27 a 36 DAP y Tiempo III: 37 a 46 DAP) en relación con la interacción de los niveles de déficit hídrico.

Post the fourth week under RDI, temporal variations significantly decreased compared to controls (17), showing stress-induced reductions in gs due to lowered leaf water potential. Figure 3 (page 21), depicts leaf transpiration levels.

According to Lamaoui *et al.* (2018a), stomatal closure results from reduced osmotic-water potential. Results also indicated melon's limited ability to maintain adequate leaf water potential, a trait some cultivars manage due to biochemical characteristics that sustain photoassimilate transport from shoots to roots, enabling enhanced water absorption (25).

For the variable transpiration, there was a double-factor interaction for times and levels of water deficit (figure 3, page 21).

At time I, plants under 100% SWA exhibited higher transpiration rates, while those under moderate deficit (80% SWA) were statistically similar ($p < 0.05$) to the control. There was a slight reduction of 3.79% in maximum transpiration capacity compared to the control treatment. By time II, plants irrigated at 100% SWA continued to display higher transpiration rates, whereas those under water stress exhibited significant reductions. Specifically, the moderate deficit treatment (80% SWA) showed approximately a 20.89% decrease in transpiration compared to its own performance at time I. Time III data indicated a decline in transpiration rates as plants matured, though the pattern observed in previous periods persisted. Water deficit consistently resulted in decreased transpiration rates, with non-stressed treatments exhibiting the highest rates. Notably, the moderate deficit treatment (80/80/80) did not significantly differ at the 5% probability level from the control. Previous studies (unpublished data) on melon physiology under varying irrigation levels suggest that applying an 80% deficit does not reduce transpiration rates.

Research by Elmaghrabi *et al.* (2017), on the Sancho melon cultivar suggested that this response might be attributed to melon metabolism, which can maintain efficient photosynthesis with reduced stomatal opening and lower intercellular CO_2 levels without compromising water use efficiency (WUE). Under optimal water conditions, melons exhibit higher transpiration rates correlated with stomatal conductance (gs), as stomata serve as the primary avenue for water loss, essential for water and mineral absorption, CO_2 uptake for photosynthesis, growth, and plant cooling (23). According to Lamaoui *et al.* (2018a), melon's high gs under normal conditions results from increased stomatal aperture, leading to higher transpiration rates. Conversely, under stress conditions, both transpiration rates and gs decrease. The authors noted that adequate irrigation induces stomatal opening, enhancing melon photosynthetic rates and mitigating stress effects.

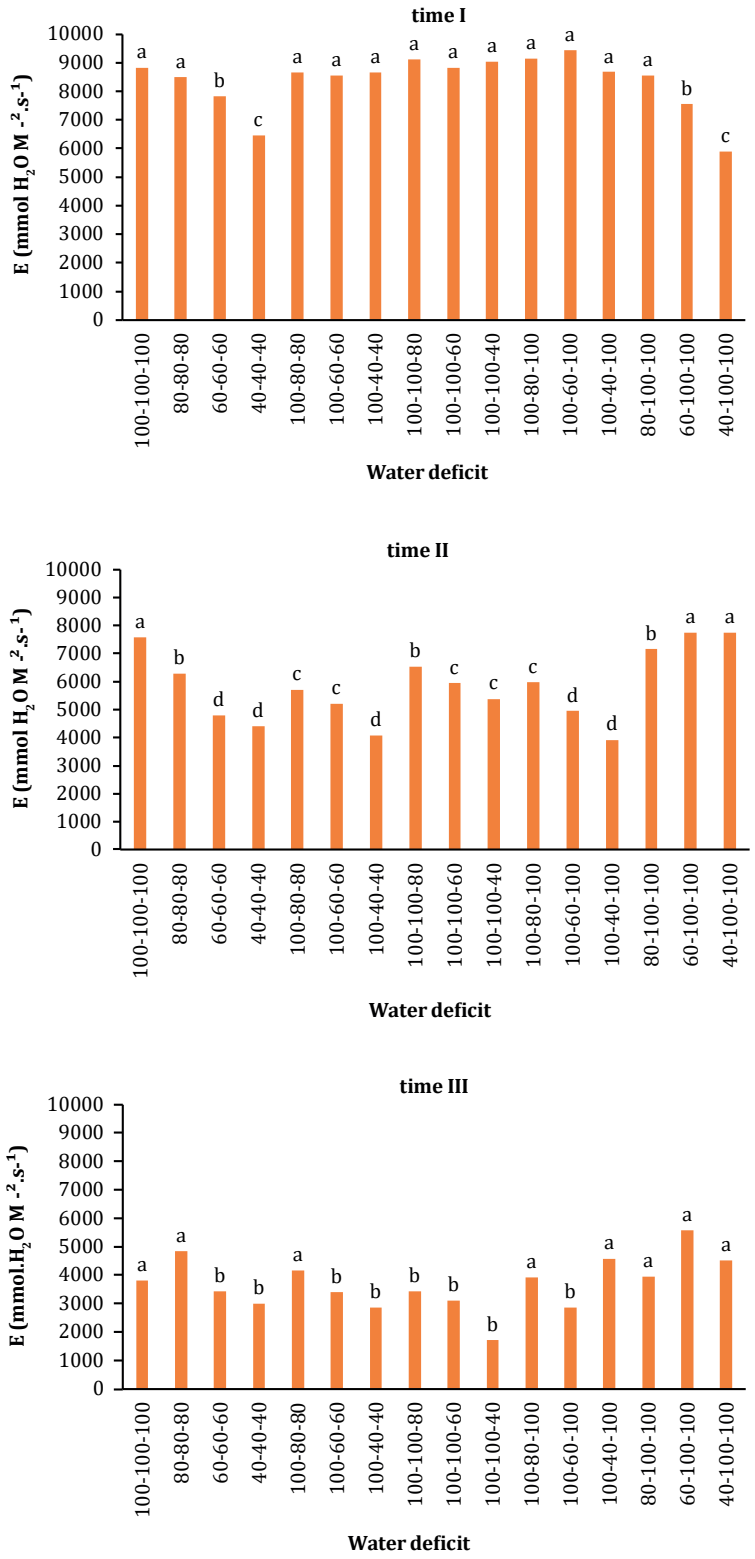
Reducing irrigation by 50% compared to conventional methods led to reduced transpiration rates primarily due to stomatal closure, a phenomenon observed in various plant species. According to Lamaoui *et al.* (2018b), hormonal signaling in shoot long-distance signaling and hydraulic conductivity control explain changes in stomatal activity.

Significant interaction effects were observed in leaf temperature results (figure 4, page 22).

Analysis across time periods (I, II, and III) indicated a gradual increase in leaf temperature over time, driven by rising ambient temperatures typical of November and December, the study period. During times I and II, most treatments receiving 100% SWA showed lower leaf temperatures compared to water deficit treatments (60% and 40% SWA). Notably, plants under 80% SWA did not exhibit increased leaf temperatures, contrasting with other stressed treatments and statistically equating to the control ($p < 0.05$). Similar findings were reported by Vieira *et al.* (2019), in studies on melon plants subjected to various water stress levels, where conductance and transpiration closely influenced leaf cooling. Water deficit indirectly raises leaf temperature by limiting cooling mechanisms through chemical signaling that prompts stomatal closure.

The WUE graph (figure 5, page 23) illustrates how WUE is directly influenced by water deficit.

Full irrigation (100/100/100) resulted in lower WUE compared to treatments subjected to higher water stress levels. Comparing the WUE of the 40/40/40 treatment with the control, there was a 14.7% increase, while the 60/60/60 treatment showed a 25.2% superiority in WUE over the control. Similar results were reported by Al-Mefleh *et al.* (2012) and Nascimento *et al.* (2011), in studies on melon subjected to varying irrigation depths (50%, 75%, 100%, and 125% ETc), highlighting that lower irrigation levels generally lead to higher WUE values.

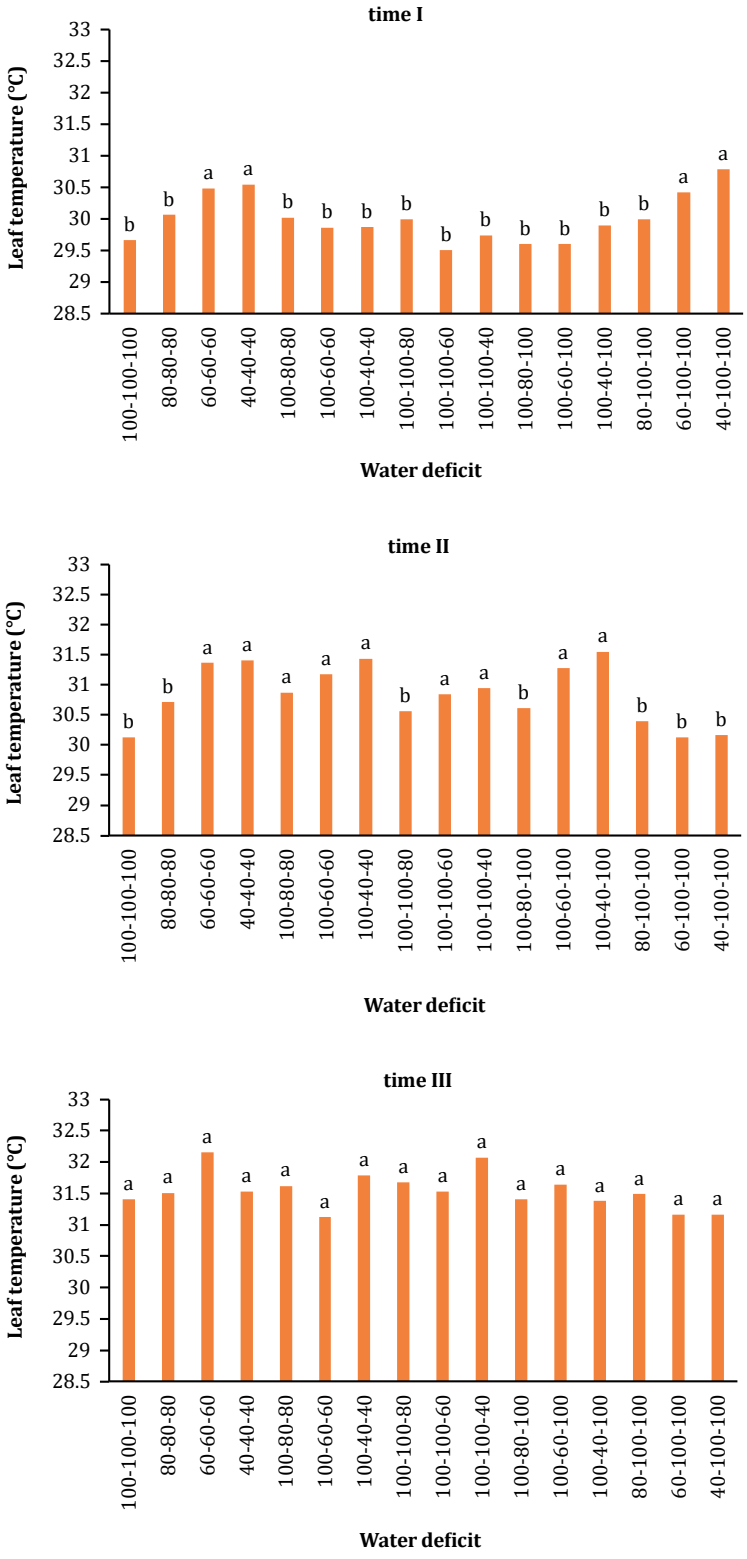


Means followed by the same letters do not differ at 5% probability level by Tukey's test.

Medias seguidas de letras iguales no difieren al nivel de probabilidad del 5% según la prueba de Tukey.

Figure 3. Changes in transpiration - (E) (mmol H_2O m^{-2} s^{-1}) at different times (time I: 17 to 26 DAP; time II: 27 to 36 DAP; and time III: 37 to 46 DAP) in relation to the interaction with water deficit levels.

Figura 3. Cambios en transpiración - (E) (mmol H_2O m^{-2} $s^{-1)}$ en diferentes tiempos (Tiempo I: 17 a 26 días después de la siembra - DAP; Tiempo II: 27 a 36 DAP y Tiempo III: 37 a 46 DAP) al interactuar con los niveles de déficit hídrico.



Means followed by the same letters do not differ at 5% probability level by Tukey's test.

Medias seguidas de letras iguales no difieren al nivel de probabilidad del 5% según la prueba de Tukey.

Figure 4. Changes in leaf temperature (°C) at different times (time I: 17 to 26 DAP; time II: 27 to 36 DAP; and time III: 37 to 46 DAP) in relation to the interaction with water deficit levels.

Figura 4. Cambios en la temperatura foliar (°C) en diferentes momentos (Tiempo I: 17 a 26 días después de la siembra - DAP; Tiempo II: 27 a 36 DAP y Tiempo III: 37 a 46 DAP) al interactuar con los niveles de déficit hídrico.

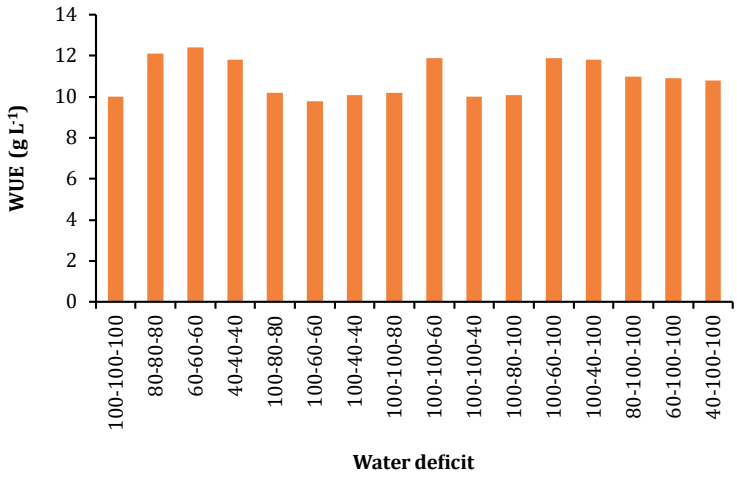


Figure 5. Variation in water use efficiency (WUE) of melon plants in relation to different water deficits.

Figura 5. Variación en la eficiencia del uso del agua (WUE) de las plantas de melón en interacción con diferentes déficits hídricos.

Conclusion

Water restriction impacts melon plants, but certain physiological traits exhibit greater tolerance and adaptive responses, notably under moderate water deficit (80% SWA), irrespective of the season. Consequently, this approach enhances water use efficiency (WUE).

However, the biostimulant applied during the cultivation period does not effectively induce adjustments in gas exchange.

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Incidence of *Fusarium graminearum* and DON in malting barley grains (*Hordeum vulgare* L.)

Incidencia de *Fusarium graminearum* y DON en granos de cebada cervecera (*Hordeum vulgare* L.)

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Abstract

Fusarium graminearum is a fungal species affecting the quality and safety of malting barley grains, one of the most important cereals worldwide. Fungal growth and mycotoxin production vary among growing seasons and sowing locations, mainly due to weather conditions. This work aimed to assess the incidence of *F. graminearum* and the contamination with Deoxynivalenol (DON) in 40 barley grain samples from different Buenos Aires, Argentina localities during the 2017 and 2018 growing seasons. *F. graminearum* was identified in 80% of the samples. It was isolated in eight of eleven localities in the first and ten in the second growing seasons, with a similar maximum incidence (20% and 17%, respectively). On the other hand, all samples were contaminated with DON, and 75% exceeded the maximum limits established by The European Union (EC 1126/2007). The level of DON contamination was significantly higher in the second growing season, which was rainier and had a higher mean temperature (an average of 2.5 ppm in 2017 and 3.75 ppm in 2018). The results obtained in the present study show the need to establish regulations in Argentina on maximum limits of *Fusarium* mycotoxins in barley.

Keywords: Barley • *Fusarium graminearum* • incidence • Deoxynivalenol • food safety

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Resumen

Fusarium graminearum es una especie fúngica que afecta la calidad e inocuidad de la cebada cervecera, uno de los cereales más importantes a nivel mundial. Tanto el crecimiento fúngico como la producción de micotoxinas varían año a año y por la región de siembra, principalmente por las condiciones climáticas. El objetivo de este trabajo fue evaluar la incidencia de *F. graminearum* y la contaminación con Deoxinivalenol (DON) en 40 muestras de cebada cervecera de distintas localidades de Buenos Aires, Argentina, durante las temporadas de cultivo 2017 y 2018. *F. graminearum* fue identificado en el 80% de las muestras. Se aisló en ocho de once localidades en la primera temporada de cultivo y en diez localidades de la segunda temporada de cultivo, con una incidencia máxima similar (20% y 17% respectivamente). Por otro lado, todas las muestras estuvieron contaminadas con DON y el 75% excedieron los límites máximos establecidos por la Unión Europea (EC 1126/2007). El nivel de contaminación con DON fue significativamente mayor en la segunda temporada de cultivo, la cual fue más lluviosa y tuvo una temperatura media mayor (un promedio de 2,5ppm en 2017 y 3,75ppm en 2018). Los resultados obtenidos en este estudio muestran la necesidad de establecer regulaciones en Argentina sobre límites máximos para micotoxinas de *Fusarium* en cebada.

Palabras clave: Cebada • *Fusarium graminearum* • incidencia • Deoxinivalenol • inocuidad

Introduction

Barley (*Hordeum vulgare* L.) is the fourth most important cereal worldwide after wheat, corn, and rice and is used for both human consumption and animal feed. The world production of barley in the 2022 growing season was 152 million tons, whereas, in Argentina, it was about 4.5 million tons, of which 3 million tons were exported. The main importing countries were China (2,335,983 tons) and Brazil (612,295 tons) (6). The use of barley has increased fundamentally in the brewing industry. In Argentina, in the 2022 growing season, malting barley occupied 98.61% of the sown area, whereas fodder barley occupied only 1.39%. Buenos Aires was the province that presented the largest sown area (26).

Malting barley is often associated with *Fusarium graminearum sensu stricto* (hereafter *F. graminearum*) infection, resulting in yield reduction, lower germination capacity, lower thousand kernel weight, and protein degradation (14, 25). Besides the several economic losses, *F. graminearum* can produce mycotoxins with the potential to cause adverse effects on human and animal health. Many studies demonstrated the ability of *F. graminearum* to produce deoxynivalenol (DON) (4, 7, 18, 22). Remnants of this mycotoxin have been observed after malting and in commercial beer (3, 11, 19, 20). DON is a trichothecene B-type which can cause vomiting, diarrhea, intestinal inflammation, reduced feed intake, and decreased absorption of amino acids and carbohydrates. Chronic diseases include anorexia and immunotoxicity (21). Regulations on maximum limits for DON vary among countries. The European Union (EU) has established a maximum DON limit of 1.25 ppm in unprocessed cereals other than durum wheat, oats, and maize (EC N° 1126/2007) (28). Instead, Brazil, one of the main importing countries in 2022, has established a maximum DON limit of 1 ppm in barley grain, malted barley, and cereal-based products (2). Argentina has established maximum DON limits for wheat and maize (1 ppm) and cereal-based foods for infants and young children (0.2 ppm). However, maximum DON limits for barley in Argentina have not yet been established (8).

Barley is the main raw material in the brewing industry. Since both fungal growth and mycotoxin production vary among growing seasons and sowing locations, this work aimed to assess the incidence of *F. graminearum* as well as the levels of contamination with DON in 40 malting barley samples from different Buenos Aires localities, Argentina, during two consecutive growing seasons (2017 and 2018).

Material and methods

Barley samples were obtained from different Buenos Aires localities, Argentina, during the 2017 and 2018 growing seasons. In the first growing season, 20 barley samples were obtained from Tandil (3 samples), Balcarce (3), Lobería (2), Necochea (1), Tres Arroyos (1), Coronel Pringles (1), Olavarría (1), Guaminí (5), Bolivar (1), San Cayetano (1), and General La Madrid (1). In the second growing season, 20 barley samples were obtained from Tandil (5), Balcarce (1), Lobería (2), Necochea (2), Tres Arroyos (1), Coronel Pringles (1), Olavarría (1), Guaminí (2), Bolivar (2), Coronel Suarez (1), and Saavedra (2) (figure 1).

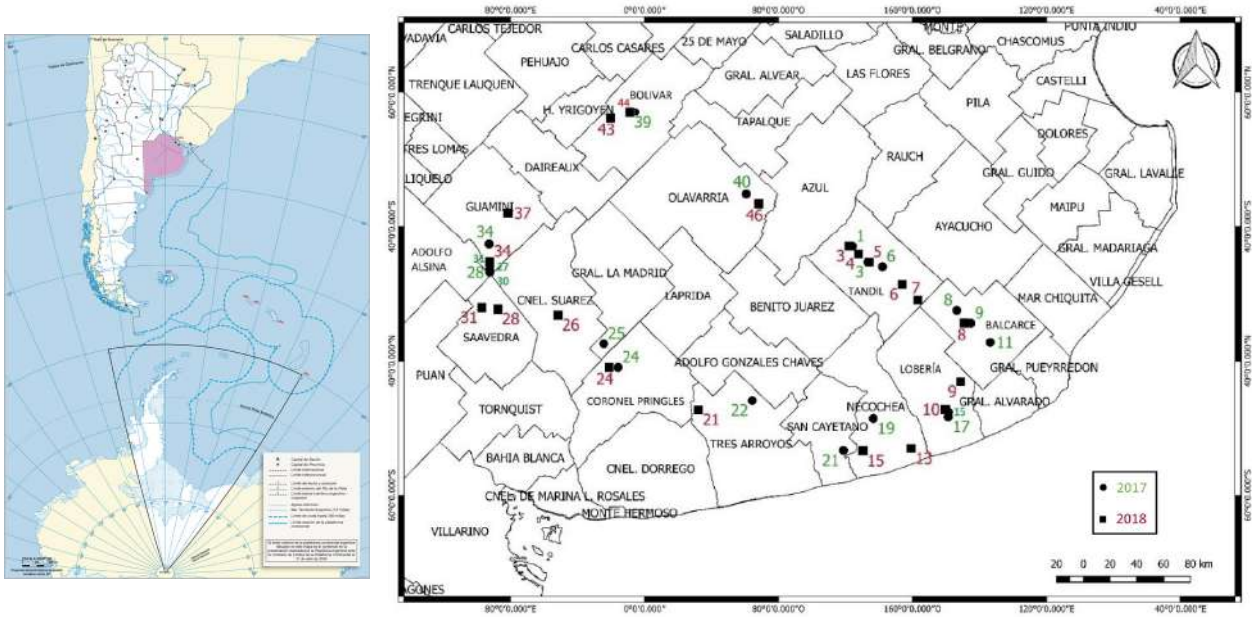

Figure 1. Geographical sampling points from different Buenos Aires localities, Argentina in two consecutive growing seasons. The numbers indicate the sample number.

Figura 1. Puntos geográficos de muestreo de diferentes localidades de Buenos Aires, Argentina, en dos temporadas de cultivo consecutivas. Los números indican el número de muestra.

Meteorological data from each sampled locality was collected during the 2017 and 2018 growing season. In particular, accumulated precipitation and mean temperature were analyzed to evaluate the eventual influence of climatic conditions on the incidence of *F. graminearum* and the levels of contamination with DON in barley grains. Meteorological data was obtained and analyzed from the Giovanni online data system, developed and maintained by the NASA GES DISC and the QGis software (v.3.16) (1). Data was collected during the period of flowering (November) in which barley is susceptible to *Fusarium* infection (figure 2, page 29).

Grain samples (500 g) were reduced with a grain divider, surfaced disinfected by washing with sodium hypochlorite 5% and ethanol 70%, subsequently, for 2 min, and washed twice with sterile distilled water for 2 min. One hundred grains were randomly plated (10 grains/plate) on potato dextrose agar 2% (PDA) with 0.25 g chloramphenicol/L and incubated at 25 °C for 4-7 days, under a 12 h light/dark cycle. Potential *F. graminearum* colonies (mycelia from white to pale orange to yellow and with red pigments in the agar) were subcultured onto PDA and carnation leaves agar (CLA) and incubated at 25 °C for 15 days, under a 12 h light/dark cycle for identification (12). To confirm morphological results, a *F. graminearum*-specific PCR was performed for a representative subgroup using primers Fg16F and Fg16R (16). Monosporic cultures of previously identified *F. graminearum* isolates were cultured for 6 days on PDA plates at 25 °C. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (27). Polymerase chain reaction (PCR) mixture (25 µL) contained 10-20 ng of genomic DNA, 10X reaction buffer, 2.5 mM $MgCl_2$, 1 µM of each primer, 30 µM of dNTPs (Genbiotech S.R.L., Argentina), 1 U of *Taq* DNA Polymerase (Inbio-Highway, Argentina), 0.014% of Cresol Red solution (Sigma-Aldrich Co. St Louis, MO), 0.0005% Tween 20®, 0.0005% Nonidet P40®.

The amplification of DNA was performed in an XP Thermal Cycler (Bioer Technology Co., China) (16). After electrophoresis separation in 5X TBE buffer at 80 V on 1.5% agarose gel containing 3-4 µL of GelRedTM, the PCR products (≈ 400 base pairs) were visualized under UV light (Biotium, Hayward, USA). The isolate 1-1 was used as positive control (7).

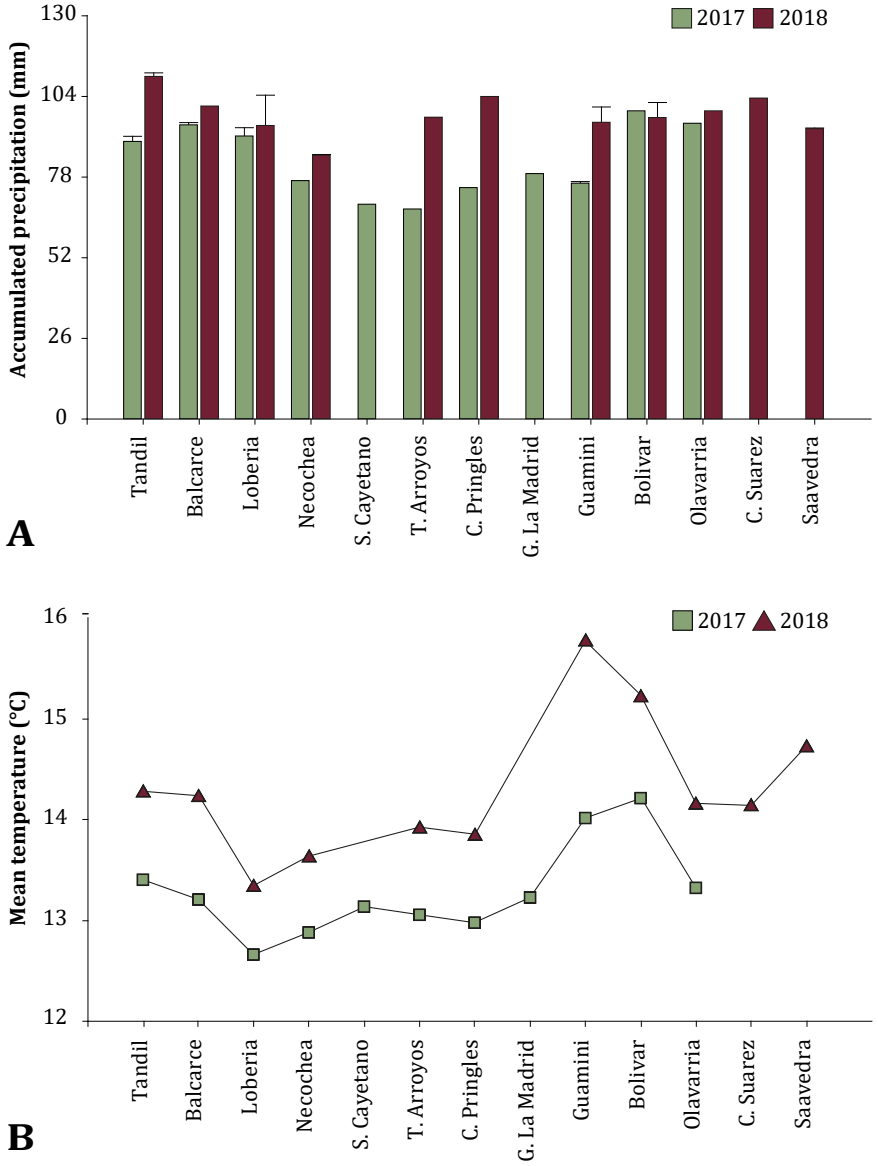

Figure 2. Accumulated precipitation (A) and mean temperature (B) from sampling localities during November in the two growing seasons. The lack of standard deviation in some localities is because only one data was obtained.

Figura 2. Precipitaciones acumuladas (A) y temperatura media (B) de las localidades muestreadas durante noviembre en las dos temporadas de cultivo. La falta de desviaciones estándar en algunas localidades se debe a que solo se obtuvo un dato.

The protein content of grain samples was measured with a NIT analyzer with a double-face monochromator (Agricheck, Bruins Instruments, Germany) to evaluate their relationship with the incidence of *F. graminearum*.

DON contamination was analyzed in a representative subsample (20 g) by enzyme-linked immunosorbent assay (ELISA) following the manufacturers' specifications (Agra-Quant DON, RomerLabs) with a detection limit of 0.2 ppm. The plate was scanned using an automatic plate reader Rayto RT-6000.

Statistical analyses were done using RStudio version 2022 (24). Normal distribution of the data was evaluated using the Shapiro–Wilks test. Analysis of variance (ANOVA) was performed to evaluate differences in *F. graminearum* incidence and DON contamination levels between growing seasons and localities. Pearson's Correlation analysis was performed between the incidence of *F. graminearum* and protein content, the incidence of *F. graminearum* and DON contamination levels, and the DON contamination levels and climatic conditions.

Results and discussion

Our results showed that 32 out of the 40 samples (80%) were contaminated with *F. graminearum*. A total of 236 isolates were morphologically identified, and the selected isolates amplified fragments of ≈ 400 base pairs, which confirmed the morphological observations. The number of samples contaminated by *F. graminearum* was greater than in other studies carried out in Buenos Aires (18), central Italy (4), and Southern Brazil (22, 23). A recent study has demonstrated that warm nights increase the incidence of *F. graminearum* in barley, so it is important to consider that the incidence observed in this study may increase in the coming years (15). The incidence (percentage of infected grains per sample) varied from 0 to 20% in the first growing season, with an average incidence of 5.3%, and varied from 0 to 17% in the second growing season, with an average incidence of 6.5% (figure 3). A similar incidence was observed in wheat samples from different Buenos Aires localities (13). Regarding distribution by locality, *F. graminearum* was isolated from eight localities in 2017 and ten localities in 2018 (figure 3). The greatest number of isolates was obtained in Necochea and Tandil in the first and the second growing seasons respectively, whereas the lowest number of isolates was obtained in Balcarce and Saavedra in the first and the second growing seasons respectively (figure 3). *F. graminearum* was not isolated from Bolivar, San Cayetano, and Tres Arroyos in the first growing season, and from Coronel Suarez in the second. Correspondent analyses showed non-significant differences in the incidence of *F. graminearum* in barley between different seasons and different localities. However, we did not have the same number of samples per locality and the same localities per growing season. Besides, an association with the favorable climatic conditions in each locality was not found.

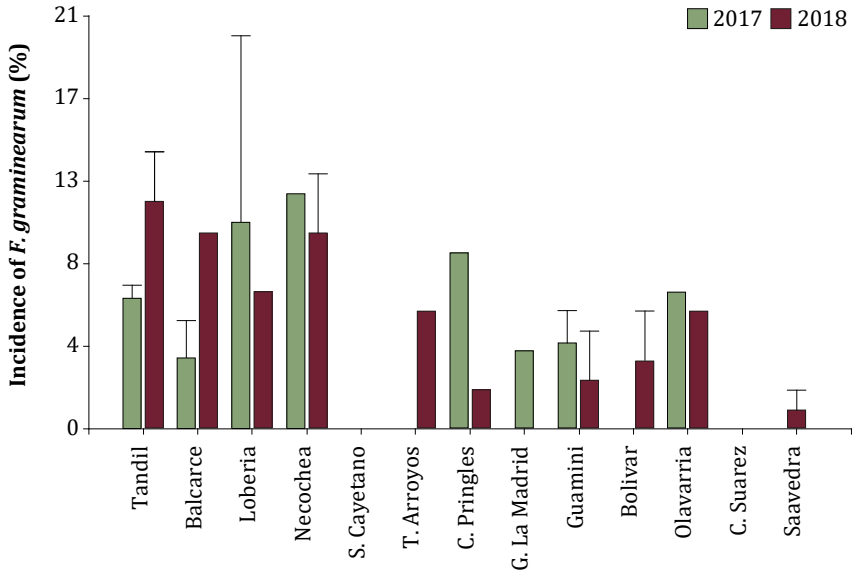

Figure 3. Incidence of *F. graminearum* (average of grains infected per sample) in malting barley samples grown in different Buenos Aires localities, in two consecutive growing seasons. The lack of standard deviation in some localities is because only one data was obtained.

Figura 3. Incidencia de *F. graminearum* (porcentaje de granos infectados por muestra) en muestras de cebada cervecera en diferentes localidades de Buenos Aires, en dos temporadas de cultivo consecutivas. La falta de desviaciones estándar en algunas localidades se debe a que solo se obtuvo un dato.

Protein content analysis showed that 67.5% of the samples did not meet the regulation (5), two samples exceeded the maximum limit of protein content (13%) and 25 samples did not reach the minimum limit of protein content (9.5%). Although the incidence of *F. graminearum* was slightly higher in the second growing season, 90% of the samples of the first growing season and 45% of the second did not meet the regulation. Pearson's Correlation analysis was non-significant and showed a low correlation between protein content and the incidence of *F. graminearum* ($r = 0.226$, $p > 0.05$). In other studies, protein content was evaluated after inoculation with *Fusarium* species, and no effects were observed, attributing the fluctuation in protein content to environmental conditions during crop development (9), and genotype evaluated (14).

All the samples analyzed were contaminated with DON in different concentrations. In barley studied in Buenos Aires (18), central Italy (4), and Southern Brazil (23), the number of contaminated samples was lower than in this study. In contrast, in wheat samples studied in Buenos Aires, the number of contaminated samples was similar to the number observed (13). Significant differences ($p < 0.05$) were found only between growing seasons. The 2018 growing season samples showed higher DON concentrations than the 2017. The second growing season was rainier and showed higher mean temperature than the first; however, only a moderate correlation was observed between DON content and accumulated precipitations ($r = 0.365$, $p < 0.05$). A similar association was observed by Gonzalez *et al*. (2008) and Piacentini *et al*. (2015). In the present study, 30 out of 40 samples (75%) exceeded the maximum limit established by the EU (1.25 ppm) and 32 out of 40 (80%) exceeded the maximum limit established by Brazil (1 ppm). These values warn of the need to establish control measures to guarantee the production of safe grains, suitable for commercialization and industrialization. As observed in figure 4, DON contamination levels greater than 5 ppm were observed in 35% of the samples in the first growing season and 55% in the second growing season, whereas DON contamination levels lower than 1.25 ppm were observed in 45% of the samples in the first growing season and 5% in the second growing season. In other studies carried out in Buenos Aires (18) and Brazil (23) DON contamination levels were similar to those observed in this study. However, some authors obtained DON contamination levels lower than our results, and even, none samples (4) and only one sample (17), exceeded the maximum limit established by the EU.

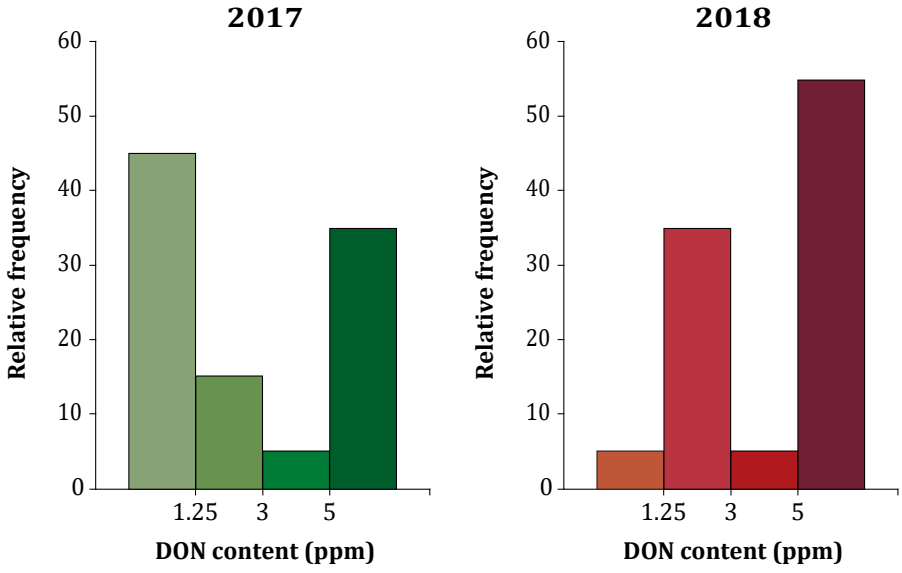

Figure 4. Relative frequency of DON content in malting barley samples grown in two consecutive growing seasons.

Figura 4. Frecuencia relativa del contenido de DON en muestras de cebada cervecera de dos temporadas de cultivo consecutivas.

Pearson's Correlation analysis was performed between the incidence of *F. graminearum* and DON contamination levels, showing a statistically significant correlation between variables ($r = 0.467$, $p < 0.05$). In the first growing season, Tandil, one of the localities with the highest DON contamination level, had an incidence of *F. graminearum* higher than average (7%). In contrast, in Bolivar, the locality with the lowest DON contamination level, *F. graminearum* was not isolated. These results may indicate the presence of other DON-producing *Fusarium* species (13, 17, 18) or that *F. graminearum* was not obtained with the isolation method used (13). In the second growing season, Tandil, one of the localities with the highest DON contamination level, had the highest *F. graminearum* incidence. In contrast, Guaminí, the locality with the lowest DON contamination level, had an incidence of *F. graminearum* lower than average (3%). The correlation between the incidence of *F. graminearum* and DON contamination levels was also demonstrated in other studies (4, 13, 22).

Conclusion

In conclusion, our results demonstrate that 80% of malting barley samples from Buenos Aires, Argentina were contaminated with *F. graminearum* in the 2017 and 2018 growing seasons. The incidence of *F. graminearum* observed was greater than in other studies in Buenos Aires in barley. The incidence of *F. graminearum* and precipitations influenced DON contamination levels. All the samples analyzed were contaminated with this mycotoxin. The results obtained in the present study show the need to establish regulations in Argentina on maximum limits of *Fusarium* mycotoxins in barley and to carry out continuous monitoring to prevent the negative impact on consumers' health.

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Morphophysiological and biochemical responses of *Schedonorus arundinaceus* to Zinc (II) excess: insights from biomarkers and elemental accumulation

Respuestas morfofisiológicas y bioquímicas de *Schedonorus arundinaceus* al exceso de zinc (II): Perspectivas sobre biomarcadores y acumulación elemental

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Abstract

Excessive levels of zinc have detrimental effects on plant physiology and morphology, hindering growth and development. This study aimed to elucidate the morphophysiological and biochemical mechanisms of *Schedonorus arundinaceus* in response to high concentrations of zinc exposure and to investigate the correlation between these parameters to identify potential stress biomarkers in this species. Plants were exposed to seven zinc concentrations (0-500-1000-1500-2000-2500-3000 µM) for 50 days. The results showed decreased dry weight, root area, photosynthetic pigments, root soluble proteins and stomatal conductance with increasing zinc concentrations. Conversely, proline, malondialdehyde and leaf-soluble protein content increased. Histological observations revealed altered stomata size and abnormalities in root tissue. Zinc accumulation exceeded phytotoxic thresholds (100-400 mg kg^{-1}) even at lower concentrations, reaching a maximum of 3432 mg kg^{-1} in shoots. Visible Zn-P crystals were observed on leaf surfaces at the highest zinc treatment. These results suggest that *S. arundinaceus* possesses a notable capacity to bioaccumulate zinc, particularly in the roots. Furthermore, the strong correlation between proline levels and zinc biomass concentration suggests its potential use as a stress biomarker for zinc-induced stress in this species.

Keywords
zinc phytotoxicity • stress adaptations • root damage • physiological response

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Resumen

Los niveles excesivos de zinc afectan negativamente la fisiología y morfología de las plantas, dificultando su crecimiento y desarrollo. Este estudio tuvo como objetivo dilucidar los mecanismos morfofisiológicos y bioquímicos de *Schedonorus arundinaceus* en respuesta a altas concentraciones de zinc y explorar la correlación entre estos parámetros para identificar posibles biomarcadores de estrés. Las plantas fueron expuestas a siete concentraciones de zinc (0-500-1000-1500-2000-2500-3000 µM) durante 50 días. Se observó una disminución del peso seco, área radicular, pigmentos fotosintéticos, proteínas solubles en raíz y conductancia estomática con el incremento de zinc. No obstante, aumentó el contenido de prolina, malondialdehído y proteínas solubles en hojas. Las observaciones histológicas revelaron alteraciones en el tamaño de los estomas y del tejido radicular. La acumulación de zinc superó los umbrales fitotóxicos (100-400 mg kg^{-1}), alcanzando un máximo de 3432 mg kg^{-1} en hojas. Se observaron cristales de Zn-P en la superficie de las hojas en las máximas concentraciones. Estos resultados sugieren que *S. arundinaceus* posee una notable capacidad para bioacumular zinc, particularmente en las raíces. Además, la fuerte correlación observada entre la prolina y la acumulación de zinc sugiere su potencial uso como biomarcador de estrés inducido por el exceso de zinc en esta especie.

Palabras clave
fitotoxicidad por zinc • adaptación al estrés • daño radical • respuestas fisiológicas

Introduction

Recently, heavy metal (HM) contamination has emerged as a pressing environmental issue. These non-biodegradable and toxic substances can accumulate in water bodies, soil, sediments, and the food chain, posing a significant risk to human health (10). The extensive use of mineral fertilizers and pesticides (38), coupled with the release of untreated urban and industrial effluents (55), has contributed to soil and water pollution (2). In Argentina, this environmental concern is particularly important in the "green belts" -peri-urban areas where commercial fresh vegetable production for urban centers is concentrated. The green belts of La Plata, Florencio Varela and Berazategui are especially significant, accounting for 82% of horticultural farms and 81% of cultivated land. These areas are crucial for fresh food production and significantly impact the local economy (32). The land use in the peri-urban area of La Plata is diverse, including residential areas, heavy industry, wastelands, intensive farmland, forests, cattle-raising areas and quarries. With urban planning lagging, this transitional zone faces competition among urbanization, industrialization and horticultural activities, leading to increased pollution and ecosystem degradation (32). Studies on HM contamination, particularly Zn, in these areas are scarce but indicate that Zn can exceed normal or toxic levels due to industrial and motor emissions (7, 13). High concentrations of Zn, Pb, Cr and Cu, often exceeding toxic limits, owe to excessive pesticide and fertilizer use in horticultural peri-urban areas (11, 41). This evidence suggests that intensive agricultural practices and urban-industrial emissions, waste production and disposal could elevate HM levels in these areas, threatening environmental health and food security (21). While certain HMs are essential for plant growth and development, their accumulation at high levels can be phytotoxic (3). Among these, zinc [Zn(II)] plays a crucial role in normal plant development. It activates multiple enzymes, participates in the synthesis and metabolism of important biomolecules and serves as a key component of the transcription factor family known as "zinc fingers", which regulates cell proliferation and differentiation processes (49). However, excessive levels result in structural and functional abnormalities negatively impacting plant performance (46). Symptoms include chlorosis, necrosis, reduced growth due to inhibited cell division and elongation, reduced CO_2 fixation and carbohydrate transport and alterations in cellular membrane permeability (28).

Within the Poaceae family, several species have demonstrated resilience in harsh environments, quickly colonizing contaminated areas (38). *Schedonorus arundinaceus* (=*Festuca arundinacea*), commonly known as tall fescue, is the most widely used temperate perennial grass throughout the Argentina Humid Pampa region (covering most

of Buenos Aires and parts of Santa Fe Cordoba and La Pampa provinces) due to its broad ecological adaptation and naturalization (45). It has been observed to thrive in soils contaminated with various heavy metals (HMs), including Pb, Cd, Cu, Zn, Ni and Cr (1, 39). Our previous studies have shown that this species can grow normally with elevated Zn(II) concentrations, indicating its potential for phytoremediation (23). Investigating the morpho-physiological and biochemical responses of Zn(II) excess on *S. arundinaceus* in this region is crucial, as it could provide an effective solution for mitigating Zn(II) contamination, thereby improving soil quality and ensuring food security in the area. To effectively manage species, it is crucial to investigate not only the uptake rate of HMs but also the underlying mechanisms that regulate stress response (26). Also, establishing correlations between plant absorption kinetics and key physiological and biochemical parameters can provide valuable insights for identifying biomarkers to assess specific plant stress responses (26). The objective of this study was to elucidate the morpho-physiological and biochemical mechanisms of *S. arundinaceus* that are elicited in response to excessive exposure to high Zn(II) concentrations. Furthermore, we aimed to investigate the correlation between these parameters in order to identify potential stress biomarkers for this species. These findings could offer promising insights into the application of *S. arundinaceus* in phytotechnologies such as phytoremediation, biofortification and phytomining.

Materials and methods

Growth conditions and treatments

This experiment was conducted between August and October in a greenhouse located in La Plata City, Buenos Aires, Argentina. *S. arundinaceus* seeds were disinfected with NaClO (10%) for 5 minutes, rinsed with sterile water and placed in plastic plug trays with 72 cells containing a perlite-vermiculite mixture (1:1 v/v). Weekly applications of half-strength Hoagland nutrient solution (25) were provided. After 14 days, the seedlings were transplanted into hydroponic containers filled with a complete Hoagland nutrient solution (pH 6.2 ± 0.5). Following a 15-day acclimatisation period to the hydroponic conditions, increasing concentrations of Zn(II) (provided as $ZnSO_4 7H_2O$) were added to obtain seven treatments: control (1µM of Zn(II) (0.065 mg L^{-1}), equivalent to the Zn(II) concentration in the Hoagland nutrient solution), 500 µM (32.69 mg L^{-1}), 1000 µM (65.38 mg L^{-1}), 1500 µM (98.07 mg L^{-1}), 2000 µM (130.76 mg L^{-1}), 2500 µM (163.45 mg L^{-1}) and 3000 µM (196.14 mg L^{-1}) of Zn(II). These various concentrations were selected based on a previous experiment (22) to screen for responses that might not be discernible at lower concentrations and to determine the maximum tolerance limit for this species. The pH of the solutions was maintained at 6.2 ± 0.5 using 0.1 N NaOH/HCl and aerated using aeration pumps. The solutions were changed every two weeks throughout the experiment. *S. arundinaceus* plants were harvested 50 days after the initial application of the Zn(II) solutions.

Morpho-physiological and biochemical parameters measured

The dry weight (DW) of roots and shoots was determined at harvest by oven-drying at 80°C until constant weight was achieved. Leaf relative water content (LRWC) was calculated based on the measurements of dry weight (DW), fresh weight (FW) and turgid weight (TW) of 1 cm diameter leaf discs (49). Maximum root length (RL) and root area (RA) were calculated using RhizoVision Explorer v2.0.3 (43, 44). Total chlorophyll and carotenoid contents were determined from a 0.5 cm diameter leaf disk; absorbance measurements were taken at 647, 664, and 480 nm (52). Soluble protein content was measured from 0.1 g of fresh leaves and roots; absorbance was read at 595 nm and protein concentration was calculated using a standard curve prepared with different concentrations of bovine serum albumin (BSA) (SiFMa Chemical Co.) (9). Malondialdehyde (MDA) content was measured from 0.2 g of fresh leaves and roots by reaction with thiobarbituric acid (24). Proline content was measured from 0.1 g of fresh leaves and roots; absorbance was read at 520 nm to calculate proline content per unit of fresh weight (4). Absorbance measurements for the aforementioned parameters were determined using a Shimadzu UV-160 spectrophotometer (Kyoto, Japan).

Stomatal density (StD) was calculated through image capture and digitalization using a Gemalux XSZ-H microscope equipped with a Motic camera and Motic Image Plus 2.0 software (15). Stomatal counting was conducted using a Leitz SM lux optical microscope with a clear drawing camera. Adaxial and abaxial stomatal conductance (StC) were determined using a Decagon SC-1 Porometer between 4 p.m. and 6 p.m. on a sunny day, in the middle portion of fully expanded and non-senescent leaves. For histological observations, roots were decolorized with 50% sodium hypochlorite and 5% chloral hydrate. The tissues were stained with an 80% safranin O alcoholic solution and mounted in gelatin-glycerin (14).

Zn(II) tolerance assessment and bioconcentration analysis

Dried root and shoot samples (0.5 g) were ground and digested using a mixture of HNO_3 and H_2SO_4 (4:1 ratio). The absorbance was read using a Shimadzu AA6650F Atomic Absorption Spectrophotometer (Japan) (8). The bioaccumulation factor (BCF), translocation factor (TF) and tolerance index (TI) were calculated using the following equations:

$$\mathbf{BCF = [Zn(II)_{biomass}]/[Zn(II)_{solution}]; TF = [Zn(II)_{shoots}]/[Zn(II)_{roots}]; TI = [(DW)_{treatment}/(DW)_{control}].}$$

Crystalline formations resembling salt crystals were exclusively observed on the leaf surfaces of plants exposed to the highest concentration of Zn(II) (3000 µM). The composition of these crystals was analyzed using energy-dispersive X-ray analysis (EDX) and examined under scanning electron microscopy (SEM).

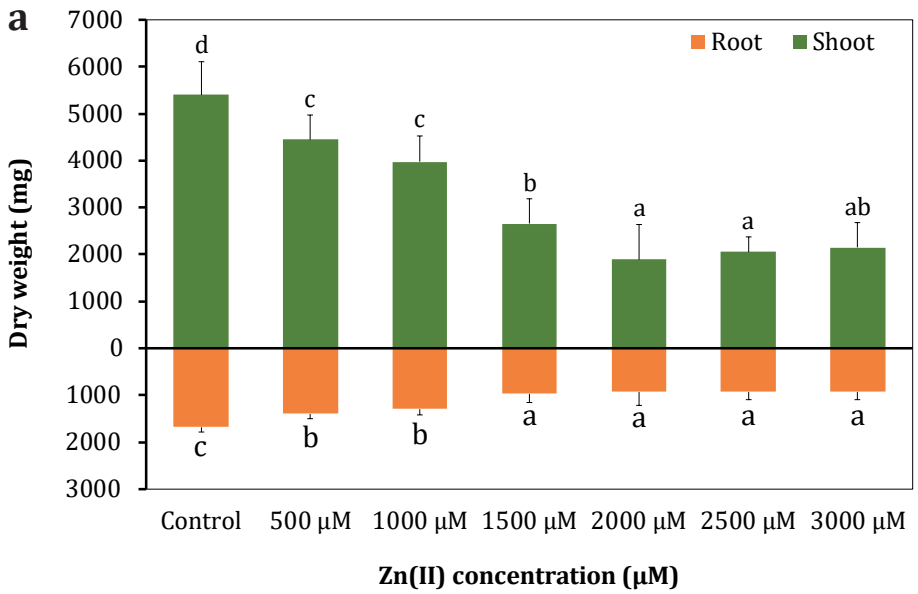
Experimental design and data analysis

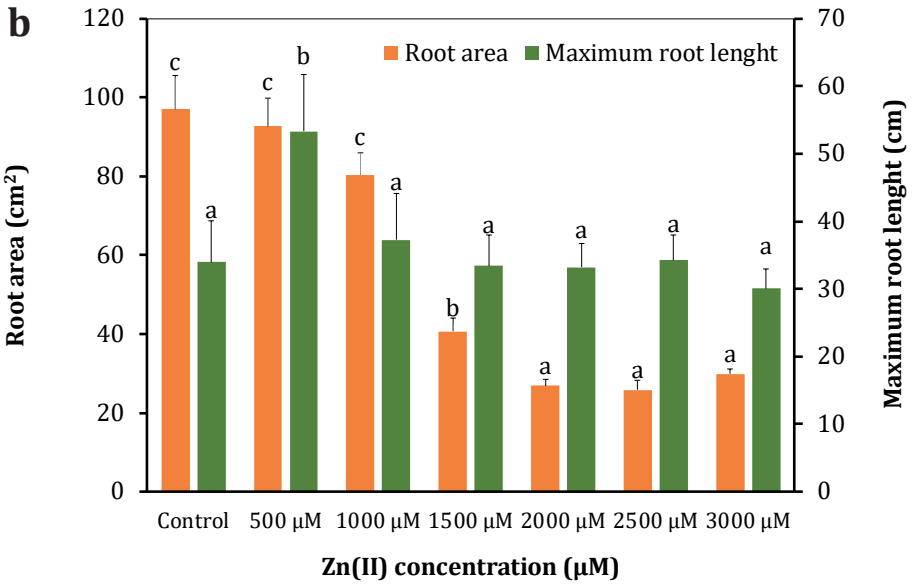
The experimental design was fully randomized, consisting of 7 treatments with 10 replicates per treatment. The following variables were analyzed in all treatments using 10 replicates per treatment: dry weight, LWRC, root length, root area, total chlorophyll and carotenoids, soluble protein, MDA, proline, Zn content, bioaccumulation and translocation factors and tolerance index. The following variables were analyzed only in the control and maximum Zn concentration (3000 µM) using 10 replicates per treatment: stomatal density and conductance and histological observations. The data were analyzed using analysis of variance (ANOVA), and means were compared using the least significant difference (LSD) test at a significance level of 5% ($p<0.05$) (16). Pearson correlations, involving 24 variables, were determined using R version 4.3.1 software. All results were expressed as mean values with corresponding standard deviations.

Results

Morpho-physiological and biochemical parameters

The DW significantly decreased as the concentrations of Zn(II) increased, with shoots and roots experiencing a reduction of 60% and 45% respectively, at the maximum concentration (figure 1a, page 38). The RA also showed a significant decrease starting from 1000 µM, while the RL exhibited a significant increase at lower concentrations (figure 1b, page 38) but remained unchanged at higher concentrations, indicating a potential reduction in secondary root formation. The LRWC remained stable, suggesting a relatively consistent water status in response to Zn(II) exposure (table 1, page 39). Total chlorophyll and carotenoid contents decreased with increasing Zn(II) concentrations, reaching values 80% and 74% lower than the control at the highest concentration (table 1, page 39). Significant changes were also observed in soluble protein content. Leaves exhibited a slight increase, being 24% higher under 3000 µM compared to the control, while roots showed a decrease of 40% under the same concentration (table 1, page 39). The levels of MDA increased in leaves under high Zn(II) stress, reaching values three times higher than the control at 3000 µM. In contrast, root MDA levels remained lower and showed no significant differences among treatments (figure 2a, page 39). Proline showed a significant increase in both leaves and roots under Zn(II) concentrations of 1500 µM and higher, reaching values in shoots that were 162 times higher than the control, while in roots, it was 13 times higher (figure 2b, page 39). These findings could indicate the activation of stress-related mechanisms in response to Zn(II) excess, as proline is believed to function as a scavenger of reactive oxygen species (ROS) or as an osmolyte, aiding in the defense against oxidative stress.





