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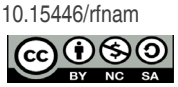

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EVALUADORES

El Comité Editorial dentro de sus políticas, envía los artículos a especialistas, con el fin de que sean revisados. Sus observaciones en adición a las que hacen los editores, contribuyen a la obtención de una publicación de reconocida calidad en el ámbito de las Ciencias Agrarias. Sus nombres son mencionados como una expresión de agradecimiento.

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EDITORIAL

Ariel Marcel: an academic who left his light

Ariel Marcel: un académico que nos dejó su luz

El profesor Ariel Marcel Tarazona Morales fue docente de la Universidad Nacional de Colombia sede Medellín desde el año 2008, siendo parte fundamental del Departamento de Producción Animal y quien además, tuvo la oportunidad de hacer su Doctorado perteneciendo a este claustro universitario en el área de Bienestar y Bioética Animal, donde como estudiante de la asignatura de inmunología (en la que fui su profesor) en un ensayo sobre la relación de inmunología y reproducción realizó un maravilloso relato de como un príncipe (el espermatozoide) alcanza a la princesa (el ovocito) cruzando terrenos inhóspitos desde un gran desierto (la vagina), pasando montañas (el cérvix), lagos y selvas (cuerpo y cuerno del útero), estrechos (istmo del oviducto), hasta encontrar a su princesa en una torre (el ámpula del oviducto), donde se besan (fertilización) y luego bajan juntos (como embrión) a construir su castillo en el cuerno uterino; en todo este camino el príncipe espermatozoide se enfrenta con fieras y dragones (inmunoglobulina A, citoquinas proinflamatorias, macrófagos y células dendríticas) y en medio de todos esos retos se logra crear un nuevo ser, se logra crear la vida. Esa era la sensibilidad de profesor Ariel.

Al egresar de su doctorado, el profesor Ariel se volvió un referente mundial en el área del Bienestar Animal por lo que contaba con múltiples invitaciones nacionales e internacionales como conferencista central en congresos de bienestar animal. En su paso por la Universidad Nacional logró que la carrera de zootecnia de nuestra sede fuera la primera en Colombia que tuviera en su malla curricular, como obligatoria, la asignatura de bienestar animal; además, sensibilizó a muchos estudiantes de pregrado y posgrado que las producciones animales se pueden hacer en el contexto del bienestar animal y que la producción de proteína de origen animal para alimentar al humano no riñe con el bienestar de los animales.

Como académico e investigador fue indudable su calidad, demostrada por su amplia productividad en el área de reproducción y bienestar animal, tanto de artículos en publicaciones indexadas, como de libros o capítulos de libros, participación en congresos nacionales e internacionales y su inmenso aporte como director o codirector de tesis de maestría o doctorado. Su calidad era reconocida tanto a nivel nacional como internacional, reflejándose con los nombramientos que tuvo en el transcurso de su vida docente hasta momentos previos a su fallecimiento como: Auxiliar de justicia del Consejo profesional de Medicina Veterinaria y Zootecnia de Colombia, Secretario Regional para Latinoamérica de la Sociedad Internacional de etología aplicada ISAE; Miembro del comité institucional para el cuidado de los animales CICUA de la Universidad Nacional de Colombia sede Medellín y de otros comités de ética para experimentación con animales de entidades universitarias de Medellín; Miembro del comité editorial de la revista de la facultad de ciencias agrarias de la Universidad de Cundinamarca y el cargo que más lo estaba llenando en sus últimos meses de vida fue el de Director de Bienestar de la Facultad de Ciencias Agrarias de la Universidad Nacional de Colombia sede Medellín, al que se entregó con toda el alma al conocer todo lo que él podía aportar como académico, profesional, docente y humano para hacer más cómodo y llevadero el paso por la universidad de los estudiantes, muchas veces en situaciones de alta vulnerabilidad económica, psicológica y personal.

Como hijo solo se puede decir, “¿Cómo hiciste para tener y criar un ser tan maravilloso e irreal?”, que fue lo que la profesora Liliana María Hoyos le preguntó a la mamá de Ariel el día que la facultad de Ciencias Agrarias hizo un pequeño homenaje póstumo de despedida. Como amigo solo puedo decir que los que lo tuvimos a nuestro lado fuimos unos afortunados y que con su partida no perdimos un amigo, sino que ganamos un ángel que nos cuidará y nos guiará con su luz siempre. Si me preguntan ¿Ariel fue un ser feliz?, les responderé que no sé, pero de lo que si estoy seguro es que fue un ser que repartió felicidad a sus seres conocidos en el mundo.

En su corta permanencia como docente de la Universidad Nacional de Colombia (15 años), Ariel pasó por todas las categorías desde: profesor auxiliar, profesor asistente, profesor asociado y la semana antes de su hospitalización había solicitado su ascenso a profesor titular, máxima categoría docente de la Universidad Nacional de Colombia, donde desafortunadamente el proceso de ascenso se vio truncado. Pero como Ariel no dejó de sorprendernos, ni aún después de su temprana partida a los 43 años (que cumplió estando en la UCI), y lo demuestra con las siguientes palabras que fueron tomadas de la introducción (de la cual Ariel fue coautor) del libro "El mundo del testículo: Fundamentos básicos y didácticos sobre el aparato reproductor masculino", libro que vio la luz después de la partida de Ariel, y que fue publicado por la Editorial de la Universidad Nacional de Colombia, cuyo autor es el profesor Yasser Lenis: "La reproducción, la sexualidad y la fertilidad han tenido un rol fundamental a lo largo de la historia de la humanidad y desde tiempos remotos en las culturas ancestrales del globo terráqueo. Los símbolos que representan a los aparatos genitales masculino y femenino han sido desde los inicios de la humanidad objeto de veneración, magia y creencias. Civilizaciones primordiales le han dado a la reproducción un lugar importante en su cosmogonía y en la comprensión de la naturaleza. Dentro de la naturaleza de los seres vivos, nacer, crecer y morir son procesos naturales; sin embargo, si un individuo llega a ser biológicamente exitoso en un ecosistema, puede lograr algo más, reproducirse. Esto último es un beneficio o un privilegio de pocos, y es el principio básico que tienen las especies animales como prueba del proceso de adaptación fisiológica y genética para permanecer a través del tiempo en un ecosistema determinado. La naturaleza es exigente, y solamente elige a los individuos más adaptados, competentes, saludables y dominantes para que transmitan la información genética a las siguientes generaciones, responsables de mantener la especie en el tiempo. De tal forma, la reproducción se convierte en un interesante mecanismo desarrollado por los seres vivos para multiplicarse".

Finalmente, con la partida de Ariel no perdimos un hijo, un hermano, un amigo, un docente, un maestro, un conocido, sino que ganamos una luz que nos guiará y acompañará hoy y siempre, ya que Ariel se representa con vida, como lo queremos recordar los compañeros de la Facultad de Ciencias Agrarias, por lo que en su acto de despedida sembramos una palma *Synechanthus fibrosus* en la entrada del bloque 11 que nos acompañará siempre.

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Resistance estimation to *Phytophthora palmivora* in cacao genotypes using artificial inoculation and natural infection in the field

Estimación de la resistencia a *Phytophthora palmivora* en genotipos de cacao empleando inoculación artificial e infección natural en campo

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ABSTRACT

Black pod disease (BPD) is a severe biotic disorder affecting cacao trees in tropical regions generating an estimated global production reduction of approximately 20 to 30%. Accordingly, this study aimed to investigate the correlation between two artificial inoculation methods for *Phytophthora palmivora* and their potential association with natural infection in cacao clones. Incidence (%) and severity in detached pods (average lesion diameter) and leaf discs (disease severity index) were evaluated. The inoculation in pods at 6 DAI (Days After Inoculation) indicated the highest lesion diameter values for clone CCN51 (9.83 cm); hence, it was categorized as the most susceptible. Conversely, clones IMC67 (5.30 cm) and PA46 (5.27 cm), with the lowest lesion diameter values, were classified as moderately susceptible. Similar outcomes were observed in the leaf disc infection test, corroborating the susceptibility categorization of all six clones at 10 DAI. The leaf disc infection method showed a significantly positive correlation with the detached pod infection method, highlighting the feasibility of employing leaf inoculation to classify clones based on their susceptibility to BPD. Significant differences in aggressiveness were established between the isolates from different Colombian cacao regions. These findings were consistently reflected in the field, where the CCN51 clone exhibited the highest susceptibility compared to TSH565 and ICS95. This research proposes using the leaf technique to assess the aggressivity of *Phytophthora palmivora* isolates in cacao trees in Colombia.

Keywords:
Average lesion diameter
Black pod disease
Cacao diseases
Disease severity index
Oomycetes

RESUMEN

La pudrición negra de la mazorca (PNM) es un desorden biótico limitante que afecta los árboles de cacao en regiones tropicales, generando pérdidas que se estiman entre el 20 y el 30% de la producción mundial. Por lo tanto, el objetivo de este estudio fue evaluar la correlación entre dos métodos frente a la inoculación de *Phytophthora palmivora* y su asociación con la infección natural en clones de cacao. Se evaluó la incidencia (%) y severidad de la enfermedad en frutos desprendidos (diámetro medio de la lesión) y en discos de hoja (índice de severidad de la enfermedad). La inoculación en frutos a los 6 DDI (días después de la inoculación), indicó los valores más altos de diámetro de la lesión para el clon CCN51(9,83 cm) por lo que fue categorizado como el más susceptible y los clones IMC67 (5,30 cm) y PA46 (5,27 cm) (con los valores más bajos de diámetro de la lesión), como moderadamente susceptibles. Resultados similares fueron encontrados en la prueba de infección en discos de hojas, corroborando la clasificación de la susceptibilidad de los cinco clones a los 10 DDI. El método de infección en disco de hoja presentó una correlación significativamente positiva con el método de infección en fruto desprendido, indicando la empleabilidad de los dos métodos para la categorización de los clones para la enfermedad PNM. Diferencias significativas en la agresividad fueron establecidas entre los aislamientos evaluados de diferentes regiones colombianas. La asociación directa se mostró en campo, donde el clon CCN51 fue el más susceptible en comparación con los clones TSH565 e ICS95. Esta investigación propone el uso de la técnica foliar para la evaluación de la agresividad de aislados de *Phytophthora palmivora* en árboles de cacao en Colombia.

Palabras clave:
Diámetro medio de la lesión
Mazorca negra
Enfermedades del cacao
Indice de severidad de la enfermedad
Oomicetos

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Several *Phytophthora* species produce cacao black pod disease (BPD) or black pod rot, which occurs worldwide in most cacao crop areas (Ploetz 2016). It has been demonstrated that without any management, cacao losses could reach up to 50-100% due to BPD (Pokou et al. 2008), considered one of the most destructive cacao diseases registered globally. *Phytophthora* species with an economic impact on cacao production are*: Phytophthora capsici* Leonian, *P. citrophthora* (R.E.Sm. & E.H.Sm.) Leonian*, P. megakarya* (Brasier and Griffin 1979), and *P. palmivora* (E.J. Butler) (Surujdeo-Maharaj et al. 2016, Marelli et al. 2019). These species have been reported in the Americas and Cameroun (*P. capsici*), Brazil (*P. citrophthora*), and West Africa (*P. megakarya*) (Adeniyi 2019; Merga 2022). *Phytophthora palmivora* (Pp) is the sole exception, colonizing every region where cacao is cultivated (Brasier and Griffin 1979; Marelli et al. 2019). In addition to this, Pp is the most prevalent species in the producing areas in Colombia (Rodríguez-Polanco et al. 2020b; Palacios Bejarano et al. 2021).

BPD symptoms progression depends on the cacao genotype (clone), abiotic factors, and *Phytophthora* species (Martins et al. 2018; Puig et al. 2018; Pokou et al. 2019; Puig et al. 2021). Disease symptoms include black spots in pods, which spread rapidly and cause internal and superficial tissue damage, affecting cacao beans (Merga 2022). In the case of the northwestern region of Colombia, during the first 2 to 3 days after field infection, symptoms initiate as diminutive black spots and then spread as brown-black lesions (4 to 7 days), colonizing all the pod with white mycelia after 7 days (Rodríguez and Vera 2015). This disease progression was recorded in ICS clones caused by the Pp pathogen. Furthermore, the causal agent can also affect other cacao plant tissues (Surujdeo-Maharaj et al. 2016), producing resistant and long-term survival structures (Ko 2003), accelerating plant tissue decomposition (Guest 2007) and increasing the negative impacts on cacao production. Technical progress is struggling against BPD dissemination, including early detection (Franco et al. 2019; Yanac Montesino et al. 2021), crop management strategies (Rodríguez-Polanco et al. 2020c; Merga 2022; Misman et al. 2022), and plant genetic programs (Pokou et al. 2019; Fister et al. 2020; Tijani et al. 2020; Mucherino Muñoz et al. 2021; Rêgo et al. 2023).

Plant breeding represents a promising alternative against BPD, increasing productivity (de Souza et al. 2021) due to resistant cacao genotypes with interest traits (Pokou et al. 2008). Cacao genetic programs typically focus on developing cultivars resistant to plant diseases while achieving exceptional crop quality and productivity (Tahi et al. 2006). However, pre-breeding activities are essential to achieve the aims of these programs, such as screening pathogen aggressiveness in different plant materials. In the case of BPD, alternative methods have been employed to assess BPD in cacao clones, with *in vitro* inoculation and field evaluation as the most common approaches (Lessa et al. 2020; Tijani et al. 2020; de Souza et al. 2021). Consequently, experiments involving artificial inoculation have been conducted on various cacao plant parts to measure their resistance against *Phytophthora* spp. (Saul-Maora et al. 2007). In this sense, environmental control is attainable using artificial inoculation in the laboratory. The benefits of laboratory methods include confining pathogens, avoiding contamination, and occupying less space compared to a greenhouse (Miller-Butler et al. 2018). An additional benefit is that the accuracy of laboratory inoculation tests shows a positive correlation with the observed resistance rates in the field, as Nyassé (1997) demonstrated. Moreover, the positive correlations between laboratory inoculation and natural infection of immature and adult pods have been demonstrated (Pokou et al. 2008).

In the case of field screening tests, sometimes results are not feasible due to abiotic (environmental) or biotic (pests, other non-target diseases) conditions, affecting error-free estimations in disease behavior (Imathiu et al. 2014). Thus, techniques under standardized conditions, such as leaf discs and detached pods, represent a suitable alternative to increase time efficiency in cacao clone selection against BPD (Nyadanu et al. 2012). However, the association between these two laboratory techniques should be explored by employing *Phytophthora* isolates from Colombia. Accordingly, the aim of this study was to assess the resistance of *Phytophthora palmivora* by employing artificial inoculation in leaves and pods to establish the utility of the leaf test in early BPD resistance selection.

MATERIALS AND METHODS

Plant material

A total of six cacao clones were tested to evaluate their resistance to Pp isolates (Table 1). Within these, two control clones, the *Phytophthora*-resistant clone PA466 and the *Phytophthora*-susceptible clone CCN51 were included (Iwaro et al. 2003; Arciniegas 2005). These clones were obtained from the cacao germplasm bank of Agrosavia – La Suiza. The cacao clones chosen for this research represent the two primary cacao types commonly cultivated in Colombia, i.e., Forastero and Trinitarian (Motamayor et al. 2008).

Table 1. Cacao clones were assessed in this study. Identification, origin, and group to which they belong.

Clone	Identification and origin	Group
CCN51†	Colección Castro Naranjal (Ecuador)	Trinitarian
ICS95	Imperial College Selection (Trinidad)	Trinitarian
EET8	United Fruit Company (Costa Rica)	Trinitarian
TSH565	Trinidad	Trinitarian
PA46†	Mixed Parinari (Peru)	Amazonian
IMC67	Iquitos Mixed Calabacillo (Peru)	Amazonian

†(Iwaro et al. 2003; Arciniegas 2005).

Phytophthora palmivora isolates

A total of 60 isolates were obtained from the C.I. Nataima collection, where they are preserved in sterile distilled water in Eppendorf tubes at a temperature of 10 °C. The molecular identification of all isolates was previously assessed (Rodríguez-Polanco et al. 2020b).

The top five most aggressive Pp isolates were selected to exhibit the highest levels of aggressiveness in screening tests, previously performed based on the detached fruits methodology, according to Rodríguez-Polanco et al. (2020a). These isolates were sampled from distinct cacao production regions in Colombia, including Tolima (TOVR 01), Huila (HURV 19), Santander (SARIO 189), Antioquia (ANYA 228), and Arauca (ARAR 153). The isolates were reactivated using pods of clone IMC67 (not susceptible) to minimize any potential influence of the host clone on pathogen aggressiveness. The environmental conditions during the experiments were maintained at a relative humidity of 90% and a temperature of 28 °C.

All the isolates were cultured in Petri dishes containing agar-V8A juice (V8A) following the methodology described by Rodríguez-Polanco et al. (2020b) and incubated under darkness conditions at 25 °C for 12 days (de Souza et al. 2021). 10 colonies were inoculated in V8A per isolate to prepare the inoculum suspensions. The final concentration of the zoospores in the suspension was set at 3.0×10^5 zoospores mL^{-1}.

Artificial inoculation of pods and leaves

Inoculation experiments of cacao pods and leaves were conducted in the Plant Pathology Laboratory at the Nataima Research Center of Agrosavia in the Municipality of Espinal, Colombia. The environmental conditions of the laboratory were maintained at an average temperature of 22 °C and relative humidity of 70%. In this trial, the *Phytophthora palmivora* isolate HURV19 was used for the artificial inoculation as it registered the highest level of aggressiveness in the previous experiment (*Phytophthora palmivora* isolates). In all experiments, clone PA46 was used as a non-susceptible control (R) and clone CCN51 as a susceptible control (S) (Iwaro et al. 2003).

Pod inoculation

The method for inoculating detached immature pods was conducted following the methodology described by Rodríguez-Polanco et al. (2020a). Two-month-old healthy cacao pods were initially covered with protective bags in the field. Once the pods were 4.5 months old, they were harvested and transported to the laboratory. Small filter paper discs with a diameter of 0.5 cm were prepared and soaked in Pp inoculum to carry out the inoculation. These filter paper discs were then placed at equal intervals in the equatorial zone of each cacao pod. Subsequently, the pods were individually incubated in a humid chamber at a temperature of 28 °C. The incidence of symptoms (absence/presence) and the severity of the infection, average lesion diameter (ALD), were determined by

measuring the lesion from two perpendicular sides of the pod. The experiment was assessed at 6 and 10 days after inoculation (DAI) following the evaluation method outlined by (Arciniegas 2005). The ALD measurement was used to determine the infection response in each clone.

Leaf inoculation

Leaf inoculation was implemented following the methodology by de Souza et al. (2021). 10 healthy leaves were collected in the morning from slightly woody branches in the field. These were placed in polyethylene bags and transferred to the laboratory. Leaves were washed in tap water for 0.5 min, followed by a rinse with distilled water. Subsequently, a circular disc with a diameter of 1.5 cm was cut from each leaf using a cutter, obtaining 20 discs per cacao material.

The discs were placed in synthetic containers with the leaf underside facing upwards. Each container was equipped with a piece (3 cm) of sterile polyurethane sponge to create a high-humidity environment, providing optimum conditions for disease development. Subsequently, a zoospore suspension of 10 µL (3×10^5 zoospores mL^{-1}) was inoculated on the middle of each disc using a micropipette. The containers were sealed and placed in a dark incubator at 25 °C for 7 days.

The leaf discs were assessed 3, 5, and 7 DAI, utilizing the Scale Degree according to Nyassé et al. (1995). The average values from the scale were then employed to calculate the disease severity index (DSI) of each clone, using the McKinney index equation (McKinney 1923) in Equation (1) as follows.

$$DSI\% = \frac{\sum(\text{Scale Degree x incidence})}{\text{Total \# discs}(40)\text{ x the highest from the scale}} \text{x } 100 \quad (1)$$

On farm contamination

BPD natural infection in the field was conducted in a commercial cacao farm in the Municipality of Palocabildo in Tolima, Colombia. The trial was established in a commercial cacao plantation located at 05°7'46.46"N and 74°59'59.9"S, and 1,235 masl. The area had adequate environmental conditions for the Pp infection, with lower temperatures at night (15 to 20 °C). These conditions favored pathogen reproduction by an increase of approximately 40% in the number of zoospores (Zentmyer 1980). Natural pod infections were assessed in 2015 for 12 months, i.e., two harvest periods. Due to the lack of cacao dissemination clones in the region, only clones CCN51, ICS565, and TSH565 were evaluated. 10 cacao trees per clone were selected randomly in the field. In each tree, all damaged pods affected by *Phytophthora* were assessed. Severity per tree was considered as the average of all pod evaluations. Pod severity evaluation was determined as the percentage of damaged area (cm^2) considering the total pod area (cm^2). Areas were determined by taking cacao pod photos and using the ImageJ program (Schneider et al. 2012). The assessment was conducted every 15 days for 12 months.

Statistical design and analysis

Pod inoculation

Two independent experiments were carried out in a completely randomized design with six cacao clones and five major aggressive Pp isolates, according to the methods stated by Rodríguez-Polanco et al. (2020a). The average lesion diameter (ALD) was evaluated (n=10). After confirming the homogeneity of variance (Cochran's test), the average data length (ADL) from both experiments was combined using Cochran's test (Gomez and Gomez 1984). The clone and the isolate were considered factors in a two-way variance analysis using ALD data. Means were compared by the t-test ($P \leq 0.05$). Statistical analyses were performed using the R program (R Core Team 2022).

Leaf inoculation and methods correlation

The experimental design and statistical analysis were carried out similarly to the ones used in the detached immature pod inoculation, except for using 20 repetitions per treatment. The inoculation method for detached immature pods was correlated with the inoculation method on leaf discs using Pearson's correlation. Statistical analyses were performed using the R program (R Core Team 2022).

Natural infection in the field

Natural tree infection was measured as severity in the field. Non-parametric analysis was conducted using Kruskal Wallis' analysis, with 10 repetitions (n=10). Severity was introduced as a variable and clones as a class variable. Pair-wise comparisons were carried out using ($P<0.05$). Statistical analyses were performed using the R program (R Core Team 2022).

RESULTS AND DISCUSSION

The aggressiveness of *P. palmivora* isolates

The average lesion diameter test inoculation in the detached pod test was assessed to test the inoculation effect of five Pp isolates (Figure 1), recording significant differences ($P \leq 0.001$). Isolate HURV19 (8.36 cm) showed the highest isolation value, and ANYA228 (2.03 cm) exhibited the lowest.

An efficient and reliable screening method must evaluate plant material with the most aggressive isolate to prevent false negative results. In addition, it has been demonstrated that different Pp isolates establish variations in BPD symptom response (Barreto et al. 2018). In the current study, isolate HURV19 (8.36 cm) displayed the highest ALD value, demonstrating its aggressiveness potential. In Colombia, several studies have characterized *Phytophthora* species associated with BPD (Villamizar-Gallardo et al. 2019; Rodríguez-Polanco et al. 2020a; Ramírez Martínez et al. 2021), demonstrating the importance of testing Pp aggressiveness from different cultivated areas in the country. Although Pp species aggressiveness in cacao is known, no population study has been conducted in Colombia, with isolates of this species obtained from different productive regions. Thus, it is crucial to consider the knowledge of the existing variability between the isolates of this pathogen for its use in cacao genetic breeding programs against BPD.

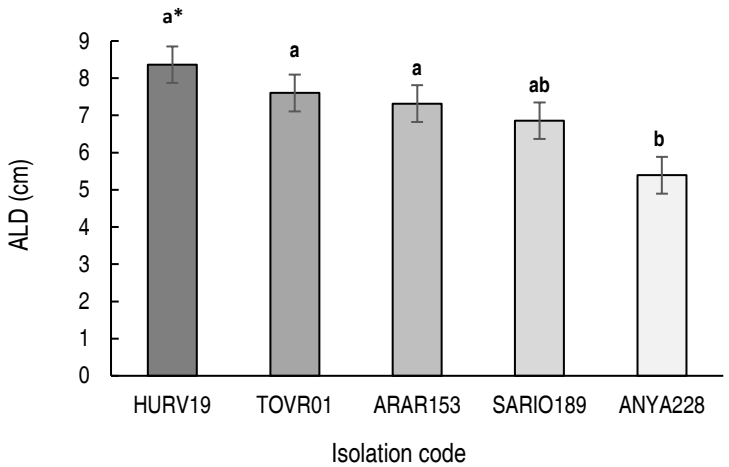

Figure 1. Aggressiveness of five *Phytophthora palmivora* isolates using the detached pod inoculation method 6 DAI (Days after inoculation). ALD: Average Lesion Diameter. *Letters above bar errors indicate statistical significance ($P<0.05$) according to Tukey's test.

Data analysis of artificial inoculation methods

The artificial inoculation methods, detached cacao pods and leaf discs were tested to evaluate the resistance level status against BPD in six different cacao clones under controlled environmental conditions (Table 2). The plant tissues assessed showed symptoms in all genotypes (clones). Regarding detached pods for the 2 days evaluated (6 and 10), statistical differences ($P \leq 0.01$) were obtained in clones and Pp isolates. Nonetheless, no significance between the interaction of these factors was obtained. Similar results were found in leaf discs. Statistical differences ($P \leq 0.0001$) in the factors host genotype x DAI (3, 5, and 7) were registered. However, the interaction significance between these two factors was not displayed (Table 2).

This is one of the first research where six different clones were assessed with two inoculation methods under controlled conditions using Colombian Pp isolates. Inoculation success in all the clones demonstrates that isolate HURV19 can infect different cacao clones in Colombia. According to the results and considering the inoculation period, 6 days for detached pods and 7 days for leaf discs, both periods were sufficient to evaluate BPD under controlled conditions. Similar results were previously reported (Arciniegas 2005). In addition to this, high genetic resistance variation observed in cacao fields may reflect difficulties in the case of researchers interested in employing screening tests in this environment. On the contrary, time and space efficiency in implementing laboratory inoculation shows prompt resistance evaluation

levels in cacao clones. Additionally, artificial inoculation methodologies demonstrate the possibility of potentiating possible resistance gene accumulation in field plant material (Mucherino Muñoz et al. 2021).

Table 2. Aggressiveness of *Phytophthora palmivora* inoculation in detached pods and leaves using ALD[§] and DSI[†], respectively, in six cacao clones.

Inoculation method	Days after inoculation	Sources of variation	df	SS	MS	F (Statistical significance)
Detached pods*	6	Clones	5	476.76	79.46	12.12***
		Isolates	4	66.48	33.24	5.07**
		Clones x Isolates	20	113.55	9.46	1.44 (NS)
		Error	180	-	-	-
	10	Clones	5	1,004.1	167.36	11.87***
		Isolates	4	117.02	58.51	4.15**
		Clones x solates	20	332.65	27.72	1.97 (NS)
		Error	180	-	-	-
Leaf discs	3, 5, 7	Clones	5	1.03	0.21	20.51***
		Days	2	0.25	0.13	12.54***
		Clones x Days	10	0.07	0.01	0.70 (NS)
		Error	70	-	-	-

[§]Average Lesion Diameter, [†]Disease severity index (Equation 1), *Data obtained from (Rodríguez-Polanco et al. 2020a; Polanco et al. 2022). NS: not significant, *P<0.05, **P<0.01, ***P<0.001.

Linear Pearson's correlation indicated a significant connection with a moderate value (r=0.52) between lesion area in detached pods and leaf disc infection (P<0.0001). The manifestation period of BPD symptoms on leaves or pods varies based on the cacao clone and the specific *Phytophthora* species implicated (Barreto et al. 2018). The direct association between laboratory inoculation methods might be due to common genes expression involved in each genotype response for distinct plant organs. However, further research must be conducted in transcriptome analysis evaluating different cacao plant organs during the early Pp infection. The non-significant isolate and host interaction has been reported from the findings by Nyadanu et al. (2012), who established the non-specificity of *Phytophthora* species with cacao genotypes. This indicates that the resistance of cacao genotypes to *P. palmivora* could be applicable to other *Phytophthora* species that cause BPD in cacao in other cacao-producing areas worldwide.

Considering the positive correlation between the two artificial methods and the 1-day difference in symptom evidence, the leaf inoculation method is recommended as the preferred methodology for tolerance screening of BPD in cacao clones in the country. By utilizing foliage instead of pods for disease evaluation, a higher number of cacao clones can be screened due to the practicality of space in the laboratory. Additionally, this approach offers the advantage of year-round availability of photosynthetic tissue, unlike the limited availability of pods due to seasonal harvests. Previous studies have demonstrated optimal results in using leave inoculation for BPD assessment in cacao clones (Nyadanu et al. 2010; Lessa et al. 2020; Tijani et al. 2020; de Souza et al. 2021).

Cacao clone tolerance against BPD results demonstrate differences between the cultivars using the two laboratory-based methods (Figure 2). The trend between detached pods and leaf discs is clear for the six clones evaluated. Statistical differences explain the susceptibility of CCN51 against the disease, with 9.83 cm (6 DAI), and 21.86 cm (10 DAI). Conversely, PA46 showed the lowest lesion tissue against BPD, with 5.27 cm (6 DAI) and, 13.25 cm (10 DAI). Cacao clones IMC 67 and PA46 did not display statistical differences in all the cases.

Overall, resistant (PA46) and susceptible (CCN51) clone controls behaved as expected in both assays. At this point, it was demonstrated that the tolerance against BPD is associated with Amazonian forastero genotypes, which, at the same time, are *amelonado* or melon-shaped pods (Rodríguez-Polanco et al. 2020a; de Souza et al. 2021). In contrast, trinitarian materials have been represented as susceptible to BPD disease, as suggest Paulin et al. (2008). In this sense, the current study demonstrated that genotype CCN51 behaves as susceptible, consistent with other studies (Ramírez 2016; Delgadillo-Durán et al. 2020; de Souza et al. 2021). For this reason, the CCN51 clone might be employed as a susceptible control in BPD research in Colombia. The variations observed in disease progression among the evaluated cultivars in this research imply the presence of quantitative resistance, following the findings from a previous study conducted by (Legavre et al. 2015).

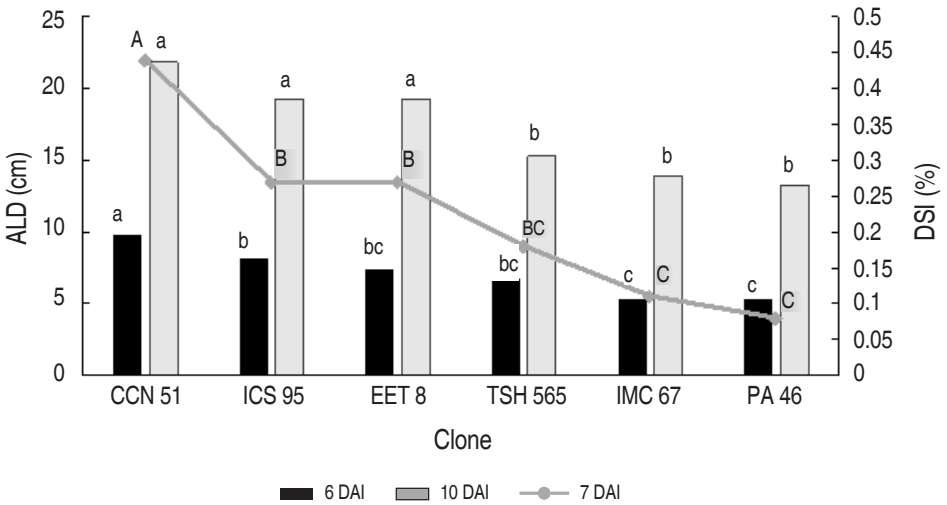

Figure 2. Aggressiveness of *Phytophthora palmivora* inoculation in detached pods and leaves. Statistical comparison between ALD[§] (detached pods) and DDI[†] (leaf discs) in six cacao clones. [§]Average Lesion Diameter. [†]Disease severity index (Equation 1). HURV19 was accepted as the Pp isolate. DAI: days after inoculation. *Means with different letters are significantly different (P<0.05) according to Tukey's test, Lowercase: ALD (Pod) analysis. Uppercase: DI (leaf) analysis.

Field experiment

In the natural inoculation in the field assessment, statistical differences were observed in the clones available in the zone, i.e., TSH565, ICS95, and CCN51. Disease severity was taken as a variable in this case (Table 3). Once again, the CCN51 clone demonstrated susceptibility to the disease due to the highest tissue damage (46.68%) compared with clones THS565 and ICS95 (13.89 and 21.29%, respectively).

Table 3. Natural infection in cacao pods in the field employing lesion area (%) in three different cacao clones in a commercial farm in Palocabildo, Tolima, Colombia.

Clone	Severity (%)
TSH565	13.89[a]*
ICS95	21.29[a]
CCN51	46.68[b]

*Means with a different letter are significantly different (P<0.05), according to the Kruskal Wallis Test.

Data shown in the current study demonstrates the high reproducibility and reliability of laboratory methods in BPD cacao resistance, being leaf infection the methodology recommended. Other studies demonstrate the positive correlation between laboratory-based methods and natural infection in cacao crops (Efombagn et al. 2011; de Souza et al. 2021). It is well known that natural field infection requires a considerable period, particularly regarding cacao pod harvest taking place twice per year. Consequently, laboratory methods boost plant breeding programs, detecting resistant and susceptible materials in less time.

In the study conducted by Pokou et al. (2008), a notable association between the response of clonal cacao to natural infection of *P. palmivora* in field conditions and leaf disc inoculation, as well as in the inoculation of detached pods were identified. Additionally, their investigations revealed

a particularly strong correlation coefficient between the inoculation of detached pods and field infection, consistent with the experiments described in the current study. A robust correlation was established between environmental control tests conducted on detached cacao pods and *in vivo* in the field, according to de Souza et al. (2021). The study highlights that these tests can be an initial screening method for evaluating germplasm. Additionally, gathering data in the field requires an adequate amount of time and favorable environmental conditions for pathogen growth.

This study represents pioneer research in Colombia by evaluating two artificial inoculation methods in various cacao clones with *Phytophthora palmivora* isolates from different cacao-producing regions in the country. The consistent success of leaf inoculation across all clones unequivocally demonstrates the effective control of environmental factors and the efficient use of space and plant tissue availability. Thus, a robust approach for screening BPD tolerance in cacao genotypes can be established, in agreement with Lessa et al. (2020). These findings contribute further to crop protection, guiding breeders in selecting tolerant cacao materials and researchers in developing integrated management strategies to combat *Phytophthora palmivora,* promoting the sustainability and productivity of cacao cultivation in the country.

CONCLUSIONS

A suitable methodology for *Phytophthora palmivora* isolates assessment in cacao clones under laboratory conditions, and its relationship under natural infection was established. All assessed clones exhibited *Phytophthora palmivora* incidence. However, the disease level was displayed distinctly in each material. The tolerance of cacao material PA46 and the susceptibility of CCN51 were validated, consolidating their respective classification. Moreover, the correlation between the two artificial inoculation laboratory methods indicates that leaf inoculation is sufficient to evaluate the aggressiveness of *Phytophthora palmivora* isolates. Further, natural field inoculation suggests a direct association between the laboratory and this *in vivo* methodology, demonstrating the possibility of selecting genotypes with the leaf disc method. This research proposes using this rapid BPD information status methodology in cacao clone materials that help boost pre-breeding programs that seek resistance against *Phytophthora palmivora* in Colombia.

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Short article

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Sex reversal in female cannabis plants as a in response to male flowering promoters

Reversión sexual de plantas femeninas de cannabis en respuesta a promotores de la floración masculina

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Keywords:
Aminoethoxy-vinyl-glycine
Gibberellic acid
1-Methylcyclopropene
Silver thiosulfate

ABSTRACT

Cannabis sativa is a widely studied species and is currently accepted worldwide due to its medicinal properties, especially those conferred to the CBD phytocannabinoid, which is synthesized mainly in the globular trichomes of female flowers. Males are undesirable and rare in commercial plantations; however, they are necessary for breeding programs. This research aimed to evaluate sexual reversion methods in female cannabis plants as a preliminary stage of a plant breeding program. A completely randomized design with eight treatments and four repetitions was used. The treatments consisted of protocols for the sexual reversion of female plants through drip application and foliar spraying of Silver Thiosulfate (STS), Aminoethoxy-vinyl-glycine (AVG), 1-Methylcyclopropene (1-MCP), and Gibberellic acid (AG_3), plus a control treatment without application. Male flower production was evaluated in female cannabis plants, and pollen viability in male flowers was determined. The AVG treatments applied to the apex by dripping, and the AG_3 applied to the foliage in the form of a spray influenced the sexual reversion of female plants and produced a total of 132 and 32 male flowers, respectively, without difference between them (P=0.08383). For AVG dripping, only male flowers were observed at the apex, where the application was made directly. Moreover, STS and 1-MCP did not induce the production of male flowers. The pollen from male plants treated with AG_3 in spray, and AVG dripping showed high viability (>50%), contrary to the low viability observed in plants treated with AGC applied in spray. The plants treated with AG_3 (spray) were higher due to the elongation of the internodes. The AVG and AG_3 compounds are effective in the sexual reversion of female cannabis and generate male flowers with viable pollen.

Palabras clave:
Aminoetoxi-vinil-glicina
Ácido giberélico
1-Metilciclopropeno
Tiosulfato de plata

RESUMEN

Cannabis sativa es una especie ampliamente estudiada y actualmente aceptada a nivel mundial por sus propiedades medicinales, especialmente las conferidas al fitocannabinoide CBD, el cual se sintetiza principalmente en los tricomas globulares de las flores femeninas. Los machos son indeseables y poco comunes en las plantaciones comerciales; sin embargo, son necesarios en los programas de mejoramiento. La presente investigación tuvo como objetivo evaluar métodos de reversión sexual en plantas femeninas de cannabis como una estrategia preliminar en un programa de mejoramiento de la especie. Se empleó un diseño completamente al azar con ocho tratamientos y cuatro repeticiones. Estos consistieron en protocolos para la reversión sexual de plantas femeninas a través de la aplicación por goteo y en aspersión foliar de Tiosulfato de plata (STS), Aminoetoxi-vinil-glicina (AVG), 1-Metil ciclopropeno (1-MCP) y Ácido giberélico (AG_3); más un tratamiento testigo sin aplicación. Se evaluó la formación de flores masculinas en plantas femeninas de cannabis y se determinó la viabilidad del polen en las flores masculinas. La aplicación de AVG en *spray* y por goteo y el AG_3 aplicado al follaje en forma de *spray* fueron efectivos en la reversión sexual de plantas femeninas y produjeron un total de 132 y 32 flores masculinas, respectivamente, sin diferencia entre ellas (P=0.08383). Para AVG en goteo solamente se observaron flores masculinas en el ápice, lugar directo donde se realizó la aplicación. Por otro lado, STS y 1-MCP no indujeron la formación de flores masculinas. El polen producido en plantas masculinas tratadas con AG_3 en *spray* y AVG por goteo presentó alta viabilidad (>50%), contrario a la baja viabilidad observada en plantas tratadas con AGC aplicado en *spray*. Las plantas tratadas con AG_3 (*spray*) presentaron mayor altura debido a la elongación de los entrenudos. Los compuestos AVG y AG_3 son eficaces en la reversión sexual de las plantas femeninas de cannabis e inducen flores masculinas con polen viable.

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The species hemp or cannabis (*Cannabis sativa* L.) belongs to the Cannabaceae family and its origin and uses date back to ancient times in Asia and the Middle East (Molina 2008; Clarke and Merlin 2013). Although it is considered a monotypic species, there are three subspecies (*sativa*, *indica,* and *ruderalis*) with highly polymorphic characteristics (Spitzer-Rimon et al. 2019). The cultivation of cannabis for medicinal or psychoactive purposes is based on the use of globular trichomes present in floral structures, mainly in female flowers, which is why the use of feminized genotypes is essential. Moreover, hermaphrodite plants produce fibers and seeds with an adequate ratio of male and female flowers (Razumova et al. 2016; Spitzer-Rimon et al. 2019).

This species is classified as a dioecious plant, which only occurs in 6% of all plant species. Both male and female inflorescences are found in different individuals. Their expression is linked to both hormonal homeostasis of the genes localized on the XX chromosomes in the case of female plants, and the genes on XY chromosomes in male plants (Negrutiu et al. 2001; Hobza et al. 2018). However, cases of hermaphroditism can occur, where both female and male flowers differ in the same individual (Salentijn et al. 2019). Both inflorescences develop orderly at the internodes in the axillary buds on the plants' main axis and lateral branches. Each phytomer (internode and axillary bud) comprises a large, webbed leaf in charge of photosynthetic processes, and a pair of bracts that support two single flowers originating from an axillary bud (Spitzer-Rimon et al. 2019). The female flowers have a simple structure comprising a perigonal bract covering the ovary. At maturity, the flower is covered with globular trichomes where Phyto cannabinoids are synthesized. It also has a pair of stigmas that emerge unevenly from the inside. Male flowers appear in hanging panicles with five sepals and five stamens; their anthers disperse pollen when they reach maturity —a process favored by the wind (Moliterni et al. 2004; Cervantes 2007).

Given the species evolution, several environmental and genetic factors are responsible for the physiological process of flowering, e.g., temperature and photoperiod, which directly influence the morphology of the genesis flower (Amaducci et al. 2008). Cannabis is considered a photoperiod plant and therefore responds to day length; when days are shortened (fewer light hours), the plant induces the flowering process and reaches the reproductive stage. Plants from seed reach maturity at the fourth phytomer, when the differentiation of the apical bud, located on the main stem, generally appears (Moliterni et al. 2004). Here, single flowers are observed on the bracts, some reach their anthesis; however, it can continue from axillary buds until the plants are subjected to short days (suspension of additional light). Some changes in the architecture and morphology of the plants are observed (Amaducci et al. 2008; Spitzer-Rimon et al. 2019).

Under appropriate age, temperature, and luminosity, the genetic expression that regulates sexual differentiation in plants —closely related to environmental interactions and hormonal synthesis— is triggered (Sarath and Mohan Ram 1979; Blazquez and Weigel 2000; Moliterni et al. 2004). Several authors found that the production of female flowers in cannabis plants is favored by increasing the concentrations of auxins, cytokinins, and ethylene. In contrast, the masculine ones were favored by increasing gibberellins (Sarath and Mohan Ram 1979; Mohan Ram and Sett 1982b; Lubell and Brand 2018).

Cannabis growers use sexual reversion methods to produce pollen with male gametes, which leads to sexual seed production when crossing them with female plants. The XX genes dominate the female's originating plant (Lubell and Brand 2018). Silver thiosulfate ($AgNO_3$) is the most used compound by seed producers to achieve sexual reversion in female plants; however, it is highly toxic for handling and to soil and water sources. Other alternatives are substances such as aminoethoxy-vinyl-glycine (AVG) and 1-Methylcyclopropene (1-MCP), whose molecules are less toxic for humans, animals, and the environment (Blankenship and Dole 2003). Studies by Mohan Ram and Sett (1982b) and Lubell and Brand (2018) demonstrated the efficacy of STS and AVG to produce male flowers with viable pollen from female cannabis plants. Therefore, the present work aims to evaluate different methods of sexual reversion in female cannabis plants as a preliminary part of the species breeding program in the search for genotypes, anticipating the industrial growth that the cultivation of cannabis could have in the country.

MATERIALS AND METHODS

Location

The trials were developed in the municipality of Bello, Antioquia, at the facilities of Breeder's of Colombia SAS. This company is registered before the Colombian Agricultural Institute (ICA by its Spanish acronym) as a cannabis breeding research unit for psychoactive cannabis, according to resolution No. 00030034 of August 14, 2018.

Experimental design

A completely randomized design with eight treatments and four repetitions was used. The treatments consisted of protocols for the sexual reversion of female plants through apex drip and foliar spraying of Silver Thiosulfate (STS), Aminoethoxy-vinyl-glycine (AVG), 1-Methylcyclopropene (1-MCP), and Gibberellic acid (AG_3), plus a control treatment without application. Each compound was prepared separately and diluted in demineralized water plus Tween-80 (0.01%) as a surfactant (Table 1).

Cuttings obtained from female cannabis plants were sown in 10 L plastic pots using the substrate of coconut fiber, rice, mushroom, and sawdust in equal proportions; then, they were placed under a plastic cover and subjected to a photoperiod of 18/6 h (Day/Night). Out of the 18 h a day, the first 12 h correspond to sunlight and the remaining 6 h were provided by 30 W and 5000 K LED bulbs. The pots were distributed in three rows with 12 plants each at 0.5 m between plants and 1m between rows.

Table 1. Dosage and application of the compounds used for the sexual reversion of female cannabis plants.

Treatment	Chemical agent	Dose	Application method
1	Silver thiosulfate (STS)	100 µg plant^{-1} + Tween-80 (0.01%)	Drip to leaf apex
2	Silver thiosulfate (STS)	100 µg plant^{-1} + Tween-80 (0.01%)	Foliar spray
3	Aminoethoxy-vinyl-glycine (AVG)	100 µg plant^{-1} + Tween-80 (0.01%)	Drip to leaf apex
4	Aminoethoxy-vinyl-glycine (AVG)	100 µg plant^{-1} + Tween-80 (0.01%)	Foliar spray
5	Gibberellic acid (GA_3)	100 µg plant^{-1}	Drip to leaf apex
6	Gibberellic acid (GA_3)	100 µg plant^{-1} + Tween-80 (0.01%)	Foliar spray
7	1- Methylcyclopropene (1-MCP)	100 µg plant^{-1}	Foliar spray
8	Control treatment	Tween-80 (0.01%)	Foliar spray

Each treatment was applied 20 µg plant^{-1} for 5 consecutive days and 100 µg of the compound per plant. The application was made 60 days after pot transplantation, when the additional light was suspended (6 h), thus modifying the relationship to 12/12 h (Day/Night).

Each compound (treatment) was applied through foliar spray (s) using a 20 µg solution in 10 mL of distilled water +0.01% Tween-80. The drip to the foliar apex (g) consisted of applying a 10 µL drop of a solution containing 20 µg of each compound with a micropipette. In the 1-MCP treatment, the application consisted only of foliar spraying. In contrast, the control treatment plants only received the Tween-80 solution (0.01%) in 20 mL of water applied in foliar spraying.

Number of male flowers and pollen viability

The male flowers in anthesis (open petals and mature anthers) were collected weekly and were counted by plant. The pollen of each male flower was extracted by vibration and deposited on a slide fixed with a drop of tetrazolium salt. After 30 seconds, the pollen grains were observed in a microscope under a 10X objective, and 200 grains were counted. Subsequently, their viability status was determined; those that presented a reddish coloration were considered viable, and those without coloration, non-viable.

Data analysis

An analysis of variance and Shapiro-Wilk test were performed for normality and the results were subjected to the Tukey Multiple comparison test (P<0.05). In cases where the normality assumption was not fulfilled, the non-parametric Kruskal-Wallis' test was used. The differences between treatments were analyzed by the Wilcoxon signed-rank test using the Agricolae package of the R statistical software Core Team 2023.

RESULTS AND DISCUSSION

Number of male flowers

Since data did not comply with the normality assumption (Shapiro-Wilk, W=0.46779, and $P<2.2x10^{-16}$), they were analyzed under the non-parametric Kruskal-Wallis' test (chi-square of 94.345, df=7 and $P<2.2x10^{-16}$). The comparisons were made by the Wilcoxon test adjusted to the Holm method (Table 2).

Table 2. *P* values comparing the median of the sexual reversion treatments of female Cannabis plants by Wilcoxon test.

Treatment	Treatment							
	1-MCP(s)	AG_3(g)	AG_3(s)	AVG(g)	AVG(s)	Control	STS(g)	STS(s)
AG_3(g)	-	-	-	-	-	-	-	-
AG_3(s)	0.0000	0.00153	-	-	-	-	-	-
AVG(g)	0.0000	0.00002	0.08383	-	-	-	-	-
AVG(s)	-	-	0.00153	0.00003	-	-	-	-
Control	-	-	0.00258	0.00014	-	-	-	-
STS(g)	-	-	0.00153	0.00002	-	-	-	-
STS(s)	-	-	0.00153	0.00002	-	-	-	-

Silver Thiosulfate (STS), Aminoethoxy-vinyl-glycine (AVG), 1-Methylcyclopropene (1-MCP), and Gibberellic Acid (AG_3). Foliar spray (s) and drip to the apex (g).

Figure 1 shows the number of male flowers produced by implementing sexual reversion methods compared to the control treatment without reversion. In this sense, the cannabis plants in the control treatment presented female flowers in their entirety, which indicates that the female character was preserved when cuttings taken from female mother plants were used. The plants treated with reversion agents, AG_3 and AVG treatments, produced male flowers due to their action on the reproductive physiology of the species.