Columns represent mean values of 10 replications, and vertical bars show standard deviation. Columns sharing different letters indicate significant differences ($p < 0.05$).

Las columnas representan los valores medios de 10 repeticiones y las barras verticales muestran la desviación estándar. Las columnas que comparten letras diferentes indican diferencias significativas ($p < 0,05$).

Figure 1. Effect of Zn(II) concentrations on the dry weight (a), root area and maximum root length (b) of *S. arundinaceus*.

Figura 1. Efecto de las concentraciones de Zn(II) sobre el peso seco (a), el área y la longitud radical máxima (b) de *S. arundinaceus*.

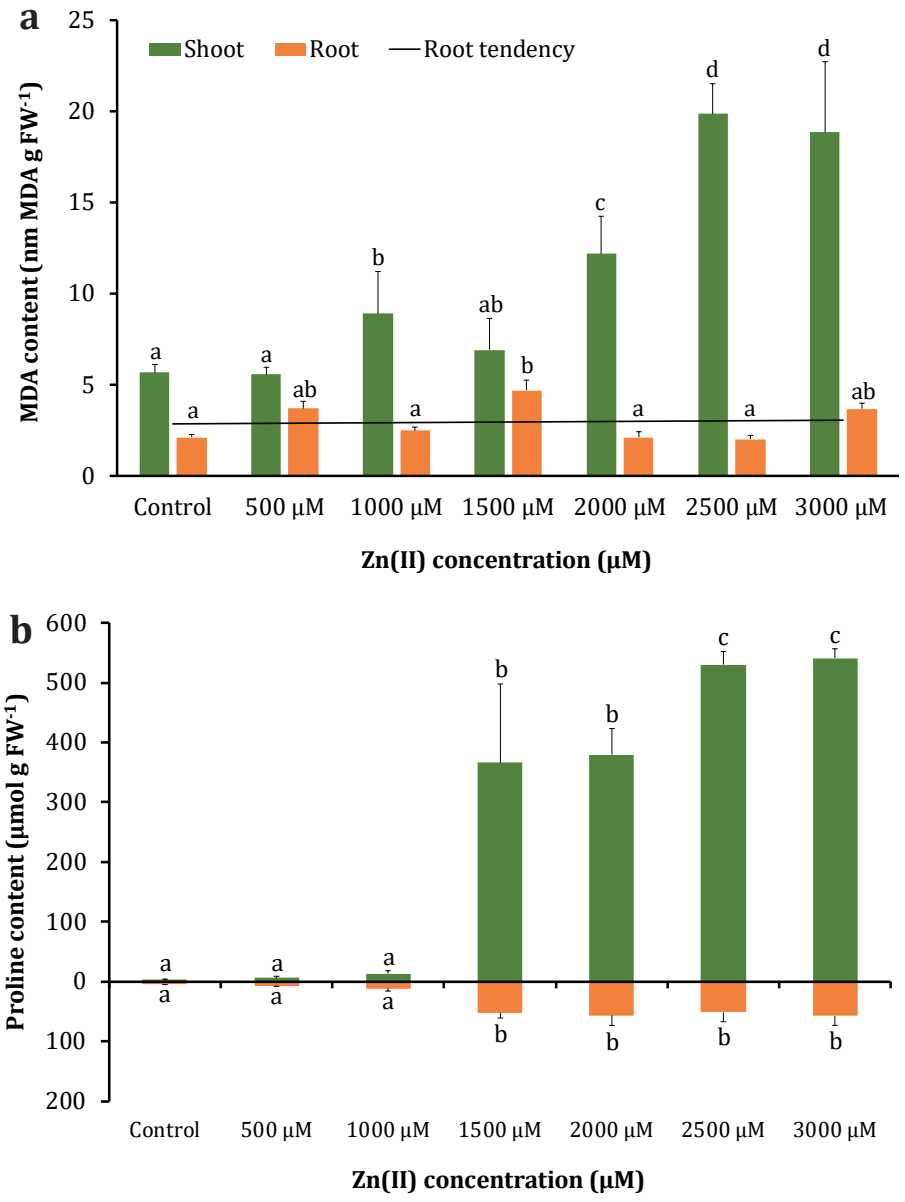
Table 1. Effect of Zn(II) concentrations on physiological and biochemical parameters of *S. arundinaceus*.

Tabla 1. Efecto de las concentraciones de Zn(II) sobre los parámetros fisiológicos y bioquímicos de *S. arundinaceus*.

Data given in the table are means of 10 replications ± standard deviation. For each parameter, data followed by different letters indicate significant differences ($p < 0.05$).

Los datos indicados en la tabla son la media de 10 réplicas ± desviación estándar. Para cada parámetro, los datos seguidos de letras diferentes indican diferencias significativas ($p < 0,05$).

Zn (II) treatment	Leaf water relative content (%)	Total chlorophyll (µg cm^{-2})	Carotenoid (µg cm^{-2})	Shoot proteins (µg mg^{-1} FW)	Root proteins (µg mg^{-1} FW)
Control	80% ± 6% ab	12.88 ± 1.42 d	1.89 ± 0.22 c	5.72 ± 1.5 ab	2.83 ± 0.15 cd
500 µM	80% ± 3% b	10.44 ± 3.13 cd	1.81 ± 0.35 c	4.51 ± 0.63 a	3.19 ± 0.16 d
1000 µM	85% ± 2% b	9.71 ± 2 c	1.32 ± 0.18 c	5.93 ± 1.39 ab	2.71 ± 0.31 c
1500 µM	84% ± 3% b	7.09 ± 1.39 b	1.36 ± 0.15 b	6.13 ± 0.74 bc	2.66 ± 0.15 bc
2000 µM	80% ± 3% ab	6.57 ± 2.04 b	1.06 ± 0.28 b	6.63 ± 0.98 bc	2.26 ± 0.21 b
2500 µM	85% ± 2% b	3.76 ± 0.17 a	0.68 ± 0.04 a	6.51 ± 0.59 bc	1.42 ± 0.2 a
3000 µM	74% ± 2% a	2.58 ± 0.18 a	0.5 ± 0.13 a	7.59 ± 0.72 c	1.68 ± 0.53 a



Columns represent mean values of 10 replications, and vertical bars show standard deviation. Columns sharing different letters indicate significant differences ($p < 0.05$).

Las columnas representan los valores medios de 10 repeticiones y las barras verticales muestran la desviación estándar. Las columnas que comparten letras diferentes indican diferencias significativas ($p < 0,05$).

Figure 2. Effect of Zn(II) concentrations on *S. arundinaceus* MDA (a) and proline (b) contents.

Figura 2. Efecto de las concentraciones de Zn(II) en los contenidos de MDA (a) y prolina (b) de *S. arundinaceus*.

Significant differences were observed in adaxial and abaxial stomatal density (StD), conductance (StC), length (StL) and width (StW) exclusively between the control and 3000 µM treatments. Hence, the subsequent results will focus solely on these specific treatments. Both adaxial and abaxial StC decreased, reaching values 2 and 4 times lower than the control under 3000 µM. Adaxial StD was significantly higher, while abaxial StD exhibited the opposite trend. However, total StD did not vary significantly. Although there were no significant differences in adaxial and abaxial StL, a noticeable decrease in abaxial StW was observed (table 2). This suggests that under higher Zn(II) concentrations, smaller and rounder stomata were present. Also, neither non-glandular trichomes nor salt glands were observed on the leaves of plants from all treatments. In terms of root anatomy, the transversal section analysis of control roots revealed a concentric cortical parenchyma composed of orderly to slightly disordered cells, with a vascular cylinder surrounded by endodermis (figure 3a). In contrast, roots exposed to 3000 µM exhibited morphological changes, including protuberances (figure 3b), dorsoventral compression of cortical parenchyma, endodermis and vascular bundles as well as signs of disintegration and medullary proliferation (figure 3c).

Data given in the table are the mean of 10 replications ± standard deviation. For each trait, data followed by different letters indicate significant differences ($p < 0.05$).

Los datos indicados en la tabla son la media de 10 repeticiones ± desviación estándar. Para cada parámetro, los datos seguidos de letras diferentes indican diferencias significativas ($p < 0,05$).

Table 2. Adaxial and abaxial stomatal density (StD), conductance (StC), length (StL), and width (StW) of *S. arundinaceus*.

Tabla 2. Densidad estomática adaxial y abaxial (StD), conductancia (StC), longitud (StL) y latitud (StW) de *S. arundinaceus*.

Zn(II) treatment	Adaxial StC (mmol m^{-2} s^{-1})	Abaxial StC (mmol m^{-2} s^{-1})	Adaxial StD (mm^{-2})	Abaxial StD (mm^{-2})	Adaxial StL (µm)	Adaxial StW (µm)	Abaxial StL (µm)	Abaxial StW (µm)
Control	190 ± 11 b	61 ± 2 a	53 ± 9 a	133 ± 8 b	55 ± 5 a	17 ± 2 a	55 ± 8 a	17 ±1 b
3000 µM	94 ± 24 a	15 ± 4 b	93 ± 8 b	97 ± 7 a	48 ± 4 a	19 ± 2 a	44 ± 7 a	12 ± 2 a

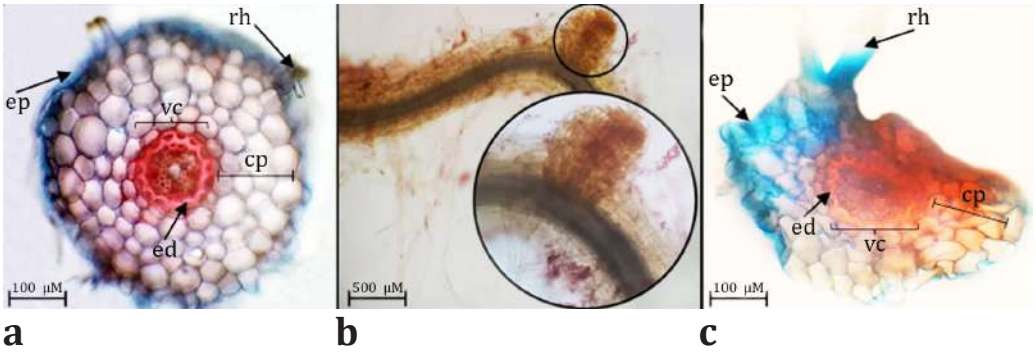


Figure 3. Control roots transversal sections (a), root protuberances observed under 3000 µM (b) and 3000 µM treated roots transversal sections (c) (ep: epidermis, rh: root hair, cp: cortical parenchyma, vc: vascular cylinder, ed: endodermis).

Figura 3. Secciones transversales de las raíces del control (a), protuberancias radiculares observadas bajo 3000 µM (b) y secciones transversales de raíces tratadas con 3000 µM (c) (ep: epidermis, rh: pelo radical, cp: parénquima cortical, vc: cilindro vascular, ed: endodermis).

Zn(II) tolerance assessment and bioaccumulation analysis

S. arundinaceus exhibited significant accumulation of Zn(II), with levels reaching 50 times higher than the control under the 3000 µM treatment, but the highest biomass concentration (7244 mg kg^{-1} DW) was observed under the 2500 µM treatment. Zn(II) distribution showed that roots accumulated more Zn(II) than shoots in the lower concentrations, but this difference became three times smaller after reaching the 1500 µM concentration (figure 4).

BCF values were only higher than 1000 under the 500 µM and 1000 µM treatments, while the TF values remained below 1 in all treatments. The TI exhibited a decrease from 81% to 43% as Zn(II) concentration increased from 500 µM to 3000 µM, respectively (table 3). Crystalline formations resembling salt crystals were exclusively observed on the leaf surfaces of plants exposed to 3000 µM. SEM analysis revealed distinct crystalline structures on the leaf surfaces and EDX analysis confirmed the predominant composition of Zn(II) and P (figure 5, page 42).

Correlation analysis

Columns sharing different letters indicate significant differences ($p < 0.05$).

Columns represent mean values of 10 replications, and vertical bars show standard deviation.

Las columnas que comparten letras diferentes indican diferencias significativas ($p < 0,05$).

Las columnas representan los valores medios de 10 repeticiones y las barras verticales muestran la desviación estándar.

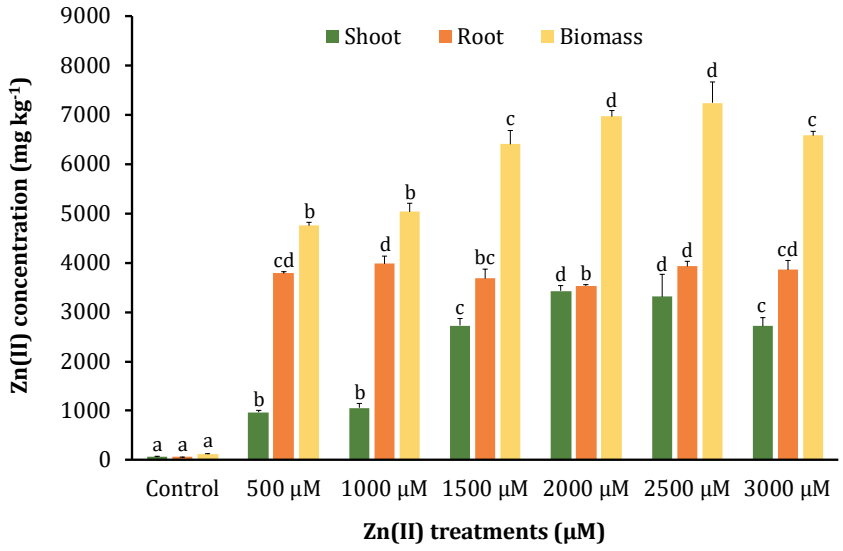


Figure 4. Zn(II) accumulation in shoots, roots, and biomass of *S. arundinaceus*.

Figura 4. Acumulación de Zn(II) en hojas, raíces y biomasa de *S. arundinaceus*.

Table 3. Bioconcentration factor (BCF), translocation factor (TF) and tolerance index (TI).

Tabla 3. Factor de bioconcentración (BCF), factor de translocación (TF) e índice de tolerancia (TI).

Data given in the table are mean of 10 replications ± standard deviation. For each trait, data followed by different letters indicate significant differences ($p < 0.05$).

Los datos que figuran en la tabla son la media de 10 repeticiones ± desviación estándar. Para cada rasgo, los datos seguidos de letras diferentes indican diferencias significativas ($p < 0,05$).

Zn (II) treatment	BCF	TF	TI (%)
Control	247 ± 9 a	0.98 ± 0.09 c	-
500 µM	3650 ± 51 e	0.26 ± 0.01 a	81% ± 14% b
1000 µM	2701 ± 95 d	0.27 ± 0.03 a	73% ± 15% b
1500 µM	415 ± 17 c	0.74 ± 0.04 b	51% ± 12% a
2000 µM	344 ± 5 b	0.97 ± 0.03 c	46% ± 15% a
2500 µM	325 ± 19 b	0.85 ± 0.12 bc	43% ± 15% a
3000 µM	237 ± 3 a	0.71 ± 0.07 b	43% ± 13% a

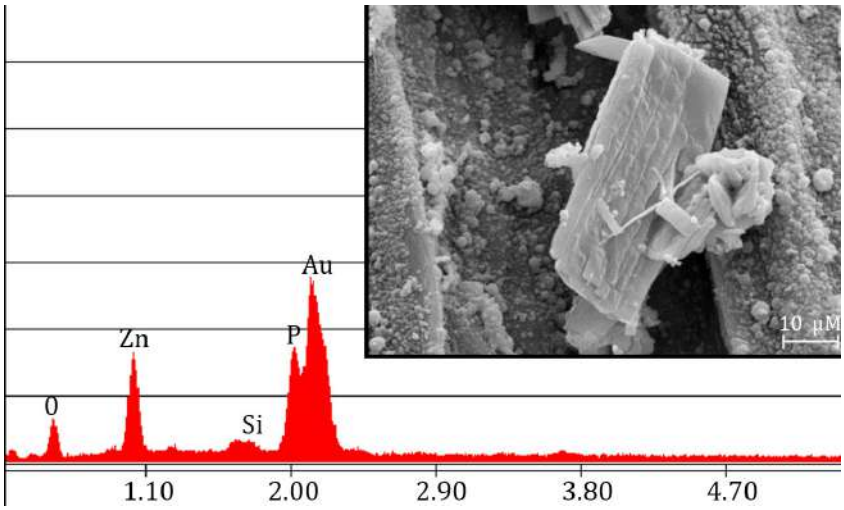
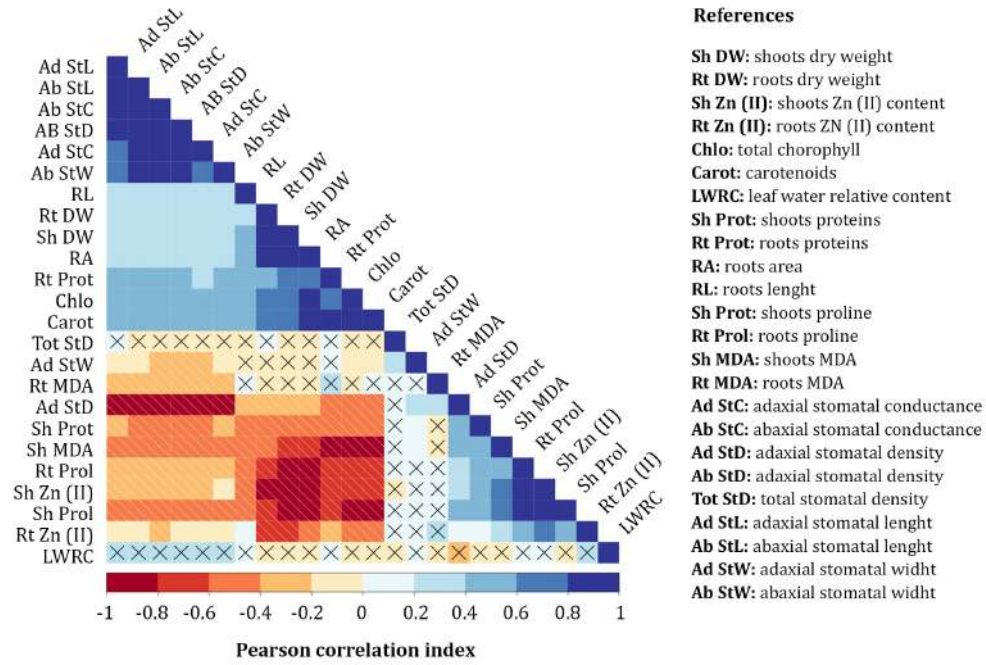


Figure 5. EDX spectra and scanning electron microscopy (SEM) image of the crystals observed on *S. arundinaceus* leaf surfaces exposed to 3000 µM.

Figura 5. Análisis EDX e imagen de microscopía electrónica de barrido (SEM) de los cristales observados en las superficies de las hojas de *S. arundinaceus* expuestas a 3000 µM.

Higher correlations were observed between Zn(II) concentrations in leaves and the examined parameters compared to Zn(II) concentrations in roots. The results revealed highly significant positive correlations between shoot Zn(II) content and MDA levels in roots, as well as proline content in shoots and roots. Conversely, shoot and root DW, photosynthetic pigments content, shoot phenolic compounds content and RA exhibited high negative correlations. Root Zn(II) content showed significantly high positive correlations with shoot and root proline content and negative correlations with shoot and root dry weight, shoot phenolics content and RA (figure 6).

Proline content demonstrated a potential association with a stress threshold



Crossed correlations indicate non-significant correlation (p >0.05%).

Las correlaciones con cruces indican correlaciones no significativas (p >0,05%).

Figure 6. Pearson correlation matrix of various morpho-physiological and biochemical parameters of *S. arundinaceus* exposed to Zn(II) stress.

Figura 6. Matriz de correlación de Pearson de varios parámetros morfofisiológicos y bioquímicos de *S. arundinaceus* expuestos a estrés por Zn(II).

bioconcentration of Zn(II) in *S. arundinaceus*. Under the 1500 µM treatment, proline content increased by 27 times in shoots and 5 times in roots compared to the preceding treatment. This significant increase was specific to proline, while other biochemical and physiological parameters displayed gradual changes with increasing Zn(II) concentrations. At 1500 µM, *S. arundinaceus* bioaccumulated 6408 mg kg^{-1} DW, which could be considered a stress threshold limit, as various growth, physiological and biochemical parameters displayed significant alterations beyond this point, indicating a higher stress condition. Furthermore, after this treatment, the shoot concentration of Zn(II) reached similar levels to that of the roots, suggesting that defensive mechanisms were incapable of preventing excessive translocation of Zn(II) to the shoots.

Discussion

S. arundinaceus bioaccumulated high concentrations Zn(II), with a maximum of 7244 ± 424 (SD) mg kg^{-1} DW at 2500 µM Zn(II). Shoot Zn(II) concentrations (970-3432 mg kg^{-1}) exceeded phytotoxic levels, even at lower treatments, as shoot concentrations above 100-400 mg kg^{-1} DW are considered phytotoxic (42). Similar findings were reported in 5-month-old *S. arundinaceus* plants in the vegetative stage, reaching Zn(II) values of 432 and 1099 mg kg^{-1} in shoots and roots, respectively (54). This difference in accumulation observed in comparison to our study could be related to the different substrates (Haplargids soil) or genotypes used in this experiment. Concerning this, other research reported higher concentrations in shoots and roots, reaching up to 6000 and 9000 mg kg^{-1} DW respectively, indicating the presence of a possible ecotype of *S. arundinaceus* and variations in Zn(II) tolerance within the same species (12).

Our study suggests that *S. arundinaceus* acted as an accumulator and phytostabilizer species, exhibiting BCF values exceeding 1000 at lower concentrations but TF values never surpassing 1. After 1500 µM, the distribution pattern of Zn(II) changed significantly, approaching TF values closer to 1. Crystalline formations were observed on the leaves of plants treated with 3000 µM Zn(II), suggesting a potential excretion mechanism. However, it has not been reported that *S. arundinaceus* produce visible Zn(II) crystals through leaf excretion. A study found that *S. arundinaceus* excreted Cd through guttation fluid (18). Some halophyte grasses have salt glands or glandular trichomes, which they use to excrete salts in large quantities, which can also excrete HMs (53). However, *S. arundinaceus* exhibited non-glandular trichomes and lacked salt glands.

We hypothesized that high Zn(II) concentrations caused crystal formation through guttation fluid with Zn-P salts, serving as a tolerance mechanism. This is also correlated with the decrease in shoots Zn(II) content observed between the 2500 µM and 3000 µM treatments. However, further investigation is needed to understand the formation and implications of these Zn-P crystals in Zn(II) tolerance.

Excessive levels of Zn(II) can have negative effects on plant physiology and morphology, including inhibition of cell division and elongation, increased production of ROS and decreased photosynthesis, nutrient uptake and water absorption (28). Our results showed a decrease in general DW, as well as RA reduction with increasing Zn(II) treatments. Moreover, TI values indicated that Zn(II) inhibited growth, reaching values below 60% at 1000 µM and higher concentrations. Higher TI values suggest plant tolerance to HMs without significant growth inhibition (48). Additionally, morphological and anatomical abnormalities, such as small protuberances, were observed in roots treated with 3000 µM Zn(II). It was found that Zn(II)-treated *Brassica napus* root epidermal cells exhibited distortion, smaller size, shrinkage and irregular alignment, which was associated with growth inhibition, reduced nutrient absorption and damage to the root apex (30).

Accumulation of HMs in root cell walls diminishes their elasticity, leading to alterations in water uptake. However, our experiment did not show significant differences in LRWC, suggesting that the observed morphological changes were insufficient to affect root water uptake. Similar findings have been reported for *Psidium guajava* exposed to high Ni levels, where enhanced vacuole volume was proposed as a contributing factor (6). Furthermore, a decrease in stomatal conductance and the presence of smaller stomata were observed

at 3000 µM Zn(II), as reported in *Citrus reticulata* (47), suggesting that these alterations may represent an adaptive response aimed at mitigating excessive water loss. Zn(II) excess leads to a deficiency in carbonic anhydrase, which affects HCO^{3-} concentration in the guard cells and K^{+} uptake, consequently resulting in alterations in guard cell morphology and stomatal shape (37). *S. arundinaceus* exhibited a reduction in photosynthetic pigments content. Similar results were reported in other plant species exposed to high Zn(II) concentrations (33). This decrease can be attributed to the inhibition of chlorophyll synthesis caused by Zn(II)-induced deficiencies of Mg(II) and Fe(II) (29).

Excessive Zn(II) bioaccumulation triggers ROS overproduction, leading to oxidative stress and membrane damage (51). Our study revealed an increase in shoot MDA levels, while root levels did not show significant differences. This finding is consistent with the observations in other plant species, exposed to high Zn(II) concentrations (17, 51). ROS overproduction disrupts the integrity of chloroplast membranes and impairs photosynthetic performance (50), which is consistent with the negative correlation observed between shoot MDA content and photosynthetic pigment levels in our experiment. Contrarily, root MDA levels suggest the activation of antioxidant defense mechanisms, indicating the ability of the root system to mitigate ROS damage (27).

The accumulation of organic osmolytes, including total soluble proteins and proline, serves as crucial indicator of stress adaptation in plants. Shoots soluble protein content of *S. arundinaceus* increased with higher Zn(II) doses, while the opposite was observed in the roots. Similar findings were reported in *T. aestivum*, where HM stress led to a significant increase in shoot-soluble protein content compared to roots (35). This could be attributed to the synthesis of stress proteins or chelators, such as glutathione, phytochelatins, metallothioneins, proline or histidine, which aid in stress tolerance and HM detoxification through compartmentalization in vacuoles (19). Reduction in protein content under Zn(II) stress has also been attributed to the increased activity of proteases or catabolic enzymes induced by HM stress (28). Additionally, in our experiment, a significant increase in proline content was observed in shoots and roots at concentrations of 1500 µM and higher.

Similarly, a higher increase in proline content in the shoots of *Solanum lycopersicum* compared to the roots under Zn(II) stress was reported (4). Proline plays a significant role in osmoregulation, osmoprotection and ROS detoxification to maintain cellular homeostasis (31, 47). Additionally, it acts as a chemical chaperone and a biomembrane protector against oxidative damage, thereby stabilizing protein structure (36). Thus, proline accumulation serves as a strategy for plants to defend against oxidative stress (40). However, the positive correlation observed between shoot MDA and proline contents in *S. arundinaceus* suggests that proline increase may not fully compensate for oxidative stress. Furthermore, findings indicated that the extent of proline accumulation and its effectiveness as an osmotic adjuster are species/cultivar specific and depend on the severity and duration of the stress (34). Biomarkers have emerged as valuable tools for environmental analysis and phytoremediation programs, complementing traditional soil chemical analysis (28). Proline content demonstrated promising characteristics as a potential biomarker for Zn(II) stress in *S. arundinaceus,* as it exhibited a strong correlation with Zn(II) bioaccumulation. Also, proline determination is a cost-effective, simple and non-destructive method that can be employed at different stages of the analysis process. However, proline levels can also be influenced by other types of abiotic stresses, such as salinity and drought, resulting in varying responses depending on the plant species (20).

Conclusion

High Zn(II) concentrations affected growth, biochemical and morpho-physiological parameters of *S. arundinaceus* (principally the dry weight, photosynthetic pigments, soluble proteins, MDA and proline content). However, this species exhibited a remarkable ability to bioaccumulate high levels of Zn(II) (3432 mg kg^{-1} in shoots), surpassing phytotoxic thresholds (100-400 mg kg^{-1}) by approximately 9 times and activating stress tolerance mechanisms such as the accumulation of proline and proteins, which could act as ROS inhibitors and HM chelators. Morphological adaptations, such as smaller stomata, played a role in maintaining a stable water content. *S. arundinaceus* performed better under 1000 and 1500 µM Zn(II),

demonstrating high biomass production and Zn(II) bioaccumulation. Proline holds potential as a possible biomarker for monitoring the status of *S. arundinaceus* in response to Zn(II) stress. However, further studies are necessary to determine if similar responses occur with other HMs in the same species.

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Different scenarios in land suitability assessment for Kernza®-intermediate wheatgrass (*Thinopyrum intermedium*), a novel perennial grain crop for Argentina

Diferentes escenarios para evaluación de la aptitud de la tierra para Kernza®-intermediate wheatgrass (*Thinopyrum intermedium*), un nuevo cultivo de granos perenne para Argentina

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Abstract

Land degradation, climate change, soil and water contamination have led to increased interest in sustainable agricultural practices. Most agricultural practices are focused on growing annual crops, which require significant amounts of synthetic fertilizers, contribute to CO_2 emissions and disrupt natural biological processes. Natural Systems Agriculture has been developed to reverse this paradigm by imitating nature through perennial grain crops. Kernza® intermediate wheatgrass *(Thinopyrum intermedium)* is a promising perennial crop producing healthy grain for direct human consumption and forage for livestock while providing multiple ecosystemic services. Given these reasons, consider its cultivation in Argentina is relevant. This research aimed to predict Kernza crop suitability in the Azul district by modeling different climatic and soil densification scenarios. The model showed that Kernza can be grown in Azul, and that southern areas were most suitable. This model allowed generating information for land use planners and farmers to consider planting in Argentina, particularly, in Azul.

Keywords
land evaluation • climate scenarios • land degradation • perennial crops

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Resumen

La degradación del suelo, el cambio climático y la contaminación del suelo y el agua han suscitado un mayor interés por una agricultura más sostenible. La mayoría de las prácticas agrícolas se centran en cultivos anuales, que requieren grandes cantidades de fertilizantes sintéticos, contribuyen a un aumento en las emisiones de CO_2 y perturban los procesos biológicos naturales. El "Natural Systems Agriculture" se ha desarrollado con el objetivo de revertir este paradigma mediante la imitación de la naturaleza a través de cultivos de granos perennes. El Kernza® intermediate wheatgrass *(Thinopyrum intermedium)* es un cultivo perenne muy prometedor porque produce grano para consumo humano, forraje para el ganado y proporciona múltiples servicios ecosistémicos. Por ello, es relevante considerar su cultivo en Argentina. El objetivo fue predecir la aptitud de Kernza mediante la modelización de diferentes escenarios climáticos y de densificación del suelo en el partido de Azul, Argentina. El modelo demostró que el Kernza puede ser cultivado en Azul, siendo las zonas del sur las más aptas. El Kernza es un cultivo muy prometedor y este modelo permitió generar información para que los planificadores del uso de la tierra y los agricultores consideren su plantación en Azul, y en Argentina.

Palabras claves
evaluación de tierras • escenarios climáticos • degradación de tierras • cultivos de granos perennes

Introduction

Land degradation, climate change, soil and water contamination have led to increased interest in sustainable agricultural practices (4, 23, 35, 54). Despite this, most agricultural practices are still focused on growing annual crops, which require significant amounts of synthetic fertilizers, labour, contribute to emissions of CO_2 and disrupt natural biological processes (2, 10). This reduces the current and potential capacity to produce goods and services, both qualitatively and quantitatively (19, 20, 21). Additionally, this causes an increase in the energy necessary to produce environmental and economic liabilities (56). In 1980, Wes Jackson published the book New Roots for Agriculture (32) to reverse this paradigm, developing the concept of Natural Systems Agriculture (NSA). In this perennial food-grain-producing system, soil erosion and agrochemical contamination decrease as fossil fuel dependency decreases (33). Its objective was to mimic nature using perennial grain crops. Unlike annuals, perennials improve soil structure and water retention capacity, contribute to climate change adaptation and mitigation, and promote biodiversity and ecosystemic functions (2, 12, 25). Additionally, they improve rural economies by reducing external inputs (*i. e.*, reducing dependence on fossil fuels and agrochemicals) and labour intensity (11, 12, 44).

Kernza® is the trade name of the intermediate wheatgrass [*Thinopyrum intermedium* (Host) Barkworth and D.R. Dewey], a novel perennial grain crop recently becoming commercially available in the USA (15, 41). Kernza´s deep root system reduces nutrient leaching while increasing water use efficiency and soil carbon content (10, 14, 25). In addition, Kernza provides a grain suitable for direct human consumption, forage for livestock, and multiple ecosystemic services for enhanced environmental quality (12, 24, 28, 49, 52).

Territory is used for different purposes, occasionally complementary but mostly conflicting (*i.e.*, they cannot be located simultaneously in the same area). For these reasons, land-use planning plays a major role in considering exploitation of natural resources; assessing requirements and land capacity, identifying and resolving conflicts among competing uses and seeking long-term sustainable solutions (19, 20, 31). Land evaluation assesses land suitability for specific purposes, constituting an integral part of land-use planning, providing information for decision-making by land-use planners (19, 20, 40). Land evaluation involves the execution and interpretation of basic studies of climate, soil, vegetation, and any aspect regarding land-use requirements (19). Several methodologies aid the development of land evaluation systems, including modeling, such as expert systems.

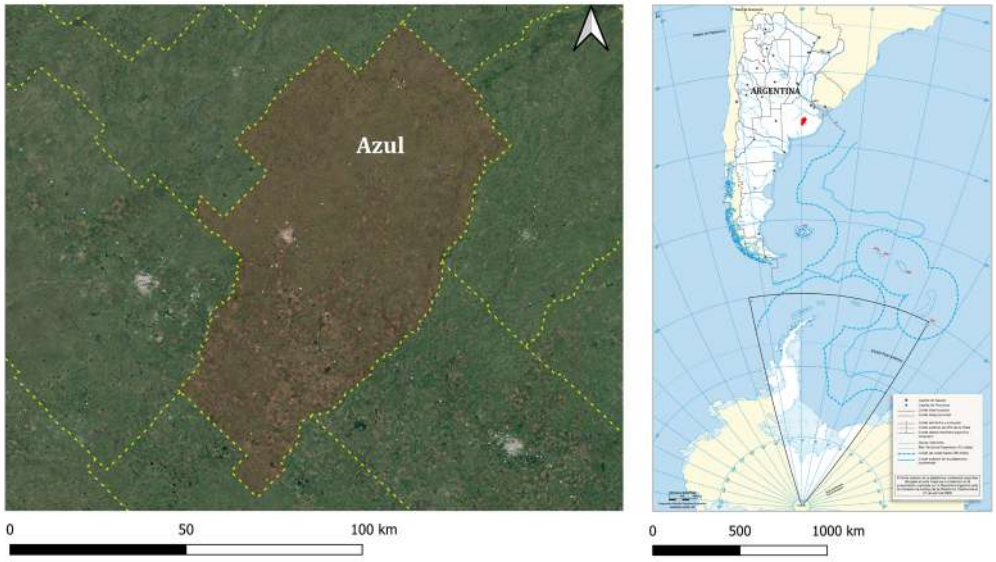
Models allow predicting outcomes under real conditions and generate new hypothetical outcomes in scenarios of change, such as different climate or soil densification scenarios. These hypothetical outcomes facilitate management and adaptation measures to future changes (31, 42, 50, 51).

While perennial grain crops are not widely cultivated worldwide (8, 36) the various agro-ecological benefits they potentially provide make them strong candidates for cultivation in Argentina. This study aimed to predict Kernza crop suitability in Azul district by modelling different climatic and soil densification scenarios in Azul district, Argentina.

Materials and methods

Study area

Azul district is located in the centre of Buenos Aires province, Argentina (36°14' S - 37°27' S and 59°8' W - 60°10' W) in the Pampa region, with an area of 6,551 km^2 (figure 1) and 70 545 inhabitants. It is divided into two large areas: southern Pampa in the south and flooding Pampa in the north (46).



(Landsat/Copernicus image. ©Google, 2022).

Figure 1. Location of Azul district in Argentina.
Figura 1. Ubicación del partido de Azul en la Argentina.

According to the Köppen classification (34), Azul has a humid temperate climate (Cfb) with oceanic influence, hot summers, and precipitations evenly distributed throughout the year (7, 43, 53). Mean annual rainfall is 921 mm (1931-2017 series). Minimum annual rainfall recorded in 1935 was 590 mm, and maximum annual rainfall in 2012 was 1449 mm. In addition, different precipitation periodicities were observed in intervals of 12 and 2.5 years (6, 53). Azul mean annual temperature is 14, 2°C (1997-2018 series). January is the hottest month with an average temperature of 21, 8°C, while August is the coldest with an average of 7 °C. Mean annual potential evapotranspiration is 752 mm. December, January and February present the higher atmospheric demand.

Soils are Argiudolls, Hapludolls, Natraquolls and Natraqualfs (30, 46, 47). Land uses are agriculture, livestock, and crop-livestock systems (57). Soybean (*Glycine max*), corn (*Zea mays*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and sunflower (*Helianthus annuus*) are the major crops. Livestock plays an important role in the district, especially in the Pampa Deprimida area (39).

Kernza crop suitability modelling

The ALES (Automated Land Evaluation System) v4.65e software (50, 51) was modelled under the FAO Land Evaluation Framework (19, 20, 21). Model inputs were Kernza requirements (table 1), soil characteristics (table 2, page 52-53), and climate characteristics (table 3, page 53). Suitability was determined by comparing Kernza crop requirements with land qualities (table 4, page 54) selected through decision trees (Supplemental data 2). The model was based on Kernza land utilization involving grain and forage production without irrigation for four years. Farming techniques include direct drilling, fertilization with nitrogen and phosphorous, phytosanitary applications and mechanized harvesting. The crop is seeded in March, with grain harvesting in January, followed by two forage harvests, one after grain harvest and another in April or May.

Table 1. Kernza land use requirements.

Tabla 1. Requerimientos del uso de la tierra para Kernza.

Requirements	Kernza	References
Seeding date	March	(41)
	May	(Locatelli, 2020*)
Vernalisation	Yes	(37, 41)
Soil moisture retention	Coarse textured soils with low water retention capacity particularly in areas with abundant rainfall, given root exploration capacity	(41)
PET, reproductive period	328 mm (October to January)	(16)
PET, vegetative period	349 mm (February to September)	(16)
Seeding density	11 a 13 kg·ha^{-1} of seeds	(16)
	16.81 kg·ha^{-1} of seeds	(45)
	13 kg·ha^{-1} or 130 seeds·m^{2} at 0.15 m away or 19.7 seeds·m^{-1} 36 seeds·m^{-2} to 145 seeds·m^{-2}	(14, 16, 22)
	Achieve a stand of 20 live plants·m^{-2}	(Locatelli, 2020*)
Seeding deep	1.2 to 2.5 cm	(16)
Row spacing	75 cm	(16)
	15 cm	(14)
	ñ30 cm to 60 cm	(28)
Fertilization	Nitrogen: 110 kg·ha^{-1} before seeding (1st year), 100 kg·ha^{-1} (2nd year), 90 kg·ha^{-1} (3rd year) then decreasing to 80 kg·ha^{-1} (4th year); Phosphorus: before seeding 10 to 20 ppm	(16)
	Nitrogen: Applied the first year during seeding 36 kg·ha^{-1}. Post-harvest and early spring 36 kg·ha^{-1}. Phosphorus: in the first year monoammonium phosphate (map, 52% P_2O_5) 67 kg·ha^{-1}	(48)
	Nitrogen: first year at seeding 50 kg·ha^{-1}, then at spring 50 kg·ha^{-1}	(14)
	Phosphorus: monoammonium phosphate (map, 52% P_2O_5) 15 kg·ha^{-1}	(48)

Most references concerning the northern hemisphere have been adapted for the southern hemisphere.

* Andrés Locatelli (Universidad de la República, Uruguay), personal communication, 2020.

La bibliografía consultada corresponde al hemisferio norte y fue adaptada al hemisferio sur.

* Andrés Locatelli (Universidad de la República, Uruguay), comunicación personal, 2020.

Requirements	Kernza	References
Grain harvest	January, with yield varying from 280.21 kg·ha^{-1} to 1500 kg·ha^{-1}. First-year yields are the highest. Then yields decline until the fourth year when it should be reseeded	(14, 16, 48)
Forage harvest	A mechanical cut 10 cm above the ground is made either once in April or immediately after grain harvest and in April	(48)
Flooding	Does not tolerate excessive moisture	(41)
Crop cycles	It has three stages: early vegetative, from regrowth after harvest until before the onset of colder temperatures (February to April); late vegetative, comprising the period of stem elongation just before flowering (May to September); reproductive stage, from flowering until harvest (November to January)	(48)

Most references concerning the northern hemisphere have been adapted for the southern hemisphere.

* Andrés Locatelli (Universidad de la República, Uruguay), personal communication, 2020.

La bibliografía consultada corresponde al hemisferio norte y fue adaptada al hemisferio sur.

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Table 2. Selected soil characteristics, classes and ranks.

Tabla 2. Características de los suelos seleccionadas, sus clases y rangos.

Code	Soil characteristics	Classes and ranks	
Prof_Ef	Soil deep (m)	0.15 - 0.30	Very slight (MuyPocoP)
		0.31 - 0.60	Slight (PocoP)
		0.61 - 0.90	Moderate (ModP)
		0.91 - 1.20	High (P)
		< 1.21	Very high (MuyP)
Agua_Util	Available water (mm/m)	0 - 50	Low (MB)
		50.1 - 75	Moderately low (B)
		75.1 - 100	Moderate (M)
		100.1 - 135	High (A)
		< 135.1	Very high (MA)
Aneg	Water logging	None (N)	
		Moderate (M)	
		High (A)	
		Very high (MA)	
CIC	Cationic exchange capacity (cmol + · Kg^{-1} soil)	0 - 16	Low (B)
		16.1 - 24	Medium (M)
		< 24.1	High (A)
Cond_Elec	Electrical conductivity (dS · m^{-1})	0 - 2	Low (B)
		2.1 - 4	Medium (M)
		4.1 - 6	High (A)
		6.1 - 9	Very high (MA)

Code	Soil characteristics	Classes and ranks	
Dren	Drainage	Very poorly drained (MPD)	
		Poorly drained (PD)	
		Somewhat poorly drained (APD)	
		Moderately well drained (MBD)	
		Well drained (BD)	
		Somewhat excessively drained (AED)	
		Excessively drained (ED)	
OM	Organic matter (%) at topsoil.	0 - 1	Low (B)
		1.1 - 2	Medium (M)
		< 2.1	High (A)
pH	Hydrogen ion concentration	0 - 5.5	Acid (A)
		5.6 - 6	Moderately acid (MA)
		6.1 - 6.5	Slightly acid (LA)
		6.6 - 7.3	Neutral (N)
		7.4 - 7.8	Slightly basic (LB)
		7.9 - 8.3	Moderately basic (MB)
		8.4 - 14	Basic (B)
PSI_Sup	PSI (%) Sodium exchangeable percentage topsoil 0 - 0.20 m	0 - 5	Low (B)
		5.1 - 10	Medium (M)
		10.1 - 15	High (A)
		< 15.1	Very high (MA)
PSI_SubS	PSI (%) Sodium exchangeable percentage subsoil 0.21 - 0.50 m	0 - 5	Low (B)
		5.1 - 10	Medium (M)
		10.1 - 15	High (A)
		< 15.1	Very high (MA)

Table 3. Climate characteristics selected, classes and ranks.
Tabla 3. Características del clima seleccionadas, sus clases y rangos.

Code	Climate characteristics	Classes and ranks	
PP_Repro	Precipitation in the reproductive period (mm)	50 - 150	Extremely low (MB)
		151 - 250	Low (B)
		251 - 350	Medium (M)
		351 - 500	High (A)
		> 501	Very high (A)
PP_ Vegeta	Precipitation in the vegetative period (mm)	100 - 250	Extremely low (MB)
		251 - 300	Low (B)
		301 - 350	Medium (M)
		351 - 500	High (A)
		> 501	Very high (A)

Table 4. Simulation of different precipitation scenarios. Different precipitation probabilities are observed for each period and each soil densification.

Tabla 4. Simulación de los diferentes escenarios de precipitación para las diferentes probabilidades de ocurrencia en cada periodo del cultivo y densificación del suelo.

Probability	Precipitation (mm)	Precipitation (mm)	Precipitation (mm)	Soil densification
	Reproductive period	Vegetative period	Annual	
20%	186	215	401	Minimum
50%	368	554	922	Minimum
80%	548	889	1437	Minimum
20%	149	172	321	Maximum
50%	294	443	737	Maximum
80%	438	711	1149	Maximum

Kernza crop requirements

Edaphoclimatic characteristics (table 2, page 52-53 and table 3, page 53) were selected, and different classes and ranks were defined (Locatelli 2020*, 2, 9, 14, 16, 22, 28, 36, 41, 48). Soil data were obtained from soil profiles of the 1:50000 soil maps (13, 30). Climate data were obtained from the climate analysis by Cassani (2020) and SMN (2018).

Available water content up to 1 meter was indirectly obtained with the Travasso & Suero model (1994), developed and validated for the southern Pampa region.

Decision trees were assembled according to edaphoclimatic characteristics and logical criteria based on expert knowledge determining land qualities (31, 40, 42). Seven land qualities were determined for model development (Supplemental data 2): Oxygen availability (Disp_O), Root depth (Exp_Rad), Nutrient availability (Disp_Nut), Exchangeable sodium percentage (%PSI), Water logging (Aneg) and Available water (Disp_Agua). Land qualities were assessed using four classes according to the FAO framework (1976, 1985, 2007), from the lowest (1) to the highest level of use limitation (4).

Climate and soil densification scenarios

Scenarios were proposed according to cumulative probabilities of precipitation for P20%, P50% and P80% (table 4). These scenarios were calculated with data obtained from Cassani (2020) and the SMN (2018).

Considering that past or current land use can affect planning and change crop suitability, scenarios of maximum and minimum soil densification were simulated for each cumulative precipitation setting. As physical degradation is the major soil degradation in Argentina (1, 5, 58), a theoretical maximum bulk density was calculated according to Duval *et al.* (2015) equation [1] as maximum soil densification. Minimum soil densification was determined as a bulk density of 1.2 g/cm^3 (47) (Supplemental data 1), simulating the occurrence of soil densifications, which decrease rainfall infiltration and percolation. On average, this led to a 20% reduction in water availability, generating different starting points for the Kernza suitability model for available water (table 4). Thus, lands with high soil densification are physically degraded, so the available water is lower. Also, in case of excess precipitation, drainage capacity is limited.

$$\textit{Maximun bulk density (g . cm}^{-3}\textit{)} = 1.766 - 0.00598 * (\%silt) - 0.0158 * (\%\ Organic\ Carbon) \quad (1)$$

Kernza suitability assessment was conducted by comparing Kernza requirements *vs.* land qualities. Kernza suitability was evaluated by considering the maximum limitation method (31) for each decision tree created (Supplemental data 2). We have expanded the four FAO categories into nine subcategories (figure 2, page 52-53; table 5, page 56) indicating suitability of each soil map unit. The assessment focuses on identifying potential limitations and risks associated with the different scenarios. In the maximum soil densification

scenario, current unsuitable land can be reverted and turned into suitable land. This is not the case for permanently unsuitable land (figure 2, table 6, page 56). Results were mapped using QGIS v3.10.8-A Coruña (49).

Soil map units identified in the 1:50000 maps (13, 30) were used as land units for this study. A total of 134 mapping units were identified, composed of 90 soil series and their phases (Supplemental data 1).

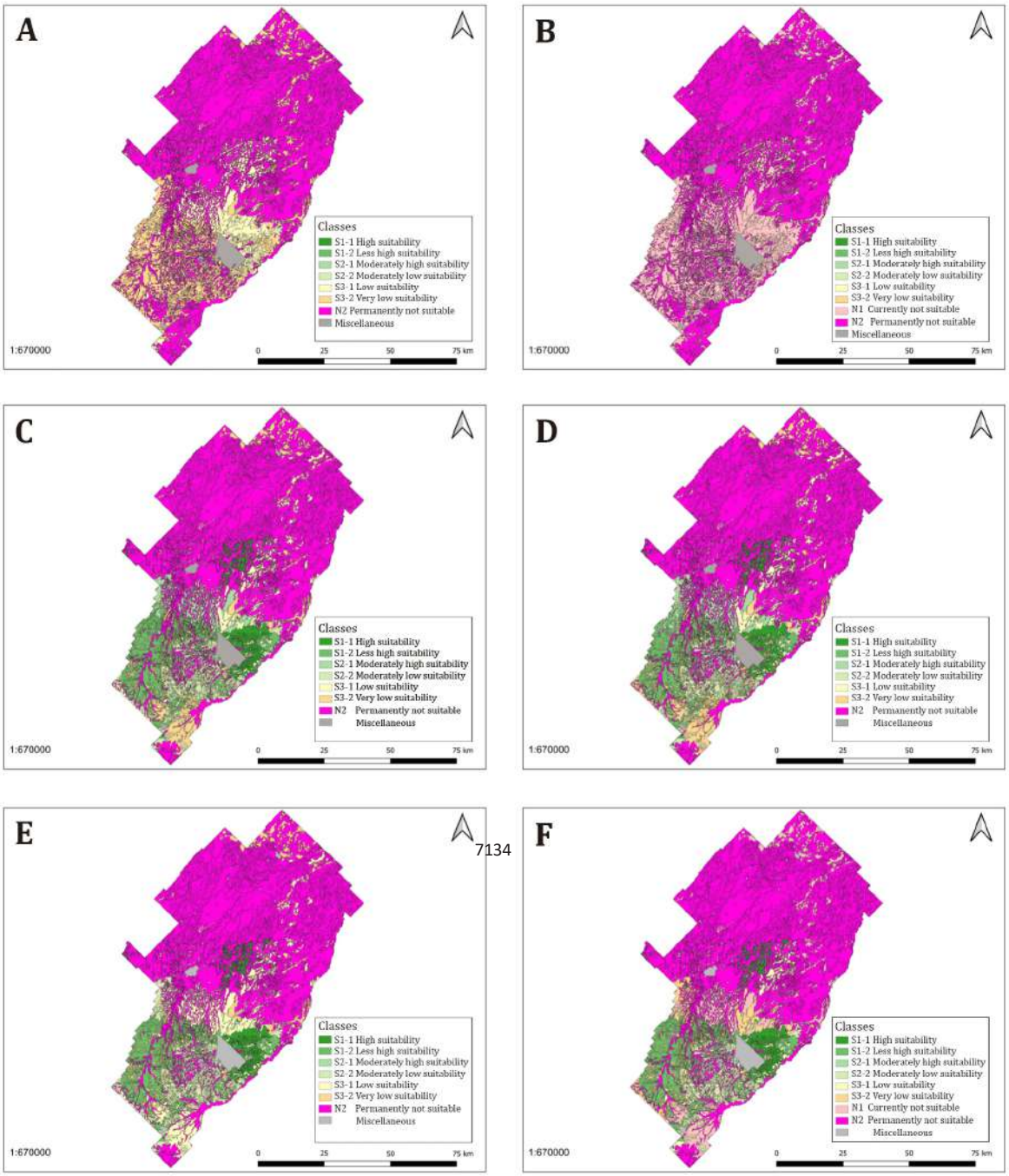

Figure 2. Land suitability for Kernza in minimum soil densification scenario for P20% (A), P50% (C) and P80% (E) scenarios, and in maximum soil densification scenario for P20% (B), P50% (D) and P80% (F).

Figura 2. Aptitud de la tierra para Kernza en el escenario de mínima densificación del suelo para los escenarios P20% (A), P50% (C) y P80% (E), y para el escenario de máxima densificación del suelo para los escenarios P20% (B), P50% (D) y P80% (F).

Table 5. Occupied land suitability for Kernza in minimum soil densification scenario for P20% (A), P50% (C) and P80% (E) climate scenarios (km²).

Table 5. Superficie ocupada por las distintas aptitudes para Kernza en el escenario de mínima densificación del suelo y P20% (A), P50% (C) y P80% (E) (km²).

P20%		P50%		P80%	
Class	**Km²**	**Class**	**Km²**	**Class**	**Km²**
High Suitability	0	High Suitability	238	High Suitability	238
Less High Suitability	0	Less High Suitability	508	Less High Suitability	495
Moderately High Suitability	0	Moderately High Suitability	236	Moderately High Suitability	5
Moderately Low Suitability	13	Moderately Low Suitability	410	Moderately Low Suitability	430
Low Suitability	652	Low Suitability	347	Low Suitability	702
Very Low Suitability	1170	Very Low Suitability	457	Very Low Suitability	294
Unsuitable	4566	Unsuitable	4205	Unsuitable	4237
Miscellaneous	150	Miscellaneous	150	Miscellaneous	150
Total	6551	Total	6551	Total	6551

Table 6. Occupied land suitability for Kernza in maximum soil densification scenario for P20% (B), P50% (D) and P80% (F) climate scenarios in Km².

Table 6. Superficie ocupada por las distintas aptitudes para Kernza en el escenario de máxima densificación del suelo y P20% (B), P50% (D) y P80% (F) en Km².

P20%		P50%		P80%	
Class	**Km²**	**Class**	**Km²**	**Class**	**Km²**
High Suitability	0	High Suitability	209	High Suitability	238
Less High Suitability	0	Less High Suitability	481	Less High Suitability	495
Moderately High Suitability	0	Moderately High Suitability	290	Moderately High Suitability	5
Moderately Low Suitability	0	Moderately Low Suitability	411	Moderately Low Suitability	320
Low Suitability	0	Low Suitability	268	Low Suitability	209
Very Low Suitability	0	Very Low Suitability	537	Very Low Suitability	287
Currently Unsuitable	1835	Unsuitable	0	Currently unsuitable	610
Permanently unsuitable	4566	Permanently unsuitable	4205	Permanently unsuitable	4237
Miscellaneous	150	Miscellaneous	150	Miscellaneous	150
Total	6551	Total	6551	Total	6551

Results

In the minimum soil densification scenario, most lands in northern Azul were classified as unsuitable considering all precipitation probabilities. By contrast, most land in south Azul was classified as suitable in all precipitation probabilities (figure 2A, 2C and 2E, page 55). In P20%, suitable lands were classified under low to very low suitabilities (figure 2A, page 55). On the other hand, in P50% and P80% suitable land was mostly classified under moderately high to very high suitabilities (figure 2C and 2E, page 55). In the P20% scenario, no area was occupied by land with high suitability, less high suitability, and moderately high suitability. A considerably small area was occupied by the moderately low and low suitability classes.

The remaining area was occupied by very low to unsuitable classes (table 5, page 56). In the P50% and P80% scenarios, classes with high and less high suitability occupied a significant area, with equal occupancy in both scenarios. In the P50% scenario, the class with moderately high suitability obtained a high occupation area concerning the P80% scenario, where it was practically insignificant. A shift was observed in the area occupied in the P80% scenario towards the class with low suitability in relation to the P50% scenario. In contrast, the class presenting very low suitability was higher in the P50% scenario than in the P80% scenario (table 5, page 56).