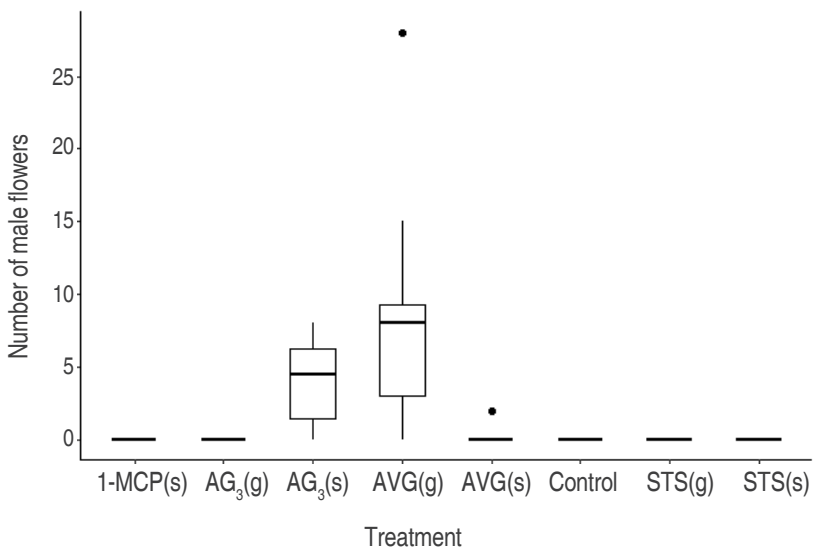

Figure 1. Boxplot of the number of male flowers in response to the treatments. Silver thiosulfate (STS), Aminoethoxy-vinyl-glycine (AVG), 1-Methylcyclopropene (1-MCP), Gibberellic acid (AG_3). Foliar spray (s) and drip to the apex (g).

The difference between successful sexual reversal treatments and those that did not differentiate between male and female flowers is clear. The treatments with AVG(g) and AG_3(s) showed positive results in the number of male flowers, with a total of 132 and 32 male flowers per treatment, respectively. Regarding plants treated with AVG(s), only one differed by two male flowers; however, no statistically significant difference was observed between AVG(g) and AG_3(s) treatments (*P*=0.08383).

Figure 2. A) Visible symptoms of AVG(g); B) Production of male flowers at 100 µg of AVG; C) Male and female flower buds in plants treated with AG_3(s); D) Morphological differences associated with the application of AG_3; E) Viable pollen obtained from reverted plants; and F) Male flowers (reduced males).

The first symptom observed in plants treated with AVG(g) was the yellowing and discoloration of the green pigments in the leaf apex where the compound was applied, without evidence of compromising the meristem (Figure 2A), unlike the treated plants with AVG(s), which did not present this symptom.

Between 25 and 30 days after applying the treatments, the formation of the first male flower nodes was observed. With 100 µg of AVG(g), the plants presented only male flowers on the apex where the application was made directly; however, some female flowers produced from the plant apex were observed at the base of the inflorescence. These observations agree with those reported by Mohan Ram and Sett (1982b), who noticed that 75 µg of AVG was sufficient to reverse female-to-male flowering in 100% of the treated plants. Unlike the results obtained in this work, those authors indicate that concentrations of 100 µg of AVG produced abscission of the leaves and inhibition of new nodes. In this study, it was not observed at that concentration of AVG(g), since the plants showed satisfactory results in sexual reversion (Figure 2B).

In plants treated with gibberellins, between the first and second week after GA_3 application, a reduction of internodes length, width, and number of leaflets, and curling of the tips were observed besides the apex damage in the plants (Figures 2C, D). However, it should be noted that despite the affectation of the foliar apex, two plants treated with AG_3(s) produced flowers at the end of the experiment, initially male flowers, followed by the differentiation of female flowers in the apical shoots (Figure 2C). Male flowers in the plants treated with AG_3(s) appeared late, between one and two weeks after the formation of flowers in the other treatments. These results

are similar to those obtained by Sarath and Mohan Ram (1979). They determined that 50 µg of AG_3 was sufficient to induce sexual reversion in female cannabis plants and that 100 µg of AG_3 only induced flowering in 50% of the treated plants, with viable pollen grains within their anthers. Likewise, Mohan Ram and Sett (1982b) reported that 75 µg of AVG applied to female Cannabis plants induces reduced males with 69% pollen viability, which could germinate between 30 and 35 min after being submerged in a sucrose solution medium.

Regarding 1-MCP —although Lubell and Brand (2018) propose evaluating this substance as a possible agent for sexual reversal in female cannabis plants— its ability to occupy ethylene receptors in cannabis plants is justified. The results in this study, this treatment under the application method did not induce the production of male flowers, nor were atypical symptoms observed in the plants concerning the control treatment.

Furthermore, none of the plants treated with the STS solution under the methods (spray or micro drip) at the evaluated concentrations expressed male flowers in female cannabis plants effectively. Contrary to that reported by Mohan Ram and Sett (1982a) and Sarath and Mohan Ram (1979), who indicate that the use of 100 µg of the silver ion ($AgNO_3$) and 50 µg of the STS compound ($Ag(S_2O_3)$) was sufficient to reverse more than 80% of the flowers in 100% of treated female Cannabis plants. In addition, Lubell and Brand (2018) state that 3 mM of STS induces between 75 and 100% of the production of male flowers in female cannabis plants. Despite the non-inductive effect of STS for sexual reversion observed in this work, it should be noted that STS is the most used method by cannabis growers to induce the production of male flowers in feminized plants.

Pollen viability

Since only three treatments (AVG(g), AVG(s), and AG_3(s)) led to the production of male flowers, the analysis for the pollen viability variable was done by comparing the percentage of viable pollen among the three treatments: those that presented a reddish coloration were considered viable (Figure 2E), and those without coloration, non-viable. The non-parametric Kruskal-Wallis' test (*P*=0.0003611) shows a difference between treatments. The Wilcoxon signed-rank test did not show significant differences between the AVG(g) and AG_3(s) treatments (*P*=0.59657), but there was a difference between these two treatments with the AVG(s).

The maximum percentage of pollen viability (96.11%) was observed in reverted male flowers produced by applying AG_3(s), followed by 51.05% viability in pollen obtained from reverted flowers with AVG(g), and 6.25% viability using AVG(s) (Figure 3).

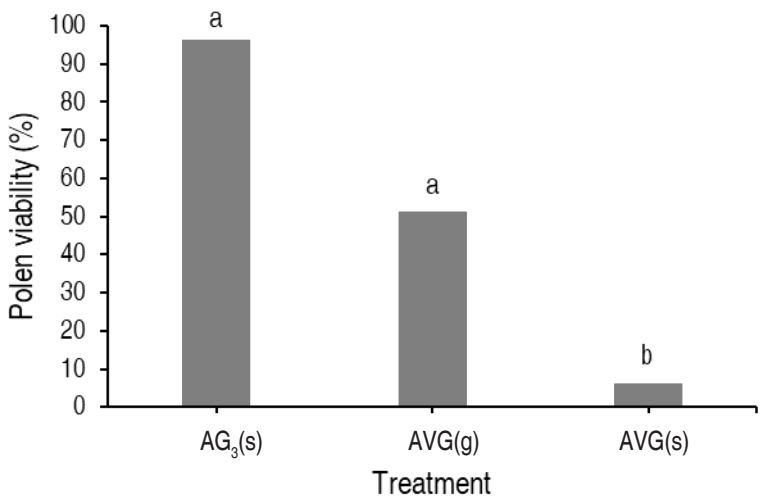

Figure 3. Percentage of pollen viability in male flower from successful reversion treatments. Aminoethoxy-vinyl-glycine (AVG) and Gibberellic acid (AG_3). Foliar spray (s) and drip to the apex (g). Different letters between treatments indicate significant differences according to the non-parametric Wilcoxon signed-rank test (*P*<0.05).

Plant height

After applying the sexual reversal treatments, a variation in plant height was observed between the moment of flower induction and the end of the experiment. The height data analyzed under the non-parametric Kruskal-Wallis' test showed differences between treatments (chi-squared=80.288, df=7, *P*-value=1.203x10^{-14}). The Wilcoxon signed-rank test shows a difference between all the treatments and the gibberellic acid treatment for this variable —AG_3(g) and AG_3(s), and even between these two treatments (Table 3).

The plants treated with AG_3(s) elongated their internodes in the main and secondary branches, unlike the treatment with AG_3(g), where this symptom was more marked in the central apex (Figure 4). In both treatments, morphological changes were previously described. The plants treated with AG_3(g) presented chlorosis followed by necrosis of the central apex and growth detection, unlike the treatment with AG_3(s), which produced flowers despite reactive apical damage. Similar observations are reported by Sarath and Mohan Ram (1979), where plants treated with AG_3 showed elongation of the stems.

Table 3. Wilcoxon signed-rank test for the plant height variable in response to sex reversal treatments.

Treatment	Treatment							
	1-MCP	AG_3(g)	AG_3(s)	AVG(g)	AVG(s)	Control	STS(g)	STS(s)
AG_3(g)	0.00000	-	-	-	-	-	-	-
AG_3(s)	0.00013	0.29265	-	-	-	-	-	-
AVG(g)	0.35994	0.00000	0.00102	-	-	-	-	-
AVG(s)	0.01082	0.00000	0.00007	0.00054	-	-	-	-
Control	0.96535	0.00002	0.00190	0.17489	0.17489	-	-	-
STS(g)	0.96535	0.00000	0.00054	0.96535	0.00036	0.29265	-	-
STS(s)	0.36826	0.00000	0.00054	0.03823	0.29265	1.96535	0.06891	-

Silver thiosulfate (STS), Aminoethoxy-vinyl-glycine (AVG), 1-Methylcyclopropene (1-MCP), and Gibberellic acid (AG_3). (s) Foliar spray and (g) Drip to the apex.

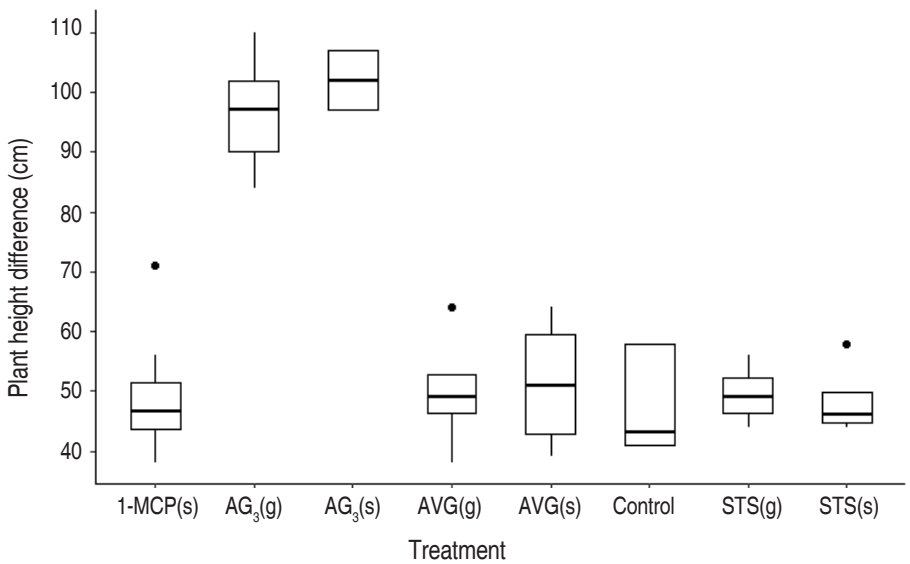

Figure 4. Boxplot of the plant's height in response to the treatments. Silver thiosulfate (STS), Aminoethoxy-vinyl-glycine (AVG), 1-Methylcyclopropene (1-MCP), and Gibberellic acid (AG_3). (s) Foliar spray and (g) Drip to the apex.

Several factors influence the floral production of plants, especially in angiosperms, e.g., environmental variations, plant age, and metabolic and epigenetic pathways; they play an essential role during meristem differentiation (Feng et al. 2020). The first stimulus for sexual differentiation in cannabis plants occurs with age. In this sense, Moliterni et al. (2004) found that in plants from sexual seed, the differentiation of vegetative to reproductive meristems can be observed from the fourth internode on, when plants produce single flowers between internodes. Since the cuttings were taken from adult plants, the results obtained in this work showed that the plants formed single flowers between the internodes. Flowering presented after two weeks after changing the photoperiod to a 12/12 ratio presented morphological changes in the architecture of the branches, as reported by Spitzer-Rimon et al. (2019). They indicated that from the beginning of the short days, a severe shortening is observed among the phytomers and there were changes in the size and number of leaflets.

Gerashchenkov and Rozhnova (2013) propose that floral morphogenesis is determined by factors that hierarchically trigger internal plant signals. Results in the genetic coding of protein synthesis and some phytohormones found in homeostasis are strongly influenced by environmental factors. The Cucurbitaceae family showed that unisexual flowers that develop individually in each internode keep ethylene reduced in the meristematic apices, thus favoring the production of male flowers. Likewise, the floral meristems produce deficient levels of ethylene, thus maintaining the androgynous condition. In contrast, the short days, low temperatures, and high levels of ethylene in the meristematic and floral apices induce the production of female flowers, considering that ethylene is a critical hormone for sexual differentiation in this species (Feng et al. 2020; Martínez and Jamilena 2021).

In the case of cannabis, a similar mechanism can occur in the synthesis and signaling of ethylene since applying agents that inhibit the biosynthesis of this hormone, such as AVG or STS and $AgNO_3$, induces the production of male flowers in this species. Different studies reported that applying Silver Thiosulfate (STS) in concentrations of 3 mM favored the production of male cannabis flowers from female plants under short-day conditions (Lubell and Brand 2018). Moreover, Mohan Ram and Sett (1982a) found that 75 µg of $AgNO_3$ was necessary to inhibit the action of ethylene in the production of only male flowers. Mohan Ram and Sett (1982b) found that the application in the apex of 75 µg of AVG induces male flowers in this species. Likewise, applying ethylene-releasing agents such as Ethephon in concentrations of 1,920 mg L^{-1} to cannabis plants subjected to sexual reversal with $AgNO_3$ or $CoCl_2$ could reverse the masculinizing effect of these compounds, thus emitting female flowers again (Mohan Ram and Sett 1982c).

Mohan Ram and Sett (1982a) evaluated the silver ion $AgNO_3$ and the STS complex to form male flowers in female cannabis plants. The authors propose a classification of the types of flowers found in the branches of treated plants. The reduced male's category coincides with the results found in this study, since the male flowers mainly presented from three to four stamens (Figure 2F); likewise, the pollen in these flowers was not easily shed by the anthers or dispersed by the wind, as occurs naturally in flowers from male plants. Similar observations are reported by Lubell and Brand (2018), who report that pollen from female cannabis plants subjected to sexual reversal treatments with STS applications is not as abundant and its natural release is not as effective. Regarding the germination percentages observed in this work, these results agree with Sarath and Mohan Ram (1979), who experimented with female cannabis plants treated with 100 µg of AG_3 and male flowers with viable pollen. Likewise, Mohan Ram and Sett (1982b) reported that 75 µg of AVG applied to female Cannabis plants reduced the production of male flowers with viable pollen, with germination of 69% between 30 and 35 min after being immersed in a sucrose solution medium.

CONCLUSIONS

The AVG and AG_3 compounds are effective in sexual reversal and enable the production of male flowers from female cannabis plants. They can reverse the feminizing effects in plants and induce male flowers with viable pollen, thus becoming an alternative that can be included in genetic breeding programs for this species.

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Phylogenetic analysis and molecular characterization of BBTV DNA-R of wild and cultivated banana isolates from East Java, Indonesia

Análisis filogenético y caracterización molecular de BBTV DNA-R de aislados de banano silvestre y cultivado de Java Oriental, Indonesia

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ABSTRACT

Banana bunchy top virus (BBTV) molecular detection and understanding its origin are important issues for mitigating future spread. The aim of this study was to molecularly detect BBTV infection and analyze the characteristic also phylogenetic of banana isolates from East Java Indonesia. Two BBTV asymptomatic wild bananas and two BBTV symptomatic banana cultivars were examined. PCR amplifications were accomplished using BBTV DNA-R primers for master replication initiation protein. Sequences evaluations were conducted in SeqScanner. Sequences identification was performed in nucleotide BLAST. Translation of ORFs was determined using ORF Finder server tool. Protein identification was conducted in protein BLAST. Sequences polymorphisms were analyzed using DnaSP6. Phylogenetic analysis was employed using Neighbor-Joining algorithm with Kimura two-parameter (K2P) substitution model in MEGA7. Results showed that BBTV DNA-R components were detected in all isolates and confirmed as Rep protein. The sequences length were varied from 616 to 1,074 bp, low GC content (42.90%) and low conservation (56.47%). Asymptomatic wild bananas generated shorter length and more variable sequences, presumably related to the resistance mechanism. Phylogenetic analysis of BBTV DNA-R East Java with other 38 homolog sequences worldwide were found clustered in Asian Group, closely related to Vietnam, Thailand, and China. Hence, it presumably originated from China mainland via Malay Peninsula route.

Keywords:
Bananas
Genetic variation
Identification
Rep protein
Virus

RESUMEN

La detección molecular virus del cogollo racimoso del banano y la comprensión de su origen son temas importantes para mitigar la propagación futura. El objetivo de este estudio fue detectar molecularmente la infección por BBTV y analizar las características filogenéticas de los aislamientos de banano de Java Oriental, Indonesia. Se examinaron dos cultivares de banano silvestre asintomáticos BBTV y dos cultivares de banano sintomáticos BBTV. Las amplificaciones por PCR se realizaron utilizando cebadores BBTV DNA-R para la proteína maestra de iniciación de la replicación. La evaluación e identificación de las secuencias se realizó en SeqScanner y un análisis nucleótido BLAST, respectivamente. La traducción de los ORF se determinó utilizando la herramienta de servidor ORF Finder. La identificación de proteínas se realizó empleando el programa BLAST. Los polimorfismos de las secuencias se analizaron utilizando DnaSP6. El análisis filogenético se empleó utilizando el algoritmo Neighbor-Joining con el modelo de sustitución Kimura de dos parámetros (K2P) en MEGA7. Los resultados mostraron que los componentes de BBTV DNA-R se detectaron en todos los aislamientos y se confirmaron como proteína Rep. La longitud de las secuencias varió de 616 a 1.074 pb, bajo contenido de GC (42,90%) y baja conservación (56,47%). Los bananos silvestres asintomáticos generaron secuencias más cortas y variables, presumiblemente relacionadas con el mecanismo de resistencia. El análisis filogenético de BBTV DNA-R East Java con otras 38 secuencias homólogas en todo el mundo se encontró agrupado en el Grupo Asiático, estrechamente relacionado con Vietnam, Tailandia y China. Por lo tanto, presumiblemente se originó en China continental a través de la ruta de la Península Malaya.

Palabras clave:
Bananos
Variación genética
Identificación
Proteína Rep
Virus

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Bananas are important global horticultural commodities ranked fourth after wheat, rice and corn; with over 130 countries in the tropical and subtropical regions of the world cultivating it for staple food (Perrier et al. 2011). Southeast Asia, including Indonesia, is considered the diversity and domestication center of bananas. There are at least 325 known banana cultivars in Southeast Asia and 200 of them are found in Indonesia (Hapsari et al. 2015). Therefore, Indonesia was categorized as ranked third as one of the world largest banana producers in 2019 with a contribution of 6.05% of the total world production (FAO 2020). Nevertheless, banana bunchy top disease (BBTD) caused by a banana bunchy top virus (BBTV) has become the most serious and destructive disease to threat the banana production in Indonesia (Hapsari and Masrum 2012).

BBTD is a viral disease, named after the symptoms, where the infected plants are stunted and have "bunchy" leaves at the top (Qazi 2016). When plants are infected early, they do not bear fruit, and when they are infected later, the fruits are poor and unmarketable. This virus infects all members of the Musaceae family, with several related families as alternate hosts such as Araceae, Heliconiaceae, Strelitzeaceae, and Zingiberaceae (Pinili et al. 2012). The banana aphid, *Pentalonia nigronervosa* (Family Aphididae; Order Hemiptera), was found to be a vector for transmitting and spreading the disease wider. Unlike fungal diseases, BBTV cannot be transmitted mechanically through garden tool use (Niyongere et al. 2012).

The virus is identified to the genus *Babuvirus* in the family Nanoviridae. It is an icosahedral virus consisting of six circular single-stranded DNA genome components i.e., DNA-R, DNA-U3, DNA-S, DNA-M, DNA-C and DNA-N (previously named DNA-1 to DNA-6, respectively) (Elayabalan et al. 2015). The sequences length of each genome component is approximately 1 to 1.1 Kb, and the transcripts have been mapped (Abdel-Salam et al. 2012). DNA-R encodes a master replication-associated protein (rep), DNA-S a capsid protein (cp), DNA-M a movement protein (mp), DNA-C a cell-cycle link protein (Clink), and DNA-N a nuclear shuttle protein (nsp) gene. Meanwhile, the function of DNA-U3 is still unknown (Stainton et al. 2015).

Understanding the origin of the BBTV is an important issue, and it is necessary to mitigate and control the future spreads. The origin of the BBTV is thought to come from the area of origin of bananas, namely Southeast Asia, including Indonesia (Perrier et al. 2011). Subsequently BBTV spread to a number of countries, including the Old World (36 countries in Africa, Asia, and Oceania) and there is no record of BBTV being found in the New World except in Hawaii and the United States (Qazi 2016). BBTV can be categorized into two groups based on their phylogenetic, including the Pacific Indian Ocean group and the Asian group (Stainton et al. 2015). However, due to human evolution and intermediary processes, new variants of BBTV possibly have emerged (Yu et al. 2019).

This study was aimed to confirm the BBTV infection on asymptomatic wild bananas *Musa acuminata* Colla varieties compared to symptomatic banana cultivars from East Java, Indonesia, through PCR-based detection using specific primers of BBTV DNA-R. The master replication initiation protein encoded by the DNA-R component of the BBTV has been successfully used for virus detection in both wild and cultivated bananas of Asian countries group (Chiaki et al. 2015; Rahayuniati et al. 2021a). Previous studies reported that wild bananas have important traits such as a relatively harsh environment and diseases resistant's, including BBTV (Hapsari and Masrum 2012), whereas banana cultivars with one or two B genomes (ABB and AAB) tend to be more tolerant to BBTD than AA and AAA (Hapsari and Masrum 2012). In addition, this study also aims to analyze molecular characteristic and phylogenetic of the BBTV DNA-R sequences with other isolates already reported in GenBank, particularly from Indonesia and Asian countries. The results of this study are expected to be useful as basic information for further mitigation and evaluation of BBTV resistant bananas.

MATERIALS AND METHODS

Plant materials

Plant materials examined in this study were four specimens of banana collection of Purwodadi Botanic Garden in Pasuruan, East Java, Indonesia. It comprised of two BBTV asymptomatic wild bananas and two BBTV symptomatic banana cultivars (Table 1, Figure 1). Asymptomatic wild bananas have a hypothesis as species that are resistant to BBTV, while symptomatic banana cultivars have a hypothesis as positive control and species that are susceptible to BBTV. For molecular study samples, young and curled leaves were taken from the living plants, stored in a cool box, and immediately transferred to the laboratory for DNA isolation.

Table 1. Plant materials examined in this study as BBTV isolate source.

No	Species/ cultivar name	Genome group	BBTV status
1	*Musa acuminata* var. *nakaii*	AAw	asymptomatic
2	*Musa acuminata* var. *rutilifes*	AAw	asymptomatic
3	*Musa acuminata* (AA) cv. Pisang Mas Mirah	AAcv	symptomatic
4	*Musa x paradisiaca* (AAB) cv. Pisang Candi	AAB	symptomatic

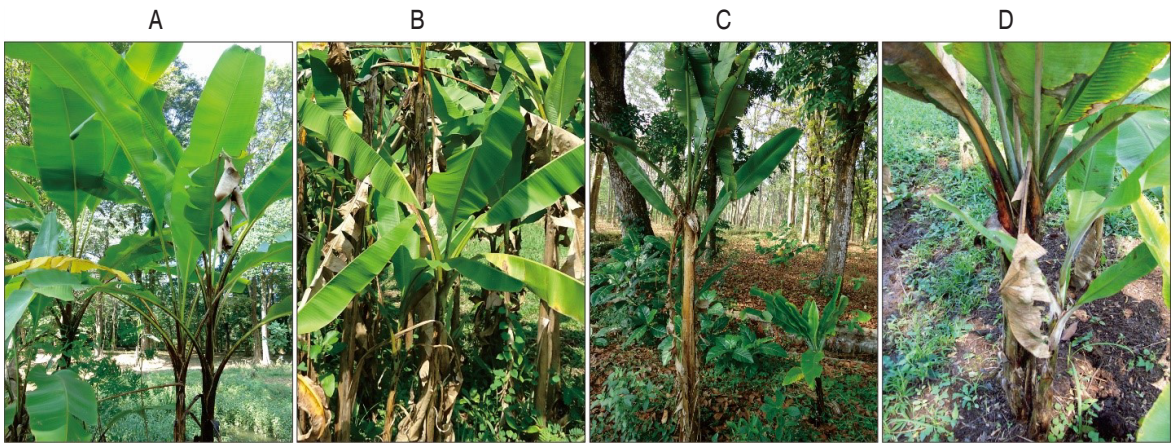

Figure 1. Documentation of BBTV status of plant materials studied: A. *Musa acuminata* var. *nakaii* (asymptomatic); B. *Musa acuminata* var. *rutilifes* (asymptomatic); C. Pisang Mas Mirah (symptomatic); and D. Pisang Candi (symptomatic).