Regarding the maximum soil densification scenario, in P20% all land was classified as unsuitable. Most northern lands were classified as permanently unsuitable. In the south, most of the lands were currently unsuitable (figure 2B, page 55). In P50% and P80%, most northern lands were classified as unsuitable. In contrast, most lands in south Azul were classified as suitable. Suitable lands were classified under very high to moderately low suitabilities. In contrast to P50%, P80% was shown as currently unsuitable land (figure 2D and figure 2F, page 55).

The largest area in the conditionally unsuitable class was in the P20% scenario, followed by the P80%. This first class was not observed in the P50% scenario. In the P50% and P80% scenarios, the highly suitable and less highly suitable classes occupied a significant area, with equal occupancy in both scenarios. In the P50% scenario, the moderately high suitable class obtained a high area of occupation in relation to the P80% scenario, where it was practically insignificant. A shift in the area occupied towards the conditionally unsuitable class was shown in the P80% scenario in relation to the P50% scenario. In contras classes with low to very low suitability were higher in the P50% scenario than in the P80% scenario (table 6, page 56).

Discussion

Precipitation probability strongly influenced results for all scenarios, notably affecting available water. Oxygen availability and exchangeable sodium percentage played a significant role given the very high levels of exchangeable sodium (%PSI) and poor drainage. As a result, most of the land in Azul was unsuitable for Kernza. However, suitable land for Kernza could be found in the Southern region, with favourable cropping conditions. Irigoin (2011) in the Pampa Arenosa region, Argentina, also documented these results, linked to the different climatic scenarios and water availability. In both Pampa Serrana and Pampa Arenosa climate fluctuations related to the ENZO (El Niño Southern Oscillation) phenomenon, are recurrent every 2-3 years (6, 34), making ENZO an important factor in land use planning for Argentina.

Maximun soil densification resulted agreed with Agostini *et al.* (2018) for southern Pampa. Furthermore, the incorporation of the currently unsuitable class was appropriate for the maximum soil densification scenario, allowing the identification of temporarily unsuitable land for Kernza. Soil densification can reversibly modify soil water dynamics, and currently unsuitable land can become suitable for Kernza when densification is removed. In the P20% maximum densification scenario, as water infiltration and percolation were restricted due to densification, annual available water was 321 mm, resulting in all land being classified as unsuitable. A quite different situation was shown in the P20% minimum soil densification scenario with 401 mm, with lands classified as suitable and unsuitable. The maximum soil densification for P80% scenario showed different land classification *vs*. P50%. Suitable lands in P50% became unsuitable in P80%. At higher precipitation, excess water due to soil densification led to higher waterlogging and lower soil oxygen availability. In Azul, suitable areas for Kernza coincide with historical wheat areas (29, 57).

However, according to Law *et al.* (2022) the environmental benefits do have trade-offs with the economic performance of Kernza, as low grain yields would require substantial price premiums to produce net returns equivalent to comparable annual crops. Kernza's current grain yield is relatively low when contrasted with annual wheat, *i.e.*, up to ~1,660 kg ha^{-1} in experimental fields (26, 36), but breeders expect IWG to achieve comparable yields soon (3, 15). The possibility to harvest forage twice a year provides an additional source of income (24, 45). Furthermore, Kernza's deep root system can explore deep soil

water, decreasing drought stress (9) due to climate change and the ENZO. Additionally, considering annual crops under fertilization (14) nitrogen leaching decreased while increasing nutrient cycling, and improving fertilizing efficiency, with a consequent reduction in costs. Moreover, bearing in mind that decarbonization is being discussed worldwide (27), carbon sequestration by roots and a lower dependence on fossil fuels for production (12) could position Kernza as the ideal crop. The crop's smaller carbon footprint could be used for carbon credits bringing in additional revenue through inclusion in programs such as the Ecosystem Services Market Consortium (2023). All these ecosystemic services must be considered in the economic equation.

After the recent commercial release of perennial rice in China, shifting from annuals to perennials seems more possible than ever (59). Given all the ecosystemic services provided, Kernza constitutes a very promising crop to consider in the Pampa region, Argentina with temperate climates and wild winters like Uruguay (38). Nextly, Kernza is to be field-tested and promoted among farmers in Azul and the rest of Argentina.

Conclusions

The land suitability model showed that Kernza can be grown in Azul and that southern areas are most suitable. These lands were mostly Argiudolls and Hapludolls, generally deep, with loamy textures, high organic matter content and granular structures in topsoil, and blocky structures in subsoil. They are well to moderately-well drained with high available water. These soil characteristics satisfy Kernza requirements, in concordance with historical wheat areas in Azul.

In addition, the different precipitation scenarios: P20%, P50% and P80%, allowed for determining land suitability. Different precipitation probabilities affect modelled performance of land units by increasing water supply and availability, while different soil densification scenarios modified available water and waterlogging. The maximum suitability expression was in P50% scenario, an average occurrence climatic scenario for both soil densification scenarios.

Given all the ecosystemic services provided, Kernza constitutes a very promising crop for land use planners and farmers in Azul and Argentina.

Supplemmentary material

https://drive.google.com/drive/folders/1O-Sc7NGpM_qOxkAXMoIH49973IwOucP-?usp=sharing

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Data availability statement

The data that support the findings of this work are in supplemental data. Also, openly available in "Zenodo" at https://doi.org/10.5281/zenodo.6884909 and https://doi.org/10.5281/zenodo.6977505.

Food labeling in Argentina. Decoding impacts on the Argentine Food Code

Rotulación y etiquetado de alimentos en el régimen argentino. El fenómeno de la descodificación y su impacto en el Código Alimentario Argentino

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Abstract

This work aims to analyze the characteristics of the legislative codification technique in Argentina, and whether, since the enactment of the Food Code to the present, this technique has been affected by a decoding normative evolution, considering the food labeling regime a particular study case. As a result, three lines of legislative alteration or modification distorting the mentioned technique re identified. This generates inconsistencies and ambiguities in the legislation, and consequent negative effects on the application, compliance and understanding of food regulations in the industry.

Keywords
codifying • decoding • food code • legislative technique • labeling

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RESUMEN

Este trabajo procura analizar las características de la técnica legislativa de la codificación en el régimen de alimentos de Argentina y si, desde la promulgación del Código Alimentario a la actualidad, dicha técnica ha sido afectada por una evolución normativa descodificante, tomando como caso de análisis particular el régimen de rotulado de alimentos. Como resultado, se identifican tres líneas de alteración o modificación legislativa que desnaturalizan claramente a la referida técnica, lo que genera necesariamente incoherencias y ambigüedades en la legislación, con los consiguientes efectos negativos en la aplicación, cumplimiento y comprensión de las normativas en la industria alimentaria.

Palabras clave
codificacion • descodificacion • código alimentario • técnica legislativa • rotulado

Introduction

Since 1969, Argentina has adopted a food regime based on the legislative codification technique. Law 18248 approves a Food Code ordered in Annex I of Decree 2126/71.

This legislative regulation strategy prioritizes a single legal text systematized in an organic unit through a specific methodology, which provides internal coherence to the set of regulations integrated as a universe. Codifying is not collecting or compiling various laws issued on the subject, but achieving a legal text systematized by a single regulatory logic.

But since its sanctioned date, the Argentine Food Code has been subject to multiple and repeated internal reforms, motivated by various needs and causes, also motivating and responding to public policies of each era – which have not always had the same objectives. Numerous special laws on food have also been issued. As external regulations, these laws separately complement the Code.

Even in the legal harmonization generated from the regional integration process of the Southern Common Market (MERCOSUR - acronym in Spanish), numerous rules on food have been issued that -when incorporated into the Code- have impacted its regulations.

This situation raises concern as to whether the proliferation of norms -often disorderly or uncoordinated-occurring for more than half a century has affected the organicity and clarity of the Code as a whole. In short, if such an impact had occurred, the initial legislative technique that organizes the Food Code would have been devalued to the detriment of legal efficiency and effectiveness, and the legal security that the regulatory system must provide.

This work seeks to analyze the characteristics of the legislative codification technique in the Argentine food regime and state whether, since the enactment of the Food Code to the present day, this technique has been affected by a decoding normative evolution, taking labeling regime as study case.

To this end, it is hypothesized that the lack of respect for the technique of legislative codification in the partial reforms of the Argentine Food Code and other food regulations issued in the last half-century can generate inconsistencies and ambiguities in the legislation, which in turn can have negative effects on the application, compliance and understanding of regulations in the food industry.

Materials and methods

The article presents a qualitative analysis of the decoding phenomenon in the Argentine Food Code; particularly considering the labeling regime stipulated in Chapter V of this Code, and the impact caused therein by formal and material decoding processes. To this end, the intrinsic modifications produced in Code and the incidence of external laws, are individualized.

An observational methodological design with a descriptive-explanatory scope analyzed the Argentine Food Code considering two well-differentiated phases.

The first phase focuses on specifying the characteristics of codification as a legislative technique for the Argentine Food regime, and the decoding risk due to normative modifications developed without considering such legislative technique. The second phase identifies the legal modifications in the labeling regime -internal and external to the Code text- and the fragmentation caused to the current regime.

Results

The argentine food code as legislative technique

The legal approval of a "Code" implies a legislative technique systematically organizing the set of regulations on a subject in a single legal text. "Codifying" means much more than compiling existing regulations; it gives organic unity to a set of norms endowed with intimate cohesion due to their meaning (12), simplifying and unifying their interpretation and application for the benefit of legal effectiveness, efficiency, and certainty.

Under this concept, dictating a code is not a mere compilation of anarchic precepts among themselves, but rather implies a coherent legal text that requires classification, distribution, and coordination of the materials with which it is constructed (20). For this reason, the legislative technique of codification should not be confused with mere "collections," "indexes," "compilations," or "recompilations" of laws by subject matter or historical era presented in order in a repertoire, or with "digests" which the current texts present in a similar way but without an organic character -typical of codification- that systematizes its content as a unit (7, 22).

Such repertoires and digests, although they allow the identification of the regulations that have been issued or even their state of validity, do not guarantee a level of coherence that allows overcoming shortcomings such as inflation and regulatory pollution, that is, the overabundance and redundancy of laws that make the regime unclear. As Campos (2018) observes, the disorderly proliferation of norms devalues the rule of law and generates a significant lack of legal security.

A code, on the other hand, is a rationally formed legal text based on harmonious and coherent principles (19). It implies a legislative technique based on a methodology that provides coherence and order to a universe of institutions and norms formed as a single legal instrument, ensuring organicity and clarity as a whole (5).

Codified regulations make it easier to verify the current law, offering clarity, concision and legal certainty (7, 21), helping to avoid antinomies and other normative failures to be overcome by scope interpretation, with the consequent interpreter bias that generates inconsistencies and ambiguities on application, compliance and understanding of regulations.

The food regime in Argentina has been implemented under the legal technique of codification since 1969, with the sanction of Law 18248 by which the Argentine Food Code was approved and put into effect. At that time, this Code systematized in a single regime the right to food, which applies to any person, commercial firm or establishment that produces, divides, conserves, transports, sells, displays, imports or exports food, condiments, beverages or raw food materials and additives.

The food regulation contained in this Code is organized into twenty-two chapters comprising 1417 articles. Seven chapters establish transversal regulations for the entire food industry -(I) General Provisions; (II) Food Factories and Businesses; (III) Food Products; (IV) Utensils, Containers, Packaging, Devices and Accessories; (V) Food Labeling and Advertising; (XX) Official Analytical Methodology; (XXI) Procedures-. The remaining chapters establish specific regulations on the different types of foods and food products -(VI) Meat and Related Foods; (VII) Fatty Foods, Food Oils; (VIII) Dairy Foods; (IX) Farinaceous Foods: Cereals, Flours and Derivatives; (X) Sugary Foods; (XI) Vegetable Foods; (XII) Hydric Beverages, Water and Carbonated Water; (XIII) Fermented Beverages; (XIV) Spirits, Alcohols, Distilled Alcoholic Beverages and Liquors; (XV) Stimulant or Fruitive Products; (XVI) Correctives and Coadjuvants; (XVII) Dietary Foods; (XVIII) Food Additives; (XIX) Flours, Concentrates, Isolates and Protein Derivatives; (XXII) Miscellaneous-.

Finally, it should be clarified that the same Code and its regulations (art. 2 Regulation approved by Decree 2126/71) have contemplated specific cases in which the codification yields specific regulations on wines and meats so that the Food Code –although valid in such matters- only applies supplementarily. This situation was initially anticipated as temporary in article 1411 of the original text ordered by Decree 2126/71 (13), contemplating a process of incorporation into the Code that was never finalized.

Modification of the Food Regime and the Danger of "Decoding"

The eventual modification of the Argentine Food Code requires legal reform, either by a new law altering the content of the Argentine Food Code or establishing new precepts without altering the Code.

However, and without prejudice to the possibility of the legislative authority to enact new regulations, article 20 of Law 18284 introduced a mechanism for permanent updating of this norm, facilitating the incorporation of industry and science advances. To this end, this provision delegated the administrative authority with the power to keep the technical normative of the Code updated, being able to resolve necessary modifications to be included in the codified text.

This legal actualization technique has provided a marked dynamism to the regulation following the contemporary concept of codification, which rejects the idea of an immovable regime and promotes constant normative adaptation (11, 17). This allows its evolution at the pace and need of each era, but always without losing internal coherence and systemic unity.

In this way, and over time, the food regime has been subject to new regulations, some produced through the actualization mechanism provided for in article 20 of the law 18284, with the administrative authority issuing resolutions substituting, deleting or adding precepts. In this sense, Guajardo (1998) identifies that in the first three decades of validity (between 1969 and 1998) the Food Code presented more than 1000 changes implemented through more than 200 modifying provisions. Updating this information, in the last two decades (1999 a 2022) more than 300 amending provisions have modified the Code.

Besides the aforementioned, there has been extensive legislative activity concerning food, with special laws being issued for certain specific aspects, as the fortification of salts (Law 17259) or flours (Law 25630), the regulation of Slaughter Establishments (Law 22375), the gluten-free products regime (Law 26588), or more recently, the Healthy Eating Promotion regime (Law 27642).

An important impact on food regulations has occurred due to the framework of the regional integration process of the Southern Common Market (MERCOSUR) initially established by Argentine, Brazil, Paraguay and Uruguay in the Treaty of Asunción of 1991, and to which Venezuela -currently suspended- and Bolivia -in the accession process- later joined. Numerous requirements on food generated within the scope of MERCOSUR have been incorporated through adjustments in the Argentine Food Code. Guajardo (1998) observed that this sector of community regulations has helped lead the unifying or harmonizing process among the different countries. Legislative action in this area of regional integration has significantly impacted the national food legislation.

This regional integration and generated regulations are important not only since, commercially, MERCOSUR increases the exchange of food products among parties while constituting a great platform for food export (18), even to other regions (4); but especially because trade governance in the 21st century is based on standards and regulations rather than on classic tariff limitations. Integration agreements provide the opportunity for developing the necessary regulatory framework (2).

Faced with the various regulatory sources currently generating food norms, *i.e.* technical adjustments to the Code, new laws on the subject and harmonization with the regional integration process, it is opportune to analyze whether practices are leading to a "decoding" or the breakdown of the unity of the Code due to the proliferation of special laws at the rate of change on the issues to be regulated (8).

Guajardo (1998) has long observed that, although the Argentine Food Code is strictly the one approved by Law 18284 and Decree 2126/71, general regulations on food are much broader, and therefore cannot be identified with the concept of Code. This implies matching the legal rules in a way that an organic and systemic whole is created.

Although, according to Guzmán Britos (1993), the decoding of a regime can occur in different ways. On the one hand, formal decoding occurs when special laws, foreign or external to the Code, alter the unity of the legislation initially found in a single normative text, generating dispersion and tension between such precepts. In this sense, Hinestrosa Forero (2014) observes that perhaps, the special legislator should be reproached for not having known or wanted to adapt the figures of the Code capable of useful application to the new demands.

Considering material decoding, modifications are introduced to the text of a Code itself, although altering the system logic that it presents (14). This is, although not every modification to the Code text implies decoding, those modifications that break rationality, harmony and coherence, denaturalizing the Code codification technique. In such cases, the Code is transformed -at least partly- into a normative index, compilation or digest of non-systematized normative precepts.

In order to analyze whether the modifications in the food regime are generating a decoding process, below we specify the impact of legal reforms focusing on the food labeling regime.

Labeling of food products

One topic in the system of the Argentine Food Code is related to "Food Labeling and Advertising Rules", developed in Chapter V (articles 220 to 246).

The Codex Alimentarius guidelines developed by FAO and WHO define labeling as "any tag, brand, mark, pictorial or other descriptive matter, written, printed, stenciled, marked, embossed or impressed on, or attached to a container of food or food product" (10). In the regional and local regime, Resolution GMC 26/01 -dictated within the scope of MERCOSUR and incorporated into the Food Code- defines labeling as any label, inscription, image or other descriptive or graphic material, written, printed, stenciled, marked, engraved in high or low relief, adhered, superimposed or fixed to the container of the prepackaged product.

Food labeling is a fundamental tool in the communication of nutritional information, potentially influencing consumer choices and eating habits. Information should be easy to read and interpret (9).

One of the actions proposed within the Global Strategy on Diet, Physical Activity, and Health adopted by the World Health Organization in 2004 is to improve people's ability to make informed decisions about nutrition through useful, easy and understandable labeling, promoting nutrition and health literacy (23).

In the aforementioned guidelines of Codex Alimentarius developed by FAO and WHO (1985), the name of the food, the list of ingredients, net content, identification and address of production companies, country of origin, batch identification, date and storage instructions are recommended as mandatory content in the labeling regulations. They also specify situations in which ingredients must be declared quantitatively, as well as when dealing with irradiated foods.

Within food labeling, the so-called nutritional labeling can be specified as a particularity, in which -to inform the consumer- a description of the food nutritional properties is provided. In certain regulations, these guidelines are related to the preparation and nutrient content of a product without indicating whether they are healthy products, making it necessary to interpret (16).

In this regard, various countries have adopted consumer protection policies through the implementation of front labeling laws, by which beyond the mere inclusion of the nutritional information, they seek to limit the marketing of food products with harmful components like saturated fats, added sugars, sodium or high calories (1).

Fragmentation and complexity of the food labeling regime

Beyond considering the limited regulation in labeling terms of the Argentine food regime (6), a complex and fragmented character also undermines the legislative technique of codification adopted by Law 18284.

An initial and basic approach to the regulations on food labeling was provided by Law 18284, a norm that, besides declaring the validity of the Argentine Food Code, stipulated in article 19 that labels, packaging and wrappers authorized according to said Code had to clearly and accurately express their hygienic-sanitary, bromatological and commercial conditions.

According to the text ordered by Decree 2126/71, the Argentine Food Code approved and dedicated Chapter V to the "Food Labeling and Advertising Standards", complemented with other labeling requirements contained in the specific regime of the various products regulated by the Code. Chapter V was composed of twenty-six precepts (arts. 220 to 246). However, over time, various alterations have affected the food labeling regime either through amending regulations or through external regulations to the Code. These alterations coexist and must be applied in a complementary manner.

Thus, Chapter V has been the subject of numerous reforms through the updating administrative mechanism provided in Article 20 of Law 18284, which has replaced and/or repealed its original text, or expanded it with new articles intercalated in the original numbering as "bis", "bis1", "bis2", "tris", "quárter" and "quinto", "sexto" and "séptimo". In this way, none of the twenty-six original articles corresponding to the labeling regime in the ordered text by the Decree 2126/71, remain to date. Instead, twelve articles have been deleted by repeal, fourteen have a replaced text, and eight new articles have been added through reiteration (such as "bis", "ter", etc.) of the original numbering.

Table 1 (page 68) details modifications introduced by updating articles of the Argentine Food Code.

These modifications are not systematic, but rather respond to isolated and temporally distant interventions, with dissimilar causes, purposes and contexts during 1980, 1994, 1998, 1999, 2002, 2004, 2005, 2007, 2013, 2017, 2021 and 2022. In this way, although each modification does not alter the internal coherence of the norm altogether eventually causes a fragmentation reducing the regime to a mere juxtaposition of isolated precepts without an organic systematization.

This internal fragmentation is strengthened since, through the same modifying mechanism, said Chapter has also been expanded in content through the incorporation of various resolutions issued within the scope of MERCOSUR. These resolutions were added as part of the Code in an effort of regional legal harmonization, although without any systematization or integration among articles, annexing the full text of said community standards before the articles that make up the Chapter. This technique corresponds to a simple normative compilation but not a codification.

Thus, the labeling regime includes the Common Market Resolutions N° 26/03 ("Mercosur Technical Regulation for Packaged Foods Labeling"), N° 46/03 ("Mercosur Technical Regulation on the Nutritional Labeling of Packaged Foods"), N° 47/03 ("Mercosur Technical Regulation of Portions of Packaged Food for the Purposes of Nutritional Labeling"), N° 31/06 (on "Nutritional Labeling of Packaged Foods"), N° 36/10 (on "Conversion Factor for Calculating the Energy Value of Erythritol"), N° 40/11 (MERCOSUR Technical Regulation on "Nutritional Labeling of Non-Alcoholic Beverages Marketed in Returnable Packaging"); N° 01/12 ("MERCOSUR Technical Regulation on Complementary Nutritional Information (Nutritional Claims)").

Table 2 (page 69), details the community standards sanctioned by MERCOSUR and incorporated in the Argentine Food Code Chapter V.

By completing this regulatory framework, the legislative authority has also issued special laws that, while regulating food matters parallely to the Code, contemplate labeling aspects for some specific products.

Although some of these special laws have been integrated into the Code through subsequent modifications (case of the labeling regime stipulated by Law 17259 for enriched salts), in other cases, such precepts are isolated, without systematization. Or even in some cases, they result antinomian, as occurs with labeling regulations of enriched flours (Law 25630 y and Decree 597/2003), labels and packaging on geographical indications and designations of origin used for the commercialization of agricultural and food origin products (Laws 25830 and 25966), whole milk powder supplied in food programs (Law 25459 and its regulations), and gluten-free products (Law 26588).

Table 1. Modifications produced in Chapter V of the Argentine Food Code.
Tabla 1. Modificaciones producidas en el Capítulo V del Código Alimentario Argentino.

Article	Status	Rule that affects it
220	Replaced	Joint Resolution SCS y SAByDR N° 26/2021
221	Replaced	Res. MS 2343, 19.4.80, Ratified by Joint Res. MSyA 149/05 y SAGPyA 683/05
222	Replaced	Res. MS 2343, 19.4.80, Ratified by Joint Res. MSyA 149/05 y SAGPyA 683/05
223	Suppressed	Repealed by Joint Res. MSyA 149/05 y SAGPyA 683/05
224	Replaced	Joint Res. MSyA 149/05 y SAGPyA 683/05
225	Replaced	Joint Res. SCS y SAGyP N° 7/2022
226	Replaced	Joint Res. SCS y SAGyP N° 7/2022
227	Replaced	Joint Res. MSyA 149/05 y SAGPyA 683/05
228	Replaced	Joint Res. MSyA 149/05 y SAGPyA 683/05
229	Replaced	Joint Res. SCS y SAByDR N° 5/2022
230	Suppressed	Repealed by Joint Res. MSyA 149/05 y SAGPyA 683/05
231	Suppressed	Repealed by Joint Res. MSyA 149/05 y SAGPyA 683/05
232	Suppressed	Repealed by Joint Res. MSyA 149/05 y SAGPyA 683/05
233	Suppressed	Repealed by Joint Res. MSyA 149/05 y SAGPyA 683/05
233 bis	Added	Res. MSyAS 659, 3.10.94, Ratified by Joint Res. MSyA 149/05 y SAGPyA 683/05
234	Suppressed	Repealed by Joint Res. MSyA 149/05 y SAGPyA 683/05
235	Replaced	Joint Res. MSyA 149/05 y SAGPyA 683/05
235 bis1	Added	Res. MSyAS N° 888, 4.11.98, Ratified by Joint Res. MSyA 149/05 y SAGPyA 683/05
235 bis2	Added	Res. MSyAS N° 005, 7.01.99, Ratified by Joint Res. MSyA 149/05 y SAGPyA 683/05
235 tris	Added	Res. MSyAS N° 005, 7.01.99, Ratified by Joint Res. MSyA 149/05 y SAGPyA 683/05
235 quárter	Added	Joint Res. SPRyRS y SAGPyA N° 2/2007 y 256/2007
235 quinto	Added	Joint Res. SPReI N° 161/2013 y SAGyP N° 213/2013
235 sexto	Added	Joint Res. SPRyRS y SAGPyA N° 136/2007 y N° 109/2007
235 séptimo	Added	Joint Res. SPReI y SAV N° 11-E/2017
236	Replaced	Joint Res. SCS y SAByDR N° 18/2021
237	Suppressed	Repealed by Joint Res. MSyA 149/05 y SAGPyA 683/05
238	Suppressed	Repealed by Joint Res. MSyA 149/05 y SAGPyA 683/05
239	Suppressed	Repealed by Joint Res. MSyA 149/05 y SAGPyA 683/05
240	Replaced	Joint Res. MSyA 149/05 y SAGPyA 683/05
241	Suppressed	Repealed by Joint Res. MSyA 149/05 y SAGPyA 683/05
242	Suppressed	Repealed by Joint Res. MSyA 149/05 y SAGPyA 683/05
243	Replaced	Joint Res. MSyA 149/05 y SAGPyA 683/05
244	Replaced	Res. 2343, 19.4.80, Ratified by Joint Res. MSyA 149/05 y SAGPyA 683/05
245	Replaced	Joint Res. MSyA 149/05 y SAGPyA 683/05
246	Suppressed	Repealed by RES. GMC N° 21/02

Table 2. MERCOSUR standards incorporated in the Argentine Food Code.
Tabla 2. Normas del MERCOSUR incorporadas al Código Alimentario Argentino.

MERCOSUR Resolution	Standards incorporated into Argentine Food Code
Common Market Group Resolution N° 26/03 "Mercosur Technical Regulation for Packaged Foods Labeling"	Joint Res. SPRyRS 149/2005 y SAGPyA 683/2005
Common Market Group Resolution N° 46/03 "Mercosur Technical Regulation on the Nutritional Labeling of Packaged Foods"	Joint Res. SPRyRS 149/2005 y SAGPyA 683/2005
Common Market Group Resolution N° 47/03- "Mercosur Technical Regulation of Portions of Packaged Food for the Purposes of Nutritional Labeling"	Joint Res. MSyA 150/2005 y SAGPyA 684/2005
Common Market Group Resolution N° 31/2006 - "Nutritional Labeling of Packaged Foods"(Resolutions Complementation GMC N° 46/03 y N° 47/03)	Joint Res. SPRyRS N° 49/2007 y SAGPyA N° 106/2007
Common Market Group Resolution N° 36/10 - "Conversion Factor for Calculating the Energy Value of Erythritol"	Joint Res. SPReI N° 12/2012 y SAGyP N° 13/2012
Common Market Group Resolution N° 40/11, Mercosur Technical Regulation on "Nutritional Labeling of Non-Alcoholic Beverages Marketed in Returnable Packaging"	Joint Res. SPReI N° 212/2012 y SAGyP N° 1197/2012

The same occurs with the legislation aimed at addressing Non-Communicable Diseases, with substantial impact on the food labeling regime. Law 26905 -and its regulatory Decree 16/17- contemplate, separately from the Food Code, that the Ministry of Health includes health warning messages on containers in which salt (sodium chloride) is marketed. Additionally, Law 27642 on Healthy Eating Promotion (also known as the Front Labeling Law), despite updates of articles 225 y 226 of the Food Code established by Joint Resolution SCS y SAGyP N° 7/2022, presents contents that exceed the codified guidelines.

Finally, certain regulations issued by regulatory authorities of other normative systems have dictated regulations on labeling that, omitting the regime stipulated in the Argentine Food Code, increase the aforementioned decoding effect. This occurs with Resolution 26/21 of the National Viticulture Institute concerning labeling of wine industry products; or with Resolution 494/01 of the National Agri-Food Health and Quality Service, labeling foods prepared with minced, ground or sliced meat, and Resolution 5-E/2018 of the same entity on marketing of bulk honey containers.

Conclusions

The Argentine Food Regime was based on the legislative technique of codification, which implies a regulatory strategy that enhances legal effectiveness, efficiency and legal security by avoiding dispersed norms without internal coherence. At the same time, codification does not necessarily imply static regulations, although its evolution must safeguard internal coherence and systemic unity.

However, based on the study of labeling regulations and modifications, it can be stated that the Argentine Food Code has three legislative action lines in tension with the codification legislative technique, affecting adoption and efficiency of the regulatory system.

On the one hand, the continuous and numerous internal modifications to the Food Code carried out individually in response to specific and independent problems, generate a clear risk for internal coherence.

This process is significantly enhanced by the notable deficiency in the legislative technique with which legal harmonizations, inherent to the regional economic integration process implemented in MERCOSUR, have been introduced into national law. In these cases, community resolutions have been attached through mere transcription, openly inconsistent with its methodology and articulation. Incorporating such texts with no conception of organic unity reduced the Code to norms without rational structure or cohesion.

A third line of the undermining process of the Code is developed through the formal decoding and enactment of various special laws and other regulations foreign to the Argentine Food Code regime, obviously affecting the normative unity of codifying.

The observed situation allows us to affirm that since the enactment of the Food Code to the present day, there has been a clear decoding normative evolution, which necessarily generates inconsistencies and ambiguities in the legislation, with the consequent negative effects on the application, compliance and understanding of the regulations in the food industry.

To counteract the observed evolution, it becomes necessary to revitalize the original legal strategy based on the codification of food matters, which implies a review and readjustment of the current legal text so that in the future it systematizes its content as a unit and regains lost internal coherence.

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Selection of fungal isolates from Buenos Aires, Argentina, as biological control agents of *Botrytis cinerea* and *Sclerotinia sclerotiorum*

Selección de aislados fúngicos de Buenos Aires, Argentina, como agentes de control biológico de *Botrytis cinerea* y *Sclerotinia sclerotiorum*

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Abstract

This work aimed to select promising microorganisms as biological control agents (BCA). Forty-one soil samples were obtained from florihorticultural farms located in Buenos Aires, Argentina. Insect trap techniques and soil serial dilutions were used to obtain isolates of entomopathogenic fungi and fungi of genera *Trichoderma,* respectively. A total of 20 isolates included five *Metarhizium* and 15 *Trichoderma*. The isolates were lyophilized and deposited as reference cultures in the Mycological Collection of the Centro de Estudios Parasitológicos y de Vectores (CEPAVE). We performed dual culture studies of the isolates collected against the pathogens *Botrytis cinerea* Pers. (1797) and *Sclerotinia sclerotiorum* (Lib.) de Bary (1884). Eleven isolates were selected for growth promotion studies in tomato plants (*Solanum lycopersicum* L.). The isolates of *Metarhizium taii* Liang & Liu (1991) CEP-722, CEP-723 *Trichoderma afroharzianum* Chaverri, Rocha, Degenkolb & Druzhinina (2015) CEP-753 and CEP-754, molecularly identified by amplification of the ITS and TEF1α zones, presented the best results in the dual culture and growth promotion tests. Subsequent studies will evaluate virulence of fungal strains in insects.

Keywords

entomopathogenic fungi • biological control agents • molecular identification • dual culture • plant growth promotion

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Resumen

El objetivo de este trabajo fue seleccionar microorganismos promisorios como agentes de control biológico (ACB). Se visitaron predios florihortícolas ubicados en Buenos Aires, Argentina, de los cuales se obtuvo un total de 41 muestras de suelo. Se utilizaron las técnicas de insecto trampa y diluciones seriadas de suelo para la obtención de aislados de hongos entomopatógenos y hongos del género *Trichoderm*a respectivamente. Se obtuvieron un total de 20 aislados, cinco pertenecientes al género *Metarhizium* y 15 aislados correspondientes al género *Trichoderma*. Los aislados fueron liofilizados y depositados como cultivos de referencia en la Colección Micológica del Centro de Estudios Parasitológicos y de Vectores (CEPAVE). Se realizaron estudios de cultivos duales de los aislados recolectados frente a los patógenos *Botrytis cinerea* Pers. (1797) y *Sclerotinia sclerotiorum* (Lib.) de Bary (1884). Se seleccionaron 11 aislados para la realización de estudios de promoción de crecimiento en plantas de tomate (*Solanum lycopersicum* L.). Los aislados de *Metarhizium taii* Liang y Liu (1991) CEP-722, CEP-723 y de *Trichoderma afroharzianum* Chaverri, Rocha, Degenkolb y Druzhinina (2015) CEP-753 y CEP-754, identificados molecularmente por medio de la amplificación de las zonas ITS y TEF1α, presentaron los mejores resultados en las pruebas de cultivo dual y promoción de crecimiento. Se espera avanzar en estudios posteriores que evalúen la virulencia de cepas de hongos en insectos.

Palabras clave

hongos entomopatógenos • agentes de control biológico • identificación molecular • cultivo dual • promoción de crecimiento de las plantas

Introduction

Stem “wet rot” caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, (1884) and “grey rot” caused by *Botrytis cinerea* Pers. (1797) stand among the economically most important diseases in tomato (*Solanum lycopersicum* L.) Control of these diseases has relied on benzimidazole and dicarboximide fungicides. However, dicarboximide-resistant isolates are commonly detected (3). In this regard, biological control programs sustained by isolation and subsequent selection of antagonists (4) constitute a valuable alternative. Among beneficial biota, nutrient-fixing and solubilizing microorganisms produce *plant growth-promoting* substances, induce plant resistance to diseases or behave as antagonists to phytopathogenic agents (32).

The genus *Trichoderma* dominates the mycobiome of various ecosystems (10) with the ability to colonize the rhizoplane, rhizosphere and roots, producing numerous metabolites with antimicrobial and biostimulant activity. The plant growth stimulating effect is probably generated by the interaction among growth hormones synthesized by *Trichoderma* spp. and plant defense hormones (8). Some entomopathogenic fungi act as fungal growth inhibitors of phytopathogens (11, 12). The genus *Metarhizium* is composed of diverse common soil fungi with multifunctional lifestyles and different nutrient acquisition modes, either saprophytes, endophytes, and/or insect pathogens (37). Classically, studies have focused on their entomopathogenic characteristics, but their ability to inhibit phytopathogens was recently determined (13). Some studies have evaluated the endophytic capacity and colonization methods of this genus (2).

Metarhizium is a genetically diverse taxon, and colony color and conidial measurements of different species are not reliable identification factors (22). Alternatively, the molecular identification of *Trichoderma* is abundant, with no standard process, except the recently proposed gene standardization system for molecular identification (7). This study intends to develop biological inputs based on native and/or naturalized strains of *Trichoderma* and entomopathogenic fungi for agricultural pest management.

Materials and methods

Collection of soil samples

Six agroecological productions located in the province of Buenos Aires, Argentina were visited. Agroecological production of Bernardo Castillo (Street 519, El Pato, Buenos Aires. -34.905505, -58.200043); Organization 1610 (Street 1610, La Capilla, Buenos Aires. -34.9046857, -58.2666433); Agroecological production Santa Elena (Road Parque Pereyra Iraola, Pereyra, Buenos Aires. -34.83699, -58.093384); M. G. Agroecológica (Esteban Echeverría, Buenos Aires. -34.8672710, -58.4608800); Cooperative UTT Jaúregui. (Luján, Buenos Aires. -34.6204249, -59.1764168); and the experimental plot of Cátedra de Horticultura, Facultad de Agronomía de la Universidad de Buenos Aires (Av. San Martín 4453, C.A.B.A. -34.594101, -58.484467). Fourty-one soil samples were obtained from cultures of cabbage (*Brassica oleracea* var. *capitata*); basil (*Ocimum basilicum*); corn (*Zea mays*); lettuce (*Lactuca sativa*); tomato (*Solanum lycopersicum*); zucchini (*Cucurbita pepo*); bell pepper (*Capsicum annuum*); cherry tomato (*Solanum lycopersicum* var. *cerasiforme*); chives (*Allium fistulosum*); leek (*Allium ampeloprasum* var. *porrum*); fennel (*Foeniculum vulgare*); beets (*Beta vulgaris*); broccoli (*Brassica oleracea* var. *italica*); brussels sprouts (*Brassica oleracea* var. *gemmifera*); chard (*Beta vulgaris* var. *cicla*); carrot (*Daucus carota*); artichoke (*Cynara cardunculus* var. *scolymus*); broad bean (*Vicia faba*); turnip (*Brassica rapa* subsp. *rapa*); kale (*Brassica oleracea* var. *sabellica*); peas (*Pisum sativum*); and arugula (*Eruca vesicaria*). Sample number and species varied by establishment. As sampling criterion, soil from more vigorous plants within the same plot was also sampled, for later obtention of growth-promoting microorganisms (20, 39, 42). Five random subsamples within a crop row were collected for each sample from the first 20 cm below ridge surface with crop roots. Then, they were mixed into a single homogeneous sample with approximately 500 g from each crop, soil and rhizosphere, holding the greatest biodiversity (24). Fungal greatest abundance is found in the superficial layers or soil horizons (21). Samples were arranged in plastic bags indicating date, culture and origin, later transported to the laboratory in a closed expanded-polystyrene container and processed within 24 hours (table 4, page 78 and table 5, page 79).

Isolation of *Metarhizium, Trichoderma, Botryris cinerea* and *Sclerotinia sclerotiorum* fungi

From the collected soil samples, the insect trap technique was used with larvae of *Tenebrio molitor* L. from stage L3 to L4 as bait insects (1). Samples were sieved and 300 g were placed in 500 ml plastic containers with five larvae each, moistened with 20 ml of sterile distilled water and incubated at 18°C 65% relative humidity and 14:10 h light-darkness photoperiod. *T. molitor* carcasses prospected after seven days. Dead larvae with external mycosis were washed with sterile distilled water and placed in a humid chamber to increase sporulation. External mycelium present in *T. molitor* corpses (figure 5A, page 82) was obtained via direct isolation from the sporulated corpses, using a previously sterilized loop and subsequent sowing in Sabouraud Dextrose Agar culture medium, SDYA (Merck, Germany) with the addition of 5% chloramphenicol inside a 90 mm diameter *Petri* dish. *Trichoderma*, fungi were isolated via serial dilution. Five grams of each soil sample were suspended in 100 ml of sterile distilled water in an Erlenmeyer and vortexed for one hour. Serial dilutions were made until reaching $x10^6$ spores/ml. Concentrations were determined with a Neubauer chamber, with each dilution inoculated in 90 mm diameter *Petri* dishes with Potato Glucose Agar, APG (Merck, Germany), and 2% streptomycin. The plates were incubated at 20-22°C for 72 hours. When fungal colonies developed, they were replicated in *Petri* dishes with APG (Merck, Germany) until purification.

Phytopathogenic fungi isolates from *B. cinerea* and *S. sclerotiorum* were obtained from the mycological bank of phytopathology (Facultad de Agronomía de la Universidad de Buenos Aires), with identification code BC18 and SS18 and pathogenicity tested on tomato (*S. lycopersicum*). After isolation and before bioassays, visual prospection of the isolates was carried out under a microscope (OLYMPUS BX51, Japan), identifying fungal types.

Monosporic isolates were preserved on sterile filter paper and were lyophilized, deposited and preserved (14) as reference cultures in the mycological collection of the "Centro de Estudios Parasitológicos y de Vectores" (CEPAVE) (CONICET-UNLP), La Plata, Argentina.

Laboratory tests in dual cultures of *Metarhizium* and *Trichoderma* isolates against *B. cinerea* and *S. sclerotiorum*

Ninety mm diameter *Petri* dishes were filled with 12 ml of APG (Merck, Germany) or 12 ml of SDYA (Merck, Germany) for *Trichoderma* and *Metarhizium* trials, respectively. Once culture medium solidified, two 10 mm diameter discs with seven-days mycelial growth were placed on the medium, 70 mm apart, one containing *Trichoderma* spp. or *Metarhizium* spp. and the other containing *B. cinerea* or *S. sclerotiorum* according to each treatment. A disk of each isolate (pathogens and antagonists) was inoculated as control against a disk of APG and/or SDYA without microorganisms. *Petri* dishes were incubated at 22°C and maintained under fluorescent lights with a 14:10 h light-darkness photoperiod in a completely randomized design, with eight replicates per treatment. Growth radius of colonies considering *Trichoderma* isolates against phytopathogenic fungi were measured with a millimeter ruler, at 1, 2, 3, 4, 5 and 6 days of trial with *B. cinerea* and at 1, 2, 3, 4 and 5 days with *S. sclerotiorum*. Considering *Metarhizium* isolates against phytopathogenic fungi, colony radius was measured at 4, 5, 6 and 7 days in both cases. The number of measurement days per trial differs according to growth rate in phytopathogen control treatments. Pathogen percentage inhibition (I) was calculated using the following equation:

$$\mathbf{I= (C-T) / C \times 100.}$$

where:
(I) = Percentage of mycelium growth inhibition
C = Pathogen growth on control plates
T = Pathogen growth in dual culture plates (19).

The data were analyzed by ANOVA and Tukey test ($p> 0.05$) with InfoStat software (version 2016e) (9).

Growth promotion assays in tomato plants var. platense (*S. lycopersicum*) inoculated with a spore suspension of isolates of the genera *Metarhizium* and *Trichoderma*

Tomato plants (*S. lycopersicum*) var. platense were inoculated with a spore suspension of *Metarhizium* sp. CEP-722, CEP-723, CEP-724, CEP-725 and CEP-726 or the *Trichoderma* sp. CEP-745, CEP-749, CEP-751, CEP-752, CEP-753 and CEP-754, selected after *in vitro* growth inhibition tests of *B. cinerea* and *S. sclerotiorum*. The test was conducted in a biotherium chamber under controlled conditions at an average temperature of 24°C, average relative humidity of 70%, and a 18-6 h light-darkness photoperiod, using high-pressure sodium vapor lamps (Philips Son T Agro 250 W, China). Seeds of tomato (*S. lycopersicum*) var. platense were sown in commercial substrate (Grow Mix Multipro, Argentina) in seedling trays with 50 x 50 mm holes and irrigated with sterile distilled water. Spore suspensions of *Trichoderma* spp. and *Metarhizium* spp. isolations were obtained in sterile distilled water standardized to a concentration of 1×10^7 spores/ml. Tomato plants (*S. lycopersicum*) were inoculated at 7, 21 and 35 days after being sown (31 days in the case of the genus *Metarhizium*) with 2 ml of conidial suspension on the substrate. Measurements were made 49 days after sowing in *Trichoderma* spp. and 37 days in *Metarhizium* spp. Plants were watered to field capacity with sterile distilled water throughout the study. The completely randomized block design had seven repetitions (seven plants) for each treatment (spore suspension of the *Metarhizium* sp. and *Trichoderma* sp. isolates selected). The variables analyzed were stem length (mm), stem diameter at cotyledon height (mm) and aerial fresh weight (g), using a millimeter rule, graduated metal caliper and precision balance, respectively. An ANOVA and means comparison with Tukey test ($p > 0.05$) were performed with InfoStat (2016e version) (9).

Morphological characterization, molecular identification and phylogenetic analysis of the isolates CEP-722, CEP-723, CEP-753 and CEP-754

Four isolates, two of the genus *Metarhizium* and two of the genus *Trichoderma,* were selected for best results on dual culture and promotion growth assays. Isolates were identified at genus level based on microscopic traits contrasted with taxonomic keys (5, 18). Once the material was mounted in lactophenol/cotton blue (0.01% w/v), shape and size of conidia, conidiogenous cells (phialide), mycelium and other traits were observed under an optical microscope (OLYMPUS BX51, Japan) and photographed with a digital camera (Sony DSCP73, Japan). Measurements were based on 25 observations per microstructure (conidia, phialide and chlamydospore) and average calculations. Molecular analysis and identification of the isolates included mycelium production in three 90 mm diameter *Petri* dishes with APG (Merck, Germany) as culture medium for *Trichoderma* and SDYA (Merck, Germany) for *Metarhizium*, kept at 23° ± 1°C for seven and 14 days, respectively. Then, mycelium was placed in 1.5 ml Eppendorf tubes. Tubes containing fungal material were placed in a container with liquid Nitrogen for eight minutes. DNA extraction was performed using the DNeasy extraction kit from Qiagen (Germany) according to manufacturer instructions. Extracted DNA was quantified using a micro-volume spectrophotometer (Nanodrop, Thermo Fisher Scientific, United States) and stored in a freezer. PCR was performed to amplify 2 DNA regions: 1) The ribosomal DNA region comprising the 3' end of the 18S gene (small ribosomal subunit, SSU). The ITS1 internal spacer sequence (internal transcribed spacer 1), the 5.8S gene, the internal transcribed spacer 2 (ITS2) sequence and the 5' end of the 28S gene (long ribosomal subunit), using the universal primers ITS4 (5' -TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (30). 2) The 5' region of the Elongation Factor 1-Alpha (TEF1α) gene with the primers EF1 983F (5'-ATGGGTAAGGARGACAAGAC-3') and EF" 2218R (5'-ATGGGTAAGGARGACAAGAC-3') (23). Amplification reactions were carried out in a final volume of 50 µl, containing 25 µl Mastermix Promega 2x (GoTaq, USA), 17 µl nuclease-free water, 2 µl PRIMER-F, 2 µl PRIMER- R and 4 µl of DNA for each isolate. Table 1 shows the thermocycling processes for the ITS1 and TEF1α regions.

Table 1. Thermocycling processes for the ITS1 and TEF1α regions.
Tabla 1. Procesos de termociclados para las regiones ITS1 y TEF1α.

ITS - 32 ciclos	CEP-722	CEP-723	CEP-753	CEP-754
Initial denaturation	ITS: 95°C 5 min. TEF1α: 94°C 2 min.	ITS: 95°C 5 min. TEF1α: 94°C 2 min.	ITS: 95°C 5 min. TEF1α: 94°C 2 min.	ITS: 95°C 5 min. TEF1α: 94°C 2 min.
Denaturation	ITS: 94°C 1,30 min. TEF1α: 94°C 30 seg.	ITS: 94°C 1,30 min. TEF1α: 94°C 30 seg.	ITS: 94°C 1,30 min. TEF1α: 94°C 30 seg.	ITS: 94°C 1,30 min. TEF1α: 94°C 30 seg.
Alignment	ITS: 60°C 1 min. TEF1α: 53°C 40 seg.	ITS: 56°C 1 min. TEF1α: 54°C 40 seg.	ITS: 58°C 1 min. TEF1α: 54°C 40 seg.	ITS: 60°C 1 min. TEF1α: 54°C 40 seg.
Elongation	ITS: 72°C 50 seg. TEF1α: 72°C 30 seg.	ITS: 72°C 1,30 min. TEF1α: 72°C 30 seg.	ITS: 72°C 1,3 min. TEF1α: 72°C 30 seg.	ITS: 72°C 50 seg. TEF1α: 72°C 30 seg.
Denaturation 35 cycles*	TEF1α: 94°C 30 seg.	TEF1α: 94°C 30 seg.	TEF1α: 94°C 30 seg.	TEF1α: 94°C 30 seg.
Final elongation	ITS: 72°C 5 min. TEF1α: 72°C 10 min.	ITS: 72°C 5 min. TEF1α: 72°C 10 min.	ITS: 72°C 5 min. TEF1α: 72°C 10 min.	ITS: 72°C 5 min. TEF1α: 72°C 10 min.
Hold	ITS: 10°C TEF1α: 8°C.	ITS: 10°C TEF1α: 8°C.	ITS: 10°C TEF1α: 8°C.	ITS: 10°C TEF1α: 8°C.

* TEF1α region only.
* Solo región TEF1α.

Electrophoresis was performed in 1% agarose gels (UNQ Biological Products, Argentina) stained with Ethidium bromide in 0.5x Buffer TBE (Roti-Gelstain, Germany), applying a voltage of 90 V for 50 min. Five µl of each reaction was mixed with 1 µl of loading buffer (Productos Biológicos UNQ, Argentina). A molecular weight marker was added (Ladder 100 bp, PB-L), to determine PCR product size. Gels were visualized with a UV transilluminator (Analytik-Jena, Alemania). The ribosomal region was expected at 530 bp, while the TEF1α gene was 620 bp. Positive reactions were stored at -20°C. Samples were then sent to Macrogen (South Korea) for purification and sequencing. The free software "Chromas" (38)

cleaned the obtained sequencing then aligned using the online software "Clustal-Omega" (35). Agreement between the base pairs replicated by the forward and reverse primers of the four isolates analyzed was verified using the free software "Genedoc" (25). The sequences obtained were compared with those available in Genbank for each molecular marker (41). Sequences were aligned with 16 homologues and contrast of reference strains of *Metarhizium* with the sequencing of the ITS and TEF1α areas of Gutierrez *et al*. (2019) (table 2) and with 15 homologous species and a contrast obtained from the "Trichokey" data software (7), for *Trichoderma* (table 3, page 78). The phylogenetic tree was constructed with "Mr. Bayes" (version 3.2.7) (17), "Tracer" (version 1.7.2) (30) and "FigTree" (version 1.4.4) softwares (29).

Table 2. ITS and TEF1α gene sequences used for molecular identification of isolates CEP-722 and CEP-723.
Tabla 2. Secuencias de genes ITS y TEF1α utilizadas para la identificación molecular de los aislados CEP-722 y CEP-723.

Species	Strain	ITS	TEF1α
Metarhizium taii.	CEP-722	OP709693	OP792040
Metarhizium taii.	CEP-723	OP709705	OP792039
Metarhizium sp.	NHJ11597	HQ165703/AY646375	HQ165683
Metarhizium sp.	NHJ11618	HQ165704/AY646376	HQ165684
Metarhizium sp.	MY00896	HQ165697	HQ165678
Metarhizium argentinense	CEP414	MF784813	MF966620
Metarhizium argentinense	CEP424	MF784814	MF966624
Metarhizium blattodeae	IP414	KU182915	KU182917
Metarhizium flavoviride	ARSEF2025	AF138269	KJ398804
Metarhizium frigidum	ARSEF4124	HM055448	DQ464002
Metarhizium minus	ARSEF1764	HM055453	KJ398800
Metarhizium album	ARSEF1942	HM055452	KJ398802
Metarhizium taii	ARSEF5714	JN049829	AF543775
Metarhizium owariense	NBRC33258	JN049883	JF416017
Metarhizium kusanagiense	TNS-F18494	JN049873	JF416014
Metarhizium martiale	HMAS197472	JN049881	JF416015
Metarhizium pseudoatrovirens	TNS-F16380	JN049870	KJ398785
Metarhizium koreanum	ARSEF2038	HM055431	KJ398805
Beauveria bassiana	ARSEF751	AY532045	AY531954

Table 3. ITS and TEF1α gene sequences used for molecular identification of isolates CEP-753 and CEP-754.
Tabla 3. Secuencias de genes ITS y TEF1α utilizadas para la identificación molecular de los aislados CEP-753 y CEP-754.

Species	Strain	ITS	TEF1α
Trichoderma afroharzianum	CEP-753	OP700049	OP792041
Trichoderma afroharzianum	CEP-754	OP709539	OP792042
Trichoderma afroharzianum	GJS04-186/TRS835	FJ442265	KP008787.1
Trichoderma arundinaceum	GJS05-180/MSX70741	EU330928.1	KY630170.1
Trichoderma asperellum	CBS433.97/TRS705	AY380912	KP009011.1
Trichoderma atroviride	693.94 UTHSC08-2443	Z48817	KJ786838.1
Trichoderma bissettii	1158	KJ174235.1	MH249948.1
Trichoderma dorothopsis	HZA8/HZA5	MH624143	MK850827.1
Trichoderma gamsii	GJS04-09/TW20050	DQ315459	KU523895.1
Trichoderma harzianum	CBS226.95/T18	AY605713	KX632606.1
Trichoderma koningiopsis	GJS93-20/18ASMA001	NR_131281	MT671922.1
Trichoderma longibrachiatum	CBS816.68/S328	NR120298	JQ685867.1
Trichoderma ochroleucum	CBS119502/GJS01-265	NR134401	DQ835494.1
Trichoderma pollinicola	LC11682/LC11686	MF939592	MF939620.1
Trichoderma reesei	ATCC26921/QM6a	KU729028	XM006963994
Trichoderma virens	CBS249.59/Tvien3	MH857855	MT081441.1
Trichoderma viride	CBS119327/GJS89-127	DQ677657	AF534585.1
Hypomyces aurantius	GJS74-69/TFC95-171	FJ442642.1	FN868743.1

Results

Isolates with access numbers CEP-722, CEP-723, CEP-724, CEP-725 and CEP-726 for *Metarhizium* spp. (table 4) and CEP-745, CEP-747, CEP-748, CEP-749, CEP-750, CEP-751, CEP-752, CEP-753, CEP-754, CEP-755, CEP-756, CEP-757, CEP -758, CEP-759 and CEP-760 for *Trichoderma* spp. (table 5, page 79) were obtained and admitted to the mycological bank of the "Centro de Estudios Parasitológico y de Vectores" (CEPAVE) (CONICET-UNLP), La Plata, Argentina.

Table 4. *Metarhizium* isolates obtained from soil samples.
Tabla 4. Aislados del género *Metarhizium* obtenidos de las muestras de suelo.

N°	Establishment	Geographic reference	Code	Crop on soil
1	Coop. UTT Jauregui	-34.6204249, -59.1764168	CEP-722	*Beta vulgaris*
2	Coop. UTT Jauregui	-34.6204249, -59.1764168	CEP-723	*Beta vulgaris* var. *cicla*
3	Prod. Bernardo C.	-34.905505, -58.200043	CEP-724	*Cynara cardunculus* var. *scolymus*
4	Prod. Bernardo C.	-34.905505, -58.200043	CEP-725	*Vicia faba*
5	Prod. Bernardo C.	-34.905505, -58.200043	CEP-726	*Brassica rapa* subsp. *rapa*

Table 5. *Trichoderma* isolates obtained from soil samples.
Tabla 5. Aislados del género *Trichoderma* obtenidos de las muestras de suelo.

N°	Establishment	Geographic reference	Code	Crop on soil
1	Coop. UTT Jauregui	-34.6204249, - 59.1764168	CEP-745	*Allium ampeloprasum* var. *porrum*
2	FAUBA	-34.594101, -58.484467	CEP-747	*Solanum lycopersicum*
3	Org. 1610	-34.9046857, -58.2666433	CEP-748	*Brassica oleracea* var. *capitata*
4	Coop. UTT Jauregui	-34.6204249, - 59.1764168	CEP-749	*Lactuca sativa*
5	Sta. Elena Agroecológica	-34.83699, -58.0933384	CEP-750	*Pisum sativum*
6	Sta. Elena Agroecológica	-34.83699, -58.0933384	CEP-751	*Lactuca sativa*
7	FAUBA	-34.594101, -58.484467	CEP-752	*Solanum lycopersicum*
8	M. G. Agroecológico	-3.8672710, -58.4608800	CEP-753	*Lactuca sativa*
9	Org. 1610	-34.9046857, -58.2666433	CEP-754	*Lactuca sativa*
10	M. G. Agroecológico	-3.8672710, -58.4608800	CEP-755	*Zea mays*
11	Org. 1610	-34.9046857, -58.2666433	CEP-756	*Beta vulgaris*
12	M. G. Agroecológico	-3.8672710, -58.4608800	CEP-757	*Ocimum basilicum*
13	Coop. UTT Jauregui	-34.6204249, - 59.1764168	CEP-758	*Beta vulgaris*
14	FAUBA	-34.594101, -58.484467	CEP-759	*Solanum lycopersicum*
15	M. G. Agroecológico	-3.8672710, -58.4608800	CEP-760	*Solanum lycopersicum*

Percentage inhibition of growth of *B. cinerea* and *S. sclerotiorum* caused by fungi of the genera *Trichoderma* and *Metarhizium*

Considering growth speed and physical conditions of the *Petri* dishes, results on dual culture trials are presented for day 4 for *Trichoderma* and day 7 for *Metarhizium*. Percentage growth inhibition of the pathogens stabilized after mycelial contact with the treatments or when an inhibition halo was generated. *Trichoderma* isolates with the highest inhibition percentages at day four of measurement in the dual culture tests against the pathogen *B. cinerea* were CEP-745, CEP-749, CEP-751, CEP-754 and CEP-756, these being 46.04; 51.75; 45.40; 45.40; and 43.18%, respectively. Inhibition percentage of *S. sclerotiorum* in *Petri* dishes on day 4 of measurement reached 55.88; 56.75; 58.25; 52.13; and 51.75% for isolates CEP-749, CEP-752, CEP-754, CEP-755 and CEP-758 respectively, presenting the highest mean values (figure 1, page 80). No significant differences were observed in growth percentage of *B. cinerea* at day seven among the different treatments of spores suspension *Metarhizium* isolates, but the highest mean values were recorded for CEP-722 and CEP-723, being 32.97 and 32.19%, respectively. The CEP-722 isolate presented the highest values in inhibition percentage of *S. sclerotiorum* at day seven, reaching a mean value of 27.34%. This was the only treatment with statistically significant differences concerning treatment CEP-726, which presented the lowest inhibition percentages (23.59%) against S. *sclerotiorum* (figure 2, page 80).

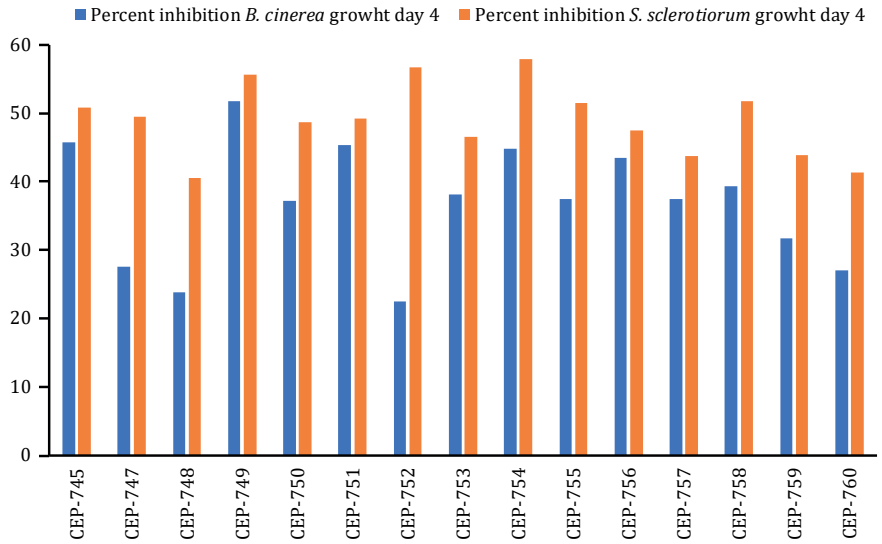


Figure 1. Growth inhibition (%) of *B. cinerea* and S. *sclerotiorum* by isolates of the genus *Trichoderma* at day 4 of measurement.
Figura 1. Medias del porcentaje de inhibición del crecimiento de *B. cinerea* y *S. sclerotiorum* generado por aislados del género *Trichoderma* al día 4 de medición.

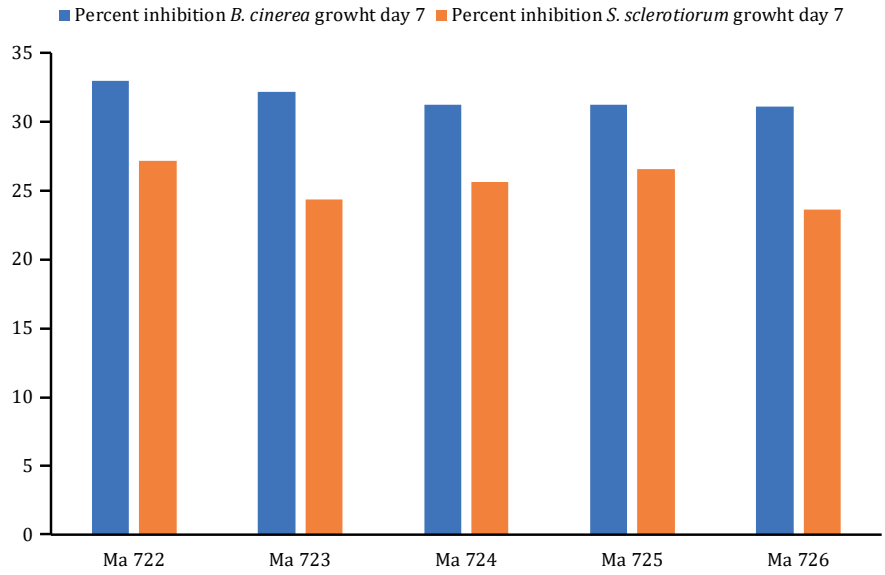


Figure 2. Growth inhibition (%) of *B. cinerea* and S. *sclerotiorum* by isolates of the genus *Metarhizium* at day 7 of measurement.
Figura 2. Medias del porcentaje de inhibición del crecimiento de *B. cinerea* y *S. sclerotiorum* generado por aislados del género *Metarhizium* al día 7 de medición.

Growth promotion of tomato plants (*S. lycopersicum*) var. platense inoculated with six isolates of *Trichoderma* and five isolates of *Metarhizium*

Considering all variables studied in the *Trichoderma* assays, control treatment presented the lowest mean values after application. Regarding stem diameter, the plant inoculated with spore suspension of the isolates CEP-751, CEP-752, CEP-753 and CEP-754 presented higher mean values than the plant inoculated with CEP-745, CEP-749 and the control. Stem length reached the highest mean value (21.37 cm) for the plant inoculated with spore suspension of the isolates CEP-754. Aerial weight mean was highest for the plants inoculated with spore

suspension of CEP-753 and CEP-754, reaching 13.02 and 12.59 g, respectively (figure 3). Stem diameter, stem length and aerial weight of all treatments with *Metarhizium* fungi differed from the control. Regarding stem diameter, treatments inoculated with a spore suspension of the isolates CEP-722, CEP-724 and CEP-726 presented differences from the control, with mean values of 5.71, 5.50 and 5.57 mm respectively. Stem length of tomato plants inoculated with CEP-724 stood out with a mean of 33.54 cm followed by treatments inoculated with CEP-723 and CEP-726, with mean values of 32.84 and 32.44 cm respectively. Regarding aerial weight, the treatment with CEP-726 presented 12.15 g, the highest mean value (figure 4, page 82).

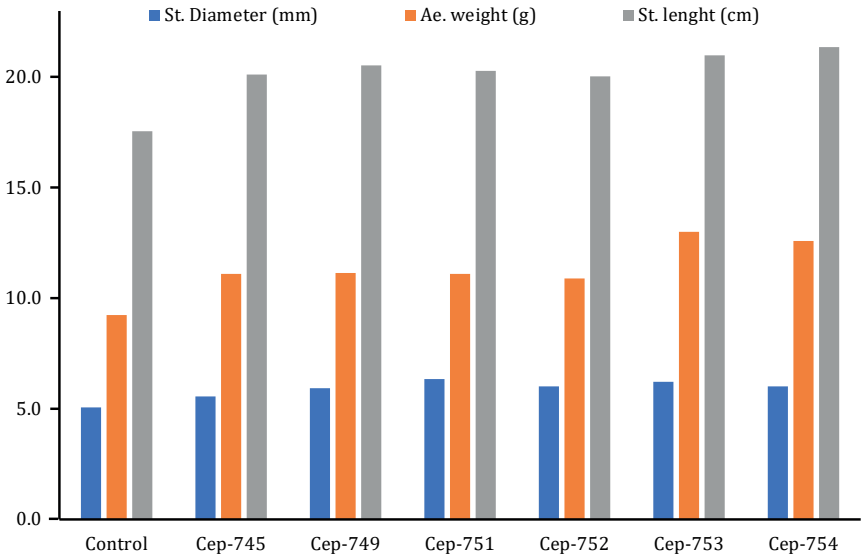


Figure 3. Mean values of stem diameter, aerial weight and stem length of tomato plants (*S. lycopersicum*) inoculated with spore suspensions of *Trichoderma* isolates in growth promotion assays.
Figura 3. Medias registradas en el diámetro de tallo, peso aéreo y longitud de tallo de plantas de tomate (*S. lycopersicum*) inoculadas con suspensiones de esporas de aislados del género *Trichoderma* en los ensayos de promoción del crecimiento.

Morphological characterization, molecular identification and phylogenetic Analysis of the isolates CEP-722, CEP-723, CEP-753 and CEP-754

Figures 5D, 5E and 5F (page 82), show microscopic traits of the isolates CEP-722 and CEP-723. Conidiogenesis occurs in a dense hymenium; conidiophores branch repeatedly at wide angles resembling candelabra; conidiogenous cells are clavate or cylindrical, with a rounded to conical apex, no obvious neck; the apical wall thickens progressively as conidia are produced in long chains, adhering laterally to form prismatic (palisade) columns. The CEP-722 isolate was the only one presenting chlamydospores (figure 5F, page 82). Microscopic measurements and morphological traits of CEP-722 and CEP-723 coincide with *Metarhizium* taxonomic keys (18). Microscopic traits of isolates CEP-753 and CEP-754 are hyaline conidiophores, smooth-walled, up to 5 µm wide near the base, gradually tapering to about 2 µm wide near the apex, with relatively conspicuous septa distant; side branches borne at right angles, singly or in whorls of 2-3, gradually increasing in length. Phialides occur in whorls of 2-5, solitary and alternate, or more irregularly arranged, particularly towards the apex of the conidiophore. Terminals are more elongated and generally not constricted at the base. Conidia are unicellular, diluted green in color, smooth-walled, short cylindrical and almost oblong, with obtusely rounded apex (figures 5B and 5C, page 82). Microscopic measurements and morphological traits of CEP-753 and CEP-754 coincide with *Trichoderma* taxonomic keys (5).

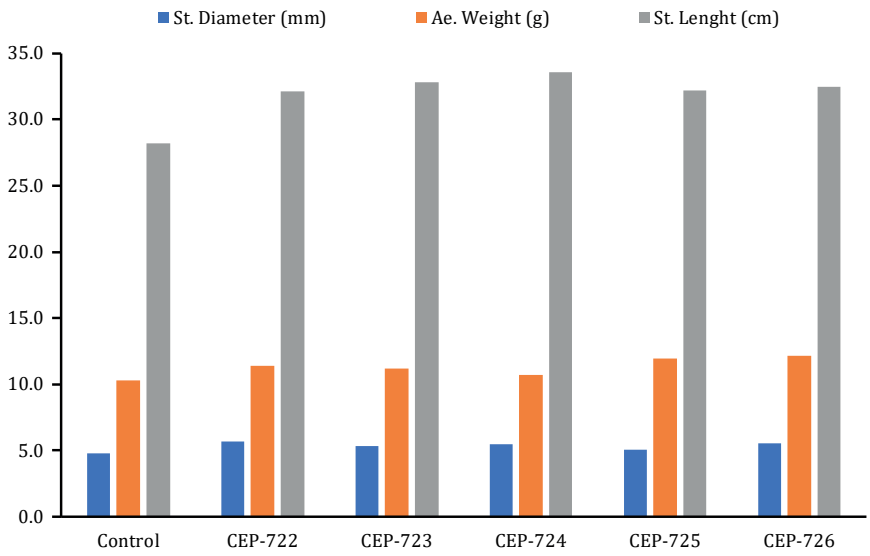


Figure 4. Mean values of stem diameter, aerial weight and stem length of tomato plants (*S. lycopersicum*) inoculated with spore suspensions of *Metarhizium* isolates in growth promotion assays.
Figura 4. Medias registradas en el diámetro de tallo, peso aéreo y longitud de tallo de plantas de tomate (*S. lycopersicum*) inoculadas con suspensiones de esporas de aislados del género *Metarhizium* en los ensayos de promoción del crecimiento.

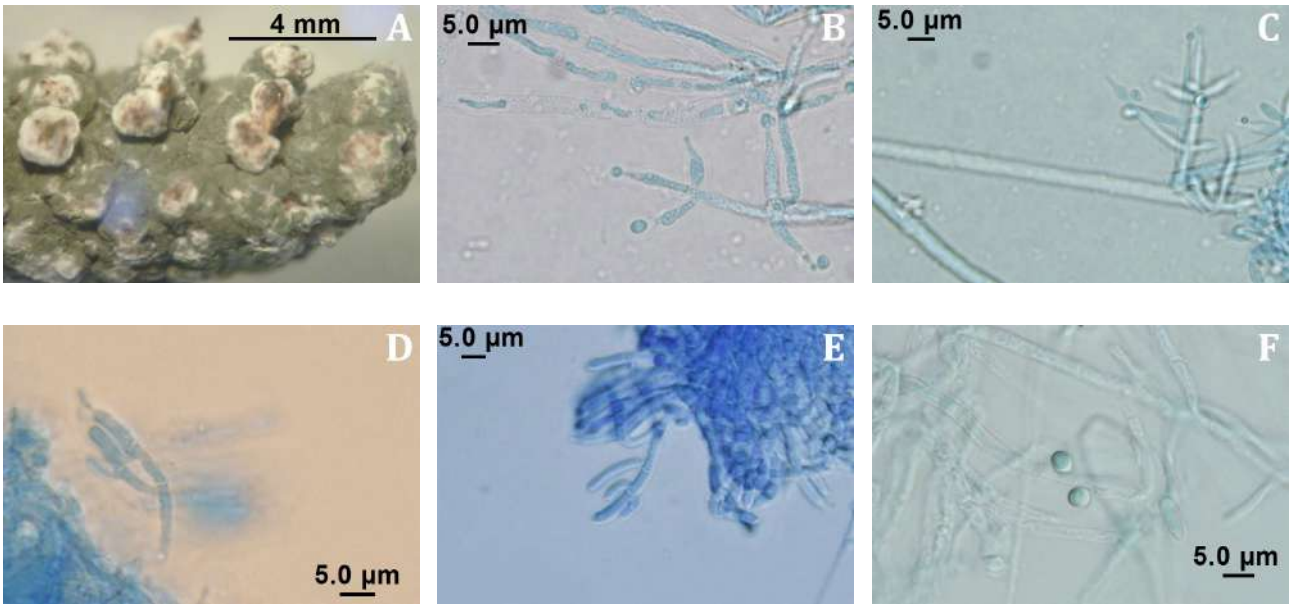


Figure 5. A) Detail of *T. molitor* larva corpse with sporulation of isolate CEP-722; B) Conidiophores, phialides and conidia of isolate CEP-753; C) Conidiophores, phialides and conidia of isolate CEP-754; D) Conidiophores, phialides and conidia of isolate CEP-722, E) Conidiophores, phialides and conidia of isolate CEP- 723; and F) Mycelium, conidia and chlamydospores of the isolate CEP-722.
Figura 5. A) Detalle de cadáver de larva de *T. molitor* con esporulación del aislado CEP-722; B)Conidióforos, fiálides y conidios del aislado CEP-753; C) Conidióforos, fiálides y conidios del aislado CEP-754; D) Conidióforos, fiálides y conidios del aislado CEP-722; E) Conidióforos, fiálides y conidios del aislado CEP-723; y F) Micelio, conidio y clamidosporas del aislado CEP-722.

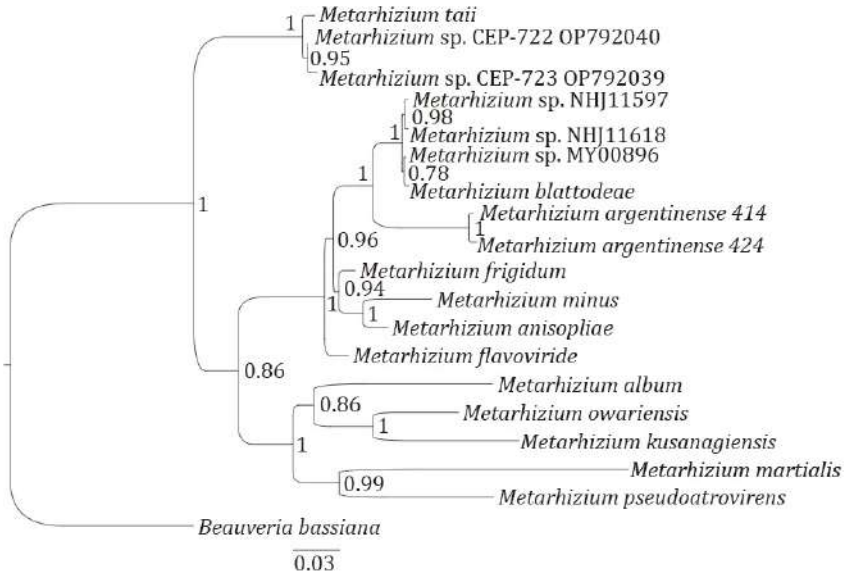
Table 6, shows average measurements of each microstructure.

Isolates CEP-722 and CEP-723 had 100% homology to each other, considering the sequences used for ITS and TEF1α markers of *Metarhizium* spp. Therefore, CEP-722 and CEP-723 correspond to the genus *Metarhizium* and present 0% genetic variability with the species *Metarhizium taii* (Genbank access code ARSEF5714). The taxonomic classification for CEP-722 and CEP-723 with GenBank reference codes ITS: OP709693/TEF1α: OP792040 and ITS: OP709705/TEF1α: OP792039, is Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreales; Clavicipitaceae; *Metarhizium* (Sorokin, 1883): *Metarhizium taii*. Isolates CEP-753 and CEP-754 presented 100% homology to each other considering reference sequences used for ITS and TEF1α markers of *Trichoderma* spp. Phylogenetic analysis shows CEP-753 and CEP-754 correspond to *Trichoderma* and present 0% genetic variability with the species *Trichoderma afroharzianum*, Genbank access code GJS04-186/TRS835. The taxonomic classification for CEP-753 and CEP-754 with reference codesITS:OP700049/TEF1α:OP792041andITS:OP709539/TEF1α:OP792042,respectively, is Fungi; Ascomycota; Euascomycetes; Hypocreales; Hypocraceae; *Trichoderma* and *Hypocrea* (Rifai, 1969): *Trichoderma afroharzianum*. Analytical "runs" of the sequences of selected microorganisms were carried out with MrBayes software (version 3.2.7). Databases were prepared according to published references. Figure 6 and figure 7 (page 84) show the phylogenetic trees for CEP-722 and CEP-723 isolates of *Metarhizium* and for CEP-753 and CEP-754 isolates of *Trichoderma*, respectively.

Table 6. Average measurements of reproductive structures of isolates CEP-722, CEP-723, CEP-753 and CEP-754.

Tabla 6. Promedios de mediciones de estructuras reproductivas de los aislados CEP-722, CEP-723, CEP-753 y CEP-754.

Strain	Average conidium size (length/width) (µm)	Average phialide size (length/width) (µm)	Average diameter of chlamydospore (µm)
CEP-722	6.45/2.83	9.12/2.66	3
CEP-723	6.99/3.07	8.76/2.20	-
CEP-753	3.16/2.58	10.24/2.88	-
CEP-754	3.22/2.52	11.04/2.78	-

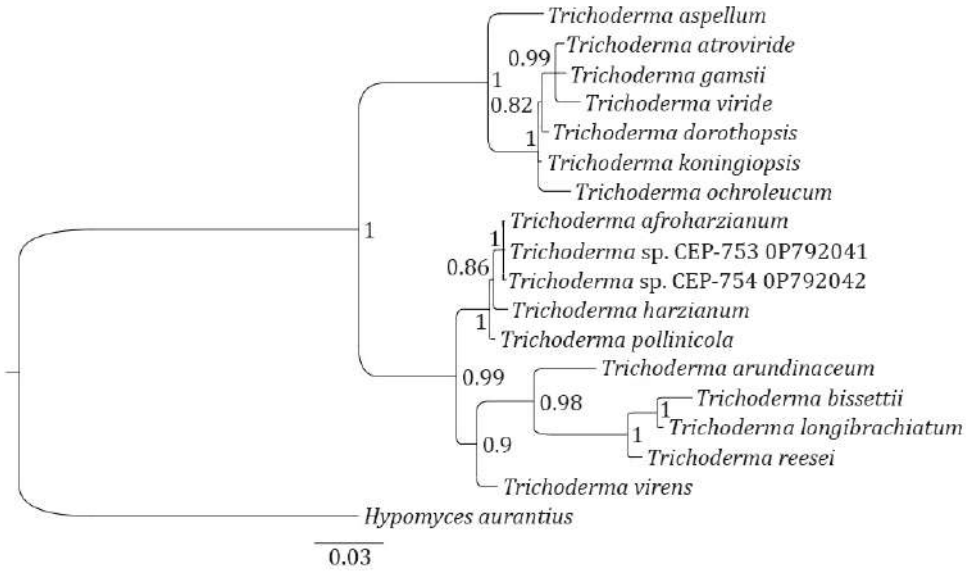


Node values represent maximum likelihood probabilities.

Los valores entre las diagonales representan el valor de probabilidad de máxima verosimilitud.

Figure 6. Phylogenetic tree of ITS and TEF1α regions of CEP-722 and CEP-723 isolates.

Figura 6. Árbol filogenético de las regiones ITS y TEF1α de los aislados CEP-722 y CEP-723.



Tree node values represent maximum likelihood probability values.

Los valores entre las diagonales representan el valor de probabilidad de máxima verosimilitud.

Figure 7. Phylogenetic tree of ITS and TEF1α regions of CEP-753 and CEP-754 isolates.
Figura 7. Árbol filogenético de las regiones ITS y TEF1α de los aislados CEP-753 y CEP-754.

Discussion

Trichoderma spp. and *Metarhizium* spp. isolates evaluated against the phytopathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum* in this study showed varying effects according to strain and isolate, as previously found (16, 34). In our *in vitro* studies, the *Metarhizium taii* strains CEP-722 and CEP-723, and the *Trichoderma afroharzianum* strains CEP-753 and CEP-754 presented different inhibition levels against different phytopathogens. This, because biological control agents of *Trichoderma* use varied mechanisms, like antifungal compounds, competition for nutrients, parasitism or pathogen inhibition, antibiosis, lytic enzymes (23) and systemic resistance (26, 28). Growth promotion of tomato plants (*S. lycopersicum*) inoculated with spore suspensions of *Metahrhizium* and *Trichoderma* fungi constitutes an important background when designing fertilization strategies in the cultivation of tomatoes (*S. lycopersicum*). The selected strains CEP-753 and CEP-754 of *T. afroharzianum* could be considered for nutritional management of crops. Our results agree with previous studies reporting a growth promotion in tomato plants (*S. lycopersicum*) var. platense inoculated with entomopathogenic fungi (33). Strains CEP-722 and CEP-723 of *M. taii* inhibit *B. cinerea* and *S. sclerotiorum*, in agreement with studies on the interaction of entomopathogenic and phytopathogenic microorganisms (11). Employing indigenous microorganisms could be a promising alternative to external inoculants, potentially reducing production costs and without introducing foreign microorganisms into the environment (6).

Conclusion

Metarhizium taii strains CEP-722 and CEP-723 and *Trichoderma afroharzianum* CEP-753 and CEP-754 were best candidates as biological control agents against *Botrytis cinerea* and *Sclerotinia sclerotiorum*. These strains constitute valuable tools for disease management and interesting ingredients for nutritional management of tomato (*S. lycopersicum*).

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First report of the causal agent of vine crown gall in Mendoza, Argentina

Primer reporte del agente causal de la agalla de corona de la vid en Mendoza, Argentina

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Abstract

Crown gall is one widespread grapevine disease worldwide, caused by *Allorhizobium vitis* (syn. *Agrobacterium vitis*) and *Agrobacterium tumefaciens* (syn. *Rhizobium radiobacter*). *All. Vitis*, is the predominant species and primary cause of the disease. This study aimed to identify and characterize molecularly the agrobacteria in plants with crown gall symptoms in Mendoza vineyards. Diseased plants with trunk-based galls were sampled from different areas of Mendoza. Two multiplex PCRs were performed for bacterial identification and characterization, demonstrating that 91.6% of the strains obtained were agrobacteria (77% *A. tumefaciens* and 23% *All. vitis*). Fifty percent of *All. vitis* and 16% of *A. tumefaciens* identified strains were pathogenic. Pathogenicity tests were also conducted on *Kalanchoe daigremontiana*, with resulting tumorigenic symptoms.

Keywords
Allorhizobium • *Agrobacterium* • *Vitis vinifera* • crown gall • Mendoza

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Resumen

Una de las enfermedades de la vid ampliamente distribuida en el mundo es la agalla de corona, que tiene como agente causal a *Allorhizobium vitis* (syn. *Agrobacterium vitis*) y *Agrobacterium tumefaciens* (syn. *Rhizobium radiobacter*), siendo la primera especie la que predomina como agente causal de la enfermedad en vid. El objetivo de este estudio fue identificar mediante técnicas moleculares las agrobacterias patógenas presentes en plantas con síntomas y determinar cuál de ellas predomina en viñedos de la provincia de Mendoza. Plantas de vid con agallas en el tronco provenientes de diversas zonas de la provincia de Mendoza se utilizaron para realizar los aislamientos. Para la identificación y caracterización molecular de los aislados se realizaron dos reacciones múltiples de PCR. Se identificó el 91,6% de las cepas obtenidas como agrobacterias (77% *A. tumefaciens* y 23% *All. vitis*). Se determinó que el 50% del total identificado como *All. vitis* son cepas patógenas, mientras que para *A. tumefaciens* sólo el 16% de los aislados dio patogenicidad positiva. También se realizaron pruebas de patogenicidad en *Kalanchoe daigremontiana*, donde se observó el desarrollo de los síntomas típicos de tumorigénesis.

Palabras clave
Allorhizobium • *Agrobacterium* • *Vitis vinifera* • agalla de corona • Mendoza

Introduction

With 207,047 hectares cultivated with grapevines (*Vitis vinífera*), Argentina leads the international wine industry. The province of Mendoza produces 70% of Argentinian wine (11) and is considered one of the Wine Capitals Worldwide. This industry, including grape growing, wine and must production, and tourism, is fundamental to the economic development of the province.

Various pests and diseases significantly reducing production quantity and quality affect grapevine cultivation. Crown gall, a disease caused by *Allorhizobium vitis* (15) and *Agrobacterium tumefaciens* (14), is among the most important and widespread vine diseases globally. These bacteria were first isolated in the United States in 1907 and were later reported in China, Japan, South Africa, and some countries in Europe and South America (4).

In grapevine, *All. vitis* is the predominant species causing the disease, while *A. tumefaciens* is found less frequently and in smaller proportions. *A. tumefaciens* is polyphagous and can affect several dicotyledon species, including *Solanaceae* and various *Asteraceae* (4, 6). Currently, rrs analysis and constitutive genes have described new species of *Agrobacterium* initially identified as *A. tumefaciens* in various hosts (7).

A. tumefaciens and *All. vitis* exist in nature as pathogenic and non-pathogenic strains. Pathogenic strains contain a non-essential tumor-inducing plasmid (pTi) involved in disease triggering (16). *All. vitis* genomic organization is characterized by two circular chromosomes. The smaller, chromosome II later classified as a chromid, is essential for disease development. *A. tumefaciens* carries one circular chromosome and a secondary linear chromid (16).

Typically, the process begins with a wound in the trunk or roots. The wound releases chemical signals that, perceived by bacteria, induce virulence (2). The disease is triggered when certain genes from the Ti plasmid are transferred to the host genome, encoding overexpression of phytohormone synthesis. This overexpression augments cell division (hyperplasia) and cell size (hypertrophy), leading to the characteristic tumor. This plasmid also contains genes encoding opine synthesis. Opines are low-molecular-weight compounds, used by agrobacteria as carbon and nitrogen source (18). According to Kuzmanović *et al.* (2020). Ti plasmids are classified into three major groups: octopine, nopaline and vitopine. Genes coding for octopine are present in 60% of the strains. About 30% of strains carry the *nos* genes (nopaline synthase), and only 10% of strains have vitopine type (4).

The development of one or more tumors around a diseased organ alters sap movement, causing chlorosis, vigor loss and decreased production. In extreme cases, it may lead to plant death, including nursery young plants or cuttings (4).

Several chromosomal genes aid in accurate identifications of pathogenic agrobacteria species. Plasmid genes determine the presence of pathogenicity-related oncogenes. Due to the importance of viticulture in Mendoza, this study aimed to define the main molecular traits of the causal agent of crown gall identifying pathogenic species. We finally aimed to determine the predominant species in Mendoza vineyards.

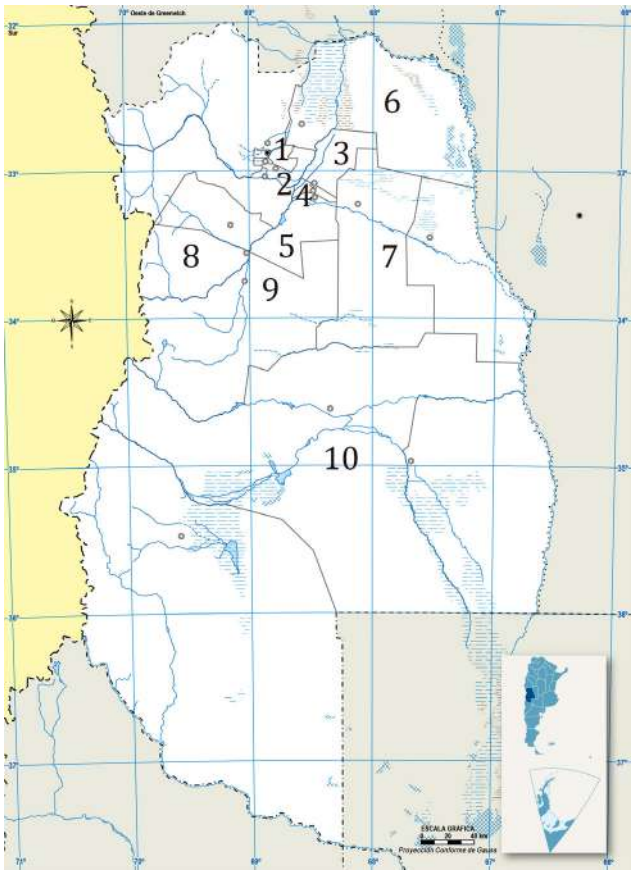
Materials and methods

Plant samples and Bacterial strains

One hundred and forty-eight symptomatic plants (figure 1) were collected from various vine-growing areas of Mendoza (figure 2). Composite samples were taken from plants within the same vineyard, resulting in 86 samples to be analyzed (table 1, page 90-91).

Figure 1. Symptoms of crown gall on Mendoza grapevines.

Figura 1. Síntomas de agalla de corona en vides de Mendoza.



1- Guaymallén
2- Maipú
3- San Martín
4- Junín
5- Rivadavia
6- Lavalle
7- Santa Rosa
8- Tunuyán
9- San Carlos
10- San Rafael

Figure 2. Mendoza, sampled departments.

Figura 2. Mendoza, departamentos muestreados.

Table 1. Strain identification, geographical origin, plant age, cultivar, number of plants, number of analyzed samples, isolation.

Tabla 1. Identificación de la cepa, origen geográfico, edad de las plantas, cultivar, número de plantas, número de muestras analizadas y aislamiento.

Strain	Geographical Origin	Age	Cultivar	Plants	Samples	Isolation
A	San Martín	2	Malbec	1	1	+
B	San Martín	8	Tannat	1	1	+
D	San Martín	4	Cereza	1	1	+
E	San Martín	2	Tannat	1	1	+
F	Guaymallén	2	Cereza	1	1	+
G	Lavalle	4	Aspirant Bouschet	1	1	+
I	San Carlos	3	Malbec	1	1	
J	Junín	3	Sauvignon Blanc	1	1	+
L	San Martín	3	Malbec	1	1	+
M	Rivadavia	2	Aspirant Bouschet	1	1	+
N	Tunuyán	2	Malbec	1	1	
Q	Rivadavia	3	Cereza	1	1	+
R	Junín	UN	UN	1	1	+
S	San Rafael	UN	UN	2	1	+
T	San Carlos	UN	UN	1	1	+
U	San Carlos	UN	UN	1	1	+
V	Tunuyán	6	Tempranillo	1	1	+
W	Tunuyán	2	Malbec	1	1	+
X	San Carlos	UN	Sauvignon Blanc	2	1	+
10	San Carlos	4	Torrontés riojano	1	1	+
19	San Carlos	2	Torrontés riojano	1	1	
21	Maipú	1	Malbec	2	1	+
23	Maipú	1	Malbec	3	1	+
37	Santa Rosa	UN	Bonarda	4	2	+
51	San Rafael	UN	Malbec	2	2	+
52	San Martín	3	Torrontés riojano	2	0	
53	San Rafael	2	Torrontés riojano	2	0	
54	San Carlos	UN	Tempranillo	3	1	+
58	San Carlos	10	Malbec	3	1	+
64	Maipú	UN	UN	5	0	
65	San Rafael	UN	Syrah	1	1	+
87	Rivadavia	4	Cereza	9	3	+
90	San Martín	3	Tempranillo	1	0	
92	San Martín	UN	Cereza/Torrontés	2	2	+
111	San Martín	UN	Cereza	5	1	+
113	Lavalle	UN	Bonarda	1	1	+
115	San Martín	UN	Cereza	3	1	+
145	Maipú	12	Syrah	1	0	
148	Maipú	1	Malbec	3	1	+
153	Tunuyán	15	Aspirant Bouschet	1	1	+
163	Santa Rosa	1	Malbec	10	1	+
165	Junín	3	Cereza	2	1	+
201A	San Martín	17	Syrah	2	1	+
201B	San Martín	6	Cereza	3	1	+
Sant2	San Martín	6	UN	1	1	+
Sant3	San Martín	6	UN	1	1	+
Sant4	San Martín	6	UN	1	1	+
Sant5	San Martín	6	UN	1	1	+
219	San Rafael	UN	UN	1	1	+
275	San Martín	1	Chardonnay	7	1	+
295	San Martín	1	Cabernet franc	8	1	+
A1	Lavalle	5	Cereza	1	1	+
A2	Lavalle	5	Cereza	1	1	+

UN: unknown/desconocido.

Strain	Geographical Origin	Age	Cultivar	Plants	Samples	Isolation
A3	Lavalle	5	Cereza	1	1	+
A4	Lavalle	5	Cereza	1	1	+
A5	Lavalle	5	Cereza	1	1	+
A6	Lavalle	5	Cereza	1	1	+
A7	Lavalle	5	Cereza	1	1	+
A8	Lavalle	5	Cereza	1	1	+
A9	Lavalle	5	Cereza	1	1	+
A10	Lavalle	5	Cereza	1	1	+
A11	Lavalle	5	Cereza	1	1	+
A12	Lavalle	5	Cereza	1	1	+
A13	Lavalle	5	Cereza	1	1	+
A14	Lavalle	5	Cereza	1	1	
L1	Lavalle	5	Cereza	1	1	
L4	Lavalle	5	Cereza	1	1	+
L5	Lavalle	5	Cereza	1	1	
L5b	Lavalle	5	Cereza	1	1	
L6	Lavalle	5	Cereza	1	1	
L7	Lavalle	5	Cereza	1	1	+
L8	Lavalle	5	Cereza	1	1	
L9	Lavalle	5	Cereza	1	1	+
L10	Lavalle	5	Cereza	1	1	
L11	Lavalle	5	Cereza	1	1	+
L12	Lavalle	5	Cereza	1	1	+
L13	Lavalle	5	Cereza	1	1	
L14	Lavalle	5	Cereza	1	1	
L15	Lavalle	5	Cereza	1	1	+
L16	Lavalle	5	Cereza	1	1	+
L17	Lavalle	5	Cereza	1	1	+
L21	Lavalle	5	Cereza	1	1	+
L22	Lavalle	5	Cereza	1	1	+
L23	Lavalle	5	Cereza	1	1	+
L25	Lavalle	5	Cereza	1	1	+
L26	Lavalle	5	Cereza	1	1	+

UN: unknown/desconocido.

Galls were washed with running water. Subsequently, they were disinfected with 1.1% sodium hypochlorite for 5 minutes in a laminar flow cabinet. After disinfection, they were rinsed twice with sterile distilled water, completely removing sodium hypochlorite. Galls were then cut into small pieces, discarding the external part to minimize contaminating microorganisms. The resulting pieces were placed in 5 ml sterile distilled water for one hour allowing diffusion of bacteria in the sample.

Each bacterial suspension was streaked onto Roy and Sasser (RS) semiselective culture medium for *All. vitis* and Schroth culture medium for *A. tumefaciens.* Culture plates were incubated at 27 °C in darkness. Colony development was observed after seven days. *All. vitis* colonies on RS medium had a dark red center with transparent or white edges. The red center is not always evident (Schaad *et al*., 2001 and Burr, T. J. personal communication, June 28, 2016). *A. tumefaciens* colonies acquire a reddish color in Schroth culture medium. Colonies with these characteristics were then transferred to Luria Bertrani (LB) culture medium.

DNA extraction and specific PCR amplification

DNA was extracted according to Khlaif, H. and Al-Karablieh (2002). *All. vitis* and *A. tumefaciens*, were differentiated according to differences in the 23S rDNA gene (20). A universal forward and two specific reverse primers were used: B1R for *A. tumefaciens* and AvR for *All. vitis* (table 2, page 92).

Table 2. Primers for identification and molecular characterization of *All. vitis* and *A. tumefaciens* strains.

Tabla 2. *Primers* usados para identificación y caracterización molecular de cepas de *All. vitis y A. tumefaciens*.

Primers	Sequence 5'-3'	Length of the Amplified Fragment	Gene	Reference
UF/B1R	GTAAGAAGCGAACGCAGGGAACT / GACAATGACTGTTCTACGCGTAA	184 bp	23S ADNr	(20)
UF/AvR	GTAAGAAGCGAACGCAGGGAACT / AACTAACTCAATCGCGCTATTAAC	478 bp	23S ADNr	(20)
iaaHF2/ iaaHR1	ACATGCATGAGTTATCGTTTGGAAT/ GCATCAAGGTCATCGTAAAAGTAGGT	420 bp	*iaaH* gene of *A. tumefaciens* and *All. vitis octopine and nopaline strains*	(3)
S4iaaM5/ S4iaaM3	CGCGTCCCCGTTTACACTA/ CGAGATCGCGCTTCAAGAT	800 bp	*iaaM* gene of *All. vitis* vitopine type	(3)

Multiplex PCR (polymerase chain reaction) used the following reagents: 1X PCR buffer, 1.5 mM MgCl2, 200 mM dNTP, 1 mM of each primer and 1U of Recombinant DNA polymerase (Invitrogen) and 5 µl of template DNA for a final reaction volume of 25 µl. The PCR consisted of initial denaturation at 94°C 1 min, 35 cycles at 94°C 1 min, 67°C 1 min, 72°C 1.5 min and 72°C 10 min, using an Eppendorf thermocycler.

Multiplex PCR with specific primers for oncogenes allowed for pathogenic strain detection. The reaction combined the primers iaaHF2/iaaHR1 and S4iaaM5/S4iaaM3 for the auxin-biosynthesis genes iaaH and iaaM, respectively. The reaction was carried out in a final volume of 25 µl, with 1X Buffer, 1.5 mM MgCl2, 0.5 µM of each primer, 200 µM of dNTP, 1.25 U of polymerase (Invitrogen Platinum DNA polymerase) and 1 µl of DNA. Amplification began with initial denaturation at 94°C for 1 min, followed by 30 cycles at 92°C 1 min, 54°C 1 min, 72°C 1.5 min and 72°C 3 min (3).

PCR-generated amplicons were detected by electrophoresis using 1% agarose gel, run at 90 volts for 1 hour and stained with ethidium bromide. The gels were visualized under UV light and photo-documented using Bio-Rad equipment and Quantity One software. Band size was compared with a 100 bp ladder molecular marker (Invitrogen).

Pathogenicity tests

PCR results were confirmed via biological tests performed on *Kalanchoe daigremontiana* plants to evaluate isolate-pathogenicity. Inoculation was carried out through punctures on the stem with a micropipette tip and 2.5 µl of bacterial suspension 10^9 cfu/ml of each strain. Each strain was inoculated in three plants via 5 stem wounds per plant. Sterile distilled water was the negative control and *All. vitis* and *A. tumefaciens* reference strains were positive controls.

The plants were kept in the laboratory at room temperature, and covered with plastic bags to maintain approximately 90 % humidity for three days. Then, bags were removed and plants were taken to the greenhouse. Observations were made every 15 days for two months (23). Isolations from tissues developed in the inoculation zone were carried out in a semiselective culture medium, using the same method as with vine galls.

Results

Molecular analysis

Sixty-nine isolates out of 86 samples analyzed resulted in 91.6% identified as agrobacteria, among which 77% were *A. tumefaciens* and 23%. *All. vitis*. Figure 3 presents a PCR with 3 isolates where A1 was *A. tumefaciens* and A2 and A3 were *All. vitis*. The multiplex PCR was performed with the combination of primers iaaHF2/iaaHR1, while S4iaaM5/S4iaaM3 determined pathogenicity. The iaaH gene was only amplified on 16% of *A. tumefaciens* strains, molecularly identified as pathogenic, and 50% of *All. vitis* isolates proved to be pathogenic. The iaaM gene did not amplify. Figure 4 shows amplification of the pathogenicity gene present in both species. Isolates A1 of *A. tumefaciens* and A2 and A3 of *All. vitis* present positive pathogenicity.

From left to right: M: marker 100 bp (PROMEGA); Bco: water; At: *A. tumefaciens* reference strain; Av: *All. vitis* reference strain.; gall isolates: A1, A2, A3.

De izquierda a derecha: M: marcador 100 pb (PROMEGA); Blanco: agua; At: cepa de referencia de *A. tumefaciens*; Av: cepa de referencia de *All. vitis*; aislados de agalla: A1, A2, A3.

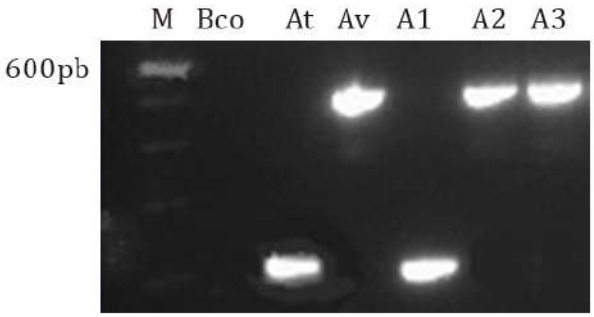


Figure 3. Multiplex PCR with primer pairs UF/B1R (184 bp) and UF/AvR (478 bp).

Figura 3. PCR múltiple con los pares de *primers* UF/B1R (184 pb) y UF/AvR (478 pb).

From left to right: M: 100bp marker (Promega), Bco: water; At: *A. tumefaciens* reference strain; Av: *All. vitis* reference strain; gall isolates: A1, A2 and A3.

De izquierda a derecha: M: marcador 100 pb (Promega), Blanco: agua; At: cepa de referencia de *A. tumefaciens*; Av: cepa de referencia de *All. vitis;* aislados de agalla: A1, A2 y A3.

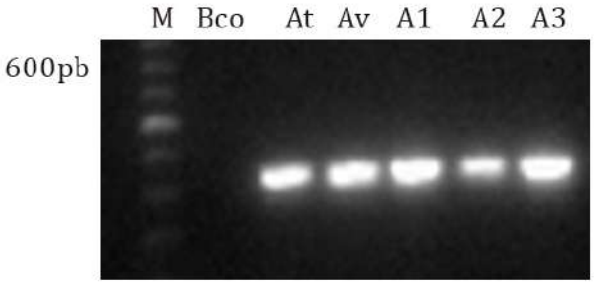
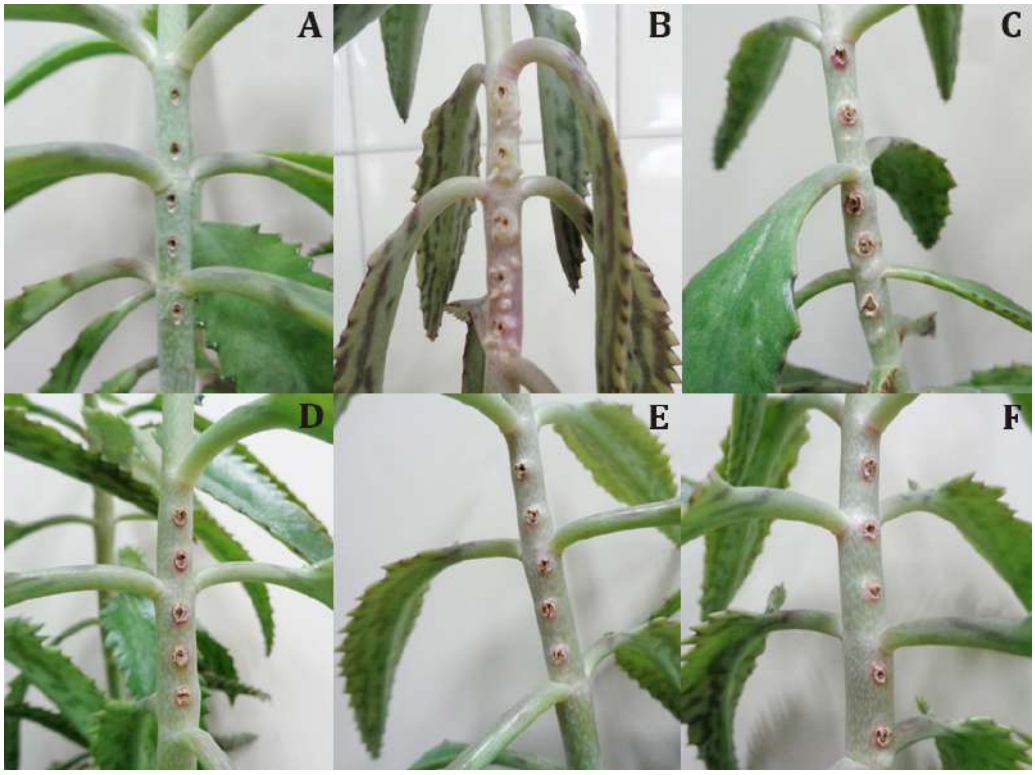


Figure 4. Multiplex PCR with primer pairs iaaHF/iaaHR (420 bp) and S4iaaM5/S4iaaM3 (800 bp).

Figura 4. PCR múltiple con los pares de *primers* iaaHF/iaaHR (420 pb) y S4iaaM5/S4iaaM3 (800 pb).

Pathogenicity Test

Two weeks after inoculation, positive results were observed in *Kalanchoe* plants. Abnormal growth and color change (redness) were similar to those in plants inoculated with reference strains. In some cases, corky tissue developed at the inoculation site (figure 5). These results became more pronounced two months after inoculation.



A: Negative control (water), B: reference strain of *A. tumefaciens*, C: reference strain *of All. vitis*, D, E and F: gall isolates: A1, A2 and A3 with positive pathogenicity result (molecular analysis).

A: Control negativo (agua), B: cepa de referencia de *A. tumefaciens,* C: cepa de referencia de *All. vitis,* D, E y F: aislado muestra con resultado de patogenicidad (análisis molecular) positivo.

Figure 5. Symptoms observed in *Kalanchoe* stems 2 weeks after inoculation.

Figura 5. Síntomas observados en tallos de Kalanchoe a las 2 semanas de la inoculación.

Bacteria isolated from inoculated plants developed typical agrobacteria colonies on semiselective culture medium, fulfilling Koch's postulates. These results align with those obtained through molecular analysis. In 79% of cases, pathogenic isolates by PCR also tested positive in the biological test.

Discussion

Bacterial genetic diversity of both species limits detection efficiency in grapevines (3). *All. vitis* strains are genetically diverse (4, 8, 9, 16, 23). Our data suggest *All. vitis* could be a species complex comprising several genomic species (16). In this study, after obtaining pure and simple isolates, successful species identification followed the molecular protocol described by Pulawska *et al*. (2006). However, since this PCR does not identify pathogenicity genes, the analysis must be complemented with additional PCR determining gene presence (1, 3, 8, 17, 19, 22). This research used specific primers iaaHF2/iaaHR1 and S4iaaM5/S4iaaM3 for iaaH and iaaM genes, respectively, showing non-pathogenic *A. tumefaciens* strains predominated over *All. vitis* strains in the analyzed grapevine samples. However, 50% of *All. vitis* isolates were pathogenic. This finding indicates that *All. vitis* is the predominant pathogenic species and main disease cause in grapevines studied in Mendoza, in agreement with prior studies (2, 5, 10, 14, 22).

The same PCR determining pathogenicity, determined opine type. We found absent vitopina type in all *All*. *vitis* isolates and presence of the octopine/nopaline types in Mendoza,

Seventy-nine percent of pathogenicity tests in inoculated *Kalanchoe* showed disease symptoms. This value is within the expected range (78-94%) (21). These data also align with Kuzmanović *et al.* (2016), who observed that some strains did not demonstrate their tumorigenic capacity in inoculated plants despite possessing pathogenicity-associated genes molecularly identified. This suggests that such isolates remain potentially tumorigenic. However, pathogenicity is influenced by plant age and environmental conditions. Absent Crown gall symptoms do not imply absent tumorigenesis genes (18), probably because no single host is infected by more than 81% of pathogenic strains and not all strains produce tumors in every host (13). According to Lamovšek *et al*. (2014), determining pathogenicity through molecular tests might replace biological tests. The PCR are less time-consuming and labor-intensive. However, given the occurrence of false negatives, pathogenicity tests remain a valuable tool in plant bacteriology.

Conclusions

This study successfully identified and characterized the causal agents of Crown gall in Mendoza vineyards using molecular methods. Our methodology enables the characterization of agrobacteria in Argentina and provides a quick and precise diagnostic tool, even for evaluating asexually propagated grapevines.

This information will help develop management strategies to reduce disease spread and incidence in our vineyards and nurseries and improve the health and productivity of their vineyards.

Finally, our results aiding bacterial identification in plant material allow for protocols to detect bacteria in asymptomatic material ensuring propagation of healthy plants from health-controlled material.

To the best of our knowledge, this is the first study identifying uncited crown gall species in Argentina.

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Laboratory evaluation of the feeding behavior of the generalist predatory mirid bug *Tupiocoris cucurbitaceus* (Hemiptera: Miridae) for the biological control of *Phthorimaea absoluta* (Lepidoptera: Gelechiidae)

Evaluación en laboratorio del comportamiento alimenticio del mírido depredador generalista *Tupiocoris cucurbitaceus* (Hemiptera: Miridae) para el control biológico de *Phthorimaea absoluta* (Lepidoptera: Gelechiidae)

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Abstract

The use of predatory insects has gained interest for reliable and environmentally safe pest management to control the South American tomato leafminer, *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae), a pest of tomato crops worldwide. Based on video tracking using EthoVision® software and static feeding multiple-choice tests, we report the prey-searching behavior and feeding preference of the Neotropical mirid bug *Tupiocoris cucurbitaceus* Spinola (Hemiptera: Miridae), a biological control agent of *P. absoluta* when presented with its eggs and other two prey species. *T. cucurbitaceus* exhibits generalist feeding behavior; the nymphs initially showed a preference for *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae) nymphs but consumed more *P. absoluta* and *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs after 24 h. *T. cucurbitaceus* males preferred *T. vaporariorum* throughout the experiment while females showed no preference for any prey. Furthermore, they did not cause significant damage to the leaves. The findings emphasize the importance of evaluating the simultaneous offer of multiple prey types to understand the effectiveness of biocontrol agents in the field. Overall, the research contributes valuable insights into the feeding habits of *T. cucurbitaceus*, supporting its potential as a biological control agent for *P. absoluta* in tomato crops.

Keywords
diet breadth • mirid bug • *Trialeurodes vaporariorum* • *Ephestia kuehniella* • biocontrol

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Resumen

El uso de insectos depredadores ha ganado interés para el manejo confiable y ambientalmente seguro de la polilla del tomate, *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae), una plaga de este cultivo en todo el mundo. Por medio de un estudio con el programa EthoVision® y de ensayos de opción múltiple, reportamos el comportamiento de búsqueda de presas y la preferencia alimentaria del mírido neotropical *Tupiocoris cucurbitaceus* Spinola (Hemiptera: Miridae), un agente de control biológico de *P. absoluta*, cuando se le presentan huevos de la plaga y simultáneamente otras dos especies de presas. *T. cucurbitaceus* exhibe un comportamiento alimentario generalista. Las ninfas del depredador mostraron inicialmente preferencia por las ninfas de *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) pero consumieron más huevos de *P. absoluta* y *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) al cabo de 24 h. Los machos de *T. cucurbitaceus* prefirieron a *T. vaporariorum* mientras que las hembras no mostraron preferencia por ningún tipo de presa. Además, los individuos no causaron daños directos a las hojas. Los hallazgos enfatizan la importancia de evaluar la oferta de diferentes presas para conocer la efectividad de los agentes de control biológico en el campo. La investigación aporta valiosos conocimientos sobre los hábitos alimentarios de *T. cucurbitaceus*, respaldando su potencial como agente de control biológico para *P. absoluta*.