DNA isolation and amplification

Total genomic DNA was extracted using Wizard® Genomic DNA Purification Kit from Promega. The DNA isolation steps follow the guidelines for plants. The amplification was using specific BBTV DNA-R primers (Wickramaarachchi et al. 2016) i.e., BBTV DNA-1F: 5'-GGA AGA AGC CTC TCA TCT GCT TCA GAG AGC-3' and BBTV DNA-1R: 5'-CAG GCG CAC ACC TTG AGA AAC GAA AGG GAA-3'. PCR amplification process was carried out in 30 µL of the total volume consisting of 3 µL (25 ng of DNA sample), 3 µL primer (10 µM), 6 µL of nuclease-free water and 15 µL of DreamTaq DNA. The PCR thermal protocol was carried out for 35 cycles consisting of initial denaturation of 94 °C for 3 min, denaturation of 94 °C for 30 seconds, annealing of 47 °C for 60 seconds, an extension of 68 °C for 30 seconds, and final extension at 72 °C for 10 min. Visualization of the amplified product was carried out by electrophoresis on 1.5% agarose gel with a voltage of 100 volts for 45 min. The PCR products were then purified and sequenced at 1st BASE Laboratories Sdn Bhd, Malaysia by Sanger dideoxy sequencing technology using an ABI PRISM 3730xl.

Data analysis

BBTV DNA-R sequences were evaluated using SeqScanner v1.0 software. The sequences identification and confirmation were performed using the nucleotide BLAST search in GenBank database (Altschul et al. 1990). Open reading frames (ORFs) and translation of ORFs was determined using ORF Finder server tool (Wheeler et al. 2003). All predicted ORF were aligned using protein BLAST search in GenBank database (Altschul et al. 1997) to identify the protein. Data sequences polymorphisms were analyzed using DnaSP6.

For further phylogenetic study, homolog sequences of BBTV DNA-R across the world were retrieved from GenBank database (Table 2). The all-DNA sequences were then aligned using ClustalW in MEGA7 software (Kumar et al. 2016), followed by manual adjustment and converted to suitable formats (FASTA). The evolutionary history was inferred using the Kimura two-parameter model (Kimura 1980) and Neighbor-Joining method (Saitou and Nei 1987) with 1,000 bootstrap replications.

Table 2. List of BBTV DNA-R isolates retrieved from GenBank database.

No.	Accession number	Total seq.	Location source	Region	Reference
1	MN055477	1104	Central Java, Indonesia	South East Asia	Rahayuniati et al. 2019
2	MN037872	1104	Papua, Indonesia	South East Asia	
3	JN003632	1103	Bali, Indonesia	South East Asia	Pinili et al. 2012
4	AB847590	1104	Sumatra, Indonesia	South East Asia	Chiaki et al. 2015
5	MN017715	1104	Sulawesi, Indonesia	South East Asia	Rahayuniati et al. 2019
6	MN089582	1104	Halmahera, Indonesia	South East Asia	
7	KM607666	1105	Philippines 1	South East Asia	Stainton et al. 2015
8	KM607595	1103	Philippines 2	South East Asia	
9	KY427063	1101	Thailand 1	South East Asia	Tantiwanich et al. 2018
10	MF039867	1104	Thailand 2	South East Asia	
11	AF416464	1105	Vietnam 1	South East Asia	Karan et al. 1994
12	AB113659	1104	Vietnam 2	South East Asia	Furuya et al. 2005
13	KM607610	1103	Taiwan 1	East Asia	Stainton et al. 2015
14	KM607668	1104	Taiwan 2	East Asia	
15	AB108456	1104	Japan 3	East Asia	Furuya et al. 2005
16	AB108452	1104	Japan 2	East Asia	
17	AB108454	1104	Japan 1	East Asia	
18	AF238875	1103	China 1	East Asia	He et al. 2000
19	AF246123	1103	China 2	East Asia	
20	MG545610	1105	China 3	East Asia	Yu et al. 2019
21	FJ463042	1106	China 4	East Asia	Feng et al. 2010
22	KR350604	1111	India 1	South Asia	Das and Banerjee 2018
23	KR350595	1111	India 2	South Asia	
24	KR350615	1111	India 3	South Asia	
25	AF416465	1111	Egypt 1	Middle East	Karan et al. 1994
26	HQ259074	1108	Egypt 2	Middle East	Abdel-Salam et al. 2012
27	JQ820453	1111	Malawi 1	Africa	James et al. 2011
28	JQ820459	1111	Rwanda 1	Africa	
29	JQ820465	1110	Rwanda 2	Africa	
30	KM607636	1111	Congo 1	Africa	Stainton et al. 2015
31	KM607637	1110	Congo 2	Africa	
32	KM607697	1109	Tonga 1	Pacific	
33	KM607691	1109	Tonga 2	Pacific	
34	KM607692	1109	Tonga 3	Pacific	
35	KM607672	1109	Samoa 1	Pacific	
36	KM607673	1110	Samoa 2	Pacific	
37	KM607599	1110	Hawaii 1	Pacific	
38	KM607660	1110	Hawaii 2	Pacific	

RESULTS AND DISCUSSION

PCR amplification and sequence analysis of BBTV DNA-R

The wild bananas examined produce normal fruits and do not show any obvious bunchy top symptoms (Figure 1A and B). Meanwhile, the banana cultivars examined showed severe bunchy top infections (Figure 1C and D). This condition was in agreement with the previous study by Hapsari and Masrum (2012) which indicated that wild banana species were more resistant to BBTV than banana cultivars. Furthermore, the genotypes of banana cultivars are correlated with resistance to BBTV where banana cultivars with one or two B genomes tend to be more tolerant. In spite of that, it seems that cultivars with AAA genomes are not all equally susceptible to BBTV. Ngatat et al. (2017) stated that Gros Michel (AAA, Cavendish sub-group) exhibits resistance to the BBTV under both experimental inoculation and field conditions.

BBTV DNA-R primers were successfully amplified in all isolates, both BBTV symptomatic and asymptomatic. This indicates that BBTV DNA-R components were detected in all isolates examined, both symptomatic and asymptomatic. Furthermore, sequencing results showed high-quality value DNA sequences (Table 1) with medium and long contiguous read length (CRL) and high trace score value. Interestingly, the amplicons show different sequence lengths and contrasting with expected amplicons size (1.0 to 1.1 kb) (Stainton et al. 2015). The sizes of the BBTV DNA-R components in this study were varied from 616 to 1,074 bases (Table 3).

Table 3. BBTV DNA-R sequences profile of banana isolates from East Java via Seqscanner.

Banana Host	Sequence quality		Size (nt)	GC Content	Predicted CDS size (location)	Predicted protein (s) (aa)	TATA box location	Poly (A) location
	Trace score	CRL						
M. acuminata (AA) cv. Pisang Mas Mirah.	56 (high)	1,053 (long)	1,074	452(42.9%)	621(1,019-399)	Rep(206)	67-83	547-552
M. acuminata (AAw) var. *nakaii.*	52 (high)	814 (long)	857	367(45%)	417(808-392)	Rep(138)	60-76	540-545
M. acuminata (AAw) var. *rutilifes.*	50 (high)	583 (med)	616	267(45.7%)	135(136-1)	Rep(44)	48-64	528-533
M x paradisiaca (AAB) cv. Pisang Candi.	56 (high)	1,051(long)	1,073	453(43.1%)	621(1,017-397)	Rep(206)	52-59	545-550

CRL=contiguous read lengh, CDS=coding sequence, GC=Guanine+Cytosine, TATA box=transcription start site.

The asymptomatic wild bananas showed shorter or partial length amplicons than symptomatic banana cultivars that showed full length (Table 3). Nevertheless, all BBTV DNA-R detected in this study were still predicted as Rep Protein (Table 3). This is presumable to be related to the resistance mechanism of wild bananas to BBTV. Since, only part of DNA-R fragments were found (not full length) so that viral master replication protein transcription failed to form and infect the plants (asymptomatic).

The characteristics of BBTV DNA-R from banana isolates in this study were differ with banana isolates from Sri Lanka as described by Wickramaarachchi et al. (2016) in the length of predicted coding sequence (CDS), protein, TATA box and polyadenylation or poly (A) location; even though the same primer was used. Furthermore, it was also differing in the absence of the conserved regions defined as stem-loop common region (CR-SL) and major common region (CR-M). In general, each component of the BBTV genome (except DNA-R) has one big (monocistronic) transcriptional active open reading frame (ORF) and two conserved regions: TATA box at 3' of the stem–loop, CR-SL and CR-M, and poly (A) (Islam et al. 2010). CR-SL is common region with the conserved nonanucleotide (TATTATTAC), an origin of virion DNA replication (Wickramaarachchi et al. 2016). Whilst the CR-M is the second common region as the binding site for ssDNA primers and a prime the synthesis of transcriptionally active dsDNA (Das and Banerjee 2018).

The absence of CR-SL and CR-M in this study may involve to the BBTV resistance mechanism in bananas.

BBTV DNA-R polymorphism

The nucleotide composition of BBTV DNA-R isolates were low in GC content, averaging 42.9% (Table 3). It is relevant to the expectation that single-stranded DNA viruses should display, on average, lower G and C frequencies compared to double stranded since ss genomes are prone to mutations toward A and T/U (Simon et al. 2021). Furthermore, sequences alignment of four isolates showed that 607 sites were conserved (56.47%), nine sites were polymorphic due to mutations, and 616 were missing data or gaps or deletions. The polymorphic sites comprised of eight singleton variables and one parsimony informative. All of the singleton variables were found in wild *M. acuminata* var. *rutilifes*, which mostly due to transversion (Ti/Tv=0.33). The singleton variables found in site positions of 20(C→A), 558(G→C), 596(T→A), 610(T→C), 613(T→C), 620(A→C), 624(A→C), and 634(A→T). The parsimony-informative were found in *M. acuminata* var. *rutilifes* and Pisang Mas Mirah at site number 351, due to transversion (A→T).

BBTV DNA-R sequences alignment and comparison of two asymptomatic wild bananas showed large gaps or missing data (241 sites; 28.82%) and moderately conserved (607 sites; 70.83%). About nine mutations were identified mostly due to transversion (Ti/Tv)=0.29) and no parsimony informatives. The singleton variables found in site positions of 13(C→A), 344(A→T), 551(G→C), 589(T→A), 603(T→C), 606(T→C), 613(A→C), 617(A→C), and 627(A→T). Meanwhile, BBTV DNA-R of two symptomatic banana cultivars showing highly conserved region up to 1,055 sites (98.14%) and only four missing data. There were 16 polymorphic sites identified mostly due to transversion (Ti/Tv=0.78) and no parsimony informatives. The singleton variables found in site positions of 4(C→A), 6(T→C), 7(C→G), 10(T→C), 12(C→T), 13(A→C), 351(T→A), 1032(T→G), 1058(C→T), 1059(T→C), 1063(G→A), 1065(T→G),1071(G→C), 1073(T→C), 1074(G→T), and 1075(A→G). These results indicated that BBTV DNA-R in wild bananas are more variables than that of banana cultivars, which thought to be the reason they show resistance (asymptomatic).

Phylogenetic tree of BBTV DNA-R isolates from East Java with others

Multiple sequences alignment and phylogenetic analysis of isolates from East Java (this study) were conducted with other 38 accessions of homologs BBTV DNA-R sequences from Asia, Africa, and the Pacific countries (Table 2). The total aligned and selected BBTV DNA-R sequences length of 42 accessions were 630 bp. The sequences were considered highly polymorphic reached 64.60% (407 sites), only about 196 positions (31.11%) were identified as conserved regions, and 27 gaps (4.29%). Furthermore, the nucleotide composition were low in GC content (43.21%).

The bootstrap consensus of Neighbour-Joining phylogenetic analysis of BBTV DNA-R in this study delineates into two large groups in relevant to many previous studies. It was separated into the Pacific Indian Ocean group/PIO (Group I) and the Asian group (Group II) with moderate to strong bootstrap support (Figure 2). BBTV DNA-R isolates from the Pacific, South Asia, Middle East and Africa countries were clustered in Pacific Indian Ocean Group and has wider distribution than Asian group that was comprised of BBTV DNA-R from East Asia and South East Asia countries. The transfer of infected banana material is thought to be the primary cause of the PIO group's wider geographic dispersion than the Asian group. Differential adaptation of PIO and Asian groups of BBTV isolates is suggested for different banana species (Wickramaarachchi et al. 2016), but this hypothesis cannot be proven because of the lack of an infectivity assay system.

Specifically, BBTV DNA-R isolates from Indonesia were found separated in three different subgroups based on the source of the materials. Isolates from Sulawesi and Halmahera Islands were clustered in sub-group 1 with the Philippines, Taiwan and Japan. It is thought to be due to its close geographical position. Phylogenetic analysis based on DNA-S and DNA-C analysis also showed that isolates from the islands of Sulawesi and Halmahera were closely related to isolates from Philippines (Rahayuniati et al. 2021b). Furthermore, isolates from Sumatera, Central Java, Bali, and Papua were separated as sub-group 2 (Indonesia only). Meanwhile, isolates from East Java (this study) were nested in sub-group 4 closely related with Thailand and Vietnam isolates (Figure 2). Hence, BBTV

DNA-R from East Java were found to be unique and has different route of evolutionary history from the other isolates from Indonesia. Their evolutionary history were presumably came from China mainland via Malay Peninsula route; while the other isolates probably came from the East Asia islands route.

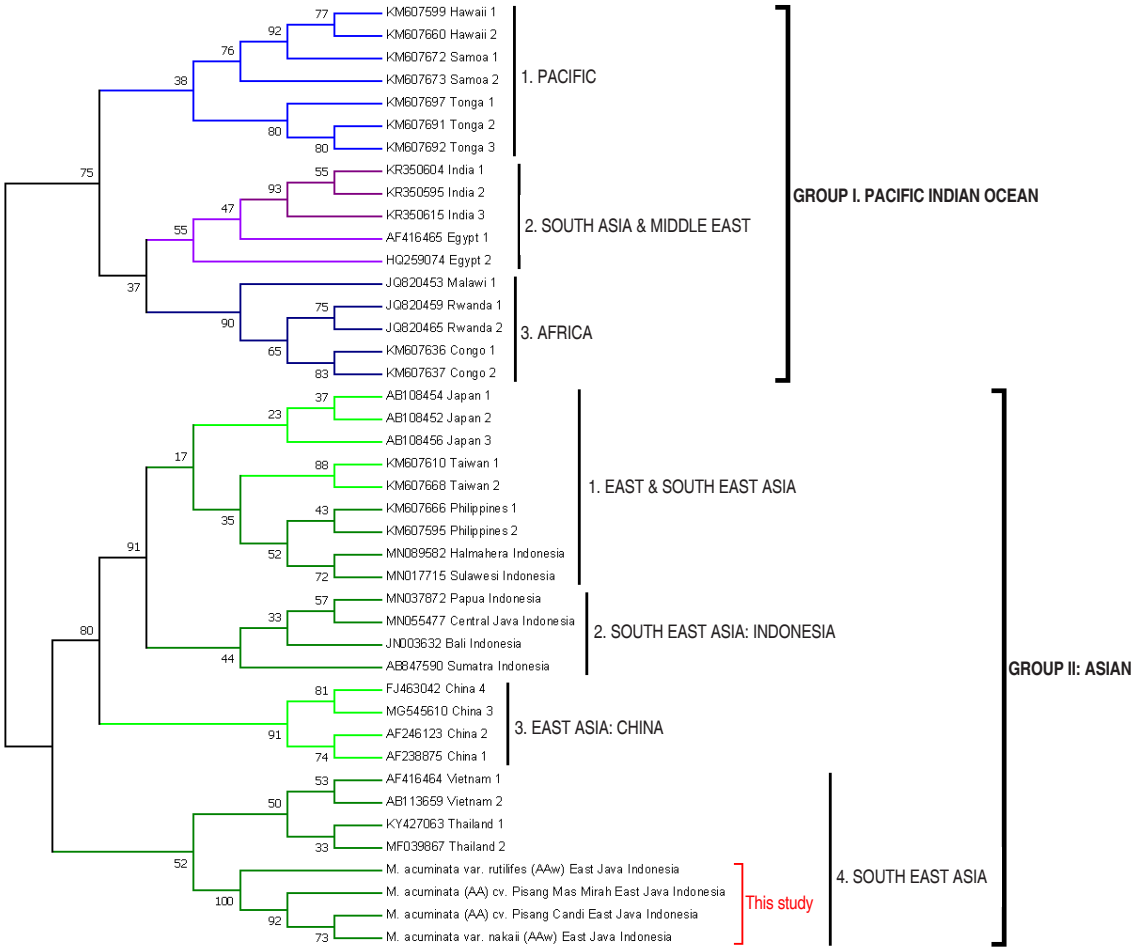

Figure 2. Neighbor-Joining phylogenetic tree of BBTV DNA-R isolates (bootstrap consensus with 50% cut-off).

CONCLUSIONS

BBTV DNA-R components were detected in all isolates examined, both symptomatic and asymptomatic. Asymptomatic wild bananas showed shorter length and more variable sequences than symptomatic banana cultivars, which presumably to be related to the resistance mechanism of wild bananas to BBTV. Phylogenetic analysis delineates into two groups i.e. Pacific Indian Ocean group/PIO and Asian group. BBTV DNA-R from East Java were clustered in Asian group. It was found to be unique and has different route of evolutionary history from the other isolates from Indonesia, presumably came from China mainland via Malay Peninsula route. Further researches on the resistance mechanism to BBTV in wild bananas are suggested.

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Proposal of descriptors to study the variability of *Vaccinium meridionale* Swartz

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Propuesta de descriptores para estudiar la variabilidad de *Vaccinium meridionale* Swartz

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ABSTRACT

Agraz, mortiño, or Andean blueberry (*Vaccinium meridionale* Swartz) is a fruit tree with high potential for national consumption since it is considered a functional food due to its high content of anthocyanins and antioxidants. The morphological description in plants involves both characterization variables that are highly heritable and easily detectable, as well as evaluation variables influenced by the environment and useful for genetic breeding that, together, are called descriptors and allow knowing the variability of the species. The aim of this study was to develop a list of morphological descriptors with the inclusion of variables to characterize and evaluate *Vaccinium meridionale* Swartz. Observations were made in natural populations of 11 municipalities in Antioquia, three in Boyacá, one in Cundinamarca, two in Nariño, and one in Santander, Colombia, as well as in the *ex situ* collection established in Rionegro Antioquia, between 2005 to 2011. A descriptor with 38 quantitative, binary, and multi-state variables was developed. Seven of these variables were obtained at the plant level, 10 from the leaf, six from the flower, 14 from the fruit, and one from the seed. The application of the morphological descriptors in *in situ* and *ex situ* conditions reported high polymorphism in the qualitative traits and high variation between individuals for the quantitative variables in the collections under study. These variables are of taxonomic and agronomic importance in the knowledge of the species and are essential for producing and marketing the fruit.

Palabras clave:
Especie sub-utilizada
Neotropical
Ericácea
Bayas
Recurso genético

RESUMEN

El agraz o mortiño (*Vaccinium meridionale* Swartz) es un frutal con alto potencial para el consumo nacional ya que se considera un alimento funcional por su alto contenido de antocianinas y antioxidantes. La descripción morfológica en plantas involucra tanto variables de caracterización que son altamente heredables y fácilmente detectables, como las de evaluación influenciadas por el ambiente y útiles para el mejoramiento genético, que en su conjunto se denominan descriptores y permiten conocer la variabilidad de las especies. El objetivo de este estudio fue desarrollar una lista de descriptores morfológicos con la inclusión de variables para la caracterización y evaluación del *Vaccinium meridionale* Swartz. Se realizaron observaciones en poblaciones naturales de 11 municipios de Antioquia, tres en Boyacá, una en Cundinamarca, dos de Nariño y una en Santander, Colombia, al igual que en la colección *ex situ* establecida en Rionegro Antioquia, entre el 2005 y el 2011. Se desarrolló un descriptor con 38 variables cuantitativas, binarias y multi-estado. Siete de estas variables se obtuvieron a nivel de planta, 10 de la hoja, seis de la flor, 14 del fruto y una de la semilla. La aplicación de los descriptores morfológicos en condiciones *in situ* como *ex situ* reportó alto polimorfismo en las características cualitativas y alta variación entre individuos para las variables cuantitativas en las colecciones en estudio. Estas variables son de importancia taxonómica y agronómica en el conocimiento de la especie e importantes para la producción y el mercadeo del fruto.

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Agraz, mortiño, vichachá and camueza in Spanish, or Andean blueberry in English, are common names of *Vaccinium meridionale* Swartz a berry plant that belongs to the family Ericaceae Juss., tribe Vaccinieae Rehb that includes about 35 genera and more than 1,000 species (Luteyn 2002). The species belongs to the Pyxothamnus section and includes other species such as *V. consanguineum* Klotzsch, *V. floribundum* Kunth, and *V. puberulum* Klotzsch (Ehlenfeldt and Luteyn 2021).

In the Neotropics, the Ericaceae family is concentrated in northwestern South America, typically in mountainous habitats with cold and humid areas, between 1,500 and 3,000 meters above sea level (masl). Species of this family are mainly found in Colombia, Ecuador, Peru, and Venezuela, where almost 50% of the species are epiphytic, and approximately 94% are endemic (Luteyn 2002). The *Vaccinium* genus has a wide geographical distribution, found in cold and temperate tropical zones with representation in most continents, except Antarctica (Luteyn 2002), Australia, and most parts of Africa (Vander-Kloet 1990). Most of the species grow in acidic and well-drained soils with high organic matter content, coastal dunes, lake, and river margins, abandoned fields, or mountain terrains (Lyrene et al. 2003).

V. meridionale is found predominantly in the coastal and Andean areas of Venezuela, on the Andes of Colombia, on the island of Jamaica and on the mountains of Peru. Its habitats include high mountain cloud forests to moor thickets between 1,000 and 2,800 masl (Ehlenfeldt and Luteyn 2021). In Colombia, according to the National Institute of Natural Sciences, the distribution of *Vaccinium* species includes the departments of Antioquia, Boyacá, Cauca, Chocó, Cundinamarca, Magdalena, Meta, Nariño, Norte de Santander, Putumayo, Quindío, Santander, and Tolima. The highest number of reports are found in Antioquia, Boyacá, and Cundinamarca. The latter has more records of *V. floribundum*, a closely related species, and Antioquia has the highest number of records for *V. meridionale* (Ligarreto 2009).

The first report of the species was found using the name "mortiño", described as a Castilian (Spain) word applied to plants of the genus *Vaccinium*, a name imposed by the Spanish in America. The first reference to the expression "mortiño" dates back to 1548, used in the Guaca region, the current province of Carchi, Ecuador, a town inhabited by the Pastos indigenous people (Patiño 2002).

V. meridionale produces a berry rich in flavonoids, mainly anthocyanins, with high antioxidant activity (Garzón et al. 2010), cardioprotective effect (Lopera et al. 2013) with ischemic lesions treatment potential (Shen et al. 2018), and antiproliferative activity (Agudelo et al. 2018), among others.

Genetic diversity is the heritable variations that occur in organisms, individuals, and between populations under more or less stable natural conditions. Population genetics and evolution study and conserve genetic diversity. Genetic variability is the basis of any breeding program, as it includes the diversity available for selection of sources for adaptation to different environments (Rimieri 2017).

Genetic resources are biological diversity are essential for the sustainable development of agriculture and to assure food security (Nass 2011). The International Treaty on Plant Genetic Resources for Food and Agriculture defines them as genetic material, whether propagated sexually or asexually, containing functional heredity units. Regardless of the genetic resources' definitions, these are the basis of plant breeding and agricultural production (Fowler and Hodgkin 2004).

Within the activities of germplasm banks, an essential task is the description of the variability of a crop in both the phenotype and genotype of the accessions conserved. However, a list of characters, called descriptors, is required to carry out the characterization process. In 1976, the basis of such a system was created, which initiated the development and formulation of a list of descriptors that are the basis of internationally standardized documentation developed by specialists in each crop (Gotor et al. 2008).

Characterization and evaluation descriptors provide insight into the variability of a crop. Characterization refers to the recording of variables with high heritability and controlled by a single or very few genes; they should be easy to measure and allow differentiation and expression of the trait in a precise and uniform way. Evaluation depends on the environment and is controlled by several genes expressing yield, agronomic productivity, stress

susceptibility, biochemical and cytological traits used in crop improvement (Romanciuc 2017).

Currently, there is no published descriptor for the species *V. meridionale* to allow for knowing and understanding its variability. Accordingly, this study aims to develop morphological descriptors for this species. This will enable the differentiation of the accessions included in a collection through specific attributes and obtain essential information that effectively allow of the domestication of this interesting plant genetic resource.

MATERIALS AND METHODS

For the construction of the preliminary version of the descriptor, the taxonomic keys of the genus *Vaccinium* and the description of the species *V. meridionale* presented by Sleumer (1941), Romero-Castañeda (1961), Berazaín-Iturralde (1991), Vander- Kloet (1996) and Luteyn (1998) were used.

A first list of proposed variables, developed by Agrosavia, was done *in situ* by Lopera (2005) in four to six plants per locality included in his study. Later Ligarreto et al. (2011), studied a total of 177 natural populations located in the departments of Antioquia, municipalities of Belmira (30), Don Matías (8 populations), El Retiro (1), Entrerrios (8), Guarne (9), La Ceja (1), Medellín, Corregimiento de Santa Helena (23), Rionegro (1), San Jerónimo (2), San José de la Montaña (11), San Pedro de los Milagros (4), Santa Rosa de Osos (41), and Yarumal (9). In Boyacá, the municipalities of Chiquinquirá (4), Ráquira (5) and Tinjaca (4) were visited, and in Cundinamarca, only the municipality of Guachetá (2). In Nariño, the municipalities of Buesaco (1) and Pasto (2), and in Santander, the municipality of California (11) were visited (Medina et al. 2009).

Subsequently, a modified version was applied in the previous process (Lopera 2005), also including the variables and states found in the natural populations of 102 accessions of the *V. meridionale*. Germplasm collection established in the Research Center "La Selva" of Agrosavia, located in Rionegro, Antioquia. The area is located at 06°08'06" N and 75°25'03" W at 2,120 masl, where the average temperature was 17 °C, average relative humidity of 78%, and photosynthetically active radiation (PAR) of 357 Watts m^{-2}.

RESULTS AND DISCUSSION

Descriptor

A descriptor for *V. meridionale* was developed with 38 quantitative, binary, and multistate variables, of which seven were obtained at the plant level, 10 from the leaf, six from the flower, 14 from the fruit, and one from the seed (Table 1). This descriptor had variables with taxonomic and agronomic importance, vital for production and marketing.

Table 1. List of variables, state, and description proposed as a descriptor for *Vaccinium meridionale* Sw.

Variable	State	Description
	Plant	
Plant height	Given in centimeters and recorded from the neck to the apex of the main stem.	
Stem diameter	Given in centimeters and measured 10 cm from the ground, on the main stem.	
Growth habit	1. Prostrate. 2. Intermediate. 3. Erect.	 1 2 3

Table 1

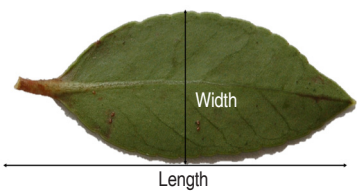
Variable	State	Description
	Plant	
Branch density	1. Scarce. 2. Intermediate. 3. Dense.	Rate the number of branches formed per plant.
Branch pubescence	0. Absent. 1. Scarce. 2. Abundant.	Observe the terminal part of the branches.
Presence of wax on the stem	0. Absent. 1. Present.	Observe in young branches.
Color in branches	0. Absent. 1. Scarce. 2. Medium. 3. High.	 0 1 2 3
	Leaf	
Leaf density	Register the number of leaves formed in 10 cm; in the middle part of a branch or shoot.	
Leaf blade length	Average length of five leaves, given in centimeters; from the middle part of the branch.	 Width Length
Leaf blade width	Average width of five leaves, given in centimeters; from the middle part of the branch.	
Leaf blade shape	1. Elliptical: ellipse-shaped, rounded, and curved, and widest in the central part. 2. Ovate: egg-shaped. 3. Lanceolate: shaped like an iron spear figure. 4. Oval: elliptical with the blade width considerably larger in the middle of the length. 5. Oblong lanceolate. 6. Other.	 1 2 3 4 5
Leaf margin shape	1. Serrated: Serrated with sharp and proximate teeth. 2. Slightly serrated. 3. Crenate. 4. Other.	 1 2 3

Table 1

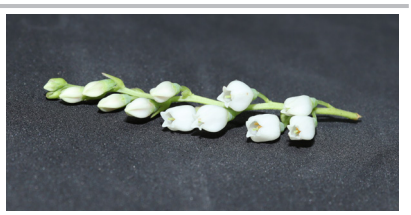
Variable	State	Description
	Leaf	
Leaf base shape	1. Cuneate. 2. Rounded. 3. Other.	 1 2
Apex shape	1. Acuminate: ending in an acumen. 2. Acute: the edges form an acute/sharp angle. 3. Very acute/sharp. 4. Obtuse: the edges form an obtuse. angle at the apex. 5. Obtuse. 6. Other.	 1 2 3 4
Presence and size of nectaries or glands on the leaf margin	0. Absent. 1. Small. 2. Big.	
Anthocyanin in the leaf margin	0. Absent. 1. Present.	
Anthocyanin in the petiole	0. Absent. 1. Present.	
	Inflorescence and flower	
Inflorescence length	Given in centimeters and recorded from its insertion with the branch to its apex.	
Number of flowers per inflorescence		Counted. In five inflorescences per plant, all the flowers of the inflorescence are counted, even those that have already fallen.
Presence of anthocyanin in the inflorescence	1. Absent. 2. Present.	
Calyx color	1. Green. 2. Red-wine. 3. Light green and dark green. 4. Green and red wine. 5. Red wine and light green and dark green. 6. Other.	

Table 1

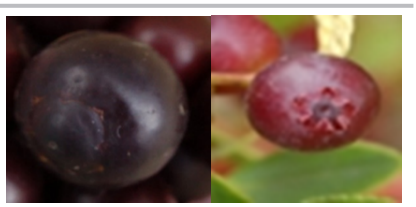
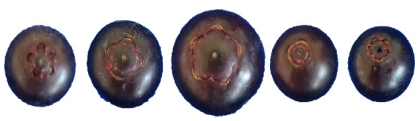
Variable	State	Description
	Inflorescence and flower	
Pedicel and bract color	1. Green. 2. Red-wine. 3. Light green and dark green. 4. 1 and 2. 5. Other.	
Color of the Corolla	1. White. 2. White tinged with pink. 3. Other.	
	Fruit	
Polar length	Measured in centimeters in five fruits per plant.	
Equatorial width	Measured in centimeters in five fruits per plant, in its widest part.	
Fruit weight	Average weight of five fruits and expressed in grams.	Find the average weight of the harvested fruits.
Number of locules per fruit	Split the fruit transversely and count the average number of locules in five fruits per plant.	
Presence of wax in the fruits	0. Absent. 1. Present.	
Color of mature fruits. See color Table of the Royal Horticultural Society (RHS)	1. Blue. 2. Purple. 3. Black.	
Persistence of the sepals when mature	0. Absent. 1. Present.	

Table 1

Variable	State	Description
	Fruit	
Fruit shape	1. Spherical. 2. Elongated round. 3. Flattened. 4. Cordiform. 5. Ovate. 6. Elliptical. 7. Other.	 1 2 3 4 5 6
Fruit cracking	0. Absent. 1. Present.	
Shape of petiole insertion in the fruit	1. At level. 2. Slightly sunken.	 1 2
Sepal scar shape	1. Flat. 2. Protuberance. 3. High protuberance. 4. Sunken. 5. Medium protuberance and deep hole.	 1 2 3 4 5
Presence of ribs in the fruit. Ribs are slight depressions around the base of the fruit.	0. Absent. 1. Present.	 0 1
Brix degrees	Measured in a refractometer.	
Fruit pH	Measured on a potentiometer.	
	Seed	
Number of seeds per fruit	Average number of seeds in five fruits.	

Qualitative variables

Five qualitative variables were included in the stem level descriptor; seven were selected for the leaf, four for the inflorescence and flower, and eight for the fruit, for a total of 24 variables (Table 1). In the *ex situ* characterization of 102 materials carried out with the final proposal of the descriptor, polymorphism in 24 qualitative traits were found, with the presence of 67 states out of the 70 included in the

list of the descriptors for the species (95.7%) (Table 2). This result indicates a broad morphological variability related to attributes of this nature. The only states not observed in this characterization were large foliar nectaries found only in one specimen deposited in the "Josep Cuatrecasas y Arumí" herbarium of Universidad Nacional de Colombia - Palmira Headquarters. The color of its calyx is red-wine, and the color of the fruit is blue.

In the *in situ* characterizations carried out in Antioquia in natural populations and plants that had already started their reproductive stage, broad qualitative variability was found, recording 46 out of the 48 states described (Medina et al. 2009) but not reporting blue-colored fruits. In the departments of Cundinamarca, Boyacá, Nariño, and Santander, 20 natural populations were characterized *in situ*, with 17 qualitative variables included in the descriptor, but not finding the following states: absence of stem pubescence (branches), light and dark green colors in the calyx, light and dark green colors in the pedicel and bracteole, and a blue fruit color (Ligarreto et al. 2011).

The blue color of the berries, not observed at the *in situ* characterizations, is reported for some species of *Vaccinium*, giving the plant the name "blueberry". For the specie *V. meridionale*, the colors of the mature fruit range from purplish and purple to dark purple, violet, and black (Buitrago–Guacaneme et al. 2015).

Various authors report that the corolla is reddish-white or pink (Romero-Castañeda 1961), and research carried out in Guachetá (Cundinamarca) and San Miguel de Sema (Boyacá) indicates that the flowers have a white or light to intense pink color (Chamorro and Nates–Parra 2015).

Table 2. List of variables and states included and reported in the *ex situ* collection.

Variable	State	
	Total reported states	**Reported in the *ex situ* collection**
Growth habit	3	3
Branch density	3	3
Branch pubescence	3	3
Presence of wax in the stem	2	2
Anthocyanin in branches	4	4
Leaf blade shape	5	5
Leaf margin shape	3	3
Leaf base shape	2	2
Leaf apex shape	4	4
Presence of foliar nectaries	3	2
Anthocyanin in the leaf margin	2	2
Anthocyanin in the petiole	2	2
Anthocyanin in the inflorescence	2	2
Calyx color	5	4
Pedicel and bract color	4	4
Flower color	2	2
Presence of wax on fruit	2	2
Fruit color	3	2
Fruit shape	6	6
Fruit cracking	2	2
Shape of petiole insertion in the fruit	2	2
Sepal scar shape	4	4
Presence of ribs in the fruit	2	2
Total	70	67
Percentage (%)	100	95.7

Quantitative variables

14 quantitative variables were included as follows: two from the entire plant, three from the leaf, two from the flower, six from the fruit, and one from the seed (Table 3). In the *ex situ* characterization of the 102 accessions, the variability between individuals for these types of attributes was evident without a defined grouping pattern between subregions from which the populations were collected or at the intrapopulation level.

On the other hand, the *ex situ* quantitative variability indicated the presence of 278 out of 280 total plants, i.e., 99% of the plants, with a distance between them or differences due to quantitative genes. Applying the proportion criterion of "different clones" of Persson and Gustavsson (2001), the value obtained was 0.99. It should be noted that, in the case of quantitative expressions, these were recorded in a common planting site with homogeneous traits and with similar management practices, removing, to a high degree, the environmental effect, as it is a response to a common locality with similar cultural management.

For the quantitative variables included in the descriptor, various authors report variability in the number of flowers per raceme (between 10-15 flowers) in mortiño or agraz populations (Romero-Castañeda 1961). In works carried out in natural populations in Guachetá (Cundinamarca) and San Miguel de Sema (Boyacá), the inflorescences measure 7.2±0.5 mm long, with 12±3 flowers per raceme (Chamorro and Nates-Parra 2015). Fruit weight and other fruit dimensions varied according to the pollination treatments evaluated and the location (Chamorro and Nates-Parra 2015). The equatorial diameter of agraz fruits ranges between 6 and 20 mm. The average fresh weight of 1.60 g, and the number of seeds per fruit ranges between 15 and 37 depending on fruit size (Magnitskiy and Ligarreto 2009). Agraz fruits harvested in Cundinamarca have about 14.13 °Brix of total soluble solids (Ávila-Rodríguez et al. 2007), and other authors report Brix degrees for fruits harvested in Antioquia between 12.6 and 15.2 and pH between 2.2 and 2.7 (Gaviria et al. 2009).

Table 3. List of quantitative variables and their measurement units, average, standard deviation (SD), and maximum and minimum values included in the descriptor and used in the *ex situ* characterization.

Variable	Average	SD	Maximum	Minimum
Stem height (cm)	67.46	32.99	148.5	13
Stem diameter (cm)	1.35	0.52	2.8	0.5
Leaf density (number of leaves*)	21.00	3.312	30	16
Leaf length (cm)	2.55	0.278	3.27	1.51
Leaf width (cm)	1.16	0.157	1.71	0.77
Inflorescence length (cm)	3.47	0.982	5.9	0.94
Number of flowers per inflorescence	15.03	3,194	22	1.06
Fruit length (cm)	0.93	0.153	2	0.56
Fruit width (cm)	0.98	0.123	1.22	0.57
Fruit weight (g)	0.68	0.287	1.93	0.16
Locus number	4.00	0.198	5	4
Brix degrees	15.93	1.923	22.2	7.2
Juice pH	2.56	0.213	3.75	2.23
Number of seeds per fruit	28.61	14,382	168	11
Weight of 200 seed (g)	0.09	0.115	1.06	0.008

*Counted on a section of 10 cm in the middle part of a branch.

Quantitative diversity shows an adaptive potential that could favor its use to combine attributes and cope with variations in potential planting environments in relation to global climate change and the diverse sites where natural populations were found. The conservation and maintenance of adequate quantitative variability is the basis for coping with environmental changes that may occur (Kramer and Havens 2009).

CONCLUSIONS

The importance of this descriptor lies in the fact that the scientific community can research with a standardized characterization protocol that favors the use and exchange of information on the species, which allows working with similar data between national and international institutions.

The descriptor proposed indicated that the species has variability that must be evaluated in any work carried out with the species.

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Correlation between leaf nutrient contents and grain, oil and protein productivities in *Jatropha curcas* L

Correlación entre el contenido de nutrientes de la hoja y la productividad de grano, aceite y proteínas en *Jatropha curcas* L

UNIVERSIDAD NACIONAL DE COLOMBIA

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Keywords:
Antagonism
Inhibition
Mineral nutrition
Nutritional interaction
Synergism

ABSTRACT

In plants, several chemical elements are found in different concentrations and formulations. Some of these elements influence each other, either through positive stimulation or inhibition. This study evaluated the correlation between nutrient contents and production components of Jatropha (*Jatropha curcas* L.). The experiment was conducted at the Federal University of Viçosa-MG, Brazil. A randomized block design with four replications was used. The treatments consisted of six Jatropha clones transplanted 4.5 years ago, from the municipalities of Janaúba and Bomfim, in Minas Gerais, Brazil. Yield was determined by harvesting the ripe and dried fruits, and the oil and protein contents in the grains were obtained by nuclear magnetic resonance. To determine nutrient contents, leaves were collected when the plants were in flowering, with yellow fruits and when the fruits were dry. There was a significant and negative association between grain yield and Mg content. Regarding foliar nutrient contents, the positive (r) significant correlations were between the following pairs: (N and S, r=0.554); (N and Cu, r=0.460); (P and Ca, r=0.420); (K and Zn, r=0.511); (K and Cu, r=0.506); (Ca and Mg, r=0.603); (Zn and Fe, r=0.662); (Zn and Mn, r=0.795); (Zn and Cu, r=0.574); (Fe and Mn, r=0.528) and (Mn and Cu, r=0.479); and the negative ones were between: (K and Ca, r=-0.596); (K and Mg, r=-0.673); (Mg and Cu, r=-0.506). Therefore, it was possible to prove the existence of nutritional interaction between some elements, as well as the effects on grain yields. This research will serve as a basis for studies to recommend fertilizer doses, plant improvement through nutritional efficiency, and studies in the area of biochemistry.

Palabras clave:
Antagonismo
Inhibición
Nutrición mineral
Interacción nutricional
Sinergismo

RESUMEN

En las plantas, varios elementos químicos se encuentran en diferentes concentraciones y formulaciones. Algunos de estos elementos se interactúan entre sí , ya sea por estimulación positiva o por inhibición. Este estudio evaluó la correlación entre los contenidos de nutrientes y los componentes de producción de Jatropha (*Jatropha curcas* L.). El experimento se realizó en la Universidad Federal de Viçosa-MG, Brasil. Se utilizó un diseño de bloques al azar con cuatro repeticiones. Los tratamientos consistieron en seis clones de Jatropha trasplantados hace 4,5 años, procedentes de los municipios de Janaúba y Bomfim, en Minas Gerais, Brasil. El rendimiento se determinó mediante la cosecha de los frutos maduros y secos, y los contenidos de aceite y proteína en los granos se obtuvieron por resonancia magnética nuclear. Para determinar el contenido de nutrientes, se recolectaron las hojas cuando las plantas estaban en floración, con frutos amarillos, y cuando los frutos estaban secos. Hubo una asociación significativa y negativa entre el rendimiento de grano y el contenido de Mg. En cuanto a los contenidos de nutrientes foliares, las correlaciones positivas (r) significativas se dieron entre los siguientes pares: (N y S, r=0,554); (N y Cu, r=0,460); (P y Ca, r=0,420); (K y Zn, r=0,511); (K y Cu, r=0,506); (Ca y Mg, r=0,603); (Zn y Fe, r=0,662); (Zn y Mn, r=0,795); (Zn y Cu, r=0,574); (Fe y Mn, r=0,528) y (Mn y Cu, r=0,479); y los negativos estuvieron entre: (K y Ca, r=-0,596); (K y Mg, r=-0,673); (Mg y Cu, r=-0,506). Por lo tanto, fue posible comprobar la existencia de interacción nutricional entre algunos elementos, así como los efectos sobre los rendimientos de grano. Esta investigación servirá de base para estudios de recomendación de dosis de fertilizantes, mejoramiento de plantas a través de la eficiencia nutricional y estudios en el área de la bioquímica.

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The high consumption and environmental impacts caused by burning petroleum diesel have driven the search for renewable energy sources. Among these alternatives, biodiesel has stood out. This fuel is obtained from vegetable oils and animal fats. Among the plant species, *Jatropha curcas* L., or popularly Jatropha, has shown promise as a raw material for obtaining biodiesel (Giraldo Ramírez et al. 2014). This species is outstanding mainly due to its high grain yield and high oil content (Laviola and Dias 2008; Cañadas-López et al. 2020). However, for the plant to reach maximum productivity in grains and oil, it must be properly nourished, with the amount of nutrients at ideal and balanced concentrations.

In the plant, nutritional balance is a very complex and dynamic mechanism due to interactions between nutrients. This is because nutrients behave in different ways, and they can also have different pathways or locations for absorption, which may be exclusive pathways or shared by more than one nutrient (Coskun and White 2022). Also, according to the authors, the interaction between nutrients can also occur due to the difference in ion charges, which may compromise or facilitate the absorption of the other.

There are basically three types of interactions between nutrients: two negative (antagonism and inhibition) and one positive (synergism). Antagonism occurs when the presence of an element decreases the absorption of another element regardless of its concentration in the medium. Inhibition occurs when the presence of an excess element decreases the absorption of another element. Inhibition can be competitive and non-competitive. Competitive is when the two elements compete for the same site (place) of absorption by the plant. Non-competitive inhibition is characterized when the absorption sites are different for each element. Finally, synergism, which occurs when the presence of one element favors the absorption of another element, provides a beneficial effect for the plant (Coskun and White 2022).

In Jatropha, Lima et al. (2011) and Maia et al. (2014) found the occurrence of antagonism, synergism and inhibition when studying nutritional interaction. These authors also verified that in addition to the effect on the nutrient content in the plant, the dry matter of both the aerial part and the root system were influenced by the interaction between nutrients.

In extreme conditions in mineral nutrition, that is, when the content of some nutrient is available to the plants in the soil solution at high or very low concentrations, the nutritional interaction can lead to toxicity, as this imbalance will negatively influence the development and consequently, crop productivity (Bell 2022).

The concentration of nutrients in the plant and the grain, oil and protein yields are directly correlated, as nutrients are inputs for obtaining these products by the plant. This source-sink relationship, in addition to the direct effect on productive aspects, also influences vegetative growth (Kirkby et al. 2022).

In view of the possible nutritional interactions that may occur in plants and their effects on productivity, the aim of this study was to verify the existence of a correlation among the foliar nutrient contents in Jatropha, and to investigate the presence of correlation with grain yield, oil, and protein contents.

MATERIALS AND METHODS

The experiment was carried out with 4.5-year-old Jatropha clones grown in the “Diogo Alves de Mello” Experimental Field (latitude 20°45'58” S, longitude 42°52'06” W and 676 m average altitude), at the Federal University of Viçosa (UFV), Viçosa, MG, Brazil. The soil of the experimental area was classified as Red-Yellow Latosol (Embrapa 2018). The climate of the region, according to the Köppen classification, is Cwa, hot and humid, characterized by dry and cold winters with minimum temperatures below 10 °C and maximum temperatures above 34 °C. The temperature during the experimental period ranged from 15.5 to 33.4 °C and the accumulated precipitation was 844.5 mm. The duration of the experiment in the field was three months, and the laboratory analyzes were carried out gradually according to the laboratory's demand.

The plants were grown in 2.5x2.5 m spacing, in a rainfed system and free from weed competition and pest and disease attacks. Chemical fertilization was carried out by chemical analysis of the soil after collecting samples in layers from 0 to 20 and from 20 to 40 cm (Table 1). The recommendation was made according to Dias et al. (2007) quoted by Silva et al. (2021), where 100 g plant^{-1} of the 20-10-15 N-P-K formula was applied at the time of planting.

In the first year of planting, 150 g plant^{-1} of formula 20-00-15 were applied and, in the three subsequent years, in installments, 200, 300 and 400 g plant^{-1} of formula 20-10-15 were applied, respectively. The soil was corrected annually by liming. During the data collection period liming was not performed.

A randomized block design with four replications was used. The treatments consisted of clones from six J. curcas populations, from the Minas Gerais municipalities of Janaúba (J1, J2, J3, J4 and J5) and Bomfim (B1). Each plot contained eight plants, and for the study the four plants from the useful area were used, thus totaling 96 plants.

Table 1. Chemical analysis of the soil in the experimental area at depths from 0 to 20 cm and from 20 to 40 cm.

Chemical Characteristics	Layer (cm)	
	0 to 20	20 to 40
pH (H_2O)	4.50	4.56
OM (dag kg^{-1})	2.93	2.00
P (mg dm^{-3})	2.60	0.90
K (mg dm^{-3})	37	25
Ca^{2+} ($cmol_c$ dm^{-3})	0.99	0.97
Mg^{2+} ($cmol_c$ dm^{-3})	0.37	0.37
Al^{3+} ($cmol_c$ dm^{-3})	0.59	0.49
H + Al ($cmol_c$ dm^{-3})	6.90	5.50
BS ($cmol_c$ dm^{-3})	1.45	1.40
$CEC_{effective}$ ($cmol_c$ dm^{-3})	2.04	1.89
CEC_{total} $cmol_c$ dm^{-3}	8.35	6.90
V (%)	17.40	20.30
m (%)	28.90	25.90
Zn (mg dm^{-3})	1.39	1.14
Fe (mg dm^{-3})	47.20	42.60
Mn (mg dm^{-3})	17.00	12.40
Cu (mg dm^{-3})	1.98	2.14
B (mg dm^{-3})	0.25	0.27
S (mg dm^{-3})	22.40	21.70

pH in water. OM: Organic Matter=C.orgx1,724 –Walkley–Black. P, K, Zn, Fe, Mn, Cu: Mehlich^{-1} extractor. Ca^{2+}, Mg^{2+} and Al^{3+}: KCl 1 mol L^{-1} extractor. H + Al: 0.5 mol L^{-1} calcium acetate extractor. BS: bases sum (Ca^{2+}+Mg^{2+}+K^{+}). $CEC_{effective}$=BS+Al^{3+}. CEC_{total}=BS+(H+Al). V: base saturation: (BS/CEC_{total})×100 m: Al. B saturation index (Hot water); S (NH_4O Acc. 0.5 mol L^{-1} and HO Acc. 0.25 mol L^{-1}).