Palabras clave

amplitud de dieta • chinche depredadora • *Trialeurodes vaporariorum* • *Ephestia kuehniella* • biocontrol

Introduction

The use of generalist arthropod predators for biological control has historically received less attention compared to parasitoids and entomopathogens, given assumed negative effects. Among those effects, the most reported are omnivory, attack of non-target species, competition, and intraguild predation on other natural enemy species present in crops (21, 32). However, important biocontrol successes have been achieved with mite and hemipteran predators since they can feed on a variety of prey and plant resources, ensuring their survival and reproduction and enhancing their establishment (39, 42). For instance, in Spain, biocontrol against the sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), is accomplished by releasing predatory mirid bugs and mites in protected sweet pepper, *Capsicum annuum* L. (Solanales: Solanaceae) crops (9, 33).

A crucial aspect when planning the use of generalist predators as biocontrol agents is to determine the range of species on which they effectively feed, *i.e.*, the diet breadth. Although most predators usually feed on various species, they may exhibit a degree of acceptance for different prey based on certain characteristics, such as nutritional quality and stage of development, which ultimately can affect their success as biocontrol agents (16). Two life traits especially defined to assess diet breadth of a predatory species are food-searching behavior and preference. The former implies predator engagement in the activity of prospecting in the environment, the recognition and acceptance of some prey. Factors such as developmental stage, sex, age, starvation, and available prey species influence food-searching behavior. Preference for food is determined by different prey's morphological, physiological, and behavioral traits to obtain enough nutrients and avoid toxic or indigestible food (3, 14, 31).

Miridae bugs (Hemiptera) are important predatory insect species. Most of them show high preying rates and are capable of finding and colonizing new habitats due to their dispersing capacity (27). Approximately 20 species are currently commercialized worldwide as biocontrol agents against whiteflies, eggs and small larvae of lepidopterans, among other horticultural pests (41). Particularly for the biological control of the South American tomato pinworm, *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae), a Neotropical pest that has invaded the European, African, and Asian continents, several mirid species are main predatory agents (4, 7, 9, 10, 19, 23, 28, 38, 40).

Tupiocoris cucurbitaceus Spinola (Hemiptera: Miridae) has been reported in the Americas preying mainly on whiteflies (17, 29). High predation rates and pest kill rates were later examined for this predatory bug over a range of prey species: the whiteflies *B. tabaci* and *Trialeurodes vaporarioum* (Westwood) (Hemiptera: Aleyrodidae), the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), and eggs of three lepidopteran species, the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), the Angoumois grain moth, *Sitrotroga cerealella* Olivier and *P. absoluta* (Lepidoptera: Gelechiidae) (8, 22, 23, 44). Besides, *E. kuehniella* eggs are also applied under a prey-enrichment technique to allow the establishment of mirids, as for the use of *N. tenuis* to control *P. absoluta* in Spain (40, 41). *T. cucurbitaceus* has been recently developed as a commercial biocontrol agent in Argentina and Uruguay against whiteflies and *P. absoluta.* To improve its establishment, releases are performed along with *E. kuehniella* eggs as supplementary food (1, 18, 35). In this research, we aimed to explore *T. cucurbitaceus* prey-searching behavior and feeding preference when offered simultaneously *P. absoluta* and *E. kuehniella* eggs, and *T. vaporariorum* nymphs. This information could help better assess the performance of this generalist predatory mirid in tomato crops as biocontrol agent of *P. absoluta*.

Materials and methods

Plant and insect materials

Tomato plants, *Solanum lycopersicon* L. (Solanales: Solanaceae), Elpida variety Enza Zaden, The Netherlands, were cultivated at the Centro de Estudios Parasitológicos y de Vectores (CEPAVE, CONICET-UNLP-Asociado CICPBA). Tomato seedlings were individually transplanted to 1 l-plastic pot, watered daily, and kept free of insect pests by protecting them inside 60 x 40 x 40 cm (length x width x height) white voile cages (BioQuip Inc., USA). Plants used in *P. absoluta*, *T. cucurbitaceus*, and *T. vaporariorum* colonies and experiments had 4-5 expanded leaves.

Tupiocoris cucurbitaceus colony was initiated with individuals collected from organic tomato crops in farms located near the La Plata Horticultural Belt (N Buenos Aires province, Argentina). Insects were maintained in a controlled environment walk-in rearing room at 25 ± 2°C, 60 ± 10% RH, 14:10 L:D. Identifications of *T. cucurbitaceus* were confirmed by taxonomists at La Plata Museum (Entomology Department, School of Natural Sciences and Museum, National University of La Plata). To obtain individuals of *T. cucurbitaceus* of known age for the tests, 30 cohorts were reared in a white voile cage along with a potted tomato plant (as described above). *Ephestia kuehniella* eggs provided by Brometán SRL (Argentina) and commercial bee pollen were spread *ad libitum* on the leaves as food, and distiller water was provided in soaked sponge pieces inside containers. Oviposition on leaves was allowed for 24 h, and later the plant was placed in a new cage to start a cohort and replaced with a fresh one. Once nymphs emerged, they were fed as adults until they reached the developmental stages needed for the trials (4-5th instar nymphs or adults) (6). To obtain *P. absoluta* eggs, a colony was initiated by maintaining bouquets of tomato leaves infested with moth larvae collected from the field placed in white voile cages. Once pupae were formed, they were transferred to a new cage until moths emerged. Adults were provided with a honey solution (70%) and allowed to mate. Potted tomato plants were offered daily as oviposition substrate and those replaced were held in clean voile cages (30). A uniform cohort of 24 h-old eggs was used in the experiments. *T. vaporariorum* nymphs used in the feeding preference experiment were obtained from adults captured in tomato crops using a manual aspirator and transported to the laboratory for identification (26). Then, they were released in white voile cages and provided with potted tomato plants, to lay eggs (37). Once they hatched, development was checked and after 15 days revised every 24 h to collect late instar nymphs for the assays.

Prey searching behavior assay

To assess *T. cucurbitaceus* food searching capacity, a three-treatment experiment was set up, considering developmental stage and sex: 1) late (4-5th instars) nymph, 2) 7-d adult coupled female, and 3) 7-d adult coupled male. Individuals were isolated from the cohort and kept starved for 24 h before performing the assays in plastic Petri dishes (diameter: 5 cm, height: 1 cm), provided with small, moistened cotton pieces as water sources.

The experimental unit consisted of a plastic container (diameter: 9 cm, height: 5 cm) with a tomato leaflet on 1-cm layer of water agar (1%) to maintain its turgidity. Three different prey patches were offered simultaneously to one *T. cucurbitaceus* individual: the target patch (TP) containing 30 *P. absoluta* eggs (24 h old) and two non-target patches (NTP) with 30 *E. kuehniella* eggs and 5 *T. vaporariorum* nymphs (late instar), respectively (figure 1). Since we were interested in determining the predatory action of *T. cucurbitaceus* on *P. absoluta*, we considered this species as a TP while the other prey species were treated as NTP. To avoid food depletion during the experiment, the quantity of *P. absoluta* and *E. kuehniella* eggs offered was estimated based on previous diet reports by Burla *et al.* (2014), López *et al.* (2019) and Duarte *et al.* (2022) for *T. cucurbitaceus*, *Macrolophus basicornis* (Stål) and *Engytatus varians* (Distant) (Hemiptera: Miridae), respectively. The number of *T. vaporariorum* nymphs used in the experiment was based on *T. cucurbitaceus* food uptake registered by Burla *et al.* (2014). Patches of prey were carefully deposited on the leaflet using a fine brush with the aid of a stereoscope microscope and placed at equidistant points. The quality of all prey items was checked before starting the trial to discard collapsed eggs or dead whiteflies. Each treatment was replicated 15 times and experimental units were not re-utilized.

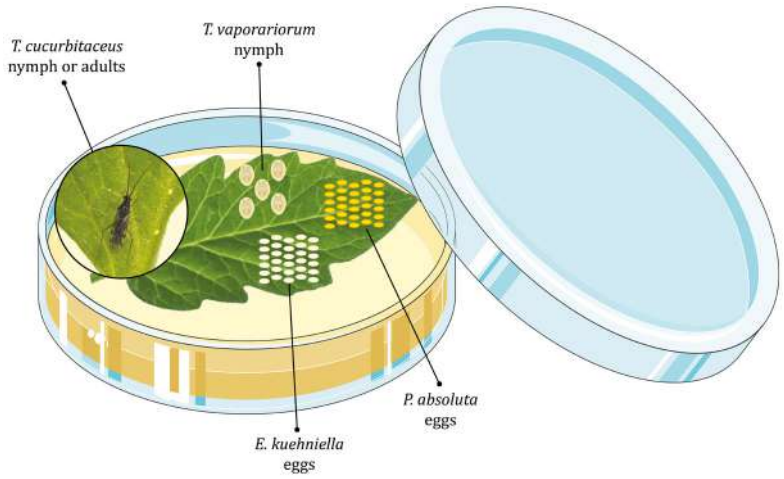

Figure 1. Assembly of the experimental unit to analyze the predation behavior of *Tupiocoris cucurbitaceus* on three types of prey exposed in patches on a fresh tomato leaflet and maintained on a layer of agar-water.

Figura 1. Esquema de la unidad experimental para analizar el comportamiento de depredación de *Tupiocoris cucurbitaceus* sobre tres tipos de presas expuestas en parches sobre un folíolo fresco de tomate y mantenidos con una capa de agar-agua.

Prey searching behavior was studied for 30 min using the software EthoVision® XT (Noldus, The Netherlands) which videotapes and analyzes animal activity inside an arena. Various steps were followed to calibrate the recording of *T cucurbitaceus* when visiting the food patches or the time they spent outside the patches (*i.e.*, clean parts of the leaflet). Observations were made between 10 am and 2 pm. Environmental conditions remained similar for all replicates during the trial (25±2°C and 60±10% RH).

Four behavioral descriptors were evaluated: 1) time spent in each of the three food patches or on the clean leaflet, 2) accumulated time of *T. cucurbitaceus* nymphs and adults in movement or non-movement, 3) visit frequency (first visit to TP and NTP), *i.e.*, the number of times that the predator entered the patch, and recurrent visit or re-visits to TP, and 4) the maximum number of times predators alternated among patches. Given our interest in mirid behavior concerning that prey, only revisits to the TP (*i.e.*, with *P. absoluta*) were considered. Thus, we set out the trial to analyze whether *T. cucurbitaceus*, after choosing TP as the first option, decided to revisit more frequently, *i.e.*, whether the predator preferred that food, or not.

Feeding preference assay

The feeding preference of *T. cucurbitaceous* nymphs, females, and males at 30 min and then for 24 h was evaluated by registering the number of preyed lepidopteran eggs or whitefly nymphs (figure 1, page 100). The number of preys consumed at 30 min was counted by removing predators from the experimental unit and keeping in labelled Eppendorf tubes, then restored to its unit. Later, all units were placed in a rearing chamber (I501PF, SEMEDIX, Argentina) at controlled temperature, relative humidity, and photoperiod conditions (25 ± 1°C, 65 ± 5% RH, 14:10 L:D) to check prey consumption at 24 h, without food replacement. After the end of the trial, the experimental units were checked to record preyed food using a stereoscopic microscope (Nikon SMZ1270) to observe and count the remains of eggs and nymphs caused by the stylets of the mirid. The occurrence of phytophagous behavior was checked by observing the presence of feeding punctures in leaflets (38). Preference was observed at 30 min since we aimed to discern whether the starvation period could influence the first food election for the predator. Instead, consumption at 24 h could bring information on prey choice when prey density decreased.

Statistical analysis

The time spent by individuals in the clean leaflet or prey patches was analyzed with a Generalized Linear Mixed Model (GLMM) using lme4 package and glmer function (2) with the type of patches and developmental stage, *i.e.*, late nymph, female and male adults as fixed factors and the individual as a random factor. The accumulated time of *T. cucurbitaceus* nymphs and adults in movement or non-movement was measured as the proportion of the time in movement / the total time of the experiment (1800 s) (response variable) and analyzed using Beta regression with betareg package (12), being the stage of predatory individuals (nymphs, female, and male adults of *T. cucurbitaceus*) the predictive factor. The frequency of the first visit to TP or NTP was analyzed using 2 x 2 contingency tables with Fisher's exact test for each developmental stage and sex of *T. cucurbitaceus* separately. Then, since the study was aimed to evaluate the feeding preference of this predator on *P. absoluta*, compared with other two possible prey items, we evaluated the frequency of those individuals who revisited the TP after visiting that TP as its first choice using 2 x 2 contingency tables with Fisher's exact test. The maximum number of food patch alternations was analyzed by Kruskal-Wallis test because the data was not normal. Then, Dunn's test checked for significant differences among factor levels with a p-value adjusted by the Benjamini-Hochberg method for multiple comparisons.

Considering the feeding preference assay, the proportions of prey eaten at 30 min were compared using Manly's Alpha index without prey replacement (25), as follows:

$$\alpha_i = \frac{ri}{ni}\left(\frac{1}{\sum_1^m \frac{rj}{nj}}\right)$$

where
αi = Manly's Alpha index for prey i
ri, rj = Proportion of prey type *i*
j in the diet (i and j = 1, 2,..., *m*)
ni, nj = Proportion of prey type *i* and *j* in the environment
m = number of prey types possible

The Manly's Alpha index varies between 0 and 1, and because in this study three types of prey were offered, values of α = 0.33 indicated no preference, greater than 0.33 a preference, and lower than 0.33 a rejection. The number of prey of each food type consumed at 24 h was calculated as the number of prey alive after 24 h - the initial number of offered prey. Then the proportion of consumed prey (*i.e.*, the number of individuals of each prey type consumed / the number of individuals of that prey type alive after 24 h) was analyzed using a logistic model (binomial family, logit link function), with the individuals (4-5th instar nymphs, females, and males of *T. cucurbitaceus*) and the type of prey (*P. absoluta* eggs, *T. vaporariorum* nymphs, and *E. kuehniella* eggs) as the predictor variables. All analyses were carried out using R software (36).

Results

Prey searching behavior

Predators spent significantly more time on the clean leaflet than in any of the food patches ($\chi 2$= 57.44; df= 3; P<0.001), with females spending more time outside of food patches than 4-5th nymphs and males ($\chi 2$= 6.5; df= 2; P= 0.04). When present on food patches, all predators spent a similar amount of time on any of the three sources (figure 2).

All bar graphs are represented by mean ± SE. Different letters show significant differences (p-value<0.05).

Las barras indican el promedio ± EE. Letras diferentes indican diferencias significativas (p valor<0,05).

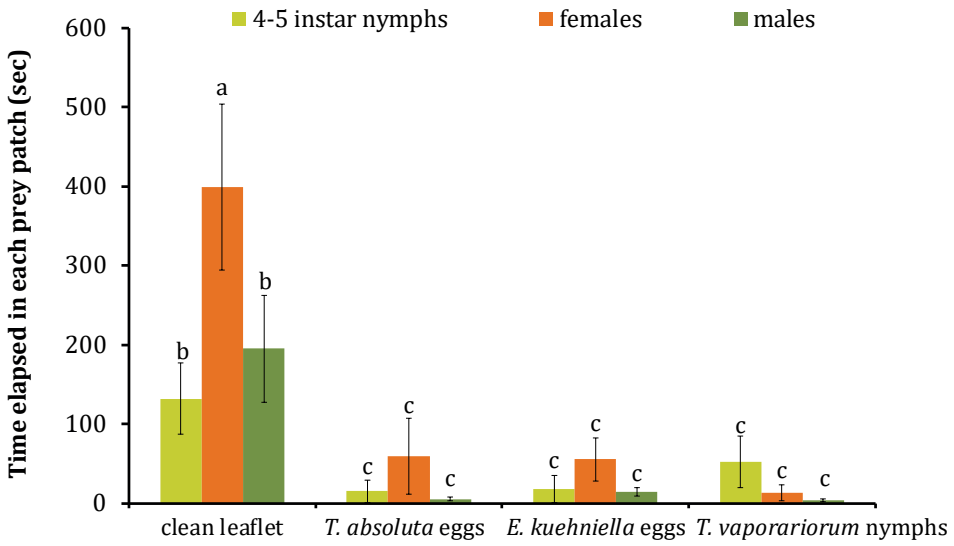

Figure 2. Time (s) elapsed in the different food patches (*P. absoluta* eggs, *E. kuehniella* eggs, and *T. vaporariorum* nymphs) by *T. cucurbitaceus* individuals (4-5th instar nymphs, females, and males) in 30 min.

Figura 2. Tiempo (seg) transcurrido por individuos de *T. cucurbitaceus* (ninfas de 4-5° estadio, hembras y machos) en los diferentes parches de alimento (huevos de *P. absoluta*, huevos de *E. kuehniella* y ninfas de *T. vaporariorum*) en 30 min de observación.

Regarding walking activity, all predatory individuals remained still for almost the 30 min tested, except for more active males ($\chi 2$= 25.07; df= 2; P<0.001). Given the small size and coloration of these insects -particularly nymphs-, on some occasions, the software was unable to detect activity responses (either moving or not moving). Failure was about 180 s for nymphs, while for adults of both sexes, the error was lower (<50 s).

The frequency of first visits to the TPs (*P. absoluta* eggs) and NTPs (*E. kuehniella* eggs and whitefly nymphs) was similar for all *T. cucurbitaceus* individuals (females: P= 0.08, males: P= 0.13, nymphs: P= 0.5). Mirid nymphs and males returned to the TPs independently of their first visit to that patch (males: P= 0.13, nymphs: P= 0.5). Females did not re-visit TPs (P= 0.04) (figure 3). In addition, males of *T. cucurbitaceus* showed a higher frequency of interchanges among patches than females and nymphs ($\chi 2$= 15.25; df= 2; P<0.001).

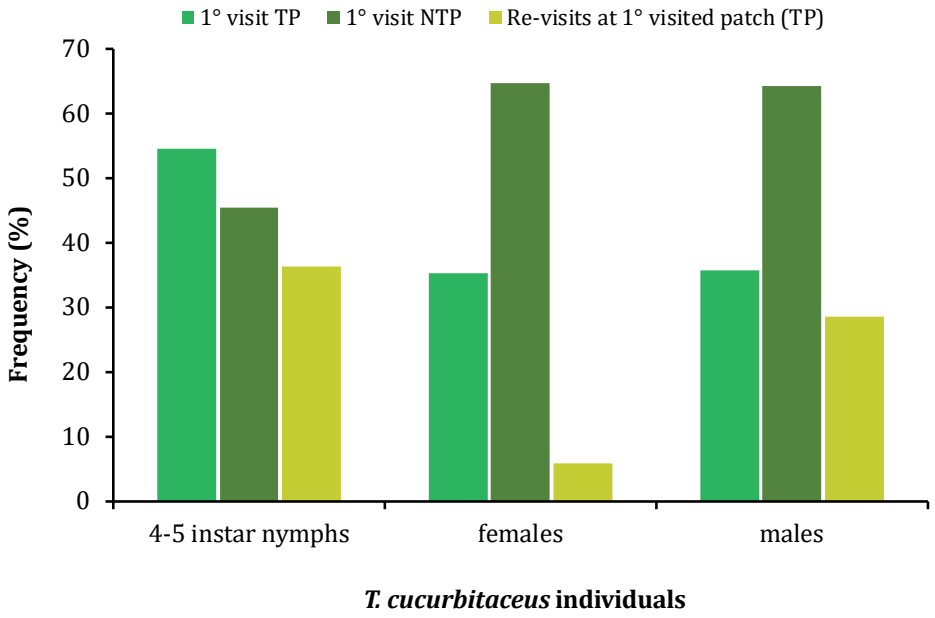

Figure 3. Frequency (%) of first visit to the target patch (*P. absoluta* eggs) and non-target patch (*E. kuehniella* eggs and *T. vaporariorum* nymphs), and re-visits to the target patch when it was the first patch visited by individuals of *T. cucurbitaceus* (4-5th instar nymphs, females, and males) in 30 min. TP: target patch, NTP: non-target patch.

Figura 3. Frecuencia (%) de primera visita al parche blanco (huevos de *P. absoluta*) y parche no blanco (huevos de *E. kuehniella* y ninfas de moscas blancas), y revisitas al parche blanco cuando este fue el primer parche visitado por individuos de *T. cucurbitaceus* (ninfas de 4-5° estadio, hembras y machos) en 30 min de observación.

Feeding preference

During the 30 min trial, all *T. cucurbitaceus* individuals tested consumed more *T. vaporariorum* nymphs, while predatory nymphs also fed on *P. absoluta* eggs and females did on *E. kuehniella* eggs. Besides, *T. cucurbitaceus* nymphs and males rejected feeding on *E. kuehniella* eggs (figure 4, page 104). After 24 h trial, *T. cucurbitaceus* nymphs ate more eggs of *P. absoluta* than females and males. Mirid nymphs also consumed a greater proportion of *E. kuehniella* eggs and *T. vaporariorum* nymphs than females and males except for the latter which mainly fed on whitefly nymphs (table 1, page 104; figure 5, page 105).

At the beginning of the experiment, all 24 h starved nymphs and adults of *T. cucurbitaceus* practiced phytophagy on the leaflet immediately after being placed in the experimental unit even in the presence of prey. However, no direct leaf injury was registered after feeding.

The black line indicates indifference (α= 0.33). All bars indicate mean ± SE.

La línea negra indica indiferencia (α= 0,33). Las barras indican el promedio ± EE.

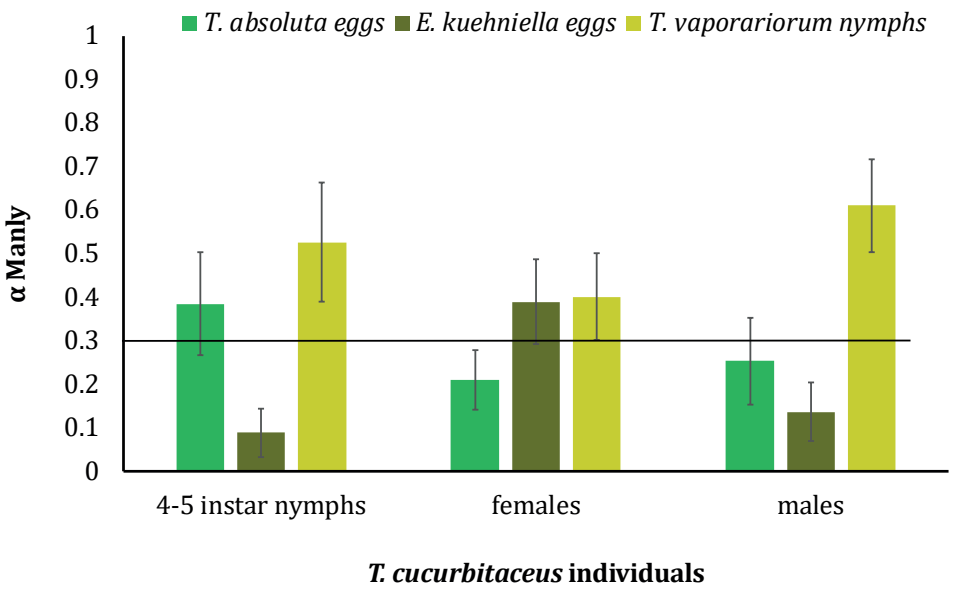

Figure 4. Preference Index (Manly's α) of 4-5th instars nymphs, females, and males of *T. cucurbitaceus* for *P. absoluta* and *E. kuehniella* eggs, and *T. vaporariorum* nymphs, after the first 30 m of the trial.

Figura 4. Índice de preferencia (α de Manly) de las ninfas de 4-5° estadio, hembras y machos de *T. cucurbitaceus* por huevos de *P. absoluta* y *E. kuehniella* y ninfas de *T. vaporariorum* luego de 30 min de iniciado el ensayo.

Table 1. Results of the ANOVA of the logistic model (binomial family) to analyze the proportion of different prey eaten by *T. cucurbitaceus* (4-5 instar nymphs, females, and males) after 24 h.

Tabla 1. Resultados del ANOVA del modelo logístico (familia binomial) para analizar la proporción consumida de las diferentes presas por *T. cucurbitaceus* (ninfas de 4-5° estadio, hembras y machos) al cabo de 24 h.

	df	deviance	resid. df	resid. deviance	P-*value*
Null model			140	1547.48	
Prey	2	9.53	138	1537.95	0.008
Individuals	2	793.63	136	744.32	<0.001
Prey x Individuals	4	86.96	132	657.37	<0.001

All bar graphs indicate mean ± SE. Asterisks denote significant differences between individuals (late instar nymphs, females, and males), and different letters show significant differences among prey items (p-value<0.05).

Las barras indican el promedio ±EE. Los asteriscos muestran diferencias significativas entre individuos (ninfas de 4-5° estadio, hembras y machos), y letras diferentes indican diferencias significativas entre ítems de presas (p valor<0,05).

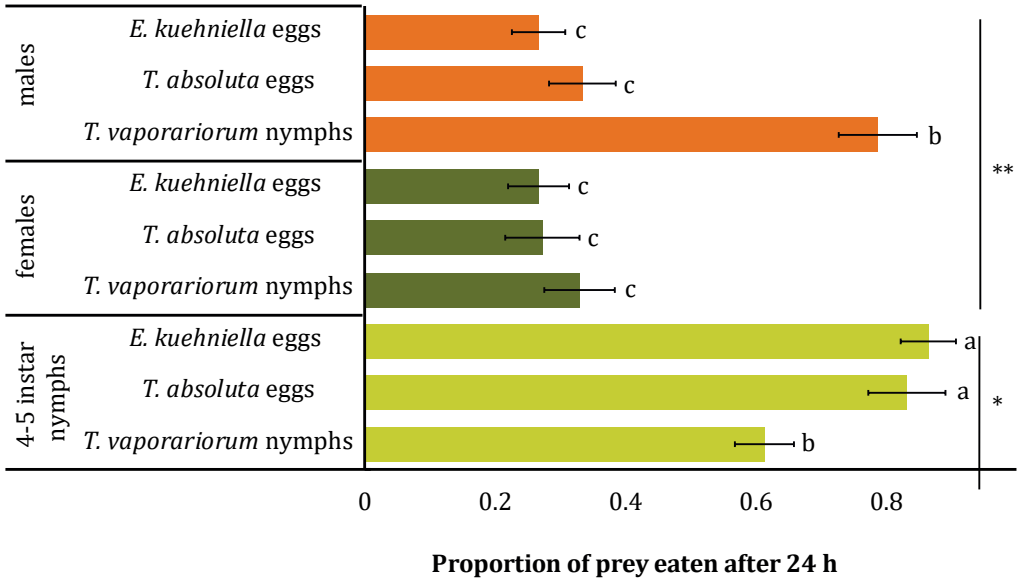

Figure 5. Proportion (mean) of each prey item eaten by *T. cucurbitaceus* individuals (4-5th instars nymphs, females, and males) in 24 h.

Figura 5. Proporción (promedio) de cada ítem de presa consumido por individuos de *T. cucurbitaceus* (ninfas de 4-5° estadio, hembras y machos) en 24 h.

Discussion

In this study, we present novel knowledge on the feeding habits of *T. cucurbitaceus*, a biocontrol agent of *P. absoluta* and whiteflies. The results confirmed the generalist feeding behavior of *T. cucurbitaceus* since the different developmental stages tested did not show clear patterns for food search. Mirid males searched for and consumed more *T. vaporariorum*. *Tupiocoris cucurbitaceus* nymphs also initially preferred whiteflies nymphs, but after 24 h consumed more lepidopteran eggs. Notably, *T. cucurbitaceus* females avoided *P. absoluta* eggs when first presented and ate all prey items in a similar proportion. As a result, the depletion of the mostly consumed prey across the experiment (observed in the 24 h trial) could force them to choose other available prey. This may indicate that, in the field, *T. cucurbitaceus* will consume the more abundant prey species. Likewise, Jaworski *et al.* (2013) showed that *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae) was able to switch feeding from *B. tabaci* to *P. absoluta* depending on their relative numbers. These results stress the importance of evaluating simultaneous prey offers to corroborate the in-field effectiveness of entomophagous biocontrol agents. Other studies proved that in a single-prey system, *T. cucurbitaceus* showed a greater consumption rate of *P. absoluta* eggs than those fed on *B. tabaci* and *M. persicae* nymphs, and *T. urticae* adults (23). Urbaneja *et al.* (2009) also found high consumption of *P. absoluta* eggs by other two mirids, *Macrolophus pygmaeus* Rambur and *Nesidiocoris tenuis* Reuter.

Zoophytophagy is an important aspect to consider when using predators as biological control agents (11, 34). Interestingly, starved 4-5th nymphs and adults of *T. cucurbitaceus* consumed plant tissue before feeding on prey but this behavior did not result in noticeably injury to the leaflet. Similarly, non-damaging feeding habit was also reported previously for this mirid species tested on tobacco *Nicotiana tabacum* L. and tomato plants without adding any prey (8). However, the potential plant injury caused by *T. cucurbitaceus* should be more thoroughly evaluated to discard effects on the crop yield.

In sum, meticulous studies on diet breadth of generalist predators should avoid failures in biocontrol programs (42). Currently, two mirid species, *M. pygmaeus* and *N. tenuis*, have proved to be successful in control programs in Europe against the tomato moth *P. absoluta* (13). In Brazil, several studies showed other hemipteran species such as *Macrolophus basicornis* (Stal), *Engytatus varians* (Distant), *Campyloneuropsis infumatus* (Carvalho), (Hemiptera: Miridae), and *Podisus nigrispinus* (Dallas) (Hemiptera: Pentatomidae) as promissory biological control agents of this pest (5, 7, 38, 43). Notably, *T. cucurbitaceus* is a dominant predatory species in northern Buenos Aires horticultural crops, co-occurring with *T. vaporariorum* and *P. absoluta* populations (29) allowing strategies for its augmentation and conservation to improve pest control. This study and others (22, 23, 24, 44) highlight the value of native beneficial fauna and the importance of preserving their natural presence in crops to contribute to IPM programs. In that context, we are currently assessing the potential of other entomophagous insects of *P. absoluta* as biological control agents, including intraguild predation interaction studies.

Conclusions

Results confirmed the generalist feeding behavior of *T. cucurbitaceus* since the different developmental stages tested in the laboratory did not show clear patterns when searching for the prey items offered. This finding could indicate that, in the field, this predatory species will consume the more abundant prey species, evidencing the importance of evaluating simultaneous prey offers to corroborate biocontrol effectiveness under crop conditions. Since we observed modest leaf consumption by *T. cucurbitaceus* in laboratory trials, the potential plant injury should be more thoroughly evaluated to discard effects on crop yield. We are currently assessing the potential of other entomophagous insects of *P. absoluta* as biological control agents, including intraguild predation interaction studies.

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Experimental ethics

Permits for insect collections were obtained from Dirección de Flora y Fauna, Ministerio de Desarrollo Agrario de la provincia de Buenos Aires. Natural resources involved in this study are the exclusive property of the Buenos Aires province, Argentina.

Conflicts of interest

This manuscript and the authors of the manuscript are not involved in any potential conflicts of interest, including financial interests and relationships and affiliations.

In vitro efficacy testing of citronella grass oil against *Tritrichomonas foetus* trophozoites

Evaluación *in vitro* de la eficacia del aceite de citronela contra trofozoítos de *Tritrichomonas foetus*

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Abstract

Tritrichomonas foetus, a sexually transmitted parasitic protozoan, causes abortion in cattle. Nitroimidazoles, such as metronidazole, treat bovine trichomonosis, but their use is precluded. Plant extracts might have antiparasitic effects. This study aimed to assess *Cymbopogon nardus* oil activity against *T. foetus* as an alternative to metronidazole. *T. foetus* trophozoites were incubated in culture medium containing serial dilutions of *C. nardus* oil. Cytotoxicity was assessed 24 hours later. *C. nardus* oil killed *T. foetus* cells. Half maximal effective concentration (EC50) was 0.4 µg/mL. These findings suggest that *C. nardus* oil could be exploited for discovery and compound isolation of plant-derived phytopharmaceuticals for bovine trichomoniasis and other protozoan diseases.

Keywords
disease • venereal • trichomonas • trichomoniasis • antimicrobial • dose-response

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Resumen

Tritrichomonas foetus es un protozoo parásito de transmisión sexual que provoca abortos en el ganado. Los nitroimidazoles, como el metronidazol, pueden ayudar en el tratamiento de la tricomonosis bovina, pero no se aconseja su uso. Los extractos de plantas pueden tener un efecto antiparasitario. El estudio actual tuvo como objetivo evaluar la actividad del aceite de *Cymbopogon nardus* contra *T. fetus* como alternativa al metronidazol. Se incubaron trofozoítos de *T. fetus* en medio de cultivo con diluciones seriadas de aceite de *C. nardus*. La citotoxicidad se evaluó a las 24 horas. El aceite de *C. nardus* mató las células de *T. fetus*. La concentración efectiva media máxima (CE50) fue de 0,4 µg/ml. Estos hallazgos sugieren que el aceite de *C. nardus* podría servir para el descubrimiento y aislamiento de fitofármacos para el tratamiento de la tricomoniasis bovina y de otras enfermedades protozoarias.

Palabras clave

enfermedad • venérea • trichomonas • trichomoniasis • antimicrobiano • dosis-respuesta

Introduction

Tritrichomonas foetus is a parasite protozoan that causes bovine trichomonosis (2). This venereal disease causes premature abortion and extended intercalving seasons. Nitroimidazoles are antibiotics used to treat infections such as trichomonosis (10). However, strain resistance and potential toxicity to meat consumers limit their use.

Bovine trichomonosis vaccines are not available and culling TF-infected animals is recommended. Between 2015 and 2019, more than 3,800 *T. foetus*-carrying bulls were culled in the United States (11). Every year in La Pampa, Argentina, more than 300 diagnosed bulls must be slaughtered (13). So far, testing and elimination programs have rarely succeeded in their purpose of eradicating *T. foetus* from cattle populations (13, 16).

New therapies for *T. foetus*-infected animals consider plant extracts as alternatives to commercial drugs. Extracts obtained from black tea, green tea, grape and pomegranate inhibit pathogenic trichomonads (9). Among natural extracts, essential oils are commonly used in traditional medicine and some studies have addressed their effect against protozoa (1, 5).

Citronella essential oil (EO) is extracted from the perennial herb *Cymbopogon nardus* (7). *C. nardus* is cultivated in tropical and subtropical regions, including northern Argentina. The oil has several biological activities, including insect-repellent, fungicidal, and bactericidal properties (7, 9, 12). To the best of our knowledge, the anti-trichomonas activity of this compound has not yet been investigated (4).

This study aimed to investigate the efficacy of *C. nardus* essential oil against the parasite *T. foetus in vitro,* in comparison with metronidazole. Increasing concentrations of *C. nardus* essential oil should significantly decrease *T. foetus* trophozoites viability.

Materials and methods

Plant material was obtained from existing monoculture plantations from El Soberbio, Misiones, Argentina (27°17'43.76" S, 54°11'46.84" W). Approximately 1,000 g of aerial parts were hydrodistilled. The chemical composition of *C. nardus* oil was determined by capillary gas chromatography on a 2014 Shimadzu GC equipped with a FID detector and a capillary column (30 m × 0.25 mm internal diameter) J&W INNOWax 19091 N-213. Nitrogen was used as carrier gas. Samples (1 µl) were diluted with acetone and injected at a 50:1 ratio. Operating conditions considered oven temperature maintained at 60°C for 8 min and gradually raised by 3°C per min, reaching 180°C, and kept for 5 min. Temperature at injection and detector ports was 250°C. *C. nardus* oil main components were citronellal (40.1%), citronellol (12.7%) Geraniol (25.3%), limonene (3.7%), trans-PMD (3.6%), cis-PMD (2.6%) and isopulegols (0.3%).

The clone *T. foetus* B1 obtained from a cow with pyometra, was used for conducting parasite-killing assays (14). Trophozoites were maintained in trypticase-yeast-maltose (TYM) medium supplemented with 1 g/L streptomycin, 1,000,000 IU/L ampicillin and 10% v/v heat-inactivated horse serum. Cultures were maintained at 37°C in sterile 2.0 mL tubes filled with media and tightly capped. Subcultures were made in 2-3 days intervals maintaining cell concentrations below 8 10^5 trophozoites/mL.

Pure cultures in antibiotic-free medium were seeded at a density of 5.10^5 *T. foetus* cells/mL in 10-fold triplicate serial dilutions containing *C. nardus* oil between 0-800 ug/mL or metronidazole (Sigma Aldrich, M1547) dilutions from 0 to 100 ug/mL. Vehicles and drugs diluted in water or 70% ethanol accounted for 1% of final volume. Incubation proceeded for 24 h at 37°C under microaerophilic conditions. All experiments were performed three times. Compound toxicity was assessed by Trypan blue exclusion on hemocytometer slides. GRmetrics' 4-parameter non-linear regression model was used to calculate IC50 and EC50 values as well as to generate dose-response curves (3). *T. foetus* growth rates were plotted against logarithmically transformed drug concentrations.

Results

Figure 1 shows best-fitted dose-response curves depicting *T. foetus* B1 cells response to *C. nardus* oil and metronidazole control. Over 24 hours, mock-treated cells doubled 4.6 times. At the same time, the vehicle (0.7% ethanol) had no visible effect on cell division or morphology. In contrast, treatment with metronidazole (positive kill control) produced a standard S-shaped curve, indicating strain sensibility. The EC50 for metronidazole was 0.016 µg/mL (16 µM). The concentration at which relative cell count was 0.5 of maximal value (IC50) was also 0.016 µg/mL (table 1, page 112).

C. nardus oil also exhibited dose-dependent inhibition, with all cells killed after 24 hours at high oil concentrations. The EC50 value for *C. nardus* oil was 0.391 µg/mL (table 1, page 112). Oil concentration required to achieve a growth rate of 0.5 was 0.65 µg/mL (table 1, page 112). Fitted curves explain over 95% of observed variation (figure 1).

Untransformed growth rate *versus* log-transformed concentration showing effects of *C. nardus* oil compared to metronidazole. Standard curves were built with Grmetrics. GR: growth rate.

Tasa de crecimiento no transformada *versus* concentración logarítmica transformada para mostrar el efecto del aceite en comparación con el metronidazol. Las curvas estándar se construyeron con Grmetrics. GR: tasa de crecimiento.

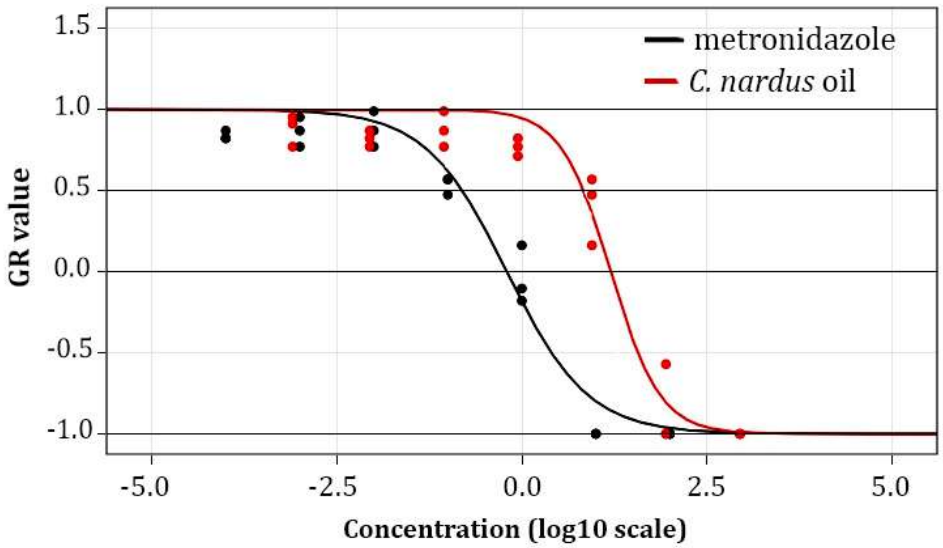

Figure 1. Dose-response curve for *Cymbopogon nardus* oil effect on *Tritrichomonas foetus.*

Figura 1. Curva dosis-respuesta del efecto del aceite de *Cymbopogon nardus* sobre *Tritrichomonas foetus.*

Table 1. *Cymbopogon nardus* oil inhibition values.
Tabla 1. Valores de inhibición del aceite de *Cymbopogon nardus*.

	r^2	GR50	IC50	EC50	h	*p*
Oil	0.96	6.78	0.391	0.391	0.337	2.47 e-08
Metronidazole	0.97	0.15	0.016	0.016	0.419	4.83 e-10

r^2 coefficient of determination indicating goodness of fit; GR50, concentration at which the effect reaches a growth rate value of 0.5 based on interpolation; EC50, the concentration at which relative cell count was 0.5 of maximal values; IC50, the concentration at half-maximal effect; h, Hill coefficient of the fitted (traditional) dose-response curve and the p-value of a F-test comparing curve fit to a horizontal line fit. Concentrations are expressed in ug/mL.

Se indican: r^2, el coeficiente de determinación para indicar bondad de ajuste de la curva; GR50, la concentración a la cual el efecto alcanzó un valor de tasa de crecimiento de 0,5 basado en la interpolación de la curva ajustada; EC50, la concentración a la que el recuento relativo de células fue 0,5 de los valores máximos; IC50, la concentración a la mitad del efecto máximo; h, el coeficiente de Hill de la curva dosis-respuesta ajustada (tradicional) y el valor p de una prueba F que compara el ajuste de la curva con un ajuste de línea horizontal. Las concentraciones se expresan en ug/mL.

Discussion

Several plant compounds have *in vitro* anti-trichomonal activity (6). Data presented here indicate that *C. nardus* oil can kill *T. foetus* in a dose-dependent manner. *C. nardus* oil could be used for local treatment. Additionally, *C. nardus* oil could constitute a basic resourse for plant-derived drugs for protozoan diseases. The main constituents of *C. nardus* oil (geranial, citronellol and elemol) show activity against *Trypanosome brucei* (9). In addition, several components have been found active against fungal strains (citronellal and linalool), Gram-positive and Gram-negative bacterial species (elemol, citronellol, citronellal) (7, 8, 15).

Conclusion

C. nardus oil affects growth and viability of *T. foetus in vitro*. *C. nardus* oil constitutes a useful resource for the discovery of plant-derived drugs for treating bovine trichomonosis and other protozoan diseases.

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Conflict of interests

The authors declare that there is no conflict of interest.

Effects of postharvest treatments based on calcium and silicon in hydro-cooling on the basic quality attributes of 'Bing' sweet cherries (*Prunus avium* L.) during storage

Tratamientos poscosecha a base de calcio y silicio en hidro-enfriamiento sobre atributos básicos de calidad en cerezas (*Prunus avium* L.) dulces 'Bing' durante almacenamiento

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Abstract

Ca^{2+} and Si^{2+} treatments confer resistance to biotic and abiotic stresses in many fruits. In sweet cherries, Ca^{2+} improves shelf life extension during storage, but only $CaCl_2$ is used. On the other hand, there is scarce information on $CaCO_3$ as a source of Ca^{2+}, which has shown increased firmness in berries. This study evaluated different treatments based on Ca^{2+} ($CaCl_2$ and $CaCO_3$) + Si^{2+} (SiO_2) alone and combined with immersion in hydro-cooling (0°C) on physicochemical characteristics of 'Bing' sweet cherries (*Prunus avium* L.) during storage at low temperature (4°C). Results demonstrate that alone or combined treatments (Ca^{2+} and Si^{2+}) with hydro-cooling significantly affected skin and flesh color of sweet cherries. Chromaticity (C^*) was increased in treated fruits, indicating an intense red color, especially in those cherries treated with $CaCl_2$. Furthermore, firmness was increased during storage in treatments with Ca^{2+}, while SiO_2 treatment increased total soluble solids (TSS). Therefore, combined treatments of Ca^{2+} and Si^{2+} with hydro-cooling might be a promising postharvest strategy to maintain desirable physicochemical characteristics in sweet cherries during low-temperature storage.

Keywords
Prunus avium • fruit firmness • shelf life • non-climacteric fruit • total soluble solids • skin color

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Resumen

Se ha demostrado que los tratamientos con Ca^{2+} y Si^{2+} confieren resistencia al estrés biótico y abiótico en muchas frutas. En cerezas dulces, el Ca^{2+} mejora la extensión de la vida útil durante el almacenamiento, pero solo se ha utilizado $CaCl_2$. Por otro lado, existe escasa información sobre el $CaCO_3$ como fuente de Ca^{2+}, que ha mostrado un aumento de la firmeza en bayas. En este estudio, se evaluaron diferentes tratamientos a base de Ca^{2+} ($CaCl_2$ y $CaCO_3$) + Si^{2+} (SiO_2) solos y combinados por inmersión en hidro-enfriamiento (0°C) sobre características fisicoquímicas en cerezas dulces 'Bing' (*Prunus avium* L.) durante el almacenamiento a baja temperatura (4°C). Los resultados demuestran que los tratamientos solos o combinados (Ca^{2+} y Si^{2+}) en hidro-enfriamiento afectaron significativamente al color de la piel y pulpa de las cerezas dulces. Se aumentó la cromaticidad (*C**) en los frutos tratados, indicando un color rojo intenso, especialmente en aquellas cerezas tratadas con $CaCl_2$. Además, la firmeza aumentó durante el almacenamiento en los tratamientos con Ca^{2+}, mientras que el tratamiento con SiO_2 incrementó la acumulación de sólidos solubles totales (SST). Por lo tanto, los tratamientos combinados de Ca^{2+} y Si^{2+} con hidro-enfriamiento podrían ser una estrategia poscosecha prometedora para mantener las características fisicoquímicas deseables en cerezas dulces durante el almacenamiento a baja temperatura.

Palabras clave

Prunus avium • firmeza del fruto • vida útil • fruto no climatérico • sólidos solubles totales • color de la piel

Introduction

Sweet cherry (*Prunus avium* L.) is one of the most appreciated fruits worldwide. Attributes such as sweetness, color, size, and flavor add up to being a rich source of antioxidants and phytonutrients (14, 39, 40, 66). In Mexico, the current demand for sweet cherries exceeds the 1,249 tons imported (17). In this country, cherry production is 144.45 tons, with only 35.5 ha established in the states of Chihuahua and Puebla (50). However, Mexico has regions with high potential for its production (4).

Fruit firmness, skin and pedicel color, acidity, and sugar content in fresh sweet cherries are major attributes influencing consumer acceptability (14). However, these attributes are often lost in between harvest, packaging, transportation, and storage, especially since sweet cherries are highly perishable and have a shorter post-harvest shelf life (40, 42, 49). Post-harvest strategies should avoid water loss, softening, color deterioration, and pedicel browning (14, 30, 53, 66). Nowadays, several technologies and practices, aimed at preserving post-harvest quality of sweet cherries, target respiration and senescence, increasing flesh firmness (10, 14, 54, 58, 66). In this regard, pre-harvest or at-harvest treatments with calcium (Ca^{2+}) and silicon (Si^{2+}) on sweet cherries extend storage life and improve flesh firmness by minimizing respiration and increasing fruit flesh resistance (14, 16, 31, 33, 46, 58, 63, 64).

Calcium is considered a critical, quality-defining nutrient in sweet cherries (63), mainly promoting firmness by acting in association with pectin molecules at cell-wall level (8, 38). $CaCl_2$ is the most widely used source of calcium in sweet cherries, both pre and post-harvest, preserving fruit quality and reducing physiological disorders like cracking (12, 14, 16, 27, 64). $CaCO_3$ is another less-known source of calcium for agriculture, shown to increase firmness of 'Shiraz' grapes after pre-harvest foliar application (32). On the other hand, silicon (Si^{2+}), although not considered an essential element for plant nutrition (7), has been suggested against various biotic and abiotic stresses in sweet cherry cultivation (2, 7, 28, 46). Si^{2+} improves strength and stiffness of plant tissues and increases wall extensibility (2, 23, 28). In addition, available literature demonstrates the safe use of physical treatments like hydro-cooling on vegetables and fruits to extend postharvest quality, especially by delaying firmness loss, reducing respiration rate and preserving fruit flavor (58, 60).

Therefore, chemical strategies like Ca^{2+} and Si^{2+} applications and physical treatments like hydro-cooling on freshly harvested sweet cherries might maintain storage quality (58, 59). However, studies considering a combination of Ca^{2+} and Si^{2+} with hydro-cooling and cool storage on post-harvest quality and shelf life of sweet cherries, are scarce (29, 53, 58).

Considering the aforementioned, the study aimed to evaluate the effect of post-harvest treatments with Ca^{2+} and Si^{2+} combined with hydro-cooling on physicochemical quality of 'Bing' sweet cherries during low-temperature storage.

Materials and methods

Fruits and chemical inputs

Sweet cherries 'Bing' (12 kg) were harvested from the commercial orchard "El Fulano" (28°26'46" N; 106°45'1.6" W and 2013 m above sea level) located in the "Tres Lagunas" ejido, in Cuauhtemoc, Chihuahua, Mex. Fruits were randomly collected from several trees on east-facing branches and from the center of the canopy. For the treatments of Ca^{2+} and Si^{2+}; food-grade $CaCl_2$, $CaCO_3$, and SiO_2 were purchased from Food Technologies Trading S.A. de C.V. Mexico.

Immersion of fruits

Before starting treatments, cherries were disinfected by immersion in a 1% (v/v) sodium hypochlorite for 5 min, washed twice with sterile distilled water, and left to dry at room temperature while packaged in commercial polyethylene boxes. Six treatments (solutions) simulated hydro-cooling, using distilled water and enough ice to keep the solutions at 0°C (58). Sweet cherries were immersed for 5 min in the evaluated solutions, all of them at 0.5% according to previous studies (58). The evaluated solutions were T1 ($CaCl_2$), T2 ($CaCO_3$), T3 (SiO_2), T4 ($CaCl_2$ + SiO_2), T5 ($CaCO_3$ + SiO_2) and a control treatment T6 (distilled water at 0°C). Thirty-two selected fruits were used in each treatment considering post-harvest evaluation dates 0, 7, 14 and 21 days after treatment. After the treatments, fruits were drained, placed on brown paper to dry at room temperature, packed in commercial polyethylene boxes (500 g) and immediately stored at 4°C with relative humidity of ~85%.

Basic physicochemical properties

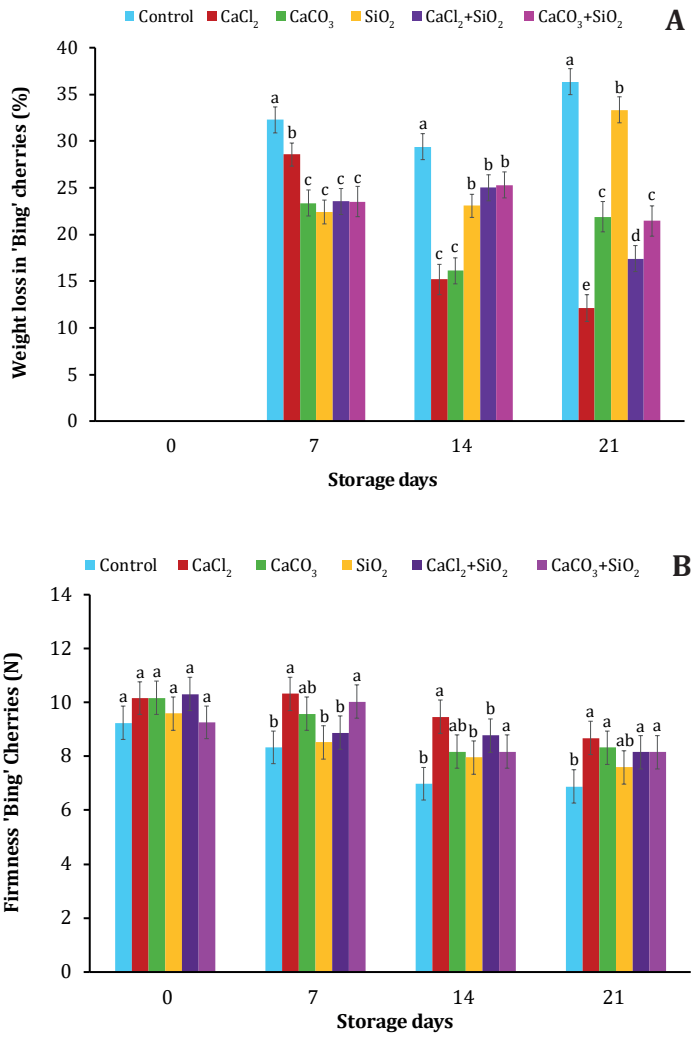
Physicochemical changes were measured by monitoring weight, firmness, color, total soluble solids (TSS; °Brix), and titratable acidity (TA). Measurements were expressed as the average of 32 fruits. The standard error (SE) was estimated at each evaluation time. Fruit weight was determined with an electronic balance, 0.01g precision, Precisa BJ 610C (Precisa Gravimetrics AG/Switzerland). Fruit firmness was evaluated as fruit resistance to a deformation of 15% of fruit diameter using a plunger of Ø=6 mm on a stationary steel plate, attached to a Universal Texture Analyzer TA-XT2i (Texture Technologies Corp. USA) according to previous studies (6). Data were expressed in Newtons (N) using the Texture Exponent Lite program. Skin color (CIELab parameters L^*, C^* and h^*) was measured at opposite sites of each fruit with a colorimeter CR-300, Minolta, (Japan). Total soluble solids content (TSS= °Brix) was determined in fruit juice with a digital refractometer PAL-1 pocket (Atago, Japan). Finally, titratable acidity (TA expressed as g 100 g^{-1} of fresh weight 'FW') was measured by diluting 1 g of flesh in 9 mL of distilled water, followed by 3 drops of phenolphthalein and titrated with 0.1 N NaOH until pH 8.2 (6). The maturity index was expressed as the ratio of TSS: TA (34).

Experimental design and statistical analysis

Results were statistically evaluated according to a split-plot in-time design. ANOVA and LSD mean tests were used to detect significant differences among treatments at $p \leq 0.05$ using SAS System for Windows 9.0 (SAS Institute. Inc. Cary, N.C., USA, 2002) after testing assumptions. All experiments were conducted using four replicates.

Results and discussion

In hydro-cooling, calcium and silicon treatments (alone or combined) significantly influenced some quality parameters and shelf life in sweet cherries during low-temperature storage (figure 1, figure 2, page 118 and figure 3, page 119). Various studies have extensively documented that Ca^{2+} applications in fruits favor storage conservation. In sweet cherries, it has been documented that Ca^{2+} delays deterioration, favorably influencing physicochemical attributes like weight, color, firmness, TSS, TA, pH, respiration rate, and anthocyanin content, especially during storage (14, 31, 57, 58, 59, 60). Shelf life extension in sweet cherries could be attributed to Ca^{2+} increase in the cell walls, favored by rapid absorption of Ca^{2+} by the fruit flesh under hydro-cooling immersion (19, 27, 59, 61).