Leaf macro and micronutrient contents, grain yield and oil and protein contents in the grains were determined. To determine the foliar nutrient contents, four leaves were collected from each of the 96 plants at random, in three samplings, at flowering, when the fruits were yellow and the last one when the fruits were dry. The leaf for the leaf analysis was chosen according to Kurihara and Silva (2015) recommendations, from between the 6th and 15th leaf of the floral branch. Fully expanded leaves between the sixth and eighth leaf below the apex free from apparent nutritional deficiency and/or pest and disease attacks were collected. These samples, as soon as they were collected, were sent to the Agroenergy Laboratory at UFV, washed with deionized water and dried in an oven with forced air circulation at 70 °C, until constant weight. Then, the samples were weighed, ground in a Willey mill and subjected to chemical analysis at the Laboratory of Mineral Nutrition at UFV.

To determine the total nitrogen content, the samples were submitted to sulfuric digestion by the Kjeldahl method. To determine the levels of phosphorus, potassium, calcium,

magnesium, sulfur, zinc, iron, manganese and copper, the leaf tissues after drying and grinding were submitted to nitric-perchloric digestion. P was determined by the phosphomolybdate reduction method by Vitamin C. K was determined by flame photometry. The contents of Ca, Mg, Zn, Fe, Mn and Cu were quantified by atomic absorption spectrophotometry. The nutrient S was determined by sulfate turbidimetry. All the macro and micronutrient analyses of leaf tissues determined in this study were obtained by the methods presented by Lana et al. (2016).

To measure grain yield, all the ripe and dry fruits of the 96 plants in the useful area were harvested; after threshing, the grains were weighed, extrapolating the value obtained to 1 ha. The oil contents in the grains were obtained by the nuclear magnetic resonance method (Oxford Instruments). For this measurement, the grains were initially heated to 40 °C in a block and these samples were placed in test tubes and weighed. The tube has a marking that corresponds to the range of magnetic rays, and its function is to delimit the amount of grains used in the analysis. On average, 10 grains weighing 0.5 g each were used in each sample, totaling a sample of 5 g.

Protein contents were obtained using a near-infrared spectrophotometer (FT-NIR). The sample preparation methodology was similar to the oil content by resonance; the difference was in the reading, as the test tube is placed on top of the lens emitting infrared rays. The FT-NIR also requires calibration prior to performing the analyses.

With the data obtained, the Pearson's correlation coefficient and the Student t test were calculated, as described by Ferreira (2018). Thus, in all possibilities, grain yield was correlated with oil, protein, and macro and micronutrient contents in leaves and fruits.

RESULTS AND DISCUSSION

Table 2 shows the Pearson's correlation coefficient (r) between grain yield, oil, protein and leaf macro and micronutrient contents. As for grain yield, statistical significance (P<0.05) was found only for leaf Mg content (r=-0.594). This association can be explained by two hypotheses: the first corresponds to the reduction of Mg levels due to fruit production, since Mg is easily translocated to regions of active growth; the second is the reduction in productivity due to the increase in the concentration of Mg in the tissues. According to Hawkesford et al. (2022), the demand for Mg is high in the reproductive growth stage, since the transport of photoassimilates is influenced by the Mg content and, the distribution of carbohydrates between the source and drain organs can be negatively affected if the Mg contents are insufficient for the fruit formation demand.

Regarding leaf nutrient contents, significant correlations (P<0.05) by the Student's t test were obtained for the following pairs: (N and Cu); (P and Ca); (K and Zn); (K and Cu); (Mg and Cu) and (Mn and Cu) and at P<0.01 for the following pairs: (N and S); (K and Ca); (K and Mg); (Ca and Mg); (Zn and Fe); (Zn and Mn); (Zn and Cu); (Fe and Mn) (Table 2).

For the levels of N and S, the correlation was positive and moderate (r=0.554) (Table 2). This effect is attributed to the high functional relationship between these ions in amino acid and protein metabolism (Vitti et al. 2018). The relationship between N and S in plant metabolism is so eminent that it is often possible to define the nutritional status of the plant based on the relationship of the contents of these two elements in dry matter (Vitti et al. 2018; Bell 2022). However, a different result was found by Maia et al. (2014) when analyzing the effect of nutrient omission in Jatropha. These researchers verified that in treatments with no nitrogen in the nutrient solution there was no reduction in the sulfur content in the shoots, when compared to those cultivated in the complete solution. They also observed that in the treatments with sulfur omission in the nutrient solution there was no reduction in the nitrogen content in the shoot.

The correlation between the N and Cu levels was positive (r=0.460) (Table 2). The occurrence of a positive interaction between these two elements was due to the fact that Cu has a strong affinity with the nitrogen atom of the amino group, indicating that soluble nitrogen compounds, such as amino acids, act as Cu carriers in the xylem and phloem (Huang et al. 2021). On the other hand, in citrus, the exaggerated supply of N caused Cu deficiency (Hippler et al. 2017; Huang et al. 2021). With the exception of the biological fixation of legumes, no results were found to explain the increase in N concentration as a result of the increase in Cu levels. However, it should be considered that a fraction of this micronutrient appears to be linked to plastocyanin and

some proteins (Dechen et al. 2018). In Jatropha, Maia et al. (2014) found that leaf nitrogen content was not affected by the omission of copper in the nutrient solution.

The correlation between leaf P and Ca contents was also positive (r=0.420) (Table 2). The increase in P content led to an increase in Ca content in chloroplasts and mitochondria, where, in these organelles, there are about 60% Ca in the leaves (Lima et al. 2018). The increase in foliar P content due to the increase in Ca concentration was due to the increase in P availability through liming (Bell 2022). Lima et al. (2011) evaluated the effect of phosphate fertilization on growth and macronutrient content in Jatropha seedlings and found reductions in Ca levels with increasing concentrations of P. Maia et al. (2014) observed that in treatments with P omission in the nutrient solution, the calcium content in Jatropha was 19.3% lower than those plants cultivated in the complete treatment of the solution. These same authors also verified that the P content in the shoot was higher than the complete treatment when the Ca was absent from the nutrient solution.

Table 2. Pearson's linear correlation matrix (r) between the means of grain yields (Yield), oil (Oil) and protein (Protein) contents in grains and macro and micronutrients in *Jatropha curcas* L leaves.

	Yield	Oil	Protein	N	P	K	Ca	Mg	S	Zn	Fe	Mn	Cu
Yield	1,000												
Oil	0.117	1,000											
Protein	-0.233	-0.354	1,000										
N	0.388	-0.221	0.097	1,000									
P	0.125	-0.193	-0.132	0.338	1,000								
K	0.385	-0.154	-0.347	0.136	0.083	1,000							
Ca	-0.296	0.151	0.034	0.052	0.420*	-0.596**	1,000						
Mg	-0.594**	0.149	0.195	-0.392	-0.082	-0.673**	0.603**	1,000					
S	-0.013	-0.262	0.063	0.554**	0.317	-0.175	0.287	0.026	1,000				
Zn	0.130	-0.020	-0.278	0.320	0.046	0.511*	-0.090	-0.242	0.044	1,000			
Fe	0.107	0.173	-0.263	0.206	-0.149	0.252	0.043	0.123	0.074	0.662**	1,000		
Mn	0.162	0.134	-0.174	0.382	0.015	0.160	0.062	-0.198	0.026	0.795**	0.528**	1,000	
Cu	0.455	0.029	-0.035	0.460*	0.293	0.506*	-0.243	-0.506*	0.044	0.574**	0.287	0.479*	1,000

Pearson's linear correlation values for nutrient contents in leaves; *Significant at 0.05 probability by the Student t test.

The correlations between the leaf contents of K and Ca, and K and Mg, were significant, but negative (r=-0.596 and r=-0.673, respectively) (Table 2). This effect is attributed to the competitive inhibition existing between the high levels of K in the tissues and those of Ca and Mg, and vice versa (Lima et al. 2018; Bell 2022). The cause of this nutritional imbalance is due to the existence of competition for the same carrier site. Lima et al. (2011) also found the nutritional imbalance between the levels of K and Ca, and K and Mg in Jatropha leaves. These authors found that while the K content in the leaves collected from the vegetative stage to senescence reduced from approximately 37.0 to 14.0 g kg^{-1}, the calcium and magnesium contents increased, respectively, by 5.0 to 48.0 g kg^{-1} and from 3.80 to 9.80 g kg^{-1}. Cañadas-López et al. (2020) also found significant interactions between K, Ca and Mg when studying the effects of pruning and fertilization on the production of Jatropha cultivated in a dry environment in the tropical region of Ecuador. These researchers found that potassium fertilization reduced the absorption of Mg and increased that of Ca.

As for the correlations between leaf K contents and the micronutrients Zn and Cu, there was a moderate positive association (r=0.511 and r=0.506) (Table 2). Considering that K is absorbed by many species in amounts higher than those necessary for vegetative metabolism ("luxury consumption"), in this condition plants may have influenced the absorption of cations, as long as they are not competing for the same carrier

site (Coskun and White 2022). Thus, it is assumed that the foliar K contents positively influenced the Zn and Cu contents. Regarding the K and Zn interaction, Maia et al. (2014) found similar effects, as they observed that in treatments with Zn omission in the nutrient solution, K levels in the shoot decreased. These authors did not find correlation between K and Cu contents, they only verified a negative interaction between Cu and Ca contents.

The correlation between Ca and Mg contents was positive and high (r=0.603) (Table 2). This result differs from those found by other authors, as high levels of Ca affect the absorption of Mg, and vice versa (Lima et al. 2018; Bell 2022). This inhibition is related to the similarity in chemical properties, such as valence and mobility, thus providing competition for sites of adsorption in the soil and absorption by roots (Guimarães Júnior et al. 2013; Coskun and White 2022). Considering that high Ca levels reduce Mg absorption, and vice versa, and that in this study the soil of the experimental area had low levels of these nutrients and low pH, the effect of reduction was not verified (Table 1). For Jatropha, Cañadas-López et al. (2020) found that Ca supply reduced Mg absorption.

For Mg and Cu, the correlation was negative and moderate (r=-0.506) (Table 2). This effect occurred due to the impairment in the absorption of one due to the increase in the other, provided by the strong competition between the cationic elements, indicating that Mg and Cu are antagonistic (Lima et al. 2018). In the literature, no associations were found between Mg and Cu contents in Jatropha.

The Zn and Fe leaf contents also showed positive and high correlation (r=0.662) (Table 2). High Zn levels in plant tissues prevent Fe reduction and may compromise its transport (Dechen et al. 2018). On the other hand, high Fe concentrations cause reductions in Zn mobility, and the presence of iron oxides and hydroxides in the soil induces low Zn mobility in the soil profile (Dechen et al. 2018; Martínez and McBride 2023). Research on associations between Zn and Fe leaf concentrations in Jatropha were not found in the literature Ferigolo (2017) verified the existence of a negative correlation when analyzing the associations between the Fe and Zn contents in the leaves of Brazilian Ginseng (*Pfaffia glomerata* (Spreng)). In maize, a negative interaction was also found for leaf Fe and Zn contents, thus indicating the existence of competitive inhibition in the absorption process promoted by excess Zn (Kume et al. 2021).

For the Zn and Mn content, the correlation was also positive and high (r=0.795) (Table 2). This effect differs from those presented by Dechen et al. (2018) and Martínez and McBride (2023), because according to these authors, plants with low Zn leaf concentrations have high Mn levels in the leaves due to Zn fixation and Mn release from the clay structure. In maize, Kume et al. (2021) found a marked reduction in leaf manganese levels caused by the increase in zinc concentration in the nutrient solution. In the literature, no associations were found between the Zn and Mn leaf contents in Jatropha.

For Zn and Cu contents, the correlation was positive and moderate (r=0.574) (Table 2). There is evidence that these elements are antagonistic (Dechen et al. 2018). In Jatropha, Maia et al. (2014) did not find a significant association between the Zn and Mn leaf contents.

The correlation between Mn and Fe leaf contents was 0.528 (Table 2). Divergent results were found in the literature, where plants with high Fe contents have low Mn concentrations due to competitive inhibition between them, due to competition for the same absorption site (Coskun and White 2022). The presence of MnO_2 oxidizes the reduced Fe to the unavailable ferric form (Dechen et al. 2018). Maia et al. (2014) found that Jatropha cultivated in treatments with Fe omission showed reductions in Mn levels. In the case of Mn omission in the nutrient solution, only a reduction in the Ca levels in the plant tissues was verified.

The correlation was positive and moderate for Mn and Cu contents (r=0.479) (Table 2). In the literature, a different effect was observed, where the reduction in leaf Cu content due to the increase in Mn concentration occurred due to the adsorption process, provided by the presence of manganese oxide and hydroxide in the soil (Martínez and McBride 2023). As for the reduction in Mn content as a function of the increase in Cu concentration, it appears that this effect is not as influenced as the opposite effect. Maia et al. (2014) did not find a significant interaction between Mn and Cu levels in Jatropha cultivated in nutrient solution.

Table 3 shows the grain, oil, and protein yields, as well as the nutrient contents in Jatropha leaves. Grain yield was 138.22 kg ha^{-1}, much lower than that estimated by Laviola and Dias (2008). The average oil content was 31.38%, very close to the 34% found by Laviola and Dias (2008). The protein content was 20.71%, relatively well below the range of 31 to 35% found by Machado and Silva (2019). It is believed that the reason for obtaining lower grain and protein yields is due to reduced micronutrient levels, since the aforementioned studies showed the highest leaf contents only for iron, manganese and copper.

Table 3. Averages of grain yield (Yield), oil (Oil) and protein (Protein) contents in grains and macro and micronutrient contents in leaves and fruits of *Jatropha curcas* L.

	Unit	Grains	Leaves
Grain productivity	(kg ha^{-1})	138.22	-
Oil content	%	31.38	-
Protein content	%	20.71	-
N content	g kg^{-1}	-	36.22
P content	g kg^{-1}	-	2.53
K content	g kg^{-1}	-	23.43
Ca content	g kg^{-1}	-	24.19
Mg content	g kg^{-1}	-	8.66
S content	g kg^{-1}	-	3.09
Zn content	(mg kg^{-1})	-	26.27
Fe content	(mg kg^{-1})	-	135.41
Mn content	(mg kg^{-1})	-	305.41
Cu content	(mg kg^{-1})	-	5.48

Regarding the nutrient contents in *Jatropha curcas* leaves, some divergences were observed when comparing the leaf contents obtained by Laviola and Dias (2008), as shown below. The nitrogen concentration was 36.22 g kg^{-1}, slightly higher than the 31.40 g kg^{-1} found by the researchers. The phosphorus content was 2.53 g kg^{-1}, a little lower than the 2.8 g kg^{-1} found. The potassium content was 58.47% higher than the study used as a reference. The leafs calcium and magnesium content were 78.64% and 55.42% higher, respectively. The S content found was almost triple that found by the authors in the study used as a reference. Regarding the zinc content in Jatropha leaves, a similarity between the values was observed, with 26.27 mg kg^{-1} in the present study and in the study by Laviola and Dias (2008). In the present study, the levels of iron, manganese and copper were lower than those obtained by the research used as a reference. The reduction was 89.97, 97.10 and 54.80% for the leaf Fe, Mn, and Cu contents, respectively.

CONCLUSIONS

A significant interaction was found between grain yield and leaf magnesium content, showing a negative correlation. No significant correlations were found between leaf contents and oil and protein yields. Significant positive interactions for nutrient content in Jatropha leaves were obtained for the following pairs of nutrients: (N and S); (N and Cu); (P and Ca); (K and Zn); (K and Cu); (Ca and Mg); (Fe and Mn) and (Mn and Cu). Negative significant interactions were obtained for the following combinations: (K and Ca); (K and Mg) and (Mg and Cu). Knowledge of the interactions between nutrients, as well as their responses in grain, oil and protein productivity can be used as a basis for studies on fertilizer recommendation, plant

improvement through nutritional efficiency, and studies in the field of biochemistry. Therefore, the mechanisms should be investigated that influence nutritional interactions.

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Short article

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Response of mint (*Mentha spicata* L.) crops to chemical and organic fertilization

Respuesta del cultivo de la menta (*Mentha spicata* L.) a la fertilización química y orgánica

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ABSTRACT

With the purpose to define the appropriate doses in the production of mint cultivation, this research was conducted in three locations (Gibraltar, Arboleda and Aguacatal) of the municipality of Jardín, Antioquia. The soils of these localities are andisols, with medium contents of organic matter, low in interchangeable bases, low in phosphorus and boron, with characteristics of low fertility. For this research, *Mentha spicata* L. (mint) was seeded at a distance of 0.3x0.3 m, in an experimental design of randomized complete blocks with four repetitions, with five increasing doses of compound fertilizer (10-30-10) (0, 60, 120, 180, and 240 kg ha^{-1}), in combination of five increasing doses of organic fertilizer (0, 1.8, 3.6, 5.4, and 7 t ha^{-1}), and one control with a biological fertilizer. In five foliage harvests, the highest dry matter (DM) yields were achieved with the application of 180 and 120 kg ha^{-1} of 10-30-10, with yields of 156 and 158 g of DM per square meter, respectively.

Keywords:
Andisols soils
Aromatic herbs
Chemical fertilization
Organic fertilization

RESUMEN

Con el propósito de definir las dosis apropiadas en la producción del cultivo de menta, se realizó la presente investigación en tres veredas (Gibraltar, Arboleda y Aguacatal) del municipio de Jardín, Antioquia. Los suelos de estas localidades son andisoles, con contenidos medios de materia orgánica, bajos en bases intercambiables, bajos en fósforo y boro, con características de baja fertilidad. Para esta investigación se sembró *Mentha spicata* L. (menta) a una distancia de 0,3x0,3 m, en un diseño experimental de bloques completos al azar con cuatro repeticiones, con cinco dosis crecientes de fertilizante compuesto (10-30-10) (0, 60, 120, 180 y 240 kg ha^{-1}), en combinación con cinco dosis crecientes de fertilizante orgánico (0, 1.8, 3.6, 5.4 y 7 t ha^{-1}), y un testigo con fertilizante biológico. En cinco cosechas de follaje, los mayores rendimientos de materia seca (MS) se lograron con la aplicación de 180 y 120 kg ha^{-1} de 10-30-10, con producciones de 156 y 158 g de MS por metro cuadrado, respectivamente.

Palabras clave:
Suelos andisoles
Aromáticas
Abonamiento químico
Abonamiento orgánico

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The Lamiaceae family is composed mainly of plants such as mints (*Mentha* spp.), Basils (*Ocimum* spp.), Rosemary (*Rosmarinus officinalis*), and thyme (*Thymus* spp.), among many others of known medicinal and culinary value (Castro et al. 2019; Velasquez et al. 2019).

Mentha spicata, popularly known as spearmint or garden mint, is a species of the genus *Mentha*, an aromatic herb widely used in gastronomy and perfumery for its intense and fresh aroma (Gallegos-Zurita 2016). Aromatic plants contain appreciable amounts of phytochemicals (phenols, their derivatives, among others), which are found in the whole plant or parts of it (Vicente-Herrero et al. 2013; Gastaldi et al. 2018; Morales et al. 2018).

Mint (spearmint and peppermint) has been grown primarily for the oil produced from its leaves. It is a perennial plant that does not produce seed, it is sown in rows, from stolons and roots of previous crops and/or rhizomes (Vacca-Molina et al. 2015). The oil is stored in leaf glands. The plant is trimmed and dried, then the dried material goes through a distillation process to recover the oil (Castro et al. 2013; Salehi et al. 2018).

Mentha spicata is among the aromatic species used in oral health, the interest in it and patenting are increasing due to its aroma and its effects as an antibiotic and sometimes as a pesticide (Ferreira et al. 2018). In addition, in recent years there has been an improvement in the processes normally used in the extraction of chemotypes, with the purpose of increasing the effectiveness of its active ingredient (Tofiño-Rivera et al. 2017).

Mint cultivation lacks technological information regarding fertilization, there are few reports in the literature on its behavior in different conditions such as cultivars, fertilization levels, climate, water availability, etc. For optimal nutritional management, it is necessary to know the nutritional requirements of the crop, which is expressed in kilograms of nutrients per ton of product, in this case as fresh product. These data are key in estimating nutrient demand, an essential parameter to assess the appropriate fertilization dose for crops (Juárez-Rosete et al. 2019). Since in this crop the aerial part is harvested and commercialized, successive harvests of shoots are carried out. This makes it a crop with a high capacity to extract nutrients from the soil (Juárez-Rosete et al. 2019).

Mint, like many other crops, extracts good amounts of nutrients, so to obtain approximately 6.7 t ha^{-1} of biomass, the crop requires at least 335 kg ha^{-1} of K_2O. To avoid a depletion of reserves of this nutrient in the soil, it is necessary to periodically supply this nutrient in the periods of greater development, growth of roots and stems (Brown et al. 2003).

During the growth period, phosphorus is an essential element in root development, for this reason an adequate supply is necessary after each harvest, so that it is a stimulus in each production cycle. It has been determined that each harvest extracts between 50 and 100 kg ha^{-1} of P_2O_5 (Arango et al. 2012).

Works carried out by Brown et al. (2003) determined that mint needs applications of N between 250 and 300 kg ha^{-1}, between 55 to 110 kg ha^{-1} of P_2O_5, and approximately 375 kg ha^{-1} of K_2O to obtain good yields. Therefore, if high yields are to be obtained with good biomass development, a sufficient supply of nutrients is necessary to avoid reducing the natural fertility of the soil (Brown et al. 2003).

Among the factors necessary for sustainable production in the cultivation of mint, there is the proper management of nutrition to obtain a high yield both in biomass and a high production and quality in the content and concentration of the oils. Normally in leaf production crops, slow-release nitrogen doses are applied periodically in each production cycle, but sufficient for good foliar development. It is estimated that 1 ha of mint produces between 95 to 125 kg of oil (Brown et al. 2003).

There is little information on the response of the crop to the application of NPK and organic matter. For this reason, El Centro de Investigación La Selva - Agrosavia had as aimed used different doses of fertilizer were used chemical (10-30-10), in combination with organic matter in the foliage production of the *Mentha spicata* variety.

MATERIALS AND METHODS

Localization

The experiment was carried out in three localities of the municipality of Jardín (Antioquia) with a moderate cold climate, located in the upper part of Gibraltar

(5°33'0.23" N, 0.75°51'0.23" O) at 1,963 m; in the lower part of Gibraltar (5°34'59.3" N, 0.75°50'57.4" O) at 1,800 m; and in La Arboleda, Aguacatales sector (5°34'0.78" N, 0.75°51'25.2" O) at 1,961 m. For the experiment, andisol soils with undulating topography, medium contents of organic matter, low in phosphorus, calcium, magnesium, and potassium were used. Regarding the minor elements, in general, they presented medium content, high in iron and low in boron (Table 1). For the present study, the variety of *Mentha spicata* was used according to the genetic description made by López-Hernández and Cortés (2022).

Statistical design

Increasing doses of compound fertilizer (10-30-10) (0, 60, 120, 180, and 240 kg ha^{-1}) were evaluated in combination with increasing doses of organic matter (0, 1.8, 3.6, 5.4, and 7 t ha^{-1}). Each plot consisted of 2 m long by 1.2 m wide; to harvest the four central plants, a randomized complete block design was used with four repetitions in a factorial arrangement where factor A were the harvests and factor B the levels of chemical fertilizer (10-30-10) in combination with the different levels of organic matter. The variation between the different treatments were analyzed using an ANOVA at each evaluation time. Multiple comparisons were made with a Tukey test with a probability of 5%.

Nutrient concentration

The dried samples were pulverized in a Thomas Scientific stainless-steel mill (Wiley Mini Mill 3383-L10), with 40 sieve (0.425 mm mesh size). The nutritional composition was determined in three composite samples, each one made up of stem and leaf tissues. The dry samples were sent to the Agrosavia-Tibaitatá soil and plant tissue chemistry laboratory (Mosquera, Cundinamarca, Colombia). There, the contents of total N (Kjedhdhal) and the extractable fractions of P (Bray II); Ca, Mg and K (1 N ammonium acetate, pH=7.0); Fe, Mn, Zn, Cu (modified Olsen), and B (monobasic calcium phosphate) were established. The protocols are described in Westermann (1990).

RESULTS AND DISCUSSION

According to Table 1, the soils have a pH between strong and moderately acidic. They have low phosphorus contents. With respect to exchangeable bases, they have low levels of calcium in two localities and low in magnesium and potassium in all localities. In terms of minor elements, they are low in all localities, except for iron. In general terms, they are soils of low to medium fertility.

Table 1. Chemical characteristics of the soils studied.

Locality	pH	MO (%)	P	CICE	Ca	Mg	K	Fe	Cu	Mn	Zn	B
			(mg kg^{-1})		(cmol kg^{-1})			(mg kg^{-1})				
1	5.58	9.32	31	5.73	5.02	0.43	0.14	72.6	3.10	2.59	2.33	0.08
2	5.76	9.86	6.88	2.19	1.53	0.33	0.19	362	3.33	8.80	3.87	0.10
3	5.34	10.55	3.80	2.04	1.02	0.32	0.15	75.6	2.08	2.44	2.87	0.11

1:Gibraltar-upper part; 2:la arboleda-aguacatal path; 3:Gibraltar lower part-La Bonaza path.

When performing a combined statistical analysis of the five crops, Table 2 shows that the dry matter production per meter and per hectare was affected by the amounts of chemical and organic fertilizer applied. Significant differences were observed between localities, being localities 1 and 2 statistically equal and different from locality three with productions of 1,055 and 1,146 kg of dm ha^{-1}, respectively. Work carried out by (Pedraza and Henao 2008) in Cundinamarca found high environmental variability (climatic and edaphic factors), it differs greatly in the nutritional requirements of the crop.

Table 2. Production of dry matter of the variety of mint (*Mentha spicata*) under different levels of 10-30-10. Three locations. Jardín.

Location	dm (m^{-1}*)	dm (ha^{-1}*)
1	146.63[a]	1055.75[a]
2	159.28[a]	1146.83[a]
3	121.18[b]	872.47[b]

*Averages followed by the same letter do not differ statistically at the 5% level, according to Tukey's test (P<0.05).

Franco et al. (2023) evaluated in mint, the effect of the application of chemical synthesis compounds and organic matter, and determined that the crop is highly extractive in nitrogen; in addition, found that the chemical composition of the soil had a direct relationship with the dry matter contents in the plants obtained at harvest, which was similar to that reported in the present research, where significant differences were obtained with increasing doses of complete fertilizer (10-30-10), in combination with applications of organic matter.

According to Table 3, the simple factor of crops, presented differences between the crops carried out, the highest production of dry matter of mint per square meter and per hectare was observed in the first harvest, 158 and 1,141 kg ha^{-1} after the first harvest showed a significant decrease in yields until harvests four and five.

Table 3. Production of dry matter of mint (*Mentha spicata*) under different levels of 10-30-10. Five harvests. Factor A.

Harvest	dm (m^{-1}*)	dm (ha^{-1}*)
1	158.55^{a}	1141.58^{b}
2	148.99^{ab}	1072.75^{b}
3	138.56^{bc}	997.61^{a}
4	140.85^{abc}	1014.14^{b}
5	124.86^{c}	899.01^{b}

*Averages followed by the same letter do not differ statistically at the 5% level, according to Tukey's test (P<0.05).

Table 4 shows the different combinations of chemical and organic fertilizer, this factor presented statistical differences between the different doses applied, being the one with the highest yield of dry matter when it was fertilized with 50% chemical fertilizer and 50% organic fertilizer (158 and 1,138 kg ha^{-1} of dm). This treatment was statistically equal to the application of only chemical (1,090 kg ha^{-1}) and to the combination of 75% chemical fertilizer and 25% organic fertilizer (1,124 kg ha^{-1} of dm). It should be noted that Henao-Rojas et al. (2022) reported that combined chemical and organic fertilization doses improve secondary metabolites and increase the yields of oils and volatile compounds, which is similar to those found in this study where the combined doses increased yields and improved the quality of the mint.

Table 4. Production of dry matter of mint (*Mentha spicata*) under different levels of 10-30-10. Five harvests. Factor B.

Chemical (%)	Organic (%)	dm (m^{-1}*)	dm (ha^{-1}*)
100	0	151.45^{ab}	1090.45^{ab}
75	25	156.24^{ab}	1124.95^{ab}
50	50	158.11^{a}	1138.37^{a}
20	75	145.12^{b}	1044.85^{ab}
0	100	136.40^{b}	982.07^{b}
0	Biologic	106.86^{c}	769.40^{c}

*Averages followed by the same letter do not differ statistically at the 5% level, according to Tukey's test (P<0.05).

In general terms, the order of nutrient extraction per ton of dry matter was N>K>Ca>Mg>P>S>Fe>Mn>Zn>B>Cu, which implies careful nutrient management to balance a high biomass growth and high production (Jiménez-alonso et al. 2012). Cano-Gallego et al. (2022) found significant differences with increases in soil fertilization (10-30-10), observing the highest expression of dry matter when between 120 and 180 kg ha^{-1} are applied; within the study carried out, it was found that dry matter increases with the increase in edaphic fertilization (10-30-10), the same as reported in this research.

According to Table 5, it is observed that as the amount of chemical fertilizer decreases, the concentration of nutrients in the tissues decreases. Work carried out by Avelar et al. (2015) in the Mentha aquatica variety, found this same situation when organic fertilizer increased. The element that presented the highest concentration in the leaves and stems was nitrogen. N is one of the nutrients responsible for vegetative growth; however, it does not follow an infinite linear trend, but the crops reach a saturation point of the element (Alejo-Santiago et al. 2015); in second order there are potassium and calcium, this agrees with that reported by Castro et al. (2005) in dandelion (*Taraxacum officinalis* Weber). However, works carried out by Giraldo and Henao (2019), in absorption curves carried out in the greenhouse, found that the element that was most extracted was Ca, followed by K and N (58.87, 36.97 and 26.92 kg ha^{-1} per production cycle, respectively).

Work carried out by Ozcan (2004) in a local market in Turkey, in leaves and flowers of *M. spicata*, found that the tissues contained among major elements such as KYP, percentages of 2.47 and 0.22%, respectively. Among secondary elements they had concentrations of 1.13% Ca, 0.5% Mg and 0.31% S, and between elements less concentrations of 97.6, 18.7, 47.6, 8.48 mg kg^{-1} of Mn, Zn, B and Cu, respectively.

Table 5. Nutrient concentration in tissues (leaves and stems) of the mint plant in Antioquia.

Dose	N	P	K	Ca	Mg	S	Fe	Cu	Mn	Zn	B
	(g 100 g^{-1})						(mg kg^{-1})				
1	3.9	0.5	2.5	1.0	0.5	0.3	84.8	10.7	182	45.3	43.2
2	3.8	0.4	2.5	1.0	0.4	0.3	80.2	8.48	129	33.8	41.0
3	3.8	0.4	3.1	0.9	0.4	0.3	88.0	10.7	187	26.7	46.0
4	3.6	0.4	2.2	0.8	0.4	0.4	89.2	15.4	140	27.4	44.3
5	3.5	0.6	3.5	0.9	0.4	0.4	87.7	14.4	92.8	25.8	50.5
6	2.6	0.4	3.1	0.7	0.3	0.3	67.5	22.4	59.7	26.0	46.0
Average	3.5	0.4	2.8	1.0	0.4	0.3	82.9	13.7	131.7	30.8	45.2

In investigations carried out by Hart et al. (2003) during 3 years in six different locations, they found that the accumulation of biomass and concentration of nutrients in *M. piperita* fluctuated between 3.85 to 4.41% for nitrogen; 0.40 to 0.63% for phosphorus; 3.65 to 4.77% for potassium; 0.36 to 0.48% for sulfur; 0.88 to 1.52% for calcium; 0.28 to 0.45% for magnesium; and for the minor elements between 20.24 to 27.8; 1.25 to 19.4; 77.5 to 119.9; and 28.0 to 45 mg kg^{-1} for B, Cu, Zn and Mn, respectively.

The minor elements are notable for the concentration of boron that exceeds zinc and copper. For phosphorus, the concentration was similar among all the applied doses, its content was between 0.38 to 0.56%. A similar situation occurred with magnesium (0.32 to 0.37%). The lowest concentration was presented for sulfur (0.34 to 0.37). Work carried out by Torres et al. (2013) on the species *Satureja macrostema* (Benth) Briq. found that the increase and yield of volatile compounds can be favored, which is closely related to the increase in biomass in plants treated with chemical fertilization. Franco et al. (2022) reported that the most relevant nutrients in the cultivation of Mentha, in their order were N, K, Ca and Mg, being similar to what was found in this study. It should be noted that the excess of Zn presented antagonism with B, showing a differential when making foliar and/or edaphic applications with this element.

In five harvests, it was determined that the removal of nutrients requires the application of at least 35 kg of N for each ton of dry matter of production; thus, 28 kg of K are required for each ton of extracted dry matter, Ca and Mg follow in their order (8.9 and 4.2%, respectively). In these soils derived from volcanic ash with low phosphorus content, a positive response to the application of this element can be expected, this is confirmed by the low concentration for the treatments of only organic and/or biological matter. To obtain good crops and maintain soil

fertility, it is necessary to apply the following elements for each ton of dry matter (N 40, P 10, K 35, Ca 45, and 35 of Mg kg). According to Brown et al. (2003), to obtain high yields the mint crop requires between 250 and 300 kg ha^{-1} of N, between 55 and 110 kg ha^{-1} P_2O_5, and about 375 kg ha^{-1} of K_2O.

CONCLUSIONS

In this research, it was found that the combination of chemical and organic fertilization in equal proportions increases crop yields. Likewise, the increase in chemical fertilization is proportional to the increase in dry matter in the mint crop, where the order of total nutrient removal per ton of dry matter was N>K>Ca>Mg>P>S>Fe>Mn>Zn>B>Cu.

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Effect of the tillage system on the floristic composition and the emergence of weeds in *Allium sativum*

Efecto del sistema de labranza sobre la composición florística y la emergencia de malezas en *Allium sativum*

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Keywords:
Garlic
Seedling emergence
Weeds diversity
Zero tillage

ABSTRACT

In fragile environments, no-tillage (0T) instead of conventional tillage (CT) is desirable to prevent agroecosystem degradation, but there is little information on its implementation in horticulture. This study aimed to investigate the effects of replacing CT with 0T on floristic composition and weed emergence dynamics in a garlic crop, under the hypothesis that the implementation of a 0T system alters the weed community during the initial stage of the transition. Two experiments were carried out following a randomized complete block design with two treatments (garlic crop grown under 0T and CT). In two subsampling per plot, biweekly destructive weed surveys were carried out. Although both tillage systems presented a similar diversity between systems, these weed communities varied by 36% in their species identity, and it was recorded a higher total weed density under CT (P>0.05). Under 0T, anemophilous Asteraceae, such as *Conyza bonariensis* and *Sonchus oleraceus*, tended to increase their presence. Under CT, there was a greater amount of indehiscent fruiting Brassicaceae such as *Raphanus sativus* and *Rapistrum rugosum*. The implementation of *Vicia villosa* as a predecessor crop led to many births due to its capacity for natural reseeding. It is concluded that there are important changes in the species composition and weed emergence patterns immediately after the implementation of 0T compared to CT, suggesting that the filtering pressures exerted by each tillage system favor certain weed species over others. By understanding weed community shifts and critical stages of weed emergence, farmers can improve herbicide application, thereby reducing the excessive use of chemicals and minimizing environmental impact. In addition, this information can help to schedule labor and machinery more efficiently, saving time and production costs.

Palabras clave:
Ajo
Emergencia de plántulas
Diversidad de malezas
Labranza cero

RESUMEN

En ambientes frágiles, la labranza cero (L0) presenta ventajas frente a la labranza convencional (LC) al disminuir la degradación de los agroecosistemas, pero se dispone de escasa información sobre su aplicación en horticultura. El objetivo del presente estudio consistió en determinar la influencia que genera la sustitución de LC a L0 sobre la composición florística y la dinámica de emergencia de malezas en un cultivo de *Allium sativum,* bajo la hipótesis de que la implementación L0 altera la comunidad de malezas durante la fase inicial de transición. Dos experimentos fueron realizados siguiendo un diseño en bloques completamente aleatorizados con dos tratamientos (cultivo de ajo bajo LC y L0). Las prospecciones de malezas fueron determinadas de manera destructiva quincenalmente en dos subáreas por parcela. Aunque ambos sistemas de laboreo presentaron una diversidad similar, la identidad de las malezas varió en un 36% y se registró una mayor densidad de plántulas bajo LC (P>0,05). Bajo L0, las Asteráceas anemófilas, como *Conyza bonariensis* y *Sonchus oleraceus,* tendieron a incrementar su presencia. Bajo LC hubo mayor cantidad de Brasicáceas de fruto indehiscente como *Raphanus sativus* y *Rapistrum rugosum*. La implementación de *Vicia villosa* como cultivo antecesor, acarreó un gran número de nacimientos dada su capacidad de resiembra natural. Se concluye que existen cambios importantes en la composición de las especies y en los patrones de emergencia de las malezas inmediatamente después de la implantación de L0 en comparación con LC, lo que sugiere que las presiones ejercidas por cada sistema de laboreo favorecen a determinadas especies frente a otras. Mediante el conocimiento de los cambios en la comunidad de malezas y los períodos críticos de emergencia de malezas los agricultores pueden mejorar los tratamientos con herbicidas, al reducir el excesivo uso de productos químicos y minimizar el impacto ambiental. Esta información puede asimismo ayudar a programar las labores manuales y mecánicas más eficientemente, reduciendo los costos de producción.

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In semi-arid regions, wind and water soil erosion is a major problem, therefore agricultural practices should aim to reduce it (Lal 2001). The implementation of conventional tillage (CT) contributes to erosion by disturbing the soil, breaking its structure, and reducing its cover, while the adoption of zero-tillage (0T) reduces the amount of sand transported near the field surface and increasing the cohesion of soil particles and the size of aggregates, making them more difficult to erode (Yang et al. 2020).

The transition from CT to 0T modifies soil environmental parameters by directly affecting water dynamics, obstructing light interception, and reducing surface layer temperature and oxygen availability (Nikolić et al. 2021). Each agricultural practice has a higher or lower potential to influence the abundance and diversity of weed species in a crop field and is a key aspect that affects both biodiversity conservation and integrated weed management (Travlos et al. 2018).

The Lower Colorado River Valley Irrigation District (VIRC; Figure 1), Argentina, presents a fragile environment, with a high susceptibility to wind and water erosion of its soils (D'Amico and Varela 2017). The economy is based on irrigated agricultural production with a strong specialization in the horticultural sub-sector, particularly bulb crops under CT, which requires a high proportion of tillage and complementary manual work (Navós López 2021; D'Amico and Varela 2017). While 0T is widespread in Argentina for extensive grain production, it has not yet been developed for vegetable production such as garlic (D'Amico and Varela 2017). Therefore, the available literature for horticultural crops produced under 0T is scarce, especially regarding the floristic composition of weeds that predominate in this new system.

Since the VIRC has a temperate climate, with summers and winters differentiated by marked thermal extremes, weed species vary according to the season in which they develop their cycle. Thus, they are classified as autumn-winter-spring (AWS), germinating in autumn, vegetating in winter, and fructifying in spring, or spring-summer (SS), which germinate in spring and culminating their cycle at the end of the summer. In turn, many species are facultative (AWS-SS) and can develop their cycle partially in both periods, so they have two main peaks of emergence, one in autumn and the other in spring (Cerazo and Conticello 2008).

Garlic (*Allium sativum* L.) is one of the main horticultural crops produced in the VIRC. The crop is sown in the region in late autumn and harvested in early summer, and as a result is invaded by AWS, SS, and AWS-SS weeds (D'Amico and Varela 2017). Considering the crop's limited competitive ability due to its slow and prolonged initial growth, upright architecture with minimal shading, and shallow, restricted root system, it becomes essential to employ an optimal management strategy to safeguard its yield from weed encroachment (Siddhu et al. 2018).

This study aimed to investigate the effects of replacing CT with 0T on floristic composition and weed emergence dynamics in a garlic crop, under the hypothesis that the implementation of a 0T system alters the weed community during the initial stage of operation. These changes in relation to various aspects of the life cycle of each species and potential management strategies were also analysed. This information helps to plan weed management strategies by reducing not only soil disturbance but also herbicide applications with their consequent ecological and economic impact.

MATERIALS AND METHODS

The VIRC has a semiarid temperate climate, with an average annual temperature of 15 °C and sandy loam-textured soils with 1% organic matter (Trillini et al. 2023). The average annual rainfall is around 500 mm with a significant water deficit condition; hence an adequate irrigation system is crucial to produce horticultural crops (Trillini et al. 2023). Two complementary experiments were carried out in an irrigated plot at the INTA-Ascasubi Experimental Station (39°23'31.8"S-62°37'43.8"W), Villarino, Buenos Aires province, Argentina (Figure 1). In these experiments, the floristic composition and weed emergence were quantified in a garlic crop planted under CT or 0T. Experiment 1 took place in 2017 where the emergence of AWS and AWS-SS weeds was counted, while experiment 2 was carried out in 2019 recording the emergence of SS and AWS-SS species.

Prior to each experiment, sowing of *Vicia villosa* Roth was carried out to homogenize the soil seed bank. For each experiment, a randomized complete block design with two

treatments (0T and CT) and six replicates (N=12) was performed. Garlic seed-cloves were planted manually at the beginning of May following a spatial planting arrangement in two-sided furrows considering each furrow as a block (Figure 2). The planting density was 20 seed teeth per linear meter at a depth of approximately 8 cm. Garlic was fertilized twice with Urea, the first one manually (20 kg N ha^{-1}) and then by fertirrigation (125 kg N ha^{-1}). The irrigation system used was drip irrigation with tapes located between crop rows with emitters separated at 30 cm and with a delivery capacity of 1 L h^{-1}. During each experiment, and for each tillage system, gravimetric moisture (GM), air temperature and soil temperature at 5 cm depth were measured. GM was determined by weight difference in soil samples taken at 7 cm depth and placed in an oven (70 °C) until a constant weight was reached.

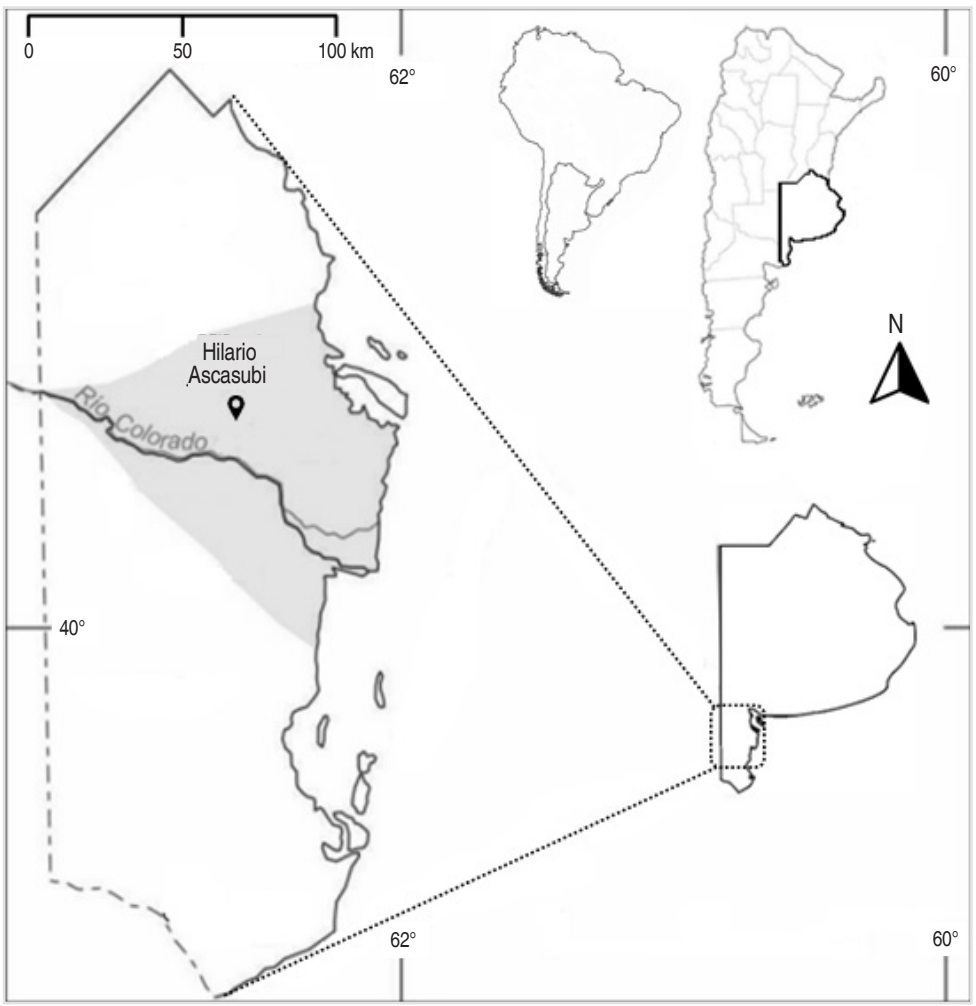

Figure 1. Location of Lower Colorado River Valley Irrigation District (Shaded area). Adapted from Torrez Gallardo (2019).

In both experiments, two quadrants of 0.25 m^2 were placed per plot where weed seedlings were counted and removed (Figure 2). This represented a typical sub-sampling design (Onofri et al. 2010). In experiment 1 the counting and removal of weed seedlings started in mid-winter and ended in mid-spring, while in experiment 2 it started in early spring and ended in early summer (Figures 5 and 6). For the 0T treatment, a chemical treatment with glyphosate was carried out at a rate of 0.83 kg Pa ha^{-1}. Under CT, weeds present prior to planting were mechanically removed by plowing the soil to a depth of 20 cm.

In each experiment, for each tillage system, weed species present were identified and density counted. A species was considered emerged when cotyledons were visible or, for perennial species, when the sprout measured more than 1 cm. Richness was calculated as the total number of species present. Density (seedlings m^{-2}) and relative frequency (%) of emerged seedlings of each species in each year and between tillage systems were analyzed with ANOVA followed by Tukey's test (P>0.05) using INFOSTAT® statistical software. When homoscedasticity and normality assumptions were not met, abundance data were previously square root transformed.

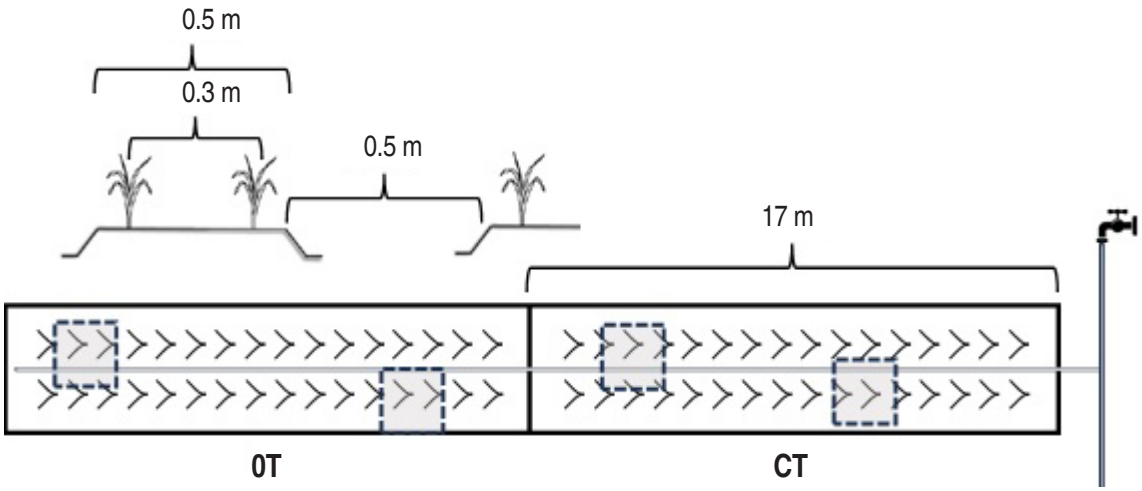

Figure 2. Schematic representation of the experiment. **Above:** garlic seed cloves planted according to a spatial planting arrangement in two-sided furrows, considering each furrow as a block. **Below:** one of the six blocks made in the experiments according to a randomized complete block design with two treatments (0T and CT) and six replicates (N=12). At the beginning of each experiment, two quadrants (sub-sampling) per plot were randomized established, and fixed, where the measurements were made.