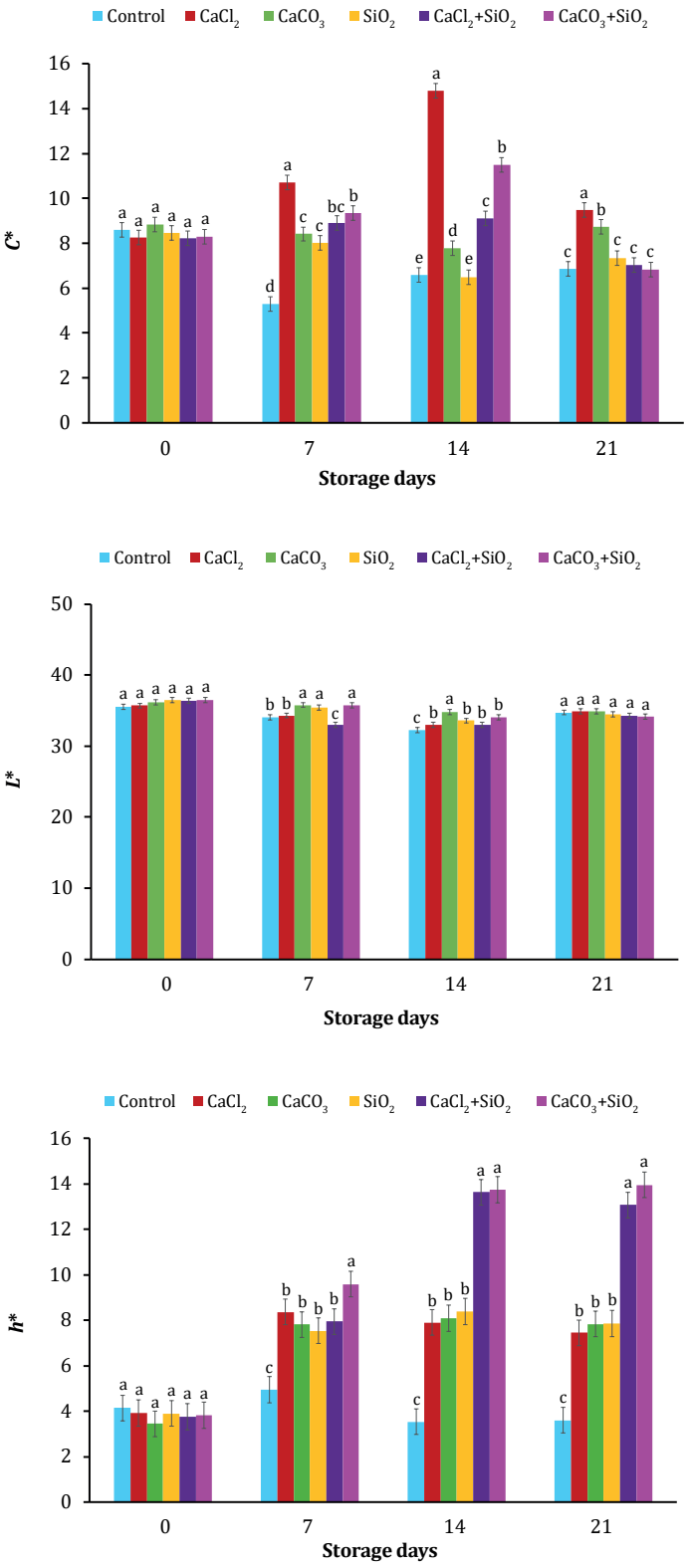


Different letters indicate significant differences ($p \leq 0.05$) between treatments for each storage date.

Las letras diferentes indican diferencias significativas ($p \leq 0,05$) entre tratamientos para cada fecha de almacenamiento.

Figure 1. Effect of post-harvest treatments based on calcium (Ca^{2+}) and silicon (Si^{2+}) sources, alone and combined with hydro-cooling on weight loss (A) and firmness (B) in 'Bing' sweet cherries during storage at low temperature.

Figura 1. Efecto de los tratamientos poscosecha basados en fuentes de calcio (Ca^{2+}) y silicio (Si^{2+}) solas y combinadas con hidro-enfriamiento sobre la pérdida del peso (A) y la firmeza (B) en cerezas dulces 'Bing' durante el almacenamiento a baja temperatura.

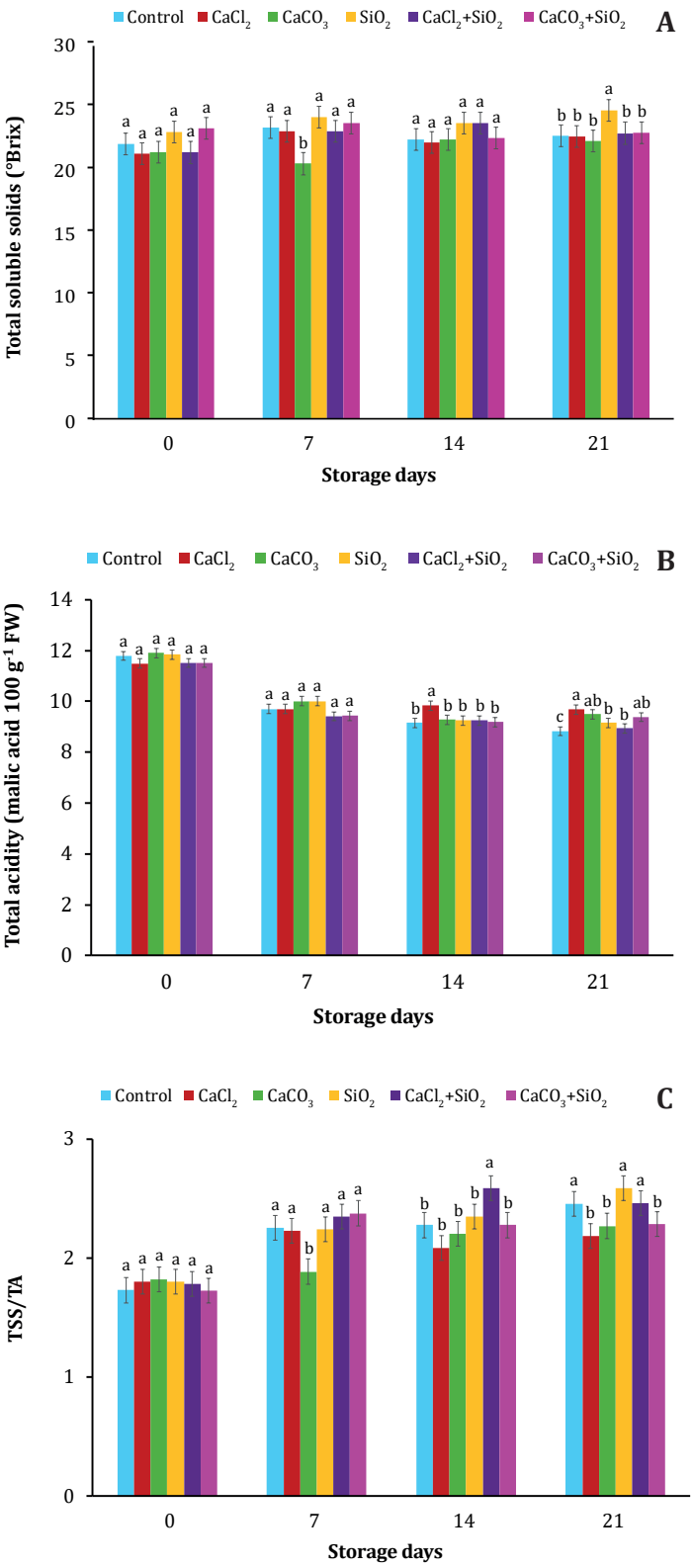


Different letters indicate significant differences ($p \leq 0.05$) between treatments for each storage date.

Las letras diferentes indican diferencias significativas ($p \leq 0,05$) entre tratamientos para cada fecha de almacenamiento.

Figure 2. Effect of post-harvest treatments of calcium (Ca^{2+}) and silicon (Si^{2+}) sources alone and/or combined with hydro-cooling on skin color ($L^* C^* h°$) in 'Bing' sweet cherries during low-temperature storage.

Figura 2. Efecto de los tratamientos poscosecha de fuentes de calcio (Ca^{2+}) y silicio (Si^{2+}) solas y/o combinadas con hidro-enfriamiento sobre el color de la piel ($L^* C^* h°$) en cerezas 'Bing' dulces durante el almacenamiento a baja temperatura.



Different letters indicate significant differences ($p \leq 0.05$) between treatments for each storage date.

Las letras diferentes, indican diferencias significativas ($p \leq 0,05$) entre tratamientos para cada fecha de almacenamiento.

Figure 3. Effect of post-harvest treatments of calcium (Ca^{2+}) and silicon (Si^{2+}) sources alone and/or combined with hydro-cooling on total soluble solids (TSS; A), titratable acidity (TA; B) and maturity index (TSS/ TA; C) in 'Bing' sweet cherries during low-temperature storage.

Figura 3. Efecto de los tratamientos poscosecha de fuentes de calcio (Ca^{2+}) y silicio (Si^{2+}) solas y/o combinadas con hidro-enfriamiento sobre los sólidos solubles totales (SST; A), la acidez titulable (AT; B) y el índice de madurez (SST/AT; C) en cerezas dulces 'Bing' durante el almacenamiento a baja temperatura.

Weight loss is the most important parameter for horticultural crops and fruit quality and shelf life. All treatments based on Ca^{2+} and Si^{2+} sources, alone and combined with hydro-cooling, affected weight loss of sweet cherries during storage (figure 1, page 117). According to previous studies (51, 66), weight loss in stored fruits mainly depends on transpiration and respiration. Interestingly, cherries treated with Ca^{2+} lost less weight during storage compared to untreated cherries (figure 1, page 117), suggesting that Ca^{2+} ions increased cell wall stability. Other studies mention increased cell wall stability after Ca^{2+} ions bind non-esterified pectins and stabilize cell membranes, preventing electrolyte leakage and consequently preventing fruit moisture and weight loss (1, 38, 41). The observed weight values in fruits treated with Ca^{2+} could have been influenced by the amount of this element absorbed through the skin (through the lenticels and peduncle pores) during the 5-minutes exposure (44). Similarly, previous studies (15) documented that combined Ca-Glu (calcium gluconate) treatment, limited weight loss in sweet cherries.

Sweet cherries treated with SiO_2 showed rapid weight loss on day 21 of storage, however less evident than for control fruits (figure 1A, page 117). Similarly, other studies (3) have documented that SiO_2 was less effective in preventing weight loss in post-harvest fruits of *Citrus* × *sinensis,* while Rombolà *et al.* (2023) found that foliar sprays with sodium silicate (Na_2SiO_3) decreased cherry weight at harvest.

Firmness is a major attribute in fruits (43). Broadly, our study showed a gradual loss of firmness concerning storage time indicating senescence, with significant differences among monitoring dates and treatments. According to previous studies (14), decreases in this parameter are more noticeable during storage. Softening of sweet cherries is attributed to enzymatic degradation of pectic compounds in the middle lamella of the cell walls by polygalacturonases, pectin methyl esterases, cellulases, and β-galactosidases (62). All sweet cherries treated with Ca^{2+} and Si^{2+} were firmer than control fruits (figure 1B, page 117). Studies have suggested that pre- and post-harvest treatments with Ca^{2+} and Si^{2+} favor greater firmness in fruits at harvest time and during storage (27, 55). Sweet cherries containing insufficient Ca^{2+} are softer, and, therefore, more susceptible to quality losses during storage (10). Fruits treated with $CaCl_2$ were the firmest compared to control fruits after 21 days of storage (figure 1B, page 117). It has been evidenced that $CaCl_2$ applied before and/or after cherry harvest increases firmness values up to 0.6 N (14, 63, 64). Our study is consistent with previous studies (10, 14, 15, 27, 55), reporting increased fruit firmness in treatments with Ca^{2+} before harvest and/or in recently harvested cherries. The treatments ($CaCO_3$ and $CaCO_3$+SiO_2) also favored greater firmness of sweet cherries but to a lesser extent than $CaCl_2$ (figure 1B, page 117). Similarly, other studies (32) documented firmer 'Shiraz' grapes after pre-harvest foliar treatment with $CaCO_3$. In our study, the treatment with SiO_2 alone was the least effective, although slightly superior to the control.

The greater firmness of sweet cherries treated with Ca^{2+} is attributed to the ability of this element to maintain cell wall mechanical properties and integrity during storage, which consequently delays softening (14, 44, 47). According to previous studies (38), Ca^{2+} acts in association with pectin molecules in fruit cell walls. It has also been suggested that Ca^{2+} maintains fruit firmness by reducing water loss and stabilizing the membrane, given this ion is responsible for binding phosphate and carboxylate groups of membrane phospholipids and proteins (62, 65).

Surface color of cherries is determined by factors such as radiation at the end of fruit development, and temperatures near harvest (13). Recently, it has been documented that color of sweet cherries is influenced by post-harvest treatments based on Ca^{2+} and Si^{2+} (14, 46). On the other hand, according to other studies (21, 36), the chromatic functions L^*, C^* and $h°$ are closely correlated with color change and anthocyanin accumulation in sweet cherries during ripening. Interestingly, after 21 days of storage, sweet cherries treated with Ca^{2+} or Si^{2+} showed increased chromaticity (figure 2, page 118), redder and intensity (C^*), especially in cherries treated with $CaCl_2$. This effect could be due to the inhibition of skin color development by Ca^{2+} or Si^{2+}. The delayed skin color darkening may be related to senescence inhibition (58, 59). Control fruits showed a darker red color attributed to chlorophyll degradation and accumulation of anthocyanins during storage (5, 18). Coincidentally, other studies (21) reported that the higher the anthocyanin content in sweet cherries, the lower the values of L^* and $h°$.

The *L** value in sweet cherries decreased during storage in all treatments, not showing significant differences among treatments (figure 2, page 118). Sweet cherries treated with $CaCO_3$+SiO_2 and $CaCl_2$+SiO_2 showed a higher *h*° angle (figure 2, page 118), indicating reduced red tones (*h*°) than control fruits and suggesting lower skin anthocyanin content (21, 37). In contrast, Rombolà *et al.* (2023) documented that Si^2+ reduced hue (*h*°), brightness (*C*), and saturation of cherry skin/flesh, while, Karagiannis *et al.* (2021) documented that foliar sprays with Si^2+ induced skin color development in apples by stimulating anthocyanin accumulation. In this experiment, sweet cherries treated with $CaCO_3$+SiO_2 and $CaCO_3$ showed higher *L** and *h*° values (figure 2, page 118) compared with control fruits, probably given to suppression of respiratory processes by $CaCO_3$, as previously established in cherries treated with Ca^2+ at harvest (14). The positive effect of $CaCO_3$ on skin and flesh color in sweet cherries is given by Ca^2+ activation of ABA biosynthesis, which influences anthocyanin biosynthesis in non-climacteric fruits such as cherries (20, 32).

The TSS concentration in sweet cherries significantly increased according to storage time in all treatments (figure 3A, page 119). Increasing TSS concentrations during storage is only frequent in climacteric fruits (22, 35). Therefore, the highest TSS concentrations in non-climacteric sweet cherries might be favored by a pronounced weight/moisture loss in SiO_2 treated and control fruits (figure 1A, page 117). The SiO_2 and $CaCl_2$+SiO_2 treatments significantly increased TSS in sweet cherries (figure 3A, page 119), like previously documented by Rombolà *et al.* (2023), who suggested that Si^{2+} forms a protective film covering fruit surface and preventing transpiration, slowing down phloem translocation, and subsequent sugar accumulation. The high concentration of TSS (figure 3A, page 119) in SiO_2-treated fruits might also be due to sugar concentration after greater weight loss (figure 1A, page 117) (11), something not observed in $CaCl_2$-, treated ones.

On the contrary, lower TSS values were observed in sweet cherries treated with $CaCl_2$ compared with control fruits. This coincides with other studies (9, 15), documenting low TSS contents in Ca^{2+}-treated cherries. Both studies attributed these results to lower respiration rates in treated cherries, leading to cell wall and membrane stabilization. This could also be attributed to delayed moisture and weight loss (figure 1A, page 117) after pectin stabilization and consequent effects on cell wall and membrane structure (32).

TA in sweet cherries also decreased over time during storage for control, Ca^{2+} and Si^{2+} treatments evidencing significant differences (figure 3B, page 119). Similar results were documented in 'Sweetheart' and 'Lapins' sweet cherries during storage (58). Low acidity mainly depends on ripeness state (45); however, during storage, organic acids might be used as carbon source during respiration (15, 25, 26, 60). After 21 days of storage, sweet cherries treated with Ca^{2+} and Si^{2+} maintained TA above values recorded for control cherries. However, the highest TA values were measured in $CaCl_2$-treated fruits (figure 3B, page 119). Sweet cherries treated with $CaCO_3$ and $CaCO_3$+SiO_2 also showed high TA values. Coincidentally, treatments with Ca^{2+} (such as $CaCl_2$ and Ca-Glu/calcium gluconate) in pre-harvest and/or before storage of sweet cherries, also preserved or retarded TA loss during storage, compared to control fruits (14, 15, 48, 55, 58).

Delayed loss of TA during storage of sweet cherries treated with Ca^{2+} sources could be due to the suppressive effect on fruit metabolic activity, especially respiration (15, 35, 56).

The maturity index TSS/TA indicates commercial and organoleptic maturity of fruits (34, 45). High contents of both TSS and TA are associated with good flavor in sweet cherries (52, 53). The TSS/TA ratios in 'Bing' sweet cherries treated with Ca^{2+} and Si^{2+} were statistically different (figure 3C, page 119), however increasing over time in all treatments and indicating a higher acid *vs.* sugar content ratio. TSS/TA ratio in sweet cherries treated with $CaCO_3$+SiO_2, $CaCO_3$, and $CaCl_2$ remained lower than control after 21 days of storage, indicating diminished respiration rates. While TSS/TA ratios in SiO_2 treatments remained above control values.

Conclusions

Immersion of freshly harvested 'Bing' sweet cherries with hydro-cooled solutions of Ca^{2+} ($CaCl_2$ and $CaCO_3$) and Si^{2+} (SiO_2) alone and combined markedly improved quality properties and extended storage capacity at low temperatures. All treatments based on Ca^{2+} and Si^{2+} alone reduced weight loss while maintaining firmness, and acidity in sweet cherries. Skin color of sweet cherries treated with Ca^{2+} and Si^{2+} was more intense than control fruits. Sweet cherries treated with $CaCl_2$ were the firmest and had the highest TA values. SiO_2 increased TSS concentration in sweet cherries, while $CaCl_2$ decreased it.

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Antibacterial activity and physicochemical characterization of bioplastic films based on cassava (*Manihot esculenta* Crantz) starch and rosemary (*Salvia rosmarinus*) essential oil

Actividad antibacteriana y caracterización fisicoquímica de láminas bioplásticas basadas en almidón de yuca (*Manihot esculenta* Crantz) y aceite esencial de romero (*Salvia rosmarinus*)

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Abstract

Bioplastics composed of renewable sources and antimicrobial components are desirable in food packaging. This study prepared bioplastic films with cassava starch and rosemary essential oil using a casting methodology. Film antibacterial activity, water vapour transmission (*Wvt*), mechanical resistance, and microstructure were measured after exposure to pathogenic bacteria such as *Salmonella enterica, Escherichia coli, Staphylococcus aureus, and Bacillus cereus. A*ntibacterial activity was evidenced against the pathogens evaluated except for *B. cereus*. The films showed average values of *Wvt* 3.6988 (10^{-14} g/Pa s m), tensile strength 8.90 MPa, young modulus 1679.72 MPa, and elongation at break 4.33%. Film microstructure showed good adhesion to bioplastic components in the matrix. Bioplastics of cassava starch and rosemary oil constitute potential food packaging solutions mainly for fruits, egg-based products or chicken.

Keywords
polymers • packaging • bacteria • water vapour

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Resumen

Los bioplásticos elaborados a partir de fuentes renovables y componentes antimicrobianos son deseables en el empacado de alimentos. Por tanto, se prepararon láminas bioplásticas con almidón de yuca y aceite esencial de romero usando el método de vaciado en placa. Se midió la actividad antibacteriana, transmisión de vapor de agua (*Tva*), resistencia mecánica y microestructura de láminas bioplásticas. Las láminas fueron expuestas a bacterias patógenas como *Salmonella enterica, Escherichia coli, Staphylococcus aureus y Bacillus cereus*. Se evidenció actividad antibacteriana para los patógenos evaluados excepto para *B. cereus*. Las láminas evidenciaron valores promedio de *Tva* 3,6988 (10^{-14} g/Pa s m), esfuerzo a tensión 8,90 MPa, módulo de young 1679,72 MPa y deformación a la rotura 4,33%. Su microestructura evidenció buena adhesión entre los componentes de la matriz bioplástica. Estos resultados muestran el potencial de los bioplásticos de almidón de yuca y aceite esencial de romero para el empacado de alimentos, principalmente de frutas o productos elaborados con huevo o pollo.

Palabras clave
polímeros • empaques • bacterias • vapor de agua

Introduction

The production of bioplastics from renewable sources is a field of research, development, and innovation of great interest worldwide (58). Bioplastics have increased from 2.4 million tons in 2021 to 7.5 million tons in 2023 (21). Applications include the packaging industry, agriculture/horticulture, consumer electronics, automobile, consumer goods, and household appliances. Package manufacturing, where rigid and flexible materials are required, is the most representative market segment (12, 23).

Bioplastics can be totally or partially obtained from natural sources (32). Fossil raw materials are generally not biodegradable. However, exceptions such as polycaprolactone can be used to make bioplastics. Polysaccharides, proteins, and fatty acids are renewable raw materials commonly used to manufacture bioplastics. Cellulose, starch, pectin, alginate, soy, wheat gluten, and gelatin are used alone or mixed with fossil polymers such as polyethylene or polypropylene (15, 46). Starch is a polysaccharide frequently used in bioplastics due to availability, costs, and biodegradable and renewable characteristics (58). Among bioplastics, starch-based bioplastics are the most widely traded (21). However, some disadvantages, mainly related to polarity, limit some applications (36, 58). Bioplastic food packaging must overcome the "polarity challenge" that implies high deterioration risks (48).

Active compounds increase biopolymers functionality for active food packaging (25). Food packaging with antimicrobial components has a positive impact on shelf life of packaged products (43, 46). These components are generally compatible with the natural raw materials used to produce bioplastic. Many studies have incorporated essential oils and plant extracts in polymer matrices to obtain bioplastics (10, 22, 25, 29, 42, 52, 57). Nevertheless, very few studies measure antimicrobial effects of rosemary oil incorporated in bioplastic films on more than three strains of bacteria or fungi. In fact, the composition of the essential oil may vary according to the place of origin affecting both bioplastic antimicrobial and physicochemical properties. To the best of our knowledge, no study has simultaneously evaluated the influence against Gram-negative bacteria (*E. coli* and *Salmonella sp.*) and, Gram-positive bacteria (*S. aureus* and *B. cereus*).

Bioplastic mechanical properties, stability against moisture and antimicrobial characteristics determine their applications. This study aimed to determine the antibacterial activity against *E. coli, S. enterica*, *S. aureus,* and *B. cereus*, physical-chemical and mechanical properties of a bioplastic film made with cassava starch and rosemary essential oil.

Materials and methods

Materials

Cassava starch (*Manihot esculenta* Crantz) was purchased from Tecnas S. A. (Cali, Colombia). Rosemary (*Salvia rosmarinus*) oil was purchased at the local market (Cali, Colombia). Food-grade glycerol was purchased from Merck (Burlington, MA, USA). All chemicals were reagent grade and purchased from Merck (Burlington, MA, USA). The American Type Culture Collection (ATCC) of the Universidad de San Buenaventura Cali (Colombia) provided bacteria. Two Gram-positive bacteria, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 15579 and two Gram-negative bacteria, *Salmonella enterica* ATCC 13314 and *Escherichia coli* ATCC 10798, were evaluated. This study was conducted at the University of San Buenaventura Cali, in Cali, Colombia.

Rosemary essential oil extraction

Rosemary leaves were placed in distilled water (mass/volume ratio 1/12). The essential oil was extracted in a hydrodistillation system for 4 hours at 100°C and stored refrigerated.

Film preparation

Cassava starch (CS) films were produced by the casting method (47) from forming suspensions (FSs). The FSs were prepared by dissolving 3 g of CS, 120 mg of rosemary essential oil, 83 mg of tween-80, and 0.75 g of glycerol in 100 mL of distilled water with heating (75 ± 5°C) and magnetic stirring. The FSs were dehydrated by convective drying at 40°C until obtaining films with 10% humidity, optimized formulation from a previous study (39) with a central composite design. The optimized formulation was validated with error values ranging from - 3.31 to 10.61%.

Antibacterial properties

Film antimicrobial activity was evaluated against Gram-negative bacteria (*E. coli* and *S. enterica*) and Gram-positive bacteria (*S. aureus* and *B. cereus*) using the disc diffusion method (54).

Mueller-Hinton agar (Sigma-Aldrich) was used to inoculate the bacteria. Then, a foil disc was placed in the center of the Petri dishes, and incubated at 37 ± 2°C for 24 hours. A calibrator (Mitutoyo, Japan) was used to measure the halo around the disc, determining inhibition percentage with Equation 1:

$$\%\ inhibition = \frac{halo\ diameter}{colony\ diameter}\ x\ 100 \quad (1)$$

Five repetitions were made for each bacteria. Chloramphenicol (Colmed, International) was used as a positive control at 100 ppn (parts-per notation).

Statistical Analysis

ANOVA and Fisher's LSD determined significant differences among treatments. Minitab 19 software was used to analyze variance with a significance level of 5%.

Rosemary essential oil

A Gas Chromatograph (AT 6890 Series Plus, Agilent Technologies, Palo Alto, California, USA) coupled to a mass selective detector (Agilent Technologies, MSD 5975 Inert XL) determined the chemical composition of rosemary essential oil operated in the full radio frequency sweep. The column was DB-5MS (J & M Scientific, Folsom, CA, and USA) [5% -phenyl-poly (dimethylsiloxane), 60mm x 0.25mm x 0.25µm]. Injection was done in Split mode (30:1) with a volume of 2µL.

Water vapour transmission

The water vapour transmission *Wvt* was measured gravimetrically following the ASTM E96-05 standard methodology (16). We used glass permeation cells filled with silica gel (0% RH). Films with a diameter of 80 mm were bonded with liquid silicone in the circular mouth of each cell. Cells were stored in airtight containers with a saturated sodium chloride solution (73 ± 2% RH) at 25°C. Weight variation in the permeation cell was plotted against time. Slopes were calculated by linear regression. The *Wvt* (g/Pa s m) was calculated by equation 2:

$$Wvt = \frac{WVTR}{PxRH} x\ l \quad (2)$$

where:

WVTR = water vapour transmission rate, calculated as the ratio between the slope of the straight line (g/s) and the permeation cell area (m^2)

P = saturation vapour pressure of water (Pa)

RH = relative humidity in the airtight container

l = mean film thickness (m). Analyses were conducted in triplicate.

Mechanical properties

A texturometer (EZ-Test L, Shimadzu, Japan) equipped with Trapezium X software conducted the test following the ASTM D882-10 standard (55). The films were cut in a rectangular shape of 20 mm wide and 100 mm long and stored for a week at 50% RH. The initial gauge was 65 mm long and test speed was 50 mm/min, using a load cell of 500 N. Tensile strength *Ts*, young modulus *Ym,* and elongation at break Eb were measured. Tests were performed ten times and the average was reported.

Scanning electron microscopy

Film surface morphology was analyzed at 20Kv scanning electron microscopy (SEM) (Jeol JSM-6490LV, USA) with backscattered electrons obtaining surface and cross-section images. Samples 5 mm wide and 5 mm long were coated with gold in a vacuum chamber (Denton Vacuum, Desk IV, USA). Images were captured at 500 and 2000 increases.

Results and discussion

Antibacterial properties

Inhibition percentages shown in table 1 indicate bioplastic films showed higher inhibition against *E. coli, S. aureus* than against *S. enterica*.

Table 1. Antibacterial inhibition percentages of bioplastic films.

Tabla 1. Porcentaje de inihibición antibacteriana de las láminas bioplásticas.

Bacteria	Inhibition (%)
E. coli	28.78 ± 0.45 ^{c}
S. enterica	18.48 ± 0.28 ^{b}
S. aureus	27.51 ± 0.75 ^{c}
B. cereus	0.00 ± 0.00 ^{a}

Different letters in the same column indicate significant differences (p<0.05).

Letras diferentes en la misma columna indican diferencias significativas (p<0,05).

The antibacterial activity of rosemary essential oil depends on ketones and monoterpene hydrocarbons that affect cell membrane permeability (5). This oil has proven antibacterial effects against *E. coli* (5, 18, 27, 30, 31), *Salmonella* (2, 33), and *S. aureus* strains (5, 6, 18, 27).

As shown in table 1, page 129, *B. Cereus* was not inhibited, probably given to bacterial rapid mutation and adaptation to different media, reaching quick resistance against antimicrobial agents (14, 53). Unlike *E. Coli, S. enterica*, and *S. aureus*, *B. cereus* is a sporulated bacterium, a mechanism that reinforces cell wall protection via environmental isolation and prevention of inhibitory interactions (40). It also generates highly resistant biofilms hindering its elimination (19).

Table 2 shows how rosemary oil incorporated in the film is mainly composed of *β*-Mircene (27.8 g/100 g), Camphor (23.9 g/100 g), and 1.8-Cineol (16.2 g/100 g). *β*-Mircene is an antibacterial monoterpene against *S. aureus, E. coli, Pseudomonas aeruginosa,* and *Proteus vulgaris* (3). Camphor is a terpenoid that affects lipoproteins and lipopolysaccharides present in bacteria cell walls, particularly gram-negative ones, generating lysis and subsequent cell death (6, 59). The third main component, 1,8-Cineol (9), is an oxygenated monoterpene (26, 35) widely used as inhibitory agent of food pathogens (9, 37). Even though many compounds have antimicrobial capacity (3), microorganisms develop defence and resistance mechanisms such as biofilms, a conglomeration of different cells allowing group protection from external factors (38). However, 1.8-Cineol inhibits biofilm formation in *S. aureus* through inhibitory agents affecting cell wall (34).

Table 2. Rosemary oil composition.

Tabla 2. Composición del aceite de romero.

Compound	Concentration (g/100 g)
Triciclene	0.2
α-Tujene	1.0
α-Pinene	4.5
Canfene	5.5
Sabinene	0.1
β-Pinene	4.6
β-Myrcene	27.8
α-Felandrene	<0.1
α-Terpinene	<0.1
p-Cimene	1.5
Limonene	2.1
1,8-Cyneol	16.2
trans-β-Ocimene	0.1
Y-Terpinene	0.3
*c*is-Sabinene Hydrate	0.3
Terpinolene	0.2
6,7-Epoxymyrcene	0.1
Linalool	0.6
trans-Sabinene Hydrate	0.2
Camphor	23.9
Borneol	0.8
$C_{10}H_{16}O$ (M^+ 152)	0.2
Terpinen-4-ol	0.7
α-Terpineol	1.1
Verbenone	0.3
Bornyl Acetate	3.4
α-Copaene	0.1
trans-β-Cariofilene	2.2
α-Humulene	0.7
Y-Muurolene	0.2
δ-Cadinene	0.2
Caryophyllene oxide	0.9

Water vapour transmission

Table 3 shows an experimental average *Wvt* of 3.6988 (10^{-14} g/Pa s m), lower than for other studies under similar manufacturing conditions. Considering that minimum *Wvt* values allow low vapour exchange between the food and the surrounding atmosphere, bioplastics for the food packaging industry should have low *Wvt* values for a longer shelf life (17).

Table 3. Physiochemical and mechanical characteristics of bioplastic films.
Tabla 3. Caracterización fisicoquímica y mecánica de las láminas bioplásticas.

Characteristic	Average values
Thickness (mm)	0.079± 0.002
Wvt (10^{-14} g/Pa s m)	3.6988 ± 0.158
Ym (MPa)	1679.72 ± 17.295
Eb (%)	4.33 ± 0.152
Ts (MPa)	8.90 ± 0.199

Wvt values of 3.11 to 8.72 (10^{-11} g Pa s m) were reported in anchovy (*Coccinia abyssinica*) starch films with cellulose nanocrystals and rosemary essential oil (27); from 2.95 to 2.7 (10^{-10} g/Pa s m) in films of polyvinyl alcohol, corn starch and cardanol oil (56); from 5.8 to 11 (10^{-10} g/Pa s m) in cassava starch films with rosemary extract (47); 4.16 to 5.27 (10^{-11} g/Pa s m) in modified cassava starch films (13); 5.8 to 12.5 (10^{-10} g/Pa s m) in cassava starch films with rosemary nanoparticles (20) and 3.9 to 8.2 (10^{-11} g/Pa s m) in biodegradable films of cassava starch with nanoclays (50). In the food industry, cellophane polymer derived from cellulose is used as wrapping film in the confectionery industry with a *Wvt* of 8.44 (10^{-11} g/Pa s m) (20). Considering conventional films, our bioplastic obtained good values.

The *Wvt* values obtained are related to film composition. The starch/tween 80 ratio constitutes a relevant factor since when its concentration allows for a continuous network, this polysorbate acts as water vapour transmission barrier (7). The network keeps the surfactant molecules dispersed, promoting a balance between the hydrophobic and hydrophilic phases and reducing *Wvt*. An excessive concentration of tween 80 will enhance the plasticizer effect, increasing the free volume inside the bioplastic structure and increasing *Wvt* (8). In addition, when starch and glycerol proportions increase, *Wvt* values may as well increase. Both starch and glycerol behave as polar components stimulating OH bonds with water molecules. Instead, the interaction between starch and rosemary oil limits the amount of water absorbed by the film (27) with covalent bonds that reduce OH groups and consequently decrease *Wvt* (49). The equilibrium among bioplastic components promoted low water values for food packaging.

Mechanical properties

Mechanical properties define bioplastic usage in food packaging. Tensile strengths and Young's modulus relate to mechanical tensile strength, while elongation at break defines ductility.

Table 3 shows an average tensile strength of 8.9 MPa, and Young's modulus of 1679.72 MPa, both higher than those reported in similar studies. Biofilms made from anchovy starch (*Coccinia abyssinica*) with cellulose nanocrystals and rosemary essential oil evidenced Ts values of 9.42 to 23.44 MPa (27). Bioplastic films made of modified starch with soybean oil oligomers reported *Ts values* of 3.35 MPa (58), while other ones made from cassava starch showed *Ts* values from 0.1 to 1.07 MPa and *Ym values* from 0.07 to 0.50 MPa (11). Films with essential oils had *Ts values* from 3 to 14 MPa (20), and bioplastic films of cassava starch with cinnamon essential oil showed Ts values ranging from 1.05 to 3.75 MPa (51). Plantain starch films had Ts values from 2.4 to 12.4 MPa and *Ym* values from 55.6 to 1482.2 MPa (41).

Bioplastic components define final mechanical properties while their concentration affects moisture gain. Starch is the major film component affecting mechanical resistance, forming hydrogen bridges with water and promoting adsorption. Water acts as a plasticizer agent, increasing mobility of polymer structure and, thus, decreasing mechanical resistance. On the other hand, the oil-starch bonds promote structural stiffness and increase polymer mechanical strength (41). However, excessive apolar components could reduce cohesion of starch binding forces and consequently, mechanical strength (24, 27).

Table 3 (page 131), shows average elongation at break (*Eb*) of 4.33%. In other studies, *Eb* values were higher, indicating low flexibility of our films. Biofilms made from anchovy (*Coccinia abyssinica*) starch with cellulose nanocrystals and rosemary essential oil reported *Eb* values between 27.71 and 73.91% (27). Others made of starch with soybean oil showed *Eb* of 58.32% (58); while films made of *Dioscorea hispida* Dennst starch and natural antimicrobial agents from turmeric extract showed *Eb* of 30.24% (28). Films made of cassava starch with cinnamon essential oil had *Eb* values between 128 and 264%; others made of corn starch with essential oils had Eb values from 30 to 170% (20). In bioplastic films made of cassava starch with cinnamon, cloves, and oregano essential oils, *Eb* values ranged between 8 and 17% (1), while films of rice starch with oregano essential oil, showed *Eb values* between 83.5% and 108.8% (45).

Bioplastic low flexibility is related to intra-structure free volume. Molecular movement of the polymer is directly proportional to intern free volume. Based on the above, we state that molecular adhesion in the assessed bioplastic matrix was high, and films had low free volume. Components promoting molecular mobility are glycerol, behaving as a plasticizer, and tween 80, a surfactant. Surfactants increase free volume into adjacent starch chains generating a flexible structure (44).

Scanning electron microscopy

Figure 1, and figure 2 (page 133), show flm cross-section and surface micrographs obtained by scanning electron microscopy. A smooth and homogeneous surface on both sides of the film indicate mixing and forming processes that allow whole matrix integration and adhesion. This indicates good bioplastic functionality for food packaging. The appropriate linkage of matrix components directly affects mechanical strength and stability against moisture. In the first case, a more compact structure could have high resistance and lower deformation or breakage capacity. Researchers stated that the finely distributed structure shown in the cross-section of corn starch films through SEM justified a better physical-mechanical behaviour and even better antibacterial response than other films without this characteristic (20).

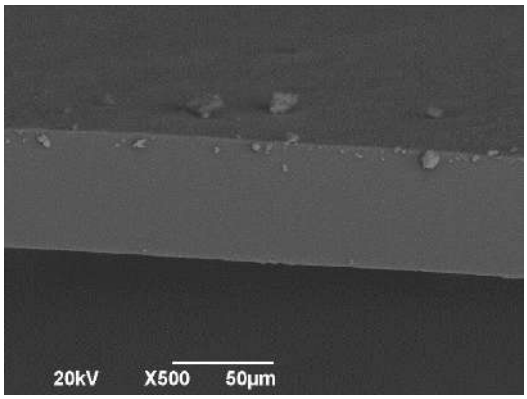

Figure 1. Scanning electron microscopic image of a cross-section of bioplastic film.

Figura 1. Imagen de microscopía electrónica de barrido de la sección transversal de la lámina bioplástica.

Structural integrity leads to good mechanical properties such as tensile strength and deformation, given by a high intermolecular interaction, components entanglement, and a continuous phase in the polymer matrix (45). Thus, interfacial interactions between mixture components and the essential oil are improved. On the other hand, when intermolecular linkage is high, the film has less porosity and empty spaces. Thus, bioplastics could have better stability against moisture.

In addition, no oil droplets were observed on film surface. In this regard, oil droplets may cause discontinuity, resulting in a cracked structure (4).

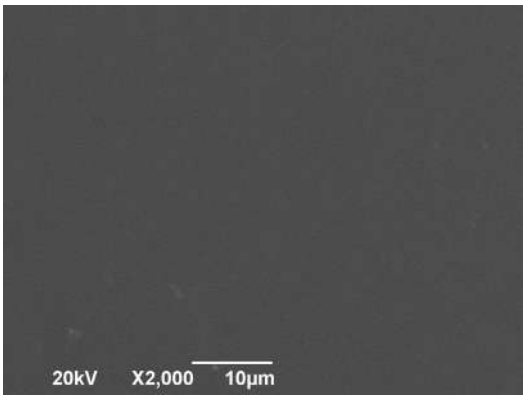

Figure 2. Scanning electron microscopic image of the surface of bioplastic film.

Figura 2. Imagen de microscopía electrónica de barrido de la superficie de la lámina bioplástica.

Conclusions

Bioplastic films based on cassava starch showed antimicrobial activity against *S. enterica, E. coli, and S. aureus,* low permeability to water vapour, good mechanical resistance, and high homogeneity in the surface and internal structure, indicating appropriate component linkage. These bioplastics constitute alternatives for packaging of susceptible foods. In this regard, these films could be used in packaging of fresh fruits or dairy products such as cheeses, where a high vapour barrier is required.

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In vitro micropropagation and physiological assessment of *Senecio bonariensis*

Micropropagación *in vitro* y evaluación del estado fisiológico de plantas de *Senecio bonariensis*

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Abstract

Senecio bonariensis is a plant native to South American wetlands. This plant has ecological importance, is used in traditional medicine, and is also popular for ornamental purposes. This study aimed to develop the first *in vitro* propagation protocol for *S. bonariensis*. Leaf explants were disinfected and placed on Murashige and Skoog (MS) agar medium supplemented with different combinations of growth regulators. We tested the effect of two different cytokinins: Kinetin (KIN) and 6-benzylaminopurine (BAP), in the presence of the auxin α-naphthalene acetic acid (NAA). All treatments with KIN resulted in root production, but only treatments with BAP induced shoot formation. As results, we determined the optimal concentration for maximum shoot production, achieving a 100% success in rustication while finding similar physiological traits among micro-propagated and wild-type plants. In conclusion, we developed a protocol for the large-scale production of *S. bonariensis* plants, providing an alternative source of bioactive compounds for medical and pharmaceutical purposes while preserving the natural habitat of this native plant.

Keywords

Margarita de bañado • *Senecio bonariensis* • plant growth regulator • *in vitro* tissue culture • conservation • OJIP-test

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Resumen

Senecio bonariensis Hook. y Arn. es una planta nativa que se encuentra principalmente en zonas de humedales de América del Sur. Esta planta tiene importancia ecológica y también se utiliza en la medicina tradicional. Además, es una opción popular con fines ornamentales. El objetivo de este estudio fue desarrollar el primer protocolo de propagación *in vitro* de *S. bonariensis*. Los explantos de hojas se desinfectaron y luego se colocaron en medio agar Murashige y Skoog (MS) suplementado con diferentes combinaciones de reguladores de crecimiento. Se probó el efecto de diferentes citoquininas: kinetina (KIN) y 6-bencilamino-purina (BAP) en presencia de la auxina ácido α-naftalenoacético (NAA). Encontramos que todos los tratamientos, incluido KIN, dieron como resultado la producción de raíces, pero solo los tratamientos que incluyeron BAP mostraron inducción de brotes. Como resultado, determinamos la concentración óptima para la mayor producción de brotes y la tasa de éxito del proceso de rusticación fue del 100%. También evaluamos el estado fisiológico de las plantas micropropagadas y observamos que los parámetros probados eran similares a los de las plantas silvestres. En conclusión, hemos desarrollado un protocolo para la producción a gran escala de plantas de *S. bonariensis*. Esto proporcionará una fuente alternativa de compuestos bioactivos para fines médicos y farmacéuticos y al mismo tiempo preservará el hábitat natural de esta planta nativa.

Palabras clave
Margarita de bañado • *Senecio bonariensis* • regulador de crecimiento vegetal • cultivo de tejidos *in vitro* • conservación • prueba OJIP

Introduction

The Asteraceae family is one of the largest families of dicotyledonous plants (5, 10, 14). *Senecio bonariensis* is a native plant species primarily found in wet zones of central and northern Argentina, Bolivia, Uruguay, and southern Brazil (10). This shrub is also known as Margarita de bañado, Pillahuincó, Bálsamo, Lampacillo, Lampaso, Lampazo, Lengua de ciervo, Margarita del agua, Margaritón de bañado, or Sanguinaria in Spanish; Margarida do banhado in Portuguese; and butterweed, groundsel, or ragwort in English (10). Considering *S. bonariensis* is traditionally used to treat skin, respiratory and osteoarticular diseases (5, 10, 14), there is growing interest in its cultivation to obtain bio-compounds for medicinal use and basic or applied research. Plant tissue culture is widely accepted for propagating native species. This technology has been adopted for conservation purposes by organizations such as The Botanic Gardens Conservation International (BGCI), which represents botanical gardens in 120 countries (4, 6, 11). However, only a few micropropagation and *in vitro* propagation protocols for other *Senecio* species have been previously reported (7, 16, 18) with protocols presenting bottlenecks at various stages of the propagation process, highlighting the need for specific protocols tailored to this genus. Considering that precise regulation of cytokinins and auxin levels strongly affects growth of stems, roots, and leaves, determining type and concentrations of particular plant growth regulators (PGRs) is essential. Poorly established protocols typically result in low shoot multiplication, low rooting frequency, morphological abnormalities, and high production costs (1). This study aimed to develop a protocol, particularly considering *S. bonariensis* for successful plant micropropagation and rustication. We assessed the effects of two cytokinins, Kinetin (KIN) and 6-benzyl aminopurine (BAP), in conjunction with the α-naphthalene acetic acid (NAA) auxin, on shoot production from leaf explants, aiming to identify the optimal combination for maximizing yield. Additionally, we evaluated physiological parameters of the micro-propagated plants utilizing the non-destructive and cost-effective OJIP test. This test analyzes the OJIP curve providing insights into thermal and photochemical phases of electron transport chain (17).

In conclusion, our study developed a protocol for large-scale production of *S. bonariensis* plants as an alternative source of bioactive compounds for medical and pharmaceutical purposes, while contributing to the preservation of the species´ natural habitat.

Material and methods

Plant material and surface sterilization of explants

Leaves of *Senecio bonariensis* were collected during springtime (2018-2022) from Laguna de Chascomús (35°35'22.92'' S 58°1'20.14'' W, Buenos Aires, Argentina). A voucher specimen was deposited in the Herbarium of the Museo de Ciencias Naturales de La Plata (Buenos Aires, Argentina), under the collection number M. G. Corigliano 1, LP 082432. Healthy young leaves were meticulously washed under running tap water to prevent damage. Subsequently, explants underwent surface sterilization using a 20% v/v solution of commercial bleach for 30 minutes, followed by four rinses with sterile distilled water in a biosafety cabinet.

Callus induction

Surface-sterilized explants were dissected into small pieces (1 cm^2) without disrupting serrated margins and subsequently placed onto sterile Murashige and Skoog (MS) basal medium (13) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. To induce callus formation, the auxin α-naphthalene acetic acid (NAA) was tested at three different concentrations (0.1, 0.5, and 1 μg ml^{-1}) in combination with two different cytokinins, BAP or KIN, each at three different concentrations (0.5, 1, and 2 μg ml^{-1}), totalling eighteen combinations. Eight leaf fragments from distinct plants were incubated in culture flasks in a growth chamber set to a 16-hour day/8-hour night photoperiod, with a photosynthetic photon flux density (PPFD) of *350 μmol quanta m^{-2} s^{-1} provided by cool-white fluorescent lamps, at a constant temperature of 24/21 ± 2°C. The percentage of callus induction (PCI) was assessed 30 days after the initial culture (d.a.i.c.), and calculated by dividing the number of explants with calli by the total cultured explants, x 100. Three independent experiments were performed.

Shoot growth study

Calli were sub-cultured onto fresh MS medium with the same hormone combination 30 d.a.i.c. The number of shoots produced per explant was evaluated at 90 d.a.i.c. We also measured shoot length and registered the tallest shoot for each treatment.

Acclimatization

Shoots obtained at 90 d.a.i.c. were transplanted into plastic pots filled with a sterile mixture of sand, soil, and perlite (1:1:1 ratio) and watered with Hoagland nutrient solution (8) every 2 days. The pots were placed in a growth chamber with a 16-hour day/8-hour night photoperiod, provided by cool-white fluorescent lamps, and maintained at a temperature of 24/21 ± 2°C. Plant survival and phenotypic variation were recorded. After 12 weeks, the plants were transferred to field conditions and flowering ability was assessed.

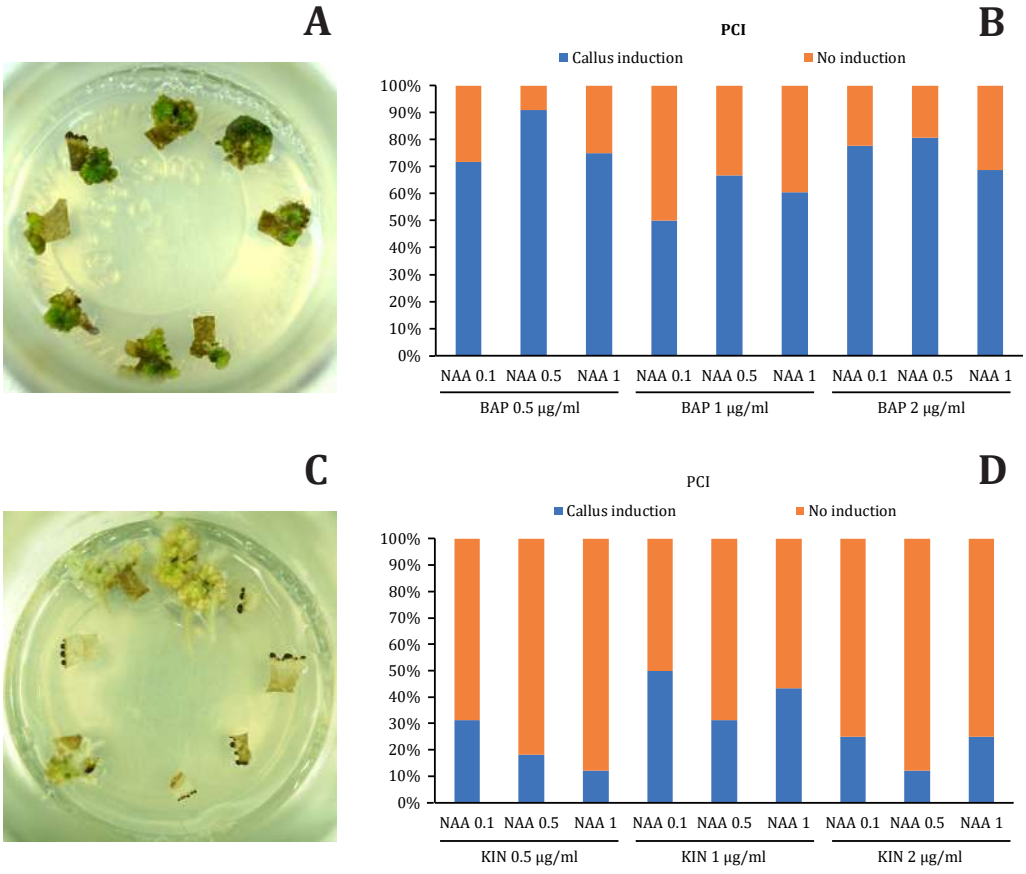
Chlorophyll fluorescence fast-transient analysis

The non-invasive OJIP test (16) was conducted on 6 to 7-month-old plants using a portable chlorophyll fluorometer (Pocket PEA v.1.1, Hansatech Instruments Ltd.), as described by Corigliano *et al.* (2019). Briefly, the youngest fully developed leaf was dark-adapted for 20 minutes before analysis. Subsequently, leaf samples were exposed to a 3-second pulse of light at an intensity of 3500 μmol photons m^{-2} s^{-1} (peak wavelength: 637 nm). Data were analyzed using PEA Plus software (Hansatech Instruments Ltd.). Maximum quantum yield of primary PSII photochemistry (Fv/Fm) and dissipation energy flux per active reaction center of PSII (DIo/RC) were determined. Additionally, we analyzed the contribution to photosynthesis regulation of two functional steps, namely ABS (absorption of light energy) and TRo (trapping of excitation energy) by RC (reaction center), and CSo (cross-section).

Results

Callus induction

Effective surface sterilization was achieved for *S. bonariensis* leaf explants. The effect of two cytokinins, BAP and KIN, in combination with the NAA auxin, was tested. Figure 1 shows calli induction 30 d.a.i.c. Although MS medium without PGR did not lead to callus generation (data not shown), callus induction was observed for all hormone combinations, either BAP-NAA (figure 1A) or KIN-NAA (figure 1C). However, the BAP-NAA combination resulted in a higher calli percentage compared to KIN-NAA in any combination. The PCI was determined for various BAP-NAA combinations. The highest PCI obtained was about 90% when calli were cultured in MS medium supplemented with NAA 0.5 µg ml^{-1} and BAP 0.5 µg ml^{-1} (figure 1B). On the other hand, the highest PCI was only 50% when calli were cultured with NAA 1 µg ml^{-1} and KIN 0.5 µg ml^{-1} (figure 1D). Initial callus induction was observed at the serrated edge of leaves (figure 1A, 1C).



Bars represent percentage of one experiment. Blue represents callus induction, while orange indicates no callus induction. The assay was performed in triplicate. Significant differences were observed between NAA 1 µg ml^{-1} - KIN 0.5 µg ml^{-1} and NAA 0.1 µg ml^{-1} - KIN 1 µg ml^{-1} (P= 0.04, X2= 16.1,8).

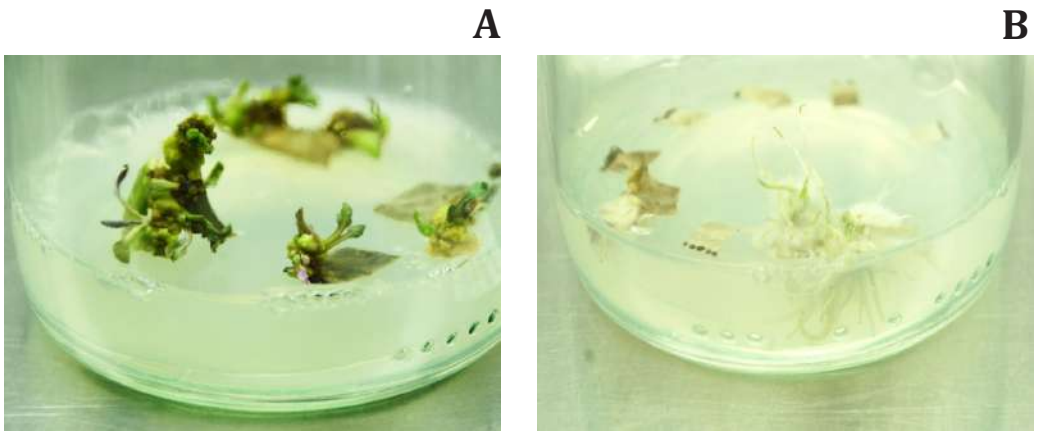
Las barras representan los promedios de un experimento y se graficaron como porcentajes. El color azul representa la inducción de callos, mientras que el anaranjado indica que no hay inducción de callos. Este ensayo se realizó por triplicado. Se observaron diferencias significativas entre NAA 1 µg ml^{-1} - KIN 0,5 µg ml^{-1} y NAA 0,1 µg ml^{-1} - KIN 1 µg ml^{-1} (P= 0,04, X2= 16,1,8).

Figure 1. Effect of NAA and BAP on callus induction. A Callus induction from leaf explants on MS medium supplemented with either 0.5 µg ml^{-1} NAA and 0.5 µg ml^{-1} BAP (C), or with 0.1 µg ml^{-1} NAA and 1 µg ml^{-1} KIN, 30 days after culture initiation. The PCI from leaves cultured on MS and supplemented with NAA at three different concentrations (0.1, 0.5, and 1 µg ml^{-1}) and combined with either BAP (B) or KIN (D) at three different concentrations (0.5, 1, and 2 µg ml^{-1}) was evaluated 30 d.a.c.i.

Figura 1. Efecto de NAA y BAP en la inducción de callos. (A) Inducción de callos a partir de explantos de hojas en medio MS suplementado con 0,5 µg ml^{-1} de NAA y 0,5 µg ml^{-1} de BAP (C) o con 0,1 µg ml^{-1} de NAA y 1 µg ml^{-1} de KIN, 30 días luego de la inducción del callo (d.l.i.c). Evaluación del porcentaje de inducción de callos en hojas cultivadas con MS y suplementadas con NAA en tres concentraciones diferentes (0,1, 0,5 y 1 µg ml^{-1}) y combinadas con BAP (B) o KIN (D) en tres concentraciones diferentes: 0,5, 1, y 2 µg ml^{-1}, a los 30 d.l.i.c.

Effects on BAP, KIN, and NAA on shoot multiplication

Treatments including BAP led to shoot induction (figure 2A). Conversely, even though different concentrations of KIN induced callus formation, they did not result in shoot production, but in roots (figure 2B). Shoots per cultured explant were counted at 90 d.p.i.c (figure 3, page 142). Even without statistical differences among groups with different BAP-NAA combinations, the highest number of shoots per explant was observed with BAP 0.5 µg ml^{-1} and NAA 0.5 µg ml^{-1} (figure 3A, page 142). Interestingly, the longest shoots were both obtained with the BAP 0.5 µg ml^{-1} and NAA 0.5 µg ml^{-1} combination (figure 3B, page 142).



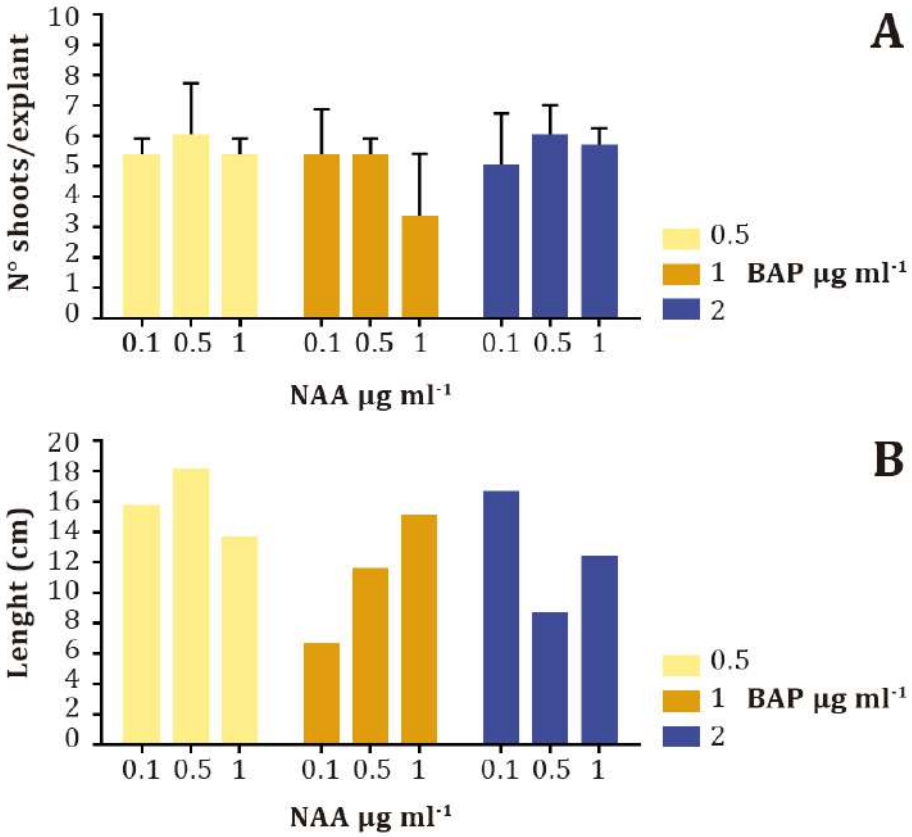
(A) Shoot induction from NAA-BAP treated calli 45 d.a.i.c. (B) Root induction from NAA-KIN treated calli 45 d.a.i.c.

(A) Inducción de brotes de callos tratados con NAA-BAP luego de 45 días. (B) Inducción de raíces tratadas con NAA-KIN luego de 45 días.

Figure 2. Effects of NAA and BAP on shoot multiplication of *S. bonariensis*.
Figura 2. Efecto de NAA y BAP en la multiplicación de brotes de *S. bonariensis*.

Micro-propagated plants displayed purple phenotype with no physiological disturbances

Shoots obtained 90 d.a.i.c. were transferred to plastic pots and placed in a plant room with a 16 h day/ 8 h night photoperiod and 24/21 ± 2°C. Plant survival and rustication were 100% successful (data not shown). A variety of physiological parameters validated the micropropagation protocol. Some plants grown *in vitro* showed purple colorations on leaf abaxial face, prompting a comparative analysis of physiological traits of green and purplish leaves from micro-propagated plants, and green leaves from non-propagated (wild-type, WT) control plants (figure 4A, page 143). We assessed maximum quantum yield of primary photochemistry (Fv/Fm) and examined photosynthesis regulation by the two functional steps, namely ABS (absorption of light energy) and TRo (trapping of excitation energy) by RC (reaction center) and CSo (cross-section). The Fv/Fm values for green, purple, and WT plants were almost 0.8, considered normal (figure 4B, page 143). Other energetic parameters (ABS/RC, ABS/CSo, TRo/RC, and TRo/CSo) showed no statistical differences among plants (figure 4B, page 143). However, the dissipation energy per reaction center (DIo/RC) was statistically lower in green and purple plants than in WT plants ($p<0.01$) (figure 4C, page 143).



(A) Assessment of the number of shoots obtained per explant using NAA at three different concentrations (0.1, 0.5, and 1 µg ml^{-1}) and combined with BAP at 0.5 µg ml^{-1} (yellow), 1 µg ml^{-1} (orange), and 2 µg ml^{-1} (blue) obtained 60 d.a.i.c. This experiment was conducted in triplicate. Statistical analysis was performed by one-way ANOVA and no statistical differences were observed among groups. (B) Shoot length at different hormone combinations achieved 60 d.a.i.c. The longest shoot per group in three independent experiments is plotted in the figure.

(A) Evaluación del número de brotes obtenidos por explanto utilizando NAA en tres concentraciones diferentes (0,1, 0,5 y 1 µg ml^{-1}) y combinado con BAP a 0,5 µg ml^{-1} (amarillo), 1 µg ml^{-1} (anaranjado), o 2 µg ml^{-1} (azul) luego de 60 días. Este experimento se realizó por triplicado. El análisis estadístico se realizó mediante análisis de varianza unidireccional (ANOVA) y no hubo diferencias estadísticas entre los grupos. (B) Gráfico de la longitud máxima de los brotes con diferentes combinaciones de hormonas alcanzada a 60 d.l.i.c. La longitud del brote más largo de cada grupo en tres experimentos independientes se midió y se representó en la figura.

Figure 3. Effects on BAP and NAA on shoot multiplication.
Figura 3. Efectos de BAP y NAA en la multiplicación de brotes.

Acclimatization of regenerated shoots

After 12 weeks, the plants were transferred to field conditions, and phenotypic variation was visually assessed. Notably, purple colorations on leaf abaxial sides disappeared after one or two weeks in the field.

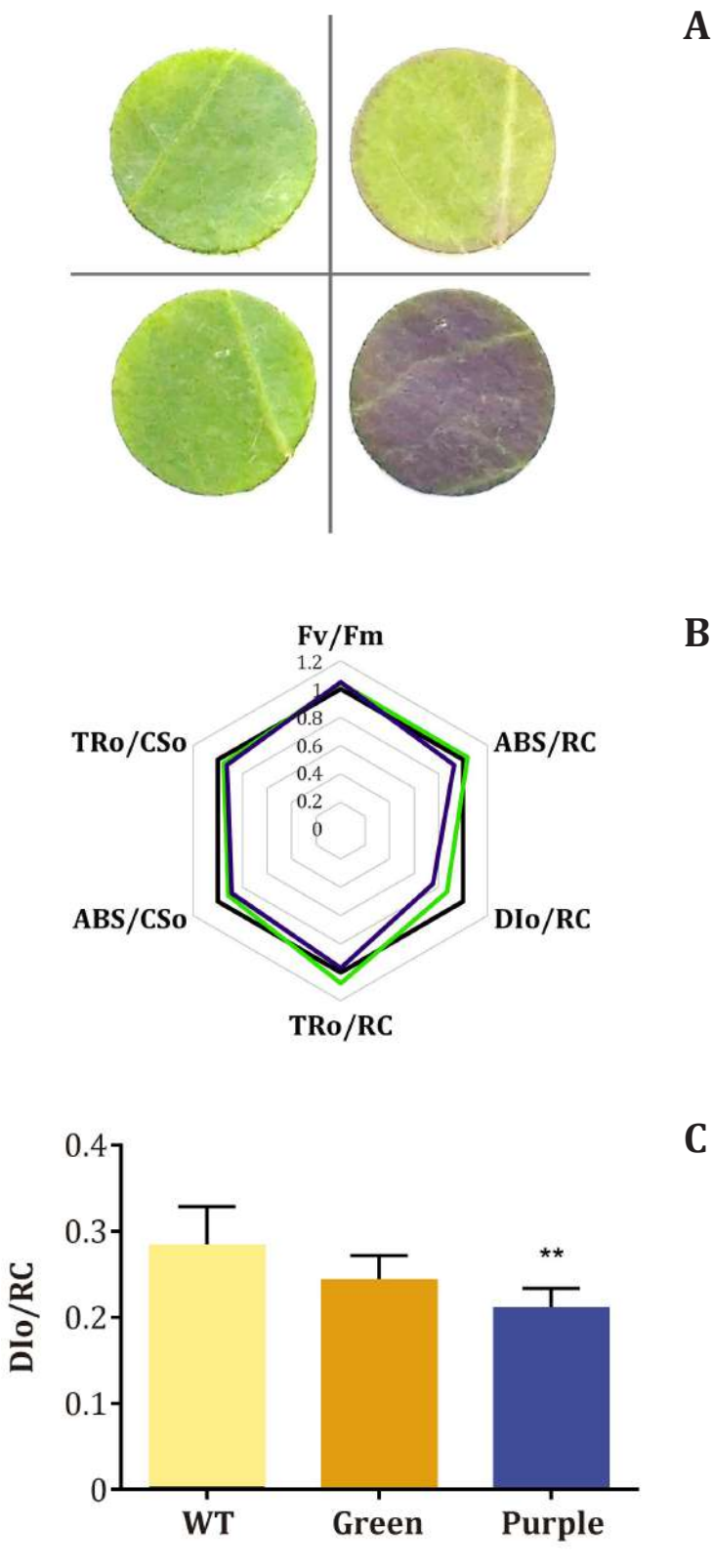


Figure 4. Physiological performance of micro-propagated *S. bonariensis* plants.
Figura 4. Desempeño fisiológico de plantas micropropagadas de *S. bonariensis*.

(A) Adaxial face (upper left and right) and abaxial face (lower left and right) of green and purple leaves from micro-propagated plants, respectively. (B) Mean values of six OJIP parameters are shown in radar charts for WT (black line), micro-propagated green leaf (light grey line), and micro-propagated purple leaf (dark grey line). Results are expressed relative to WT, assigned as 1. Each parameter is defined in the text. (C) Dissipation energy per reaction center determination in WT (yellow), micro-propagated green leaf (orange), and micro-propagated purple leaf (blue). Results are means of 7 biological replicates ± SD. Statistical analysis was performed by one-way ANOVA followed by Tukey's Multiple Comparison Test using Prism 5 (GraphPad Software, CA, USA). ** $p < 0.01$.

(A) Cara adaxial (arriba a la zquierda y derecha) y cara abaxial (abajo a la izquierda y derecha) de hojas verdes y moradas de plantas micropropagadas, respectivamente. (B) Los valores promedios de 6 parámetros OJIP se muestran en gráficos de radar para WT (línea negra), hoja verde micropropagada (línea gris claro) y hoja púrpura micropropagada (línea gris oscuro). Los resultados se expresan en relación con WT, que se asignó a 1. La definición de cada parámetro se proporciona en el texto. (C) Energía de disipación por determinación del centro de reacción en WT (amarillo), hoja verde micropropagada (anaranjado) y hoja morada micropropagada (azul). Los resultados muestran el promedio de 7 réplicas biológicas ± DS. El análisis estadístico se realizó mediante análisis de varianza unidireccional (ANOVA) seguido de una prueba de comparación múltiple de Tukey utilizando Prism 5 (GraphPad Software, CA, EE. UU.). **p<0,01.

Discussion

The growing interest in medicinal bio-compounds of *S. bonariensis* underscores the necessity for a large-scale production protocol. While micropropagation and *in vitro* propagation protocols have been documented for other Senecio species (7, 16, 18), a particular protocol for *S. bonariensis* is currently lacking. We successfully identified the optimal conditions for producing *S. bonariensis* plants by investigating eighteen combinations of two cytokinins and one auxin on shoot production. All tested combinations resulted in callus formation, while the percentage of callus induction (PCI) varied according to hormone group. Remarkably, we found that MS medium lacking plant growth regulators did not induce shoot formation. However, combinations of NAA-BAP proved more effective in inducing callus compared to NAA-KIN. In a study by Hariprasath *et al.* (2015), *S. candicans* exposed to NAA 0.5 µg ml^{-1} and either 1 or 2 µg ml^{-1} BAP resulted in 37% and 47% average PCI, respectively. In contrast, we achieved a 1.7-fold higher callus induction (66% and 80%, as shown in figure 1B, page 140). Notably, MS supplemented with NAA 0.5 µg ml^{-1} and BAP 0.5 µg ml^{-1} achieved 90% PCI. Other authors observed no differences in PCI for *S. candicans* between NAA-BAP and NAA-KIN combinations. However, for S*. bonariensis*, PCI was lower when MS was supplemented with NAA-KIN (figure 1D, page 140). We observed a twofold lower PCI with NAA-KIN combinations (0.5 µg ml^{-1} NAA - 2 µg ml^{-1} KIN and 1 µg ml^{-1} NAA - 2 µg ml^{-1} KIN), although similar PCI was observed when KIN was tested at 1 µg ml^{-1}. Notably, these calli did not produce shoots. After obtaining calluses, we evaluated shoot induction. NAA-BAP combinations resulted in 100% shoot induction for *S. bonariensis* (figure 2A, page 141), as previously found on *S. macrophyllus* M. Bieb, with 100% shoot induction (18), and *S. cruentus* cv. Tokyo Daruma, with 86.4% to 98.4% shoot induction (16). In contrast, shoot formation in *S. candicans* ranged from 48% to 76% (7). All NAA-BAP combinations were highly effective for inducing *S. bonariensis* shoots. In contrast with other Senecio species, *S. bonariensis* did not produce shoots from calli treated with NAA-KIN (figure 2B, page 141), (7, 16, 18). This may be attributed to KIN being a weaker cytokinin than BAP (1, 2). Further studies should consider the effect of different concentrations of KIN to determine optimal conditions for significant shoot production.

Shoot number per explant was assessed after 60 days of incubation. Shoot induction occurred in the presence of NAA-BAP. Mean shoot number per explant ranged from 3.3 to 6, similar to Trejgell *et al.* (2010). Shoot length was comparable to other Senecio species (7, 16, 18).

Leaf purplish coloration during rustication could constitute a form of plant photoprotection, of the immature photosynthetic apparatus, dissipating high irradiance and mitigating potential damage from solar radiation (15). We evaluated *in vitro*-propagated plants using a non-invasive OJIP test, ideal for researching valuable plant material that should not be destroyed. Considering our results regarding physiological and energetic parameters, *i.e.* Fv/Fm, ABS, TRo, RC and Cso in green and purple leaves under high-light stress, the maximum quantum yield of photosystem II (PSII) (Fv/Fm) decreases due to photo-oxidative damage, as previously reported (9, 12). However, the non-significantly different physiological state among leaves, followed by a significant decrease in DIo/RC in purple leaves of micro-propagated plants compared to unpropagated (WT) and green leaves, indicated that purple coloration did not provide photoprotection. This is because dissipation prevents photodamage. Interestingly, some leaves of micro-propagated plants displaying purple phenotype showed no physiological disturbances. This phenotype disappears one or two weeks after being transplanted to the field. Further studies are required to understand these findings. Notably, micro-propagated plants successfully flowered and attracted pollinators.

Conclusion

We created a straightforward and efficient procedure for the large-scale propagation of *S. bonariensis*. This protocol holds promise for diverse applications, ranging from medicinal research and ecological studies to commercial landscaping. Furthermore, it offers valuable insights into other Senecio species.

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Declarations

All the authors declare that they have no conflicts of interest.

REVIEW

Trends and research hotspots in principal genera of Platypodinae-fungi association: a bibliometric analysis on *Euplatypus, Megaplatypus* and *Platypus* (Coleoptera: Platypodinae)

Tendencias y principales áreas de investigación en los principales géneros de Platypodinae-hongos asociados, un análisis bibliométrico sobre *Euplatypus*, *Megaplatypus* y *Platypus* (Coleoptera: Platypodinae)

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INDEX

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Abstract

Ambrosia beetles of the subfamily Platypodinae are symbiotically associated with fungi, which provide them with food and benefit their establishment and growth. In the present study, our interest centers on the principal genera of Platypodinae: *Euplatypus*, *Megaplatypus* and *Platypus*, the most relevant symbionts being species of *Fusarium*, *Graphium* and *Raffaelea*. The objective of this work is to update the description of fungal associations on those species of interest to the scientific community, ONGs and funding institutions. An exhaustive search was performed to cover all scientific studies from 1900 to 2024 on the co-occurrence or relationship between members of the above-mentioned Platypodinae and fungi. Records of insect and fungal species, host plants and geographic locations were collected. A bibliometric analysis was conducted to characterize the overall status, general trends and current research hotspots of fungi associated with these ambrosia beetles. Eighty percent of the publications retrieved explored the association of *Platypus* spp. with different fungi. *Raffaelea* was the fungal genus showing the highest number of records and worldwide distribution. Five countries from four continents currently lead research on these associations. However, greater insights into these interactions would improve decision-making on managing these pests.

Keywords

ambrosia • beetles • *Fusarium* • *Graphium* • *Raffaelea* • Google Scholar • Scopus • Web of Science

Resumen

Los escarabajos de ambrosía de la subfamilia Platypodinae están asociados simbióticamente con hongos que les proporcionan alimento y benefician su establecimiento y crecimiento. En el presente trabajo, los principales géneros de interés de Platypodinae son *Euplatypus*, *Megaplatypus* y *Platypus*, y los simbiontes más relevantes son especies de *Fusarium*, *Graphium* y *Raffaelea.* El objetivo del trabajo es ofrecer una síntesis actualizada de la información sobre esas especies de interés, para la comunidad científica, las ONG e instituciones financiadoras. Se realizó una búsqueda exhaustiva de todos los estudios científicos desde 1900 hasta 2024 con datos sobre coocurrencia o relaciones entre los Platypodinae antes mencionados y hongos, recogiéndose los registros de especies de insectos y hongos, planta hospedante y localización geográfica. Se realizó un análisis bibliométrico para caracterizar el estado global, las tendencias generales y las áreas de investigación con mayor incidencia sobre hongos asociados a estos escarabajos de ambrosía. El ochenta por ciento de las publicaciones recuperadas exploran la asociación de *Platypus* spp. con diferentes hongos. *Raffaelea* fue el género fúngico con mayor número de registros, con distribución mundial. Cinco países, de cuatro continentes, lideran actualmente la investigación de estas asociaciones. Sin embargo, un mayor conocimiento de estas interacciones ayudaría en la toma de decisiones sobre la gestión de estas plagas.

Palabras clave

ambrosia • escarabajos • *Fusarium* • *Graphium* • *Raffaelea* • Google Scholar • Scopus • Web of Science

Introduction

Platypodinae (pinhole borers) is a subfamily of wood-boring beetles that belong to the Curculionidae family. Most species of this subfamily are members of the artificial group named ambrosia beetles, which form a mutualistic symbiosis with ambrosia fungi, in which fungi are vectored and inoculated directly into wood by the beetle. Timber quality is affected by the staining produced by ambrosia fungi and gallery systems that extend deep into wood (10). Ambrosia beetles feed on fungi growing on sapwood, which provide nutrients and suitable moisture for the development of larvae and pupae (8).

Several Platypodinae species are of particular importance to forest health and have a significant economic impact on forest and fruit tree production in tropical and subtropical countries. Polyphagous species of *Euplatypus* S.L. Wood (*Euplatypus parallelu*s Bright & Skidmore), *Megaplatypus* S.L. Wood (*Megaplatypus mutatus* Chapuis) and *Platypus* J.F.W. Herbst (*Platypus cylindrus* J.C. Fabricius*, Platypus koryoensis* Wood & Bright and *Platypus quercivorus* Murayama) cause high economic loss after attacking forest or fruit plantations (9, 29, 30, 31, 34, 95).

Examples of fungi found in association with platypodine beetles include *Fusarium* spp. (17, 18), whose functional roles as a source of nutrition or essential compounds for beetle development have been proposed (15, 33, 68). Species of *Graphium* Corda (Microascales) have been related to Platypodinae (17, 65). For example, *Graphium basitruncatum* (Matsush.) Seifert & G. Okada has been linked with *M. mutatus* males and galleries (18), suggesting that this fungus is a regular associate rather than a primary nutritional ambrosia fungus (17, 19). The synnematous anamorphs of *Ophiostoma* Syd. & P. Syd. and *Pseudallescheria* Negr. & I. Fisch., among other genera, which can be dominant in bark beetle galleries, were often classified as *Graphium* in the past (21).

Raffaelea Arx & Hennebert (Ophiostomatales) is another important associate of Platypodinae. This genus is one of the most widespread ambrosial mutualist genera. The genus has colonized many independent beetle groups throughout its evolution (24). *Raffaelea* species constitute three clades: *Raffaelea sensu stricto*, *Raffaelea lauricola* complex and *Raffaelea sulphurea* complex. Several of these species belong to the genus *Harringtonia* (22). *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva, the etiological agent of laurel wilt, is associated with several ambrosia beetles and attacks lauraceous species (38, 75). Within the *R. sulphurea* complex, *Raffaelea quercivora* Kubono & Shin. Ito is responsible for Japanese oak wilt and associated with *P. quercivorus* (59).

Although numerous studies are exploring fungi associated with *Euplatypus* spp., *Megaplatypus* spp. and *Platypus* spp., these Platypodinae genera, here referred to as EMP, no comprehensive overview of these interactions has yet been compiled.

Scientific databases enable modern research, offering a major reservoir of knowledge and insights across disciplines. They have organized vast amounts of scholarly literature, experimental data and research findings. This is the first time that an EMP and associated fungi database is created to provide an updated database that compiles trends and research hotspots among EMP species within the genera *Fusarium*, *Graphium* and *Raffaelea* and other fungi, enhancing data-sharing to advance science.

Methodology

A search was conducted in the Global Biodiversity Information Facility (GBIF) database (www.gbif.org) and the Bark and Ambrosia Beetles of North and Central America (BAB) database (www.barkbeetles.info) to identify world records of species of the genera EMP. An extensive literature search was performed using Google Scholar (www.scholar.google.com), Scopus (www.scopus.com) and Web of Science (WoS) (www.mjl.clarivate.com) until March 2024. The search included all species previously retrieved from GBIF-BAB and was based on keywords: [each species] *Euplatypus* OR [each species] *Megaplatypus* OR [each species] *Platypus*, plus (+) '*Fusarium*' OR '*Graphium*' OR '*Raffaelea*' OR "Fungi', the last including other fungal genera (e.g. minus (-) '*Fusarium*' - '*Graphium*' - '*Raffaelea*') (figure 1, page 149).

An additional investigation of GBIF was performed to find synonyms for EMP species. Only English-written publications were included in this analysis. In the present study, current species of *Harringtonia*, such as *Harringtonia lauricola* (T.C. Harr., Fraedrich & Aghayeva) Z.W. de Beer & M. Procter (23) were considered within the genus *Raffaelea* due to their historical relevance. Searches covered the last 124 years.

Retrieved publications were screened to meet specific criteria for EMP, which included: 1) record(s) of the beetle-fungus association; 2) geographical reference; and 3) the identity of plant hosts attacked by the insect. The results of this bibliographic research were analyzed using R Studio (2021) on a curated dataset.

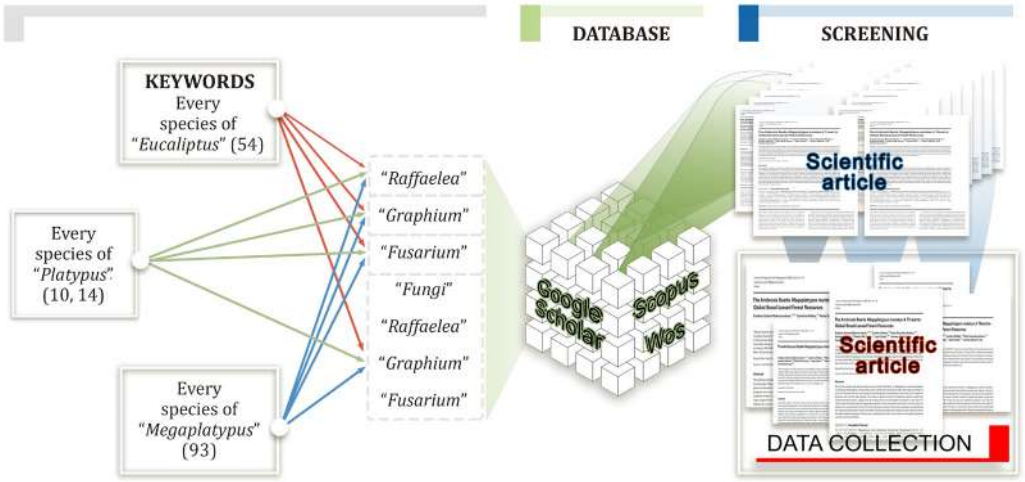


Figure 1. Workflow of search combinations in database search. Numbers in parentheses indicate the number of species within each genus.

Figura 1. Flujo de trabajo realizado mediante combinaciones de búsqueda en diferentes buscadores. Los números entre paréntesis indican el número de especies dentro de cada género.

All the publications of the bibliographic search were grouped under the following categories: *genus of the beetle, species of the beetle, fungal genus, fungal species, region, author, year, longitude* and *latitude*. Categories of fungal species were *Fusarium, Graphium, Raffaelea* and '*Other fungi*', which included the rest of fungal genera. The category *Author* comprised the author of each record or the author of the publication when the record was not registered by the finder. Category *Year* included year of registration or year of publication. The longitude and latitude of the distribution data were obtained using Google Earth 7.1.3. Several reports included only the country; yet, some cases, the province or city was also informed. The map of records was drawn on the MapChart website (www.mapchart.net), where cities, provinces or countries were painted on the map. An additional pie chart was added to each record on the map to show the percentage of fungal records.

VOSviewer version 1.6.19 (2023) was used to build up a network with leading authors, countries, institutions and keywords; it was set up as follows: co-authorship analysis, without clusters, no-normalization, attraction 8 and repulsion 1; co-occurrence analysis for all keywords, without clusters Linlog/modularity, attraction 5 and repulsion 0 (23). In the network map made by this software, each node represents elements, such as authors or countries; the size of nodes represents frequency of occurrence; the color of nodes shows affiliation according to co-authorship and co-occurrence analysis, respectively. The links between two nodes establish collaborative relationships between elements, such as authors or institutions. The thickness of the connecting lines increases with higher collaboration frequencies, and papers with no connections were disregarded by the software.

We selected for further analysis only studies where the association between beetles and fungi was described; studies where the beetle-fungi association was not reported as a new record were dismissed.

Sources and Results

Searches with Google Scholar retrieved more results than those with Scopus or WoS (figure 2). Google Scholar found fifty-six species of *Euplatypus,* ninety-five species of *Megaplatypus* and over one thousand species of *Platypus* in 2,481 publications: 219 publications for *Euplatypus*, 205 for *Megaplatypus,* and 2,057 for *Platypus.* Scopus retrieved 166 publications: 21 for *Euplatypus*, 11 for *Megaplatypus* and 134 for *Platypus*. WoS showed 124 publications: 20 for *Euplatypus,* 16 for *Megaplatypus* and 88 for *Platypus* (figure 2).

Google Scholar found sixty-eight publications on fungi related to EMP, Scopus showed fifty-two and WoS retrieved forty-two. All publications found via Scopus and WoS were also identified by Google Scholar. Most (55 publications) were related to *Platypus* spp. and their associated fungi (82%). Nine publications reported *Euplatypus* and their associated fungi (12%) and the remaining five publications (6%) dealt with *Megaplatypus* and their associated fungi (figure 3).

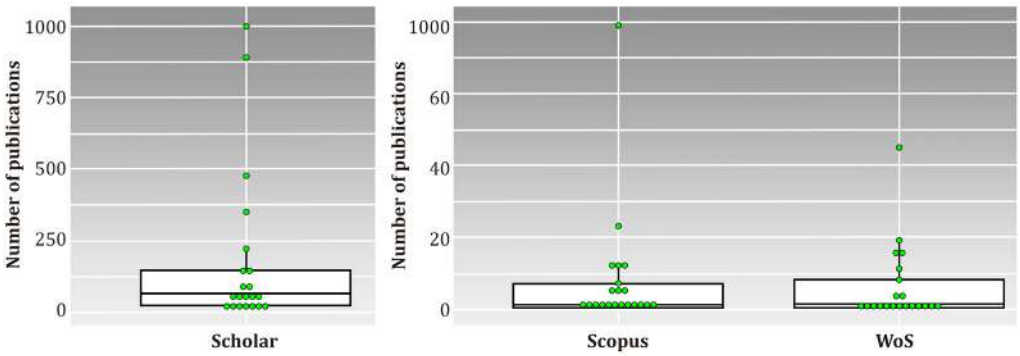


Figure 2. Publications shown for each search combination (figure 1, page 149) in each search engine (Google Scholar, Scopus and WoS).

Figura 2. Número de publicaciones obtenidas para cada combinación de búsqueda (figura 1, pág. 149) en cada buscador (Google Scholar, Scopus and WoS).

Available data range from 1945 to date (no records from 1900-1940 are available; data not shown); (B) Number of publications on *Euplatypus* and *Megaplatypus*; (C) Pie chart represents the percentage of publications for each beetle genus.

Los datos disponibles abarcan desde 1945 hasta la actualidad (no se dispone de registros para el periodo 1900-1940, datos no mostrados); (B) Número de publicaciones de *Euplatypus* y *Megaplatypus*. (C) El gráfico de torta indica el porcentaje de publicaciones para cada género de escarabajos.

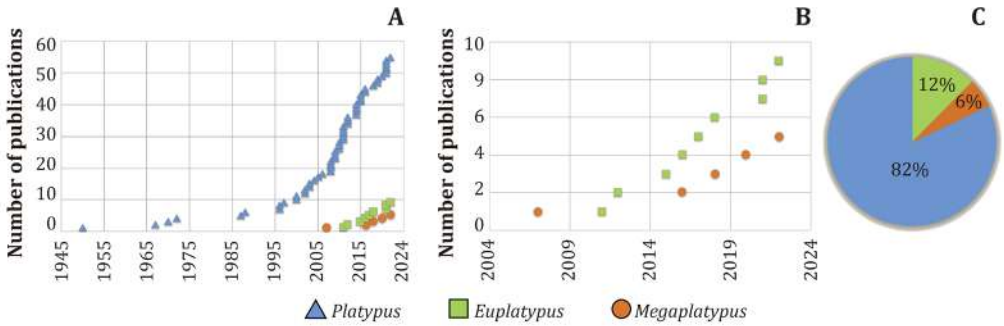


Figure 3. (A) Publications on the association between *Fusarium-Graphium-Raffaelea* (fungi) and *Platypus-Euplatypus-Megaplatypus* (Platypodinae genera).

Figura 3. (A) Publicaciones registradas sobre asociaciones entre *Fusarium-Graphium-Raffaelea* y *Platypus-Euplatypus-Megaplatypus* (géneros de Platypodinae).

Most publications focused on the association of Platypodinae with *Raffaelea,* followed by studies on the association of Platypodinae genera with *Fusarium* spp. The specific *Platypus-Raffaelea* combination was reported in the largest number of publications, followed by the *Platypus-Other fungi* combination (figure 4). Conversely, research on Platypodinae and *Graphium*, especially *Euplatypus-Graphium* and *Megaplatypus-Graphium*, is limited (figure 4).

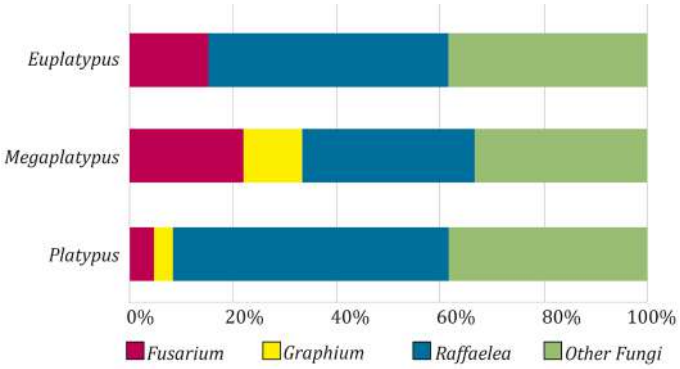


Figure 4. Percentage of publications for each beetle genus–fungal genus combination retrieved from Google Scholar.

Figura 4. Porcentaje de publicaciones para cada combinación de géneros de escarabajo-género fúngico recuperadas de Google Scholar.

Nine publications associated *Euplatypus* with fungi; the beetle species were *Euplatypus compositus* Say & T.*, Euplatypus longius* Bright & Skidmore*, E. parallelus, Euplatypus segnis* Bright & Skidmore and other undescribed species of *Euplatypus*. Four publications linked *M. mutatus* or *Megaplatypus godmanii* Bright & Skidmore to fungi, *M. mutatus* being the species most widely studied within the genus. Fifty-five publications related *Platypus* to fungi. Ten species of *Platypus* were included in these studies*: Platypus calamus* Blandford*, P. cylindrus, Platypus externedentatus* Faimare*, Platypus flavicornis* Dalman*, P. koryoensis, Platypus oxyurus* Dufour*, P. quercivorus, Platypus solidus* F. Walker*, Platypus subgranosus* Schedl.*, Platypus wilsoni* J. M. Swaine*,* together with undescribed species of *Platypus.*

Fungi associated with EMP were *Fusarium oxysporum* Schltdl.*, Fusarium solani* (Mart.) Sacc. and undescribed species of *Fusarium*; *Graphium basitruncatum* (Matsush.) Seifert & G. Okada and undescribed species of *Graphium*; *Raffaelea albimanens* D.B. Scott & J.W. du Toit, *Raffaelea ambrosiae* Arx & Hennebert, *Raffaelea campbellii* D.R. Simmons, A. Campb. & R.C. Ploetz*, Raffaelea canadensis* L.R. Batra, *Raffaelea cyclorhipidii* D.R. Simmons & Y.T. Huang, *Raffaelea hennebertii* D.B. Scott & J.W. du Toit, *R. lauricola, Raffaelea montetyi* M. Morelet, *Raffaelea quercina M*.L. Inácio, E. Sousa & F. Nóbrega, *R. quercivora, Raffaelea quercus-mongolicae* K.H. Kim, Y.J. Choi & H.D. Shin, *Raffaelea rapaneae* Musvuugwa, Z.W. de Beer, Dreyer & Roets, *Raffaelea santoroi* Guerrero*, Raffaelea subalba* T.C. Harr., Aghayeva & Fraedrich and undescribed species of *Raffaelea.* Other genera were also sporadically obtained: *Penicillium* Link and *Ceratocystis* Ellis & Halst.*,* among others (figure 5, page 152).

Most of the studies retrieved in this research involved interaction between *P. quercivorus* - *R. quercivora* (72 records, 17 publications) and *P. koryoensis* - *R. quercus-mongolicae* (62 records, 7 publications): two specific associations*.* Another well-documented association was found between *P. cylindrus-R. ambrosiae*, *R. canadensis* and *R. montetyi* (53 records, 15 publications). *Platypus cylindrus* has been reported in Algeria, Canada, England, France, Portugal and Tunisia.

Darker colors denote more publications in each combination.

La intensidad del color refleja un mayor número de publicaciones en cada combinación.

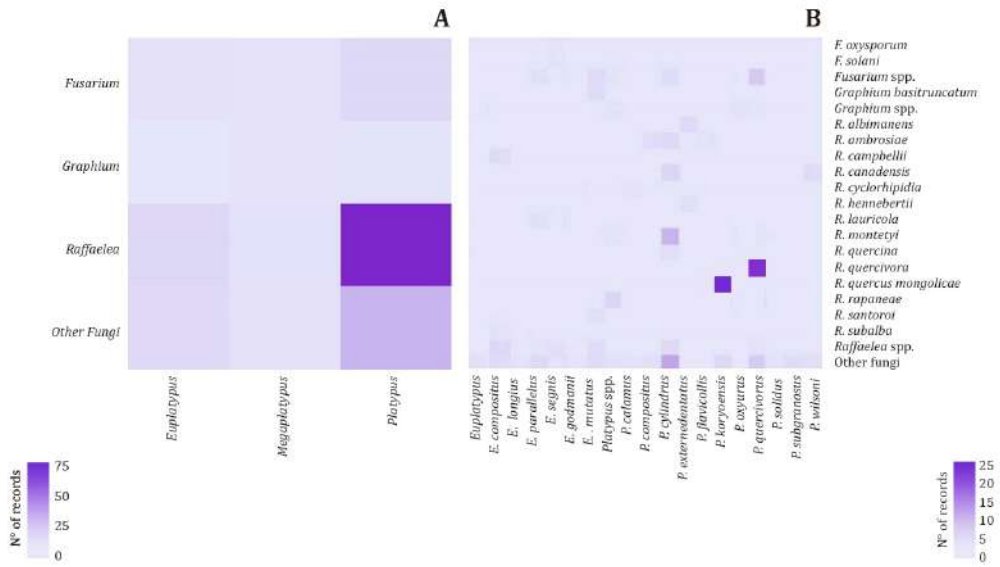


Figure 5. Heatmap of the relationship between (A) beetle genus-fungal genus and (B) fungal species and beetle species.

Figura 5. Mapa de calor sobre asociaciones entre: (A) géneros de escarabajo-género fúngico; (B) especies de escarabajo-especie fúngica.

Concerning *Euplatypus,* most of the records of this genus include species of *Fusarium* and *Raffaelea*. *Euplatypus compositus* (16 records, 6 publications) has been related to *Raffaelea* spp. and other fungi, while *E. paralellus* (9 records, 6 publications) and *E. segnis* (5 records, 2 publications) have been linked to *Fusarium* spp., *Raffaelea* spp. and other fungi. Most records of *Euplatypus* come from the USA and Mexico, with a few from Southeast Asia and Central America (Belice).

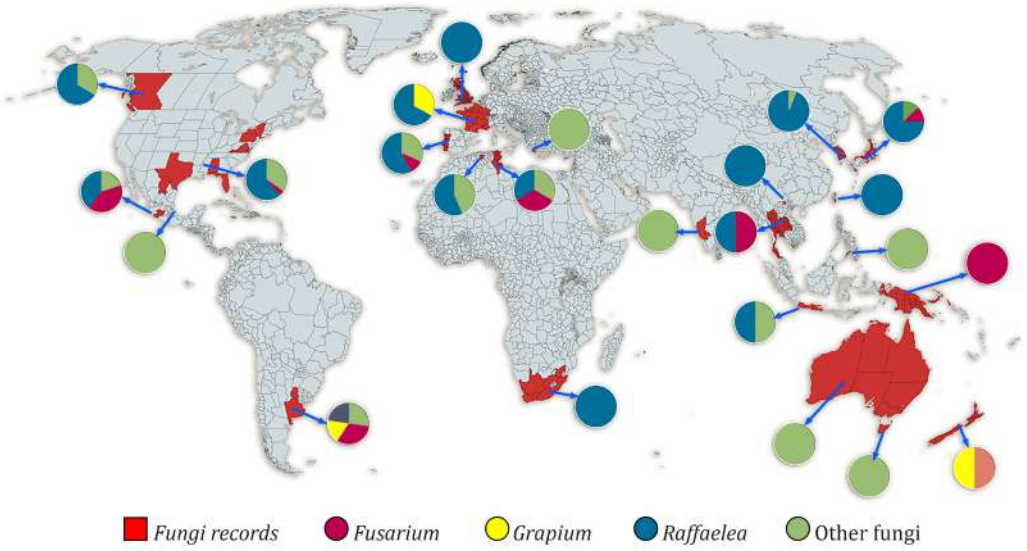
In the case of *M. mutatus,* several studies have related this beetle to undescribed species of *Fusarium* and *Raffaelea, R. santoroi* and *G. basitruncatum* (2, 17, 18, 19). This beetle has been found in many countries; however, reports of its associated fungi are currently limited to Argentina (2). Although this genus has 95 registered species, association with fungi (23 records, 5 publications) has only been found in two, *M. godmani* and *M. mutatus*, both considered as forest pests. The remaining species have not been registered as pests or linked with fungi.

After reviewing the results of bibliometric analysis for countries and authors, our findings suggest that Argentina, Japan, Portugal, South Korea and the USA (alphabetically) are the five countries leading research into ambrosia fungi-Platypodinae (figure 6, page 153).

A total of 23 plant genera were recorded as affected by the ambrosia beetle-fungi association (table 1, page 153-154-155), mainly angiosperms, with *Carya* Nutt., *Castanopsis* (D. Don) Spach, *Casuarina* L., *Nothofagus* Blume, *Persea* Mill., *Populus* L. and *Quercus* L. being the genera most commonly cited. However, most records belong to *Quercus*, with 198 records distributed across 13 countries worldwide.

Through a map of density on Vosviewer, 17 countries seemed to be the most relevant for authors and institutions. A higher density shown by a more intense color and larger bubbles represented greater participation of that country in studies on ambrosia beetles and their associated fungi. Japan, Portugal, South Korea and the USA showed the highest number of publications on ambrosia beetle-fungi within Platypodinae (figure 7, page 156).

As a result of the keyword analysis, the nine most important words were identified: Fungi, Coleoptera, Platypodidae, Scolytinae, Curculionidae, classification, phylogeny, *Platypus quercivorus* and Ophiostomaceae (figure 8, page 156).



Co-authorship was the type of analysis. Countries were the units of analysis. Density view was selected.

La coautoría fue el tipo de análisis. Los países fueron las unidades de análisis. La visualización fue elegida por vista de densidad.

Figure 6. Map based on bibliographic data from selected publications.
Figura 6. Mapa basado en datos bibliográficos, a partir de publicaciones seleccionadas.

Table 1. Summarizes the records from 69 publications, with 53 authors of records, from 22 countries, covering the period from the beginning of the last century to the present.
Tabla 1. Resume la información sobre los registros contenidos en 69 publicaciones, con 53 autores de registros, entre 22 países, abarcando el período desde el inicio del siglo pasado hasta la actualidad.

Beetle genus	Beetle species	Fungal species	Country	Host plant	Reference
Euplatypus	*E. compositus*	*R. ambrosiae*	USA	*Quercus* sp.	(8)
		R. ambrosiae	USA	*Carya* sp.	(37)
		R. campbellii	USA	*Quercus* sp.	(62)
		R. campbellii	USA	--	(85)
		R. subalba	USA	--	(85)
		Raffaelea sp.	USA	*Quercus* sp.	(62)
		Other fungi	USA	*Liquidambar* sp.	(8)
		Other fungi	USA	*Quercus* sp.	(8, 62)
	E. longius	Other fungi	Belice	*Quercus* sp.	(6)
	E. paralellus	*Fusarium* sp.	Thailand	*Pterocarpus* sp.	(41)
		Fusarium sp.	USA	*Quercus* sp.	(62)
		R. lauricola	USA	*Persea* sp.	(67, 74)
		Other fungi	Indonesia	*Pterocarpus* sp.	(89)
		Other fungi	USA	*Quercus* sp.	(6, 62)
	E. segnis	*F. oxysporum*	Mexico	*Carya* sp.	(3)
		F. solani	Mexico	*Carya* sp.	(3)
		Raffaelea sp.	Mexico	*Persea* sp.	(5)
		Other fungi	Mexico	*Carya* sp.	(3)
	Euplatypus sp.	Other fungi	Philippines	*Pinus* sp.	(63)

Detailed data for each record can be found in Table 1S.

Los datos detallados de cada registro se pueden encontrar en la Tabla 1S.

Beetle genus	Beetle species	Fungal species	Country	Host plant	Reference
Megaplatypus	*M. godmanii*	Other fungi	Belice	*Quercus* sp.	(6)
	M. mutatus	*Graphium* sp.	Argentina	*Populus* sp.	(18)
		G. basitruncatum	Argentina	*Populus* sp.	(17, 19)
		G. basitruncatum	Argentina	*Casuarina* sp.	(19)
		F. oxysporum	Argentina	*Casuarina* sp.	(18)
		Fusarium sp.	Argentina	*Casuarina* sp.	(18, 19)
		Fusarium sp.	Argentina	*Populus* sp.	(17, 18, 19)
		R. santoroi	Argentina	*Populus* sp.	(2)
		Raffaelea sp.	Argentina	*Populus* sp.	(17, 19)
		Raffaelea sp.	Argentina	*Casuarina* sp.	(19)
		Other fungi	Argentina	*Populus* sp.	(17, 18, 19)
		Other fungi	Argentina	*Casuarina* sp.	(18, 19)
Platypus	*P. calamus*	*R. cyclorhipidii*	Japan	*Acer* sp.	(82)
		Other fungi	Japan	*Acer* sp.	(82)
	P. cylindrus	*Fusarium* sp.	Portugal	*Quercus* sp.	(12, 43)
		Fusarium sp.	Tunisia	*Quercus* sp.	(12)
		R. ambrosiae	Canada	*Quercus* sp.	(1)
		R. ambrosiae	England	*Quercus* sp.	(35)
		R. ambrosiae	UK	--	(39)
		R. ambrosiae	USA	*Quercus* sp.	(8)
		R. canadensis	Algeria	*Quercus* sp.	(4, 12)
		R. canadensis	Portugal	*Quercus* sp.	(47)
		R. montetyi	Algeria	*Quercus* sp.	(4, 11, 12)
		R. montetyi	Portugal	*Quercus* sp.	(12, 47)
		R. montetyi	Tunisia	*Quercus* sp.	(12)
		R. quercina	Portugal	*Quercus* sp.	(48)
		Raffaelea sp.	Portugal	*Quercus* sp.	(16, 44, 46)
		Other fungi	Algeria	*Quercus* sp.	(4, 11, 14)
		Other fungi	European country	*Ceratonia* sp.	(69)
		Other fungi	Greece	*Mussa* sp.	(86)
		Other fungi	Portugal	*Quercus* sp.	(12, 16, 45, 46, 49)
		Other fungi	Spain	*Quercus* sp.	(80)
		Other fungi	Tunisia	*Quercus* sp.	(14)
		Other fungi	USA	*Quercus* sp.	(8)
	P. externedentatus	*R. albimanens*	South Africa	--	(39)
		R. albimanens	South Africa	*Ficus* sp.	(70, 71, 83)
		R. hennebertii	South Africa	*Ficus* sp.	(70, 71, 83)
	P. flavicornis	*R. ambrosiae*	USA	*Quercus* sp.	(8)
	P. koryoensis	*R. quercus-mongolicae*	South Korea	*Quercus* sp.	(53, 54, 60, 61, 87)
		Other fungi	South Korea	--	(40)
		Other fungi	South Korea	*Quercus* sp.	(87, 94)
	P. oxyurus	*Graphium* sp.	France	*Quercus* sp.	(16)
	P. quercivorus	*Fusarium* sp.	Japan	*Quercus* sp.	(79)
		R. quercivora	Indonesia	*Castanopsis* sp.	(59)
		R. quercivora	Japan	*Castanea* sp.	(57)
		R. quercivora	Japan	*Pasania* sp.	(57)

Detailed data for each record can be found in Table 1S.

Los datos detallados de cada registro se pueden encontrar en la Tabla 1S.

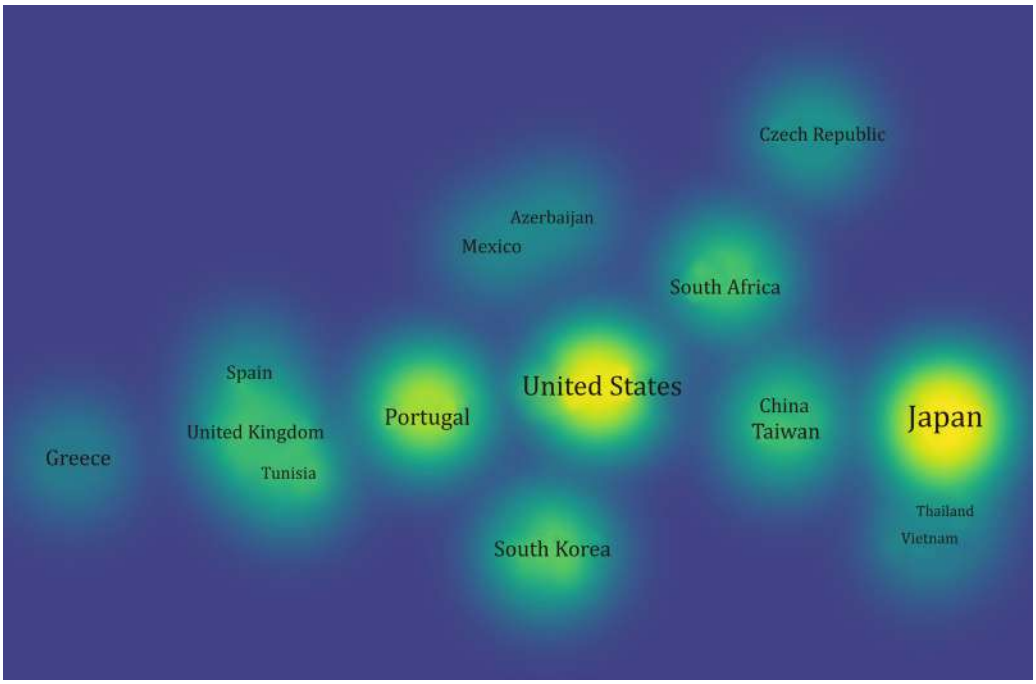
Beetle genus	Beetle species	Fungal species	Country	Host plant	Reference
Platypus	*P. quercivorus*	*R. quercivora*	Japan	*Quercus* sp.	(28, 32, 43, 50, 56, 57, 59, 72, 84, 88, 93)
		R. quercivora	Taiwan	*Castanopsis* sp.	(59)
		R. quercivora	Thailand	*Podocarpus* sp.	(59)
		R. quercivora	Vietnam	*Lithocarpus* sp.	(59)
		R. quercivora	Vietnam	*Quercus* sp.	(59)
		Raffaelea sp.	Japan	*Quercus* sp.	(55)
		Other fungi	Japan	--	(25)
		Other fungi	Japan	*Castanopsis* sp.	(26, 27)
		Other fungi	Japan	*Quercus* sp.	(26, 27, 28, 55, 79)
	P. subgranosus	Other fungi	Australia	--	(7)
		Other fungi	Australia	*Nothofagus* sp.	(52)
		Other fungi	Tasmania	*Nothofagus* sp.	(51)
	P. solidus	Other fungi	India	*Quercus* sp.	(8)
	P. wilsoni	*R. canadensis*	Canada	*Pseudotsuga* sp.	(8)
		R. canadensis	Canada	*Tsuga* sp.	(37, 82)
		Other fungi	Canada	*Pseudotsuga* sp.	(8)
		Other fungi	Canada	--	(78)
	Platypus sp.	*Fusarium* sp.	New Guinea	*Theobroma* sp.	(81)
		Graphium sp.	New Zealand	*Nothofagus* sp.	(77)
		R. montetyi	France	*Quercus* sp.	(65)
		R. rapanae	South Africa	*Rapanea* sp.	(70, 71)
		Other fungi	India	--	(8)
		Other fungi	New Zealand	*Nothofagus* sp.	(66)

Detailed data for each record can be found in Table 1S.

Los datos detallados de cada registro se pueden encontrar en la Tabla 1S.

Some of these keywords were repeated throughout all the publications considered in this survey, thus underlying the importance of certain groups of words identified in this analysis. Fungi and Coleoptera appeared as prominent words in all these articles, which were our areas of interest in this study. The term Platypodidae was associated with an older taxonomic classification of this taxon, now placed at the rank of subfamily Platypodinae. The frequent usage of this keyword is probably attributed to the fact that it has been used for a long time. Another prominent word is Curculionidae, the family that includes (both) Platypodinae and Scolytinae.

Another keyword was ophiostomatoid, an artificial fungi group of Ascomycota which includes important tree pathogens that cause tree mortality and can develop a symbiotic relationship with EMP, as in the species of *Graphium* and *Raffaelea* (28, 39, 57). This group is also composed of many of the fungal genera analyzed in this study, categorized as '*Other fungi*', along with these two key genera that were our primary focus.

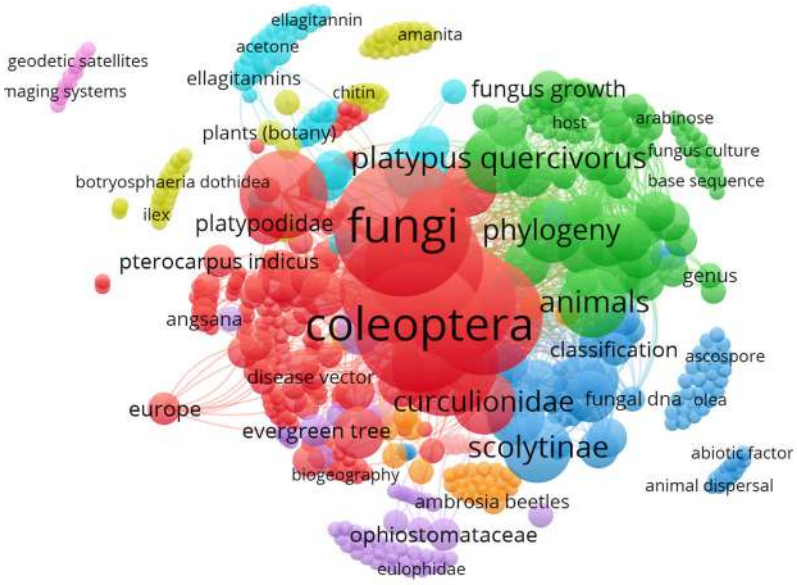


Co-authorship was the type of analysis. Countries were the units of analysis. Density view was selected.

La coautoría fue el tipo de análisis. Los países fueron las unidades de análisis. La visualización fue elegida por vista de densidad.

Figure 7. Map based on selected bibliographic data.