To compare floristic composition for each year and for each tillage system, Simpson's index (Travlos et al. 2018) was calculated according to Equation (1):

$$D = 1 - \frac{\sum_{i-1}^{S} n_i(n_i - 1)}{N(N-1)} \quad (1)$$

Where S is the number of species present, n is the density of each species and N is the sum of the densities of all species.

To measure the similarity of species composition between tillage systems Jaccard's coefficient (Travlos et al. 2018) was applied according to Equation (2):

$$J = \frac{a}{a + b + c} \quad (2)$$

Where a is the total number of species present in both tillage systems, b is the number of species present only in LC, and c is the number of species only in L0.

RESULTS AND DISCUSSION

As shown in Figure 3, in experiment 1 under 0T soil temperature was on average 3 °C lower and GH 2% higher. Records were similarly repeated in experiment 2 (data not shown). In soils prepared for planting, lower temperature and higher moisture are normally cited in 0T systems relative to CT, primarily because plant residues remain on the surface (Tuesca et al. 2001).

Crop yields, in terms of bulb weight and size distribution, did not evidence differences between treatments (INTA-EEA Ascasubi personal communication), coinciding with previous trials (D'Amico and Varela 2017).

In both experiments, the total density of emerged weeds was higher under CT (P<0.05), being on average 386 (CT) and 239 (0T) seedlings m^{-2} in experiment 1 and 201 (CT) and 170 (0T) seedlings m^{-2} in experiment 2. There were mainly annual broadleaf species, varying the dominant weeds between experiments and between systems (Table 1). In experiment 1 the richness of AWS species was higher

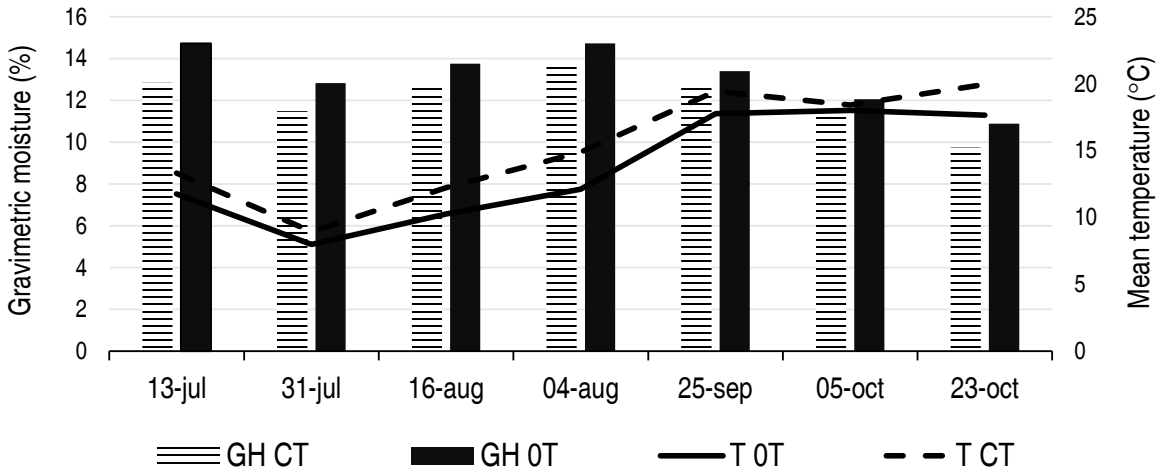

Figure 3. Gravimetric moisture (GH) and soil temperature (T) measured at 7 and 5 cm depth, respectively, for the no-till (L0) and conventional tillage (CT) treatments in experiment 1.

under CT than under 0T (18 and 13 species, respectively), while in experiment 2 both systems presented 18 SS and AWS-SS species. Values obtained by Simpson's index were 0.85 (CT) and 0.82 (0T) in experiment 1, and 0.79 (CT) and 0.80 (0T) in experiment 2. These values are relatively high and indicate a similar weed community diversity in all contexts, which was also observed by Alarcón et al. (2018) after a similar but long-term study. Jaccard's coefficient was 0.63 and 0.64 for experiments 1 and 2, respectively, suggesting that although both tillage systems showed a similar abundance structure in both experiments, weed communities varied by 36% in their species identity. This could be important in relation to weed management strategies, mainly because by varying the species composition, herbicides efficiency could be different.

Table 1. Relative frequency (%) of weeds in the garlic crop under conventional tillage (CT) or zero tillage (0T) for experiments 1 and 2.

Species		Experiment 1		Experiment 2	
		CT	0T	CT	0T
Amaranthus hybridus L.	annual SS	-	-	0.30	0.15
Ammi majus L.	annual AWS	1.30	0.42	-	-
Avena fatua L.	annual AWS	2.85	-	-	-
Centaurea calcitrapa L.	annual AWS	-	-	0.15	0.15
Chenopodium album L.	annual SS	3.89	1.67	1.95	4.13
Cichorium intybus L.	perennial AWS	-	-	0.15	-
*Cirsium vulgar*e (Savi) Ten.	annual AWS	-	0.42	0.50	-
Conyza bonariensis (L.) Cronquist	annual AWSS	15.29	33.47	-	12.95
Cynodon dactylon (L.) Pers.	perennial SS	0.26	-	2.72	1.89
Cyperus rotundus L.	perennial SS	-	-	-	0.63
Digitaria sanguinalis (L.) Scop.	annual SS	5.70	-	32.98	17.88
Diplotaxis tenuifolia (L.) DC.	perennial AWSS	3.11	5.02	24.57	30.21
Echinochloa crus-galli (L.) P. Beauv.	annual SS	-	-	4.25	5.44
Euphorbia serpens Kunth	annual SS	-	-	0.50	-
Gamochaeta filaginea (DC.) Cabrera	annual AWS	2.85	10.46	-	-

Table 1

Species		Experiment 1		Experiment 2	
		CT	0T	CT	0T
Hirschfeldia incana (L.) Lagr. Foss.	annual AWS	-	-	11.11	5.29
*Lamium amplexicaul*e L.	annual AWS	0.78	2.09	0.30	0.44
Medicago sp.	annual AWS	0.52	-	-	-
Poligonum aviculare L.	annual AWS	0.78	0.84	-	-
Portulaca oleracea L.	annual SS	2.07	-	5.30	0.49
Raphanus sativus L.	annual AWS	24.35	8.37	1.36	0.83
Rapistrum rugosum (L.) All.	annual AWS	5.44	5.86	0.30	-
Rhaponticum repens (L.) Hidalgo	annual AWS	-	-	0.17	0.29
Rumex crispus L.	perennial AWS	0.26	-	-	0.29
Salsola kali L.	annual SS	-	-	-	0.49
Senecio vulgaris L.	annual AWS	0.52	2.93	1.21	0.15
Sonchus asper L.	annual AWS	-	-	-	1.60
Sonchus oleraceus L.	annual AWS	8.29	11.72	8.06	15.45
Tribulus terrestris L.	annual SS	-	-	0.55	0.29
Vicia villosa Roth	annual AWS	21.76	16.74	5.60	0.97
Total		100	100	100	100

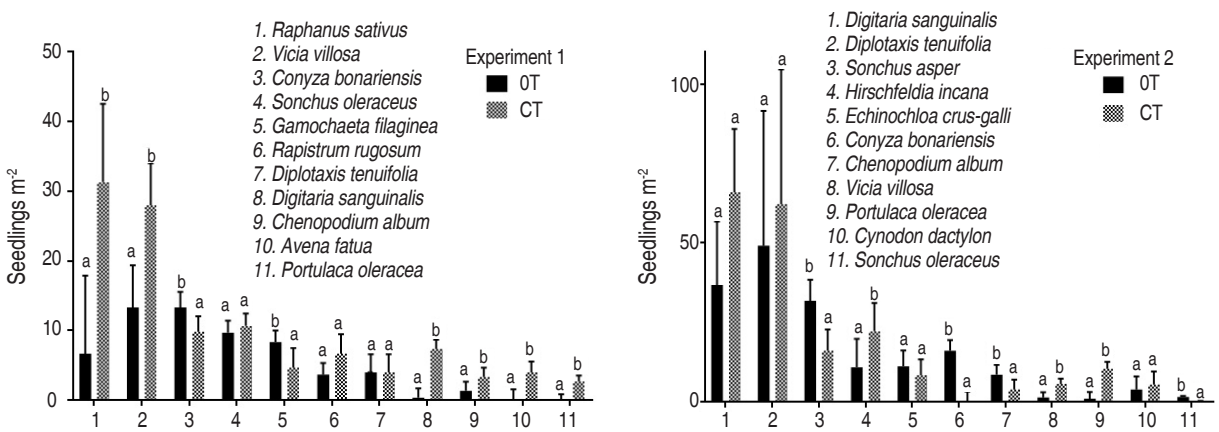

Figure 4. Density (seedlings m^{-2}) of weeds in experiments 1 and 2 under conventional tillage (CT) and no-tillage (0T). Different letters indicate differences in the density of a given species between tillage systems according to Tukey's test ($P<0.05$).

In experiment 1, 24.3% of the seedlings that emerged under CT belonged to *Raphanus sativus* and 33.5% under 0T to *Conyza bonariensis* (Table 1), a similar result was observed by D'Amico and Varela (2017) where the dominant species under CT was also a Brassicaceae (*Hirschfeldia incana*), and under 0T *C. bonariensis*. In experiment 2, mostly SS species were recorded, resulting dominants under CT *Digitaria sanguinalis* (33.0%) and under 0T *Diplotaxis tenuifolia* (35%).

As described by Zanin et al. (1997) and Tuesca et al. (2001), the consequences of the transition from CT to 0T involve treatments that lead to a repetitive re-colonization of niches generating a weed community composed mainly of anemophilous dispersal therophyte species. Likewise, in the present study, a higher abundance of *C. bonariensis* and *G. filagina* was observed under 0T (Table 1, Figure 4). These Asteraceae have small cypsela with hairy pappus that facilitates wind dispersal over long distances. Also,

they fructify for a long period, and their seeds have low or no dormancy and an intermittent and prolonged emergence period, being imperative their control on roads and bordering lots to avoid their dispersal (Loura et al. 2020). In a conservationist system, as 0T, seed emergence of anemophilous species accumulated on the soil surface could be reduced by including strategic tillage or, when dealing with photoblastic species, by implementing mulching or cover crops that leave residues on the surface (Chauhan et al. 2006b, Loura et al. 2020).

Vicia villosa, *R. sativus*, *S. oleraceus*, *R. Rugosum*, among others, are AWS species that show in both tillage systems a main peak of emergence at the beginning of winter (Figure 5). In order to avoid production losses, autumn-winter peaks must be controlled, or they will strongly impact garlic crop growth and development (Siddhu et al. 2018). On the other hand, the control operation of SS seedlings, that emerged in late spring, such as *D. sanginalis* and *C. album* (Figure 6) will depend on the abundance and size of the plants, since they could hinder bulb harvesting (Siddhu et al. 2018). Thus, if no control measures are taken, species will complete their reproductive cycle, contributing to the seed bank and becoming a potential problem in future years (Fernández et al. 2014). This is important in contexts dominated by species with high reproductive potential and seeds that remain viable in the soil for years, such as *C. bonariensis* and *R. sativus* (Wu et al. 2007; Shrestha et al. 2008).

Weed in agroecosystems is defined as any plant that interferes with the use of crop resources (Fernández et al. 2014). *Vicia villosa* was considered as such in the present context, despite being a cultivated forage species. Thus, having been used as a predecessor crop and possessing high natural reseeding capacity, in experiment 1 it was one of the most abundant species during cultivation in both tillage systems (Table 1), presenting higher seedling recruitment under CT (P<0.05; Figure 4). Seed dormancy breaking of *V. villosa* occurs after a short post-dispersal after-ripening with the accumulation of 25 to 30 °Cd (degreedays) during summer emerging in autumn (Renzi et al. 2014). Therefore, after a vetch crop, a prolonged autumn fallow with high control pressure could be included in the rotation. Since vetch seedlings emerge from more than 10 cm deep (Renzi et al. 2014), if the fallow is mechanical, it may be necessary to supplement with chemical control.

The most abundant Brassicaceae found in this study were *R. sativus* and *R. rugosum* (Table 1). In experiment 1, *R. sativus* showed similar emergence patterns in both systems with a marked peak in early winter (Figure 5). However, three times higher emergence was recorded under CT (Figure 4; P=0.022), possibly because under 0T germination was inhibited by higher seed exposure to light and lower pericarp rupture in the absence of mechanical control (Vercellino et al. 2019). In experiment 2 the quantified emergence was almost null given that winter temperatures induce secondary dormancy in *R. sativus* (Vercellino et al. 2019; Table 1). On the contrary, in experiment 1 *R. rugosum* showed a differential behavior depending on the tillage system. Under 0T, two emergence peaks of similar magnitude were quantified: the first at the end of July and the second at the end of September; while under CT there was only one winter peak that doubled in magnitude the one observed in 0T (Figure 5). In both *R. sativus* and *R. rugosum*, the embryo presents nondeep physiological dormancy with germination being mechanically restrained by the silique (Manalil et al. 2018; Vercellino et al. 2019), so the fruit walls must be abraded for the radicle to pass through them. Naturally, a large proportion of the pericarp is physically, chemically, and/or biologically degraded during the summer. Thus, considering that tillage implements break the fruit in *Raphanus* spp. (Vercellino et al. 2019), the higher emergence observed under CT could be explained.

Hirschfeldia incana is also an AWS Brassicaceae, and then, the emergence peak recorded in spring in experiment 2 is due to its facultative behavior (Figure 6). This fact is feasible given that the optimal germination temperatures of *H. incana* range between 20 to 35 °C (Castro et al. 2016). In turn, a higher emergence was quantified under CT (P<0.05; Figure 4) possibly due to the rupture of fruit walls caused by mechanical tillage.

Unlike the Brassicaceae described above, *Diplotaxis tenuifolia* is a perennial SS species with dehiscent fruit and a wide distribution in the region under study (Vigna and Fernández 2018). Among other attributes, it has a high reproductive rate, vegetative propagation by gemmiferous roots, and high adaptability to the wide range of environmental conditions prevailing in the southern region of Buenos Aires province (Vigna and Fernández

2018). In experiment 2, a peak with a high number of weed emergences was recorded in *D. tenuifolia* towards the end of spring in both CT and 0T (Table 1; Figure 6), As *D. tenuifolia* is a perennial species, it is expected that over the years weed abundance will increase in 0T systems (Tuesca et al. 2001).

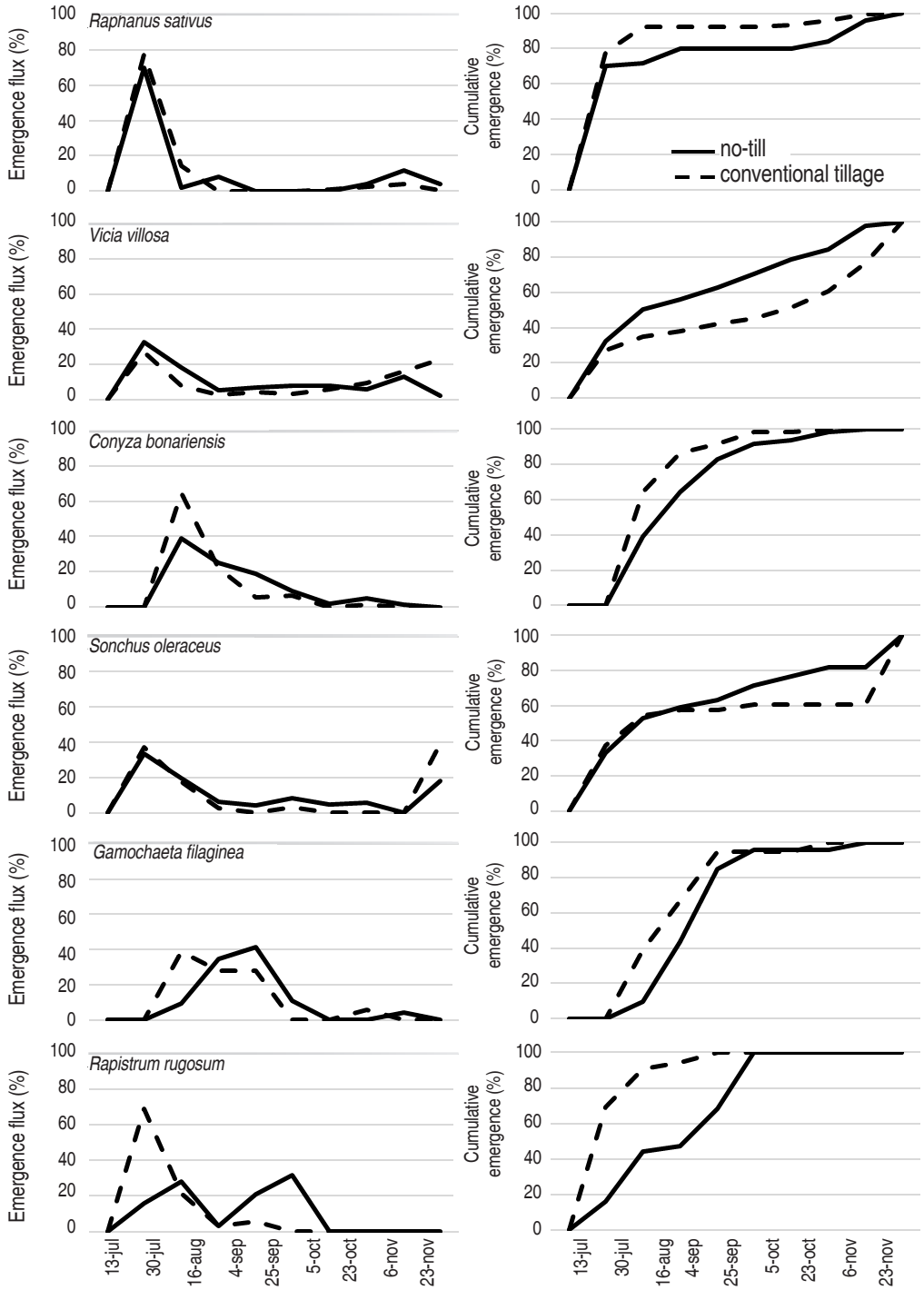

Figure 5. Percent emergence flux for each sampling date (left) and cumulative percent emergence (right) of the most abundant weeds under no-till and conventional tillage for experiment 1.

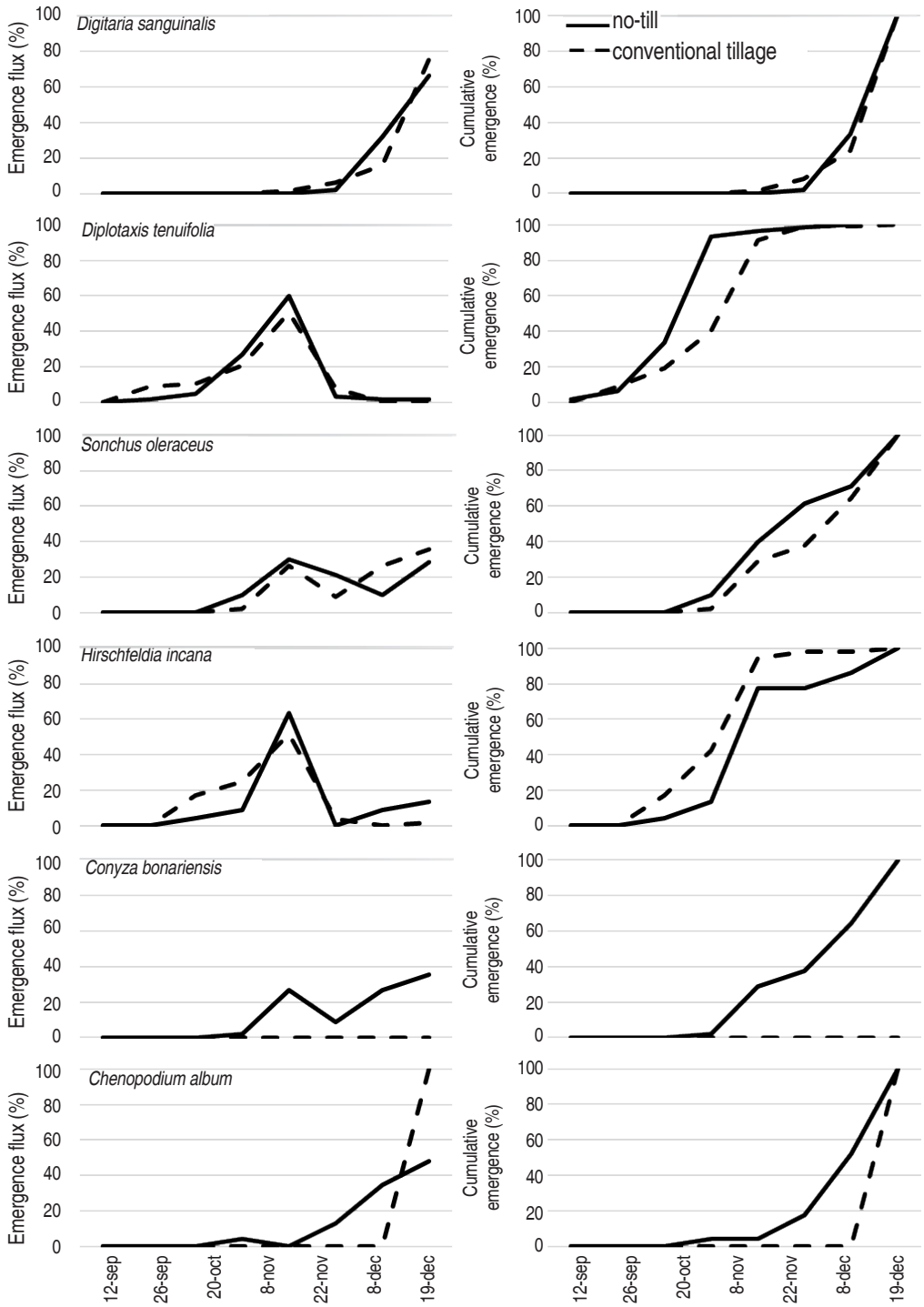

Figure 6. Percent emergence flux for each sampling date (left) and cumulative percent emergence (right) of the most abundant weeds under no-till and conventional tillage for experiment 2.

In both experiments, the most abundant Asteraceae were *C. bonariensis* and *S. oleraceus* showing similar emergence patterns throughout the garlic crop cycle, since they can germinate in a wide range of temperatures and

water conditions (Chauhan et al. 2006b; Wu et al. 2007; Widderick et al. 2010). In experiment 1, they showed similar emergence patterns among systems (Figure 5; *P*>0.05). However, in experiment 2 there was a higher recruitment under 0T (Figure 4 and 6; *P*=0.0014) coinciding with Chauhan et al. (2006a). For both species, emergence is higher when seeds are near the soil surface, possibly due to their photoblastic character (Chauhan et al. 2006b; Wu et al. 2007; Widderick et al. 2010). Due to *S. oleraceus* seed viability is low on the soil surface, Widderick et al. (2010) hypothesize that under 0T if emergence flows are controlled for eight months without allowing plants to form seeds, the species will reduce its abundance drastically. On the other hand, strategic tillage could reduce emergence by affecting the vertical distribution of the seed bank in the soil. This would reduce light exposure and reduce the vigor of seedlings and their competitive ability (Chauhan et al. 2006a). In these cases, it would not be recommended to make new soil mechanical operations immediately so that the seeds do not return to the surface.

Chenopodium album is a troublesome weed in the area and showed opposite results between 0T and CT in both experiments (Table 1, Figure 4). In experiment 1, there was a greater number of seedlings under CT than 0T, as was also observed by Alarcón et al. (2018). In experiment 2, seedling density was greater than in experiment 1, given that seedling count continued until the beginning of summer, being the optimum period for emergence (Table 1). At the same time, seedling emergence under 0T was earlier in time (Figure 6), probably because seeds were spread shallow in the soil (Tang et al. 2022). An interesting situation was reported by Nikolić et al. (2021) who observed that in a dry spring, in the presence of abundant wheat stubble, the emergence of *C. album* was earlier than in bare soil but was reduced by 44%. Consequently, considering that most *C. album* seeds are photoblastic, it is recommended to ensure