Figura 7. Mapa basado en datos bibliográficos seleccionados.



Co-occurrences were the type of analysis. Keywords were the units of analysis; the size of each word indicates frequency of occurrence and interaction with the different articles analyzed.

Las coocurrencias fueron el tipo de análisis. Las palabras clave fueron las unidades de análisis; el tamaño de cada palabra indica su frecuencia de aparición y su interacción con los diferentes artículos analizados.

Figure 8. Map based on selected bibliographic data.

Figura 8. Mapa basado en datos bibliográficos seleccionados.

Discussion

In the present study, bibliometric information was used to analyze literature on fungi associated with three genera of Platypodinae, from the beginning of the nineteenth century to the present, to gain a clearer understanding of current research, trends and hotspots of these associations. More publications were found on Google Scholar than on Scopus or WoS. Accordingly, further analysis was performed using Google Scholar.

The first studies on *Platypus* and their associated fungi were published in 1945; however, before 2000, no publications were found on *Euplatypus* or *Megaplatypus* with their associated fungi. Since 2000, the number of publications has rapidly increased, especially since the 2010s. This could be linked an increasing interest in understanding the relationship between the dispersion of ambrosia beetles, including Platypodinae, and their impact on global economies, while also addressing the influence of international trade and climate change on these beetle-fungi interactions (36, 64, 76, 90). An increase in global temperature and the occurrence of extreme meteorological events might contribute to changing population outbreaks and propagating non-native ambrosia beetles outside their native range (76). Most fungal records correspond to *Raffaelea*, which has a worldwide distribution. *Raffaelea* is a crucial genus in most platypodine ambrosial associations, and a few of its species are regarded as important phytopathogens (20). *Raffaelea quercivora* plays a causal role in mass mortality syndrome in Japanese oaks (57); it has been described throughout Japan as associated with *P. quercivorus.* This beetle species is a prominent keyword due to its economic relevance in Japan and the substantial number of associated studies (57). The pathogenicity of *R. quercus-mongolicae* has not been fully confirmed (54); nevertheless, its association with *P. koryoensis* has been intensively studied in South Korea. Several other species of *Raffaele*a have been found to be associated with *P. cylindrus* (4, 11, 13, 45, 47, 48, 49).

Fusarium species, on the other hand, seem to be relevant for the establishment of forest pests (20). Reports of *Fusarium* concentrate on the Northern Hemisphere and are more common than those of *Graphium*. Note that the presence of this genus might be underestimated in diversity studies, especially when using culture-based methods that benefit the growth of more competitive taxa (42). Publications on fungi are found mostly in Argentina, Japan, South Korea, Portugal and the USA. Finally, *Graphium* species have been associated with mycangial platypodines and can also be present in galleries and male exoskeletons, as in the case of *M. mutatus*-*G. basitruncatum*. It has been proposed that *G. basitruncatum* is one of the first colonizers of the host plant, particularly in newly excavated portions (20).

The almost skewed distribution of the publications analyzed shows that only those countries where EMP genera have been registered as forest pests have further studied their associated fungi. This distribution underlines the importance of these microorganisms at the time of pest settlement and the concentrated research efforts aimed at gaining a deeper understanding of these interactions for effective control *i.e.* as made for other biological models (73, 91).

In the co-occurrence network, which indicates collaboration between countries, it appears that there are limited collaborative relationships among these five countries. A relevant example is the promising and collaborative research program known as the 'Bark Beetle Mycobiome', dedicated to defining research priorities for the widespread insect-fungus symbiosis involving bark beetles. However, these programs are currently absent in the context of three genera of Platypodinae and of ambrosia beetles-fungi symbiosis. Initiating and strengthening these collaborations is essential to address knowledge gaps in this area.

Conclusion

This bibliometric analysis was successful in establishing the state-of-the-art publications on the relationship between EMP and fungi, indicating the most widely studied genera of beetles and fungi. The significance of ambrosia fungi as drivers of ecological interactions has been increasingly recognized. However, the present results suggest that ambrosia mycobiota is still underrepresented in research. Gaining thorough understanding of these interactions will shed light on the interconnectedness of species, contributing to our overall understanding of ecosystem dynamics and resilience.

Our analysis shows that Argentina, Japan, Portugal, South Korea and the USA (alphabetically ordered), among many other countries, have been conducting researches on fungal ambrosia, with limited collaboration between them. Despite successful collaborative initiatives internationally, there is a growing need for more effective partnerships to deepen the knowledge of South American ambrosia beetle-fungi symbiosis.

Supplemmentary material

https://docs.google.com/spreadsheets/d/1rwPDmqyY_Wo3aA0pOjdRNHybABWNSK0T/edit?usp=sharing&ouid=111310786017351827239&rtpof=true&sd=true

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REVIEW

Pseudocereals dietary fiber. Amaranth, quinoa, and buckwheat fiber composition and potential prebiotic effect

Fibra dietaria en pseudocereales. Composición y potencial efecto prebiótico de la fibra de amaranto, quinoa y trigo sarraceno

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INDEX

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Abstract

Amaranth (*Amaranthus*), buckwheat (*Fagopyrum esculentum*), and quinoa (*Chenopodium quinoa*) crops have limited production and agro-industrial development both in Argentina and globally. As the demand for functional ingredients and foods grows, developing products from these pseudocereals could offer substantial economic benefits. This study aims to analyze the dietary fiber content and composition of amaranth, quinoa, and buckwheat, and to investigate the relationship between dietary fiber structure and its potential prebiotic effects. Gaining insights into these aspects would provide valuable information for developing foods based on these pseudocereals and could enhance their future applications in the food industry.

Keywords: Pseudocereals • dietary fiber • prebiotic effect • microbiota

Resumen

Los cultivos de amaranto (*Amaranthus*), el trigo sarraceno (*Fagopyrum esculentum*) y la quinoa (*Chenopodium quinoa*) tienen una producción y desarrollo agroindustrial limitados tanto en Argentina como a nivel mundial. Dado que la demanda de ingredientes y alimentos funcionales está en aumento, el desarrollo de productos a partir de estos pseudocereales podría ofrecer beneficios económicos sustanciales. Este estudio tiene como objetivo analizar el contenido y la composición de la fibra dietética del amaranto, la quinoa y el trigo sarraceno, y examinar la relación entre la estructura de la fibra dietética y sus posibles efectos prebióticos. Obtener información sobre estos aspectos proporcionaría datos valiosos para el desarrollo de alimentos basados en estos pseudocereales y podría potenciar sus aplicaciones futuras en la industria alimentaria.

Palabras clave: Pseudocereales • fibra dietaria • efecto prebiótico • microbiota

Pseudocereals

The pursuit of healthy lifestyles and more nutritious foods adaptable to various climatic conditions has spurred interest in underutilized or alternative crops, leading to a resurgence in pseudocereals. The World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), and the scientific community are working together to identify foods that can meet the needs of a growing global population. With a current population of 8 billion and projections reaching 10.9 billion by 2050, urgent solutions are needed to address the impending food crisis (7). The agroindustry faces the challenge of ensuring a sufficient food supply while upholding high productivity and quality standards.

According to the FAO, "food security exists when all people, at all times, have physical, social, and economic access to sufficient, safe, and nutritious food that meets their daily energy needs and dietary preferences for an active and healthy life." Currently, global food security relies on a few cereal varieties, with over 50% of caloric intake provided by wheat, maize, and rice (50). Pseudocereals are nutritionally superior to traditional cereals. They have higher protein content and are rich in essential amino acids, including lysine, arginine, tryptophan, and histidine. Furthermore, pseudocereals exhibit higher digestibility, bioavailability, and protein efficiency ratios (PER), comparable to milk casein (50). Unlike wheat, oats, barley, and rye, which contain gliadin, pseudocereals are gluten-free and safe for celiac patients (43).

Ongoing research in food science uncovers new, healthy food components. Bioactive peptides, found in various foods including pseudocereals, exemplify this discovery. These peptides, along with other beneficial components, classify pseudocereals as functional foods (45). Lipids, another crucial nutritional component, exhibit high unsaturation levels in pseudocereals (75-86%). Linoleic acid (omega-6) is the predominant fatty acid, followed by oleic and palmitic acids, with notable amounts of linolenic acid (omega-3) (54). Both linoleic and linolenic acids are essential for the body, offering benefits such as cardiovascular disease prevention and improved insulin sensitivity (43). Pseudocereals also provide substantial dietary fiber, akin to whole grains.

This fiber supports gastrointestinal health, aids in weight management, and reduces the risk of non-communicable diseases like diabetes and cardiovascular conditions (2). Additionally, pseudocereals are richer in minerals such as magnesium, calcium, zinc, iron, copper, and phosphorus compared to cereals (table 1) (50).

Table 1. Centesimal composition and mineral content of amaranth, quinoa, buckwheat pseudocereals, and wheat.

Tabla 1. Composición centesimal y contenido de minerales de amaranto, quinoa, trigo sarraceno y trigo.

		Pseudocereals			Cereal
		Amaranth	**Quinoa**	**Buckwheat**	**Wheat**
Proteins*		14.0 - 16.5	11.0 - 16.5	10.9 - 15.2	11.6 - 14.3
Carbohydrates*		55.1 - 67.3	64.2 - 69.0	58.5 - 69.4	61.0 - 78.4
Lipids*		5.6 - 8.8	4.1 - 7.5	1.3 - 3.4	1.7 - 2.3
Fiber*		11.1 - 20.6	6.72 - 19.7	6.7 - 29.5	12.6 - 22
Ash*		2.8 - 3.3	2.7 - 3.8	1.4 - 3.9	1.4 - 2.2
Minerals†	**Calcium**	180.1	32.9	60.9	34.8
	Magnesium	279.2	206.8	203.4	96.4
	Zinc	1.6	1.8	1.0	1.2
	Iron	9.2	5.5	4.7	3.3

*Data taken from Haros and Schoenlechner (2017), and Serna-Saldívar and Sanchez-Hernandez (2020b). Data expressed as g/100 g of dry weight.

*Data taken from Alvarez-Jubete *et al.* (2010). Data expressed as mg/100 g of dry weight.

*Valores tomados de Haros y Schoenlechner (2017) y Serna-Saldívar y Sanchez-Hernandez (2020b). Datos expresados como g/100 g en peso seco.

*Datos tomados de Alvarez-Jubete *et al.* (2010). Datos expresados como mg/100 g en peso seco.

Despite the potential benefits that pseudocereals offer, several factors still hinder the incorporation of these crops into global agri-food systems. These factors are diverse and include social aspects, economic factors like low market participation and lack of integration into mass consumer products, as well as agronomic factors like yield and lack of technology applied to these crops. Knowledge regarding pseudocereals yield and quality is restricted to small-scale systems with low investment cultivated in rustic ways, and therefore cannot be compared to mass crops knowledge. The abundance of technology and research available for traditional cereals, combined with numerous and established marketing channels, leads producers to choose not to invest in underutilized crops.

Pseudocereals centesimal composition

Table 1 presents the proximate composition ranges for wheat and pseudocereals such as amaranth, quinoa, and buckwheat for comparison. Their compositional values and technological and culinary behaviors are similar (4). Starch, which forms semi-crystalline granules, is the primary component in both pseudocereals and wheat. Although cereals generally have lower protein content, pseudocereals lack gluten-forming proteins, making their flour unsuitable for traditional baked goods.

Lipid content is typically higher in amaranth and quinoa compared to cereals. These pseudocereals have stable lipids due to high concentrations of tocopherols (4). As shown in table 1, dietary fiber content is similar between pseudocereals and wheat. Fiber levels largely depend on whether the seed is hulled, as most fiber is located in the outer coverings. Both cereals and pseudocereals can be consumed as whole grains, whole grain flour, or processed products. While whole seeds are becoming more popular, most wheat and rice consumed by humans are dehulled, resulting in lower fiber content.

Table 1 displays the mineral content in wheat and pseudocereals. Pseudocereals generally contain higher levels of calcium, magnesium, and iron. According to the Argentine Food Code (CAA) Recommended Daily Intake (RDI) values, a 50 g serving of wheat provides only 2% of the RDI for calcium, while the same serving of amaranth provides 9%. For magnesium, a 50 g serving of wheat provides 19% of the RDI for men (260 mg/day), whereas 50 g of pseudocereals provide 40% to 54% of the RDI. Amaranth is particularly notable for its iron content, offering 16% of the RDI for women (29 mg/day) and 33% of the RDI for men (14 mg/day) in a 50 g serving. These values demonstrate that pseudocereals are a valuable source of minerals.

Dietary Fiber

Dietary fiber intake is increasingly recognized for its importance in human nutrition. Research shows that fiber supports proper intestinal function and helps prevent cardiovascular diseases, obesity, diabetes, and certain cancers (2).

The Food and Drug Administration (FDA) recommends adults consume 25 g of fiber daily in a 2000 kcal diet, which aligns with the National Cancer Institute (NCI) recommendation of 20-30 g/day to prevent colon cancer (36). However, statistics from Argentina show that the population fails to meet these recommendations. The 2007 National Nutrition and Health Survey reported that 97.2% of women aged 10 to 49 did not meet the daily fiber intake recommendation, with a median intake of 9.4 g/day. Similar deficiencies were observed in children, with 97.8% not meeting adequate fiber intake (42). Data from the 2019 National Nutrition and Health Survey suggest that the situation has not improved. Food consumption patterns reveal that 30-40% of respondents consume only one fruit or vegetable per day, indicating that dietary fiber intake remains significantly below recommended levels (42).

There are several definitions of dietary fiber. According to the CAA, "dietary fiber is any edible material not hydrolyzed by the endogenous enzymes of the human digestive tract." Compaore-Sereme *et al.* (2022) define it as "the edible parts of plants or carbohydrates resistant to digestion and absorption in the human small intestine, with complete or partial fermentation in the large intestine," highlighting its fermentability by large intestine microorganisms. Dietary fiber consists mainly of non-starch polysaccharides in plant cell walls and can be classified based on water-holding capacity into insoluble and soluble fiber. Insoluble fiber includes cellulose and hemicelluloses, while soluble fiber encompasses pectins, β-glucans, gums, mucilages, oligosaccharides, and inulin. Additionally, dietary fiber includes indigestible non-polysaccharide compounds such as lignin, proteins resistant to gastrointestinal digestion, phenolic compounds, waxes, saponins, phytates, and phytosterols (52). Health benefits are linked to the solubility of dietary fiber. Soluble dietary fiber (SDF) forms viscous gels upon contact with water, which delays gastric emptying and nutrient absorption in the intestines. This increases satiety and reduces caloric density, lowering the long-term risk of obesity (72). The delay in nutrient absorption also helps prevent glycemic spikes in diabetic patients. Additionally, SDF improves insulin sensitivity in both type 2 diabetes patients and healthy individuals (69), and lowers blood cholesterol levels, particularly LDL cholesterol, thereby reducing cardiovascular disease risk (45). This effect may result from SDF binding bile acids, altering micelle formation, preventing bile acid reabsorption in the enterohepatic circulation, and promoting their elimination in feces (74). Consequently, new bile acid synthesis in the liver is stimulated, which lowers blood cholesterol levels (8). Another important aspect of fiber is its fermentability by intestinal microorganisms. The degree of fermentability correlates with fiber solubility and particle size. For example, fructooligosaccharides are highly fermentable, whereas large cellulose or lignin polymers remain unchanged throughout the large intestine. Clinical studies show that intestinal microorganisms utilize different types of dietary fiber, with the remainder excreted in feces (18). Soluble polysaccharides fermented by intestinal microbiota produce short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate. SCFAs offer various health benefits, primarily at the intestinal level. Butyrate, for instance, strengthens the intestinal epithelial barrier by inducing tight junction protein expression and redistribution within the membrane. A loss of intestinal barrier integrity, leading to increased permeability, is linked to chronic inflammation associated with obesity, insulin resistance, and type 2 diabetes (9). Propionate also benefits individuals with obesity by inhibiting hepatic cholesterol synthesis, decreasing lipogenesis in adipose tissue, and reducing appetite (9). Insoluble dietary fiber (IDF) travels through the gastrointestinal tract with minimal modification. Its effects are largely due to mechanical interactions (69). IDF retains water and adds bulk to feces, enhancing intestinal regularity. Additionally, it reduces caloric density by acting as a physical barrier that slows the transit of digestive products through the enterocytes' brush border (69). This characteristic of insoluble dietary fiber (IDF) also impedes the absorption of other components, such as cholesterol, and promotes its excretion via feces. Studies have shown that the lignin fraction, commonly found in the outer layers of seeds, enhances bile acid binding capacity, leading to reduced blood cholesterol levels (46).

Sabbione *et al.* (2023a) reported that amaranth IDF has significant bile acid binding capacity, suggesting that its adsorptive effect may contribute to a potential hypocholesterolemic effect by sequestering bile acids. Additionally, IDF reduces the concentration and contact time of potentially carcinogenic compounds with the colon mucosa, thereby lowering the risk of colon cancer (5). Regarding other cancers, research indicates that total or insoluble dietary fiber from legumes reduces prostate cancer risk, while soluble dietary fiber decreases breast cancer risk (64).

Pseudocereals dietary fiber composition

Numerous studies have assessed the relative amounts of total dietary fiber (TDF), soluble dietary fiber (SDF), and insoluble dietary fiber (IDF) in pseudocereal whole grains and flours (table 2). These values vary widely due to differences in genotypes, environmental conditions, and laboratory techniques used for fiber quantification and characterization. While dietary fiber content is crucial, the ratio between insoluble and soluble fractions is also significant, as an appropriate balance enhances health benefits. The FDA recommends an IDF/SDF ratio close to 3 for optimal fiber balance (45). Understanding the polysaccharide structures in pseudocereal cell walls is important, as these structures influence both techno-functional and biological properties. This information can predict the behavior of fiber in the body and its effects on various food matrices.

Table 2. Amaranth, quinoa, and buckwheat dietary fiber content, expressed as total dietary fiber (TDF), soluble dietary fiber (SDF), and insoluble dietary fiber (IDF).

Tabla 2. Contenido de fibra dietaria total (TDF), soluble (SDF) e insoluble (IDF) en amaranto, quinoa, y trigo sarraceno.

Data are expressed as g fiber/100 g of seeds on a wet basis. *$IFD^{average}$/$SFD^{average}$

Valores expresados como g fibra/100 g de semillas. *IFD^{media}/SFD^{media}

Pseudocereal	TDF	SDF	IDF	IDF/SDF	Bibliographic source
Amaranth	12.5-14.8	1.5-2.2	11.0-12.6	6.4*	Repo-Carrasco *et al.* (2009)
	15.8	2.2	13.6	6.2	Glorio *et al.* (2008)
	13.6	5.7	7.9	1.4	Collar and Angioloni (2014)
	9.9	2.1	7.8	3.7	Lamothe *et al.* (2015)
	10.8	2.2	8.6	3.9	Sabbione *et al.* (2023a)
Quinoa	11.8-14.0	1.3-1.4	10.5-12.7	8.6*	Repo-Carrasco *et al.* (2011)
	14.5	5.4	9.1	1.7	Collar and Angioloni (2014)
	9.0	2.0	7.0	3.5	Lamothe *et al.* (2015)
Buckwheat	11.9	6.12	5.81	1.0	Collar and Angioloni (2014)
	7.5-7.9	2.2-2.7	5.0-5.6	2.2*	Izydorczyk and Head (2014) (dehusked)
	5.3	2.4	2.9	1.2	Wefers *et al.* (2015) (dehusked)
	28.1	1.4	26.7	19.0	Dziedzic *et al.* (2012) (with husk)
	82.1	0.5	81.6	163.2	Dziedzic *et al.* (2012) (only husk)
	86.8	16.0	70.3	4.4	Zhu *et al.* (2014) (only husk)

Over the past decades, various studies have focused on extracting and quantifying dietary fiber (DF) from fruits and vegetables to identify rich sources, assess their health benefits, and evaluate their functional properties for product development. Notable achievements have been observed in citrus fruits, tropical fruits, berries, and various vegetables.

Cell wall constituents

Dietary fiber components are primarily located in the cell wall, which provides shape and structural integrity to plant cells. The cell wall consists of a complex mixture of polysaccharides and other polymers arranged in a three-dimensional network. It also includes structural proteins, enzymes, phenolic polymers, and other materials that influence its physical and chemical properties. The characteristics of the cell wall, such as thickness, matrix arrangement, and the types and proportions of molecules, vary depending on the plant tissue (41).

Among the cell wall constituents is *cellulose*, a linear polysaccharide made up of D-glucose molecules linked by β-D (1-4) bonds. Its degree of polymerization varies significantly, with molecules consisting of 2,000 to 15,000 units, depending on the specific region of the cell wall (66). These linear polymers form microfibrils through intermolecular hydrogen bonds, which aggregate into larger structures and interact with other components such as hemicelluloses, pectins, and lignin. This interaction creates strongly hydrated matrices with high mechanical resistance. *Hemicelluloses* are a diverse group of polysaccharides with long linear chains that may have short side chains as substituents. They have a lower molecular weight than cellulose and are generally more soluble. However, their physicochemical properties, such as solubility, viscosity, and gel-forming abilities, vary based on their chemical structure, molecular size, interactions, and spatial arrangement. At least 250 types of hemicellulose polymers have been identified, including xyloglucans, xylans, mannans, glucans, and β-(1-3,1-4) glucans (53). *Lignins*, another cell wall component, are complex biopolymers and are the second most abundant in the plant kingdom after cellulose. These polyphenolic biopolymers consist of phenylpropane units and form a matrix through the condensation of three primary phenolic alcohols (47). *Pectins* are structural polysaccharides primarily composed of α-D-galacturonic acid. They form chains that can be linear, known as homogalacturonans (HG), or include rhamnose. Two typical structures containing rhamnose are rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II), which differ in structure, linkage types, and complexity. Although pectins vary in solubility, the pectin in the middle lamella of cell walls is insoluble and considered calcium pectate (75). These compounds have significant nutritional and technological value for the food industry due to their prebiotic potential and gelling ability (66). *Fructans*, which are reserve carbohydrates and the most abundant non-structural polysaccharides in nature, include inulin, oligofructose, and fructooligosaccharides (FOS). They are relatively soluble in water and remain intact in the upper gastrointestinal tract. However, in the colon, fructans are fully utilized by microorganisms, providing a prebiotic effect. Inulin, FOS, and oligofructose are commonly used as functional ingredients (70). These compounds also offer valuable technological properties, such as sweetening ability and gel formation, which enhance the body and palatability of certain foods. *Oligosaccharides*, which are short chains of sugars containing 3 to 20 monosaccharides, cannot be digested by the human body and are classified as dietary fiber. Due to their small size and capacity to form hydrogen bonds with water, they are highly soluble. The *raffinose family of oligosaccharides* (RFOs), including raffinose, stachyose, and verbascose, as well as related compounds, have shown potential prebiotic effects by promoting the selective growth of beneficial bifidobacteria (35). *Resistant starch* (RS) is a type of starch that retains its structural characteristics and remains undigested as it passes through the gastrointestinal tract. It reaches the colon, where it can be fermented by the microbiota or excreted in feces. Although RS is not a cell wall component, it functions similarly to fermentable soluble fiber, providing associated health benefits (34).

Amaranth

Table 2 (page 167) presents the values of total dietary fiber (TDF), insoluble dietary fiber (IDF), and soluble dietary fiber (SDF) for amaranth, quinoa, and buckwheat, expressed on a wet basis. The TDF values align with those reported for pseudocereals in table 1 (page 165). The data show a predominance of IDF over SDF. Specifically, 12% to 42% of the TDF corresponds to soluble fiber, while 58% to 86% corresponds to insoluble fiber.

Bunzel *et al.* (2005) reported that the primary cell walls of dicotyledons like amaranth are rich in pectins, xyloglucans, and cellulose. Lamothe *et al.* (2015) examined the composition of the insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) fractions in amaranth. They found that 5% of the IDF on a dry basis was lignin. Their analysis revealed that the IDF primarily consists of galacturonic acid, arabinose, xylose, glucose, and galactose. A significant portion of the glucose was attributed to cellulose and xyloglucans, which retained the characteristic bonds of these structures. Glucose from cellulose accounted for 7% of the IDF, though this value might be underestimated due to the analytical method used. The estimated proportion of xyloglucans was 30%, with a high degree of branching indicated by the elevated Xyl/Glc ratio. This finding is consistent with Bunzel *et al.* (2005), who noted a high number of terminal xylose units in amaranth, likely part of the xyloglucan side chains.

Sabbione *et al.* (2023a) confirmed that galacturonic acid is the primary monosaccharide in insoluble dietary fiber (IDF) and reported the same monosaccharides identified by Lamothe *et al.* (2015) (60). Lamothe *et al.* (2015) found that pectins constitute 59% of the IDF, with rhamnose present in low amounts. This suggests that the pectins are predominantly homogalacturonans (HG) with small amounts of rhamnogalacturonan I (RG-I). The bonding patterns in galactose and arabinose residues suggest they are part of RG-I pectic side chains. The predominance of HG over RG-I is consistent with Mohnen (2008), who noted that HG makes up to 65% of the pectin in plant cell walls, while RG-I represents 25-35%. Additionally, Lamothe *et al.* (2015) indicated that xylose is involved not only in xyloglucan structures but also in arabinoxylans, another type of hemicellulose found in the IDF, together with pectic polysaccharides that would be the majority. Regarding amaranth soluble dietary fiber (SDF), Lamothe *et al.* (2015) reported that the primary monosaccharides are galacturonic acid, galactose, and arabinose, indicating the presence of pectic substances. These pectic substances contribute 34% to the SDF (38). The predominance of galacturonic acid and the absence of rhamnose suggest that homogalacturonans (HG) are the major pectin component. Significant amounts of mannose were also reported, though it is notably lower compared to other monosaccharides. This mannose is attributed to galactomannans, with an estimated content of 0.3%. Additionally, xylose and glucose units from xyloglucans also contribute to the SDF, comprising 60-70% of this fiber fraction. Lamothe *et al.* (2015) describe a high Xyl/Glc ratio in these samples, reflecting a high level of branching. The side chains of these polysaccharides include di- and tri-saccharides, which may consist of xylose, glucose, and possibly arabinose. Sabbione *et al.* (2023a) detected galacturonic acid, xylose, arabinose, mannose, and glucose/galactose in amaranth SDF, with xylose and arabinose being the most prevalent. Villacrés *et al.* (2013) also reported significant amounts of soluble arabinoxylans in the SDF.

Capriles *et al.* (2008) studied resistant starch content in amaranth seeds and found that raw seeds possess 0.5% RS on a dry basis. However, they observed that after cooking, this value reduced to 0.2%. The authors concluded that the RS decrease might have occurred due to the starch granules' small size and their tendency to completely lose the crystalline structure during thermal treatment. Since a food considered a good source of resistant starch should possess an RS/total starch ratio of at least 4.5% (58), amaranth cannot be included in that group of foods since its ratio is 0.86%.

Guzmán-Maldonado and Paredes-López (1998) reported low levels of raffinose and stachyose in amaranth, with concentrations of 1.65% and 0.15%, respectively. Gamel *et al.* (2006) found even lower levels of raffinose, around 0.4%, and similar stachyose content. Both studies confirm that amaranth seeds are not a significant source of raffinose family oligosaccharides. Various authors have also assessed the presence of FODMAPs, which include fermentable oligosaccharides, disaccharides, monosaccharides, and polyols like lactose, fructose, sorbitol, and mannitol. These non-digestible carbohydrates can trigger symptoms in individuals with conditions such as irritable bowel syndrome, causing abdominal distension, diarrhea, and pain. The impact of these carbohydrates is limited to those with specific intolerances or diseases. Békés *et al.* (2017) classified pseudocereals, including amaranth, buckwheat, and quinoa, as low in FODMAPs, indicating that their FOS content is also low. Furthermore, Habus *et al.* (2022) analyzed the FODMAPs content in amaranth bran and reported that the sum of fructans and galacto-oligosaccharides (GOS) is 0.96% on a dry weight basis.

Quinoa

Table 2 (page 167) presents the total dietary fiber (TDF), insoluble dietary fiber (IDF), and soluble dietary fiber (SDF) content in quinoa seeds. Across different studies, IDF consistently exceeds SDF, with soluble fiber ranging from 10% to 37% of TDF, and insoluble fiber ranging from 63% to 90% of TDF. Zhang *et al.* (2020) analyzed the dietary fiber content of quinoa seeds, reporting that it contains 41% hemicellulose, 52% cellulose, 4.7% pectins, and 1.7% lignin (77). Lamothe *et al.* (2015) studied the soluble and insoluble fiber composition in quinoa seeds, finding that the IDF contained 9% lignin. This finding aligns with the data from Repo-Carrasco-Valencia and Serna (2011), who reported lignin values between 6% and 7%.

Based on the types of bonds present in the IDF xylose and glucose units, these monosaccharides are likely part of xyloglucans, as suggested by Serna Saldívar and Ayala Soto (2020). These authors identified xyloglucans as the primary hemicellulose components in quinoa seeds. The xyloglucans found in quinoa are similar to those in amaranth, characterized by branching with di- and trisaccharide side chains and a significant degree of branching. In their analysis of IDF, Lamothe *et al.* (2015) reported that glucose associated with cellulose constituted 6%, a value comparable to that found in amaranth. However, this finding is much lower than the 52% TDF described by Zhang *et al.* (2020), suggesting that the lower cellulose content could be attributable to the analytical methods employed (38).

The primary monomeric unit identified in quinoa TDF by Lamothe *et al.* (2015) is galacturonic acid with β-(1,4) linkages, indicating that pectic polysaccharides are predominant in both IDF and SDF fractions. According to these authors, these pectic polymers constitute approximately 55% of IDF, predominantly as homogalacturonans (HG) and, to a lesser extent, as rhamnogalacturonan I (RG-I) compounds, which are branched with arabinans and galactans. Cordeiro *et al.* (2012) also described similar pectic structures in quinoa dietary fiber, reinforcing these findings. Furthermore, the pectic content in SDF constitutes 55%, but unlike the pectins found in IDF, the SDF pectins are composed exclusively of branched homogalacturonans (HG), as rhamnose was absent from this fraction. Lamothe *et al.* (2015) identified arabinose, glucose, galactose, and, to a lesser extent, xylose and mannose in SDF. Most of the arabinose is part of the pectic branches, specifically as arabinans. Galactomannans in quinoa SDF account for 0.5%, while xyloglucans range from 40-60%, exhibiting a low Xyl/Glc ratio and a relatively low degree of branching. Additionally, Villacrés *et al.* (2013) reported significant amounts of soluble arabinoxylans as part of quinoa SDF.

The resistant starch (RS) content in quinoa seeds, as reported by Kraic (2006), is 12.6 g/kg on a dry basis, translating to an RS/total starch ratio of approximately 2%. Consequently, quinoa is not considered a significant source of resistant starch. However, a study by Linsberger-Martin *et al.* (2012) demonstrated that applying high hydrostatic pressures to quinoa seeds could increase the RS content by up to 18 times, presenting a promising method for developing functional ingredients in the food industry. In their analysis of the FODMAP profile of various grains, Ispiryan *et al.* (2020) found that quinoa has a low content of fructans and oligofructans (OFR). Fructans were below the detection limit of their method, and the RFOs were present in minimal amounts (0.09% on a dry basis).

Buckwheat

Table 2 (page 167) presents the total dietary fiber (TDF), soluble dietary fiber (SDF), and insoluble dietary fiber (IDF) contents of both husked and dehusked buckwheat seeds, as well as those of the husk alone. The values for TDF, SDF, and IDF vary among studies, partly due to the inclusion or exclusion of the husk in the analyzed samples. The buckwheat husk is particularly rich in insoluble fiber, and flours made from buckwheat that include the husk typically have high levels of insoluble fiber (19). According to Zhang *et al.* (2020), the fiber composition of buckwheat seeds includes 39.2% hemicellulose, 38.8% cellulose, 20.2% lignin, and 1.8% pectin. Based on the data presented in table 2 (page 167), it is inferred that the buckwheat seeds analyzed likely contained the husk, given the high content of cellulose and lignin, which are characteristic of the husk's insoluble polysaccharides. Wefers *et al.* (2015) analyzed the IDF monosaccharides in dehusked buckwheat and identified significant amounts of galacturonic acid, arabinose, galactose, and to a lesser extent, rhamnose. Many of the monosaccharides found in buckwheat IDF, such as galacturonic acid, arabinose, and galactose, are attributed to the presence of pectic arabinans and galactans, which are linked to rhamnogalacturonan-I (RG-I) segments. The observed galacturonic acid/rhamnose ratio indicates that homogalacturonan (HG) also contributes to buckwheat IDF pectin, though to a lesser extent compared to quinoa and amaranth. This study also suggests a relatively low content of cellulose in buckwheat. The presence of terminal xylose and glucose with β-(1-4) linkages implies that xyloglucans may be present, although xylose could also be part of xylans, albeit in low proportions. Regarding SDF, Izydorczyk and Head (2010) noted that it primarily consists of pectic polysaccharides, xyloglucans, and arabinogalactans. The SDF monosaccharide composition analyzed by Wefers *et al.* (2015) supports the presence of RG-I and indicates higher amounts of HG compared to the IDF.

The authors also identified arabinans in the SDF fraction, which are part of the pectin branches. Additionally, glucose and xylose monosaccharides with linkages consistent with xyloglucan structures were found. A high amount of mannose could be attributed to mannan content. Dziedzic *et al.* (2012) further reported that buckwheat husks are predominantly composed of fiber, with high proportions of insoluble fiber, particularly lignin and cellulose.

Regarding buckwheat's resistant starch content, Kraic (2006) reported an RS content of 38 g/kg on a dry basis and an RS/total starch ratio of 6.5%. Thus, buckwheat can be considered a good source of resistant starch compared to other pseudocereals. The high RS values are likely due to buckwheat's elevated amylose content (27).

Ispiryan *et al.* (2020) found that buckwheat is low in FODMAPs, indicating that it contains a low proportion of both fructans and raffinose family oligosaccharides (RFOs).

Table 3 summarizes the composition of IDF and SDF in amaranth, quinoa, and buckwheat seeds.

Table 3. Polysaccharides in the soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) fractions in amaranth, quinoa, and buckwheat.

Tabla 3. Polisacáridos presentes en la fibra dietaria soluble (SDF) y en la fibra dietaria insoluble (IDF) de amaranto, quinoa y trigo sarraceno.

Pseudocereal	SDF	IDF
Amaranth	Highly branched XYLOGLUCANS HOMOGALACTURONANS ARABINOXYLANS GALACTOMANNANS (low proportion)	HOMOGALACTURONANS with small RG-I sections branched XYLOGLUCANS CELLULOSE LIGNIN XYLANS (low proportion)
Quinoa	Sparsely branched XYLOGLUCANS branched HOMOGALACTURONANS ARABINOXYLANS GALACTOMANNANS (low proportion)	HOMOGALACTURONANS with small RG-I sections branched XYLOGLUCANS CELLULOSE LIGNIN
Buckwheat Husked	Highly branched RG-I PECTINS HOMOGALACTURONANS ARABINOGALACTANS XYLOGLUCANS	RESISTANT STARCH highly branched RG-I PECTINS HOMOGALACTURONANS (low proportion) XYLOGLUCANS CELLULOSE (low proportion) XYLANS (low proportion)

*Data from Bunzel *et al.* (2005); Cordeiro *et al.* (2012); Dziedzic *et al.* (2012); Izydorczyk and Head (2010); Lamothe *et al.* (2015); Repo-Carrasco-Valencia and Serna (2011); Sabbione *et al.* (2023a); Serna Saldívar and Ayala Soto (2020a); Villacrés *et al.* (2013); Wefers *et al.* (2015); Zhang *et al.* (2020).

The data on dietary fiber content in pseudocereals show considerable variability among studies. However, some general trends can be identified. Pseudocereals have TDF levels comparable to those found in wheat (table 1, page 165). The predominant fiber fraction is IDF, reflected in an IDF/SDF ratio greater than 1 (table 2, page 167). An ideal balance, suggested to be around 3, is approached by amaranth, quinoa, and cereals, with some husked buckwheat samples exhibiting even higher ratios. Regarding fiber composition, amaranth and quinoa share similarities in the proportion and types of dietary fiber structures (table 2 page 167 and table 3). The main fiber components in these pseudocereals are pectic polysaccharides, with a lesser amount of xyloglucans. These polysaccharides vary in complexity and branching, contributing to the distinct characteristics of the dietary fiber in each pseudocereal. In contrast, husked buckwheat is notable for its high cellulose and lignin content. The removal of the husk reduces the IDF content, which is reflected in the lower IDF values compared to buckwheat with the husk (table 2, page 167). Overall, while the general composition of dietary fiber in pseudocereals follows certain patterns, the specific characteristics and proportions can vary significantly depending on the pseudocereal and its processing.

Probiotics and prebiotics

The human gastrointestinal tract hosts a vast number of microorganisms that interact symbiotically with the host. These microorganisms perform essential functions, such as utilizing non-digestible components to produce health-beneficial compounds, maintaining the epithelial barrier, regulating host metabolism, preventing pathogen colonization, and modulating the immune and nervous systems (65). Certain microorganisms, known as probiotics, are used as food supplements to improve health. Probiotics must survive gastrointestinal transit, establish in the intestine, and proliferate. The International Scientific Association for Probiotics and Prebiotics (ISAPP) defines probiotics as "live strains of strictly selected microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (30). Foods containing probiotics are classified as functional foods. Common probiotic microorganisms include *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium spp*. However, other probiotic species also exist, such as *Lactococcus spp.*, *Streptococcus spp.*, and certain strains of *Saccharomyces* yeast (65). Probiotics and many intestinal microbes utilize dietary fiber. Different types of fiber selectively promote the growth of beneficial microbial colonies and are known as prebiotics. The ISAPP defines prebiotics as "substrates that are selectively utilized by host microorganisms and confer a health benefit" (21). This broad definition encompasses non-digestible dietary carbohydrates and other types of substrates. Prebiotics and probiotics enhance host health by modulating intestinal flora. Research aimed at developing healthier foods has led to the emergence of new functional foods, including synbiotics. Synbiotics are defined as "a mixture of probiotics and prebiotics intended to increase the survival of health-promoting bacteria and to modify intestinal flora and its metabolism" (48).

Human intestinal microbiota

The term "human microbiota" refers to the community of microorganisms residing in the body. Over 70% of the human microbiota is found in the digestive tract, which displays significant variability in microbial diversity and quantity across different regions. The intestinal microbiota contains approximately 100 trillion microorganisms from at least 1,000 different species, and it is estimated to weigh about 200 grams in adults. Only one-third of these microorganisms are common to most individuals, while the remaining two-thirds are unique to each person (17).

According to data from the Human Microbiome Project (National Institutes of Health, USA), the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*, which together account for over 90% of the total microbiota (table 4), predominantly inhabit the intestine. The remaining microbiota includes *Fusobacteria*, *Verrucomicrobia*, archaea, yeasts, phages, and protists. The human colon also contains low quantities of pathogens such as *Campylobacter jejuni*, *Salmonella enterica*, *Vibrio cholerae*, and certain strains of *Escherichia coli*.

Table 4. Main bacterial phyla and genera present in the human intestine (17).
Tabla 4. Principales *phylum* y géneros de bacterias presentes en el intestino humano (17).

Phylum	Percentage	Genus
Firmicutes	38.8	*Clostrudium* (anaerobes) *Bacillum* (fermentatives) *Lactobacillus/Enterococcus*) *Mollicutes*
Bacteroidetes	27.8	*Cytophaga (Cytophaga hutchinsonii)* *Flavobacterium* *Bacteroidetes (Prevotella)*
Actinobacteria	8.2	*Bifidobacterium*
Proteobacteria	2.1	*Escherichia coli*
Verrucomicrobia	1.3	*Akkermansia muciniphila*
Euryarcheota	0.9	*Methanobrevibacter smithii*

Digestive tract colonization begins at birth. The quantity and type of microorganisms evolve until age 3, influenced by environmental factors and dietary patterns. After this period, the intestinal microbiota resembles that of adults in composition, diversity, and functionality. It can be altered during adolescence due to hormonal changes but remains relatively stable in adulthood. After age 65, the microbiota composition shifts, with increased abundance of the *Bacteroidetes phylum* and *Clostridium* from the *Ruminococcaceae* family, in contrast to younger individuals, where *Clostridium* from the *Lachnospiraceae* family is more common. In older adults, the microbiota becomes less diverse and more dynamic, characterized by a higher *Bacteroides*/*Firmicutes* ratio, an increase in *Proteobacteria*, and a decrease in *Bifidobacterium* (68). The intestinal microbiota performs numerous functions and influences various biological processes, both locally and distantly through its metabolites. The bioactive products and metabolites produced by microorganisms during fermentation, known as postbiotics, include short-chain fatty acids (SCFAs), enzymes, vitamins, bioactive peptides, and components of microorganisms or their remnants (63). The microbiota contributes to maintaining mucosal barrier integrity, providing essential nutrients like vitamins, and protecting against pathogens (17). Additionally, the interaction between the microbiota and the immune system in the colonic mucosa is essential for proper immune function. Microbial molecular pattern recognition receptors or microbiota-derived metabolites activate barrier functions and mediator synthesis, regulating intestinal immune cell responses to tolerate beneficial microorganisms and prevent pathogen overgrowth (3). The interaction between the human microbiota and the gut-brain axis is a bidirectional and dynamic communication pathway between the intestine and the brain, mediated through nervous, endocrine, and immune signaling mechanisms (62). The enteric nervous system (ENS) in the intestine comprises over 100 million neurons responsible for basic digestive functions, including motility and mucosal secretion. It communicates with the central nervous system (CNS) primarily via the vagus nerve. The intestinal microbiota stimulates vagus nerve afferent pathways, promotes cytokine release, and modulates the production of neurotransmitters, hormones, and metabolites such as SCFAs (23). Microbiota influences the hypothalamic-pituitary-adrenal axis, regulating cortisol release. Research indicates that high levels of *Lactobacillus rhamnosus* are associated with lower corticosterone levels and improved stress and depression management. Conversely, stress can alter the microbiota profile (23). Dysbiosis may disrupt molecules essential for proper CNS function, potentially linking microbiota imbalances to neurological diseases such as Alzheimer's, Parkinson's, multiple sclerosis, autism spectrum disorders, depression, and anxiety (1).

Colon microorganisms primarily ferment dietary carbohydrates that resist digestion in the gastrointestinal tract. Species such as *Bacteroides*, *Roseburia*, *Bifidobacterium*, and *Enterobacter* produce SCFAs, which serve as an energy source for enterocytes or enter the bloodstream to affect distant organs. Many intestinal anaerobes produce acetate, while *Bacteroidetes* predominantly produce propionate and *Firmicutes* produce butyrate (68). Butyrate is recognized for its anti-inflammatory and anticancer properties; it promotes the proliferation of colonocytes in the crypts and enhances apoptosis and exfoliation in the areas closer to the lumen. Additionally, butyrate supports barrier function regulation and reduces bacterial translocation by contributing to tight junction assembly and mucin synthesis (68). SCFAs also impact hepatic lipid and glucose homeostasis. Propionate, besides serving as an energy source, regulates blood glucose levels by modulating gluconeogenesis in the liver. It enhances insulin sensitivity and reduces cholesterol synthesis rates (29). Acetate, used as an energy source in the intestine, can be transported to peripheral organs or the liver, where it serves as a precursor for cholesterol and long-chain fatty acids. Additionally, SCFAs increase intestinal hormone levels that promote satiety and enhance insulin action on glucose uptake in muscle and adipose tissue. They inhibit *de novo* lipogenesis and lipolysis, reducing plasma-free fatty acids and aiding in body weight control (29).

Microbiota Modulation: Probiotics and Prebiotics

Microbiota modulation involves deliberately altering the composition and activity of intestinal microorganisms to improve health. This can be achieved through dietary changes, probiotics, prebiotics, synbiotics, or, occasionally, antibiotics, either alone or in combination.

Such interventions can enhance the diversity and abundance of beneficial bacteria, restore balance, or reduce pathogenic microorganisms.

Probiotics are incorporated into the diet through fermented foods or dietary supplements. Commonly used probiotic genera include *Lactobacillus* and *Bifidobacterium*. Strains such as *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*, and *Bifidobacterium lactis* are naturally present in the intestine. These strains, along with those from *Saccharomyces*, can reduce pathogen adherence to the mucosa (39). Additionally, the metabolic activity of beneficial microorganisms increases SCFA concentrations and decreases colonic pH, which inhibits the growth of pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Salmonella enteritidis* (59).

Prebiotics can naturally occur in foods or be added to enhance functional properties. Various dietary fibers serve as energy sources for intestinal microorganisms, but only some selectively promote the growth of beneficial microbiota and probiotics. Prebiotics exert health benefits by stimulating beneficial microorganisms' growth, modulating immune function, and protecting against pathogens. They primarily increase populations of *Bifidobacterium* and *Lactobacillus*, but also stimulate less well-studied beneficial bacteria such as *Akkermansia*, *Eubacterium*, *Propionibacterium*, *Roseburia*, and *Faecalibacterium* (59). The rate and extent of fiber fermentation by microorganisms are crucial for their prebiotic potential. These factors are influenced by fiber solubility, chain size, total surface area, and the structure of the cell wall or food matrix containing the fiber (59). Fructans such as inulin, oligofructose, and FOS, as well as galactans (GOS), are well-recognized prebiotics due to their low degree of polymerization and high solubility. Additionally, oligosaccharides derived from hemicelluloses, such as mannanoligosaccharides (MOS), arabinoxylanoligosaccharides (AXOS), and xylooligosaccharides (XOS), have been proposed as prebiotics because they enhance the growth of beneficial microorganisms and promote SCFAs production (33). Conversely, RFOs have shown prebiotic effects by increasing *Bifidobacteria* and *Lactobacillus* populations while reducing the adhesion and colonization of enteric pathogens (35). The degree of polymerization of polysaccharides and their interactions with other polysaccharides in food matrices can affect their metabolism. Some non-starch polysaccharides, such as pectins and certain hemicelluloses, are considered potentially prebiotic (59). Microorganisms must possess enzymes to degrade glycosidic bonds in polysaccharides into smaller molecules for utilization as carbon sources. For example, xyloglucans are degraded by *Clostridium* in the colon due to its microbiota-specific enzymes, converting them into fermentable oligosaccharides that positively impact the colon microbiota (24). β-glucans enhance the growth of *Bifidobacterium* and *Lactobacillus* strains and modulate SCFA production by increasing *Clostridium histolyticum* and *Prevotella* (65). Arabinoxylans in cereals and pseudocereals can generate AXOS and XOS through enzymatic hydrolysis with xylanases and arabinofuranosidases. Although hydrolysis yields various structures depending on the plant source, arabinoxylans are considered potential prebiotics. Their consumption has been linked to positive immunomodulation and selective growth of probiotic microorganisms such as *Lactobacillus cellobiosus*, *Lactobacillus paracasei*, and *Bifidobacterium spp*. (65). Pectins, a complex family of fermentable polysaccharides, can also promote the growth of *Lactobacillus* and *Bifidobacterium* in the intestine. The effectiveness of pectin utilization by microorganisms depends largely on colon pH and the degree of methoxylation, with low methoxyl pectins fermenting more rapidly (49). *In vivo* studies have shown an increase in *Clostridium* species capable of producing acetate and butyrate in the presence of pectin (65). Additionally, research in rats fed with pectins revealed increased SCFAs and the presence of pectic oligosaccharides as intermediates. These oligosaccharides result from the action of bacterial enzymes such as pectate lyase, polygalacturonase, and pectin esterase (49). Resistant starches are also potentially prebiotic, with promising results from both *in vitro* and *in vivo* studies. Several studies indicate that dietary RS increases the number of beneficial microorganisms, particularly *Bifidobacterium*, and elevates SCFA concentration in the colon (13). This proliferation is attributed to the fermentation of resistant starch degradation products. *Ruminococcus bromii* is a key species for initiating RS degradation, enabling other bacteria to utilize its fermentation products (65).

Potential modulatory and prebiotic effect of pseudocereals' dietary fiber

Amaranth

The prebiotic potential of amaranth remains under investigation. Although literature is limited, results are promising and have generated increased interest in these seeds. Gullón *et al.* (2014) assessed the *in vitro* prebiotic potential of amaranth by quantifying SCFAs, monitoring pH changes, and evaluating microbial population dynamics using adult women's fecal inoculums for fermentation. Amaranth seeds were cooked in water and subjected to simulated gastrointestinal digestion. The study revealed significant modifications in bacterial composition, with notable growth of *Bifidobacterium* spp., *Lactobacillus*, and *Enterococcus*, which are beneficial, as well as *Bacteroides* and *Prevotella*, which produce propionate. An increase in *Clostridium coccoides* and *Eubacterium rectale*, both butyrate producers, was also observed. SCFA production showed a progressive increase over time, with higher concentrations of acetate, followed by propionate and butyrate, indicating high fermentability of the medium where amaranth carbohydrates serve as a carbon source. pH decreased over time, correlating with the production of lactate and formate SCFAs (24). Sabbione *et al.* (2023b) investigated the ability of dietary fibers from three amaranth products to modulate children's fecal microbiota using an *in vitro* fermentation model. They observed changes in fecal microbiota and SCFAs after 24, 48, and 72h of fermentation. Sequencing results at 24h revealed a significant decrease in *Fusobacterium* and enterobacteria compared with the basal medium, accompanied by a notable increase in *Bacteroides* and *Parabacteroides*. These findings confirm that amaranth fibers are fermented by children's fecal microbiota, leading to changes indicative of a potentially prebiotic effect.

Currently, no studies confirm which specific amaranth fiber carbohydrates can beneficially modulate the microbiota or increase SCFA production. However, several components show potential prebiotic effects. Given the high content of pectin and arabinoxylans in amaranth, these compounds could be considered potential prebiotics. Additionally, xyloglucans are abundant in both soluble and insoluble fractions of amaranth dietary fiber, suggesting various hemicellulose sizes and conformations (10, 38). The observed prebiotic effects may be linked to xyloglucans, which can be hydrolyzed by microbiota enzymes into smaller carbohydrates used as a carbon source by beneficial microorganisms.

Quinoa

Quinoa, extensively studied as a pseudocereal, has a well-documented prebiotic effect on its dietary fiber. Gullón *et al.* (2014) reported results similar to those observed with amaranth. Authors described an increase in beneficial bacterial groups, although Faecalibacterium prausnitzii grew less in quinoa compared to amaranth. SCFA levels increased significantly over time, with a higher concentration in quinoa compared to amaranth. The study concluded that quinoa provides a highly fermentable medium conducive to the growth of SCFA-producing beneficial bacteria. In agreement, Zeyneb *et al.* (2021) found a marked increase in SCFAs and a decrease in pH during *in vitro* fermentation of cooked and raw quinoa following simulated gastrointestinal digestion. The authors found that propionate and butyrate were the SCFAs present in the highest concentrations after 24h of fermentation, while acetate was present in lower amounts. This low concentration of acetate, which is typically the most abundant SCFA, may suggest its degradation by other bacteria (76). Additionally, the study reported a positive shift in microbial diversity post-fermentation, with increased levels of beneficial species such as *Bifidobacterium* and *Collinsella*, indicating a potential prebiotic effect. Zeyneb *et al.* (2021) also examined polysaccharides extracted from quinoa and found a greater prebiotic effect compared to digested samples, particularly enhancing the growth of *Bifidobacterium*.

Several carbohydrates in quinoa may contribute to potential prebiotic effects. Cao *et al.* (2020) isolated a quinoa fiber carbohydrate composed of glucose and arabinose units, which exhibited a modulatory effect on the microbiota of rats fed a high-fat diet. A decrease in the *Firmicutes*/*Bacteroidetes* ratio (F/B) was noted, which is favorable as a high F/B ratio is linked to metabolic diseases. Additionally, levels of *Clostridium* and *Proteobacteria*, associated with inflammation and metabolic disorders, also decreased. The authors attributed these effects to the reduction in hyperlipidemia induced by the high-fat diet (42).

Other polysaccharides in quinoa, such as pectin or xyloglucans, may also be fermented by colon microorganisms following enzymatic hydrolysis in the intestine. Multiple carbohydrates can contribute to the observed modulatory effects, with SCFAs produced from fermentation playing a role in this process.

Buckwheat

The prebiotic potential of buckwheat has been investigated in various studies. Préstamo *et al.* (2003) examined the effects of incorporating buckwheat into the diet of rats. Their findings revealed a significant increase in Lactobacillus and *Bifidobacteria*, while potentially pathogenic strains, such as *Clostridium* and enterobacteria, decreased, suggesting a prebiotic effect of buckwheat. In a more recent study, Ren *et al.* (2021) showed that buckwheat supplementation in rats on a high-fat diet positively modulates the microbiota, reducing the *Firmicutes*/*Bacteroidetes* ratio and increasing microbial diversity, which helps reverse dysbiosis. Zhou *et al.* (2019) explored the impact of buckwheat RS on the microbiota by supplementing a high-fat diet with this component. The authors reported increased levels of *Lactobacillus*, *Bifidobacteria*, and *Enterococcus*, alongside inhibition of *Escherichia coli*. Additionally, supplementation with buckwheat RS led to increased SCFA production and significantly lower plasma levels of cholesterol, triglycerides, and glucose. The study concluded that buckwheat RS supplementation inhibited inflammation and prevented insulin resistance and hypertriglyceridemia. Given these findings, RS, a prominent component in buckwheat, may play a key role in its prebiotic effects. RS can be degraded by bacterial amylases into simpler carbohydrates that are fermented by intestinal microorganisms. This fermentation can directly influence the microbiota or produce by-products that benefit other microorganisms, leading to positive modulation. Additionally, other polysaccharides in buckwheat, such as pectins, arabinogalactans, and xyloglucans, may also contribute to these effects.

Conclusions

An exhaustive review was conducted on the structure of dietary fiber in amaranth, quinoa, and buckwheat, focusing on the relationship between their components and potential prebiotic effects. The current literature is limited regarding the modulatory effects of dietary fiber from these pseudocereals on human microbiota. Nonetheless, both *in vitro* and *in vivo* studies have evaluated the prebiotic potential of pseudocereal dietary fiber, showing promising results. These studies consistently demonstrate significant increases in beneficial microbial species, reductions in potentially pathogenic species, and enhanced SCFA production. Overall, the findings underscore the potential prebiotic effects of dietary fiber from amaranth, quinoa, and buckwheat. However, Argentine legislation, specifically Article 1390 of the Argentine Food Code (CAA, Chapter XVII), mandates the identification of functional components with potential prebiotic effects. Promoting and incorporating pseudocereals into processed food products would not only enhance the nutritional value of consumers' diets but also diversify raw material sources. Their consumption could offer health benefits, boost the regional economy of pseudocereal-producing areas, support food sovereignty, and provide consumers with options that align with their health needs and personal values.

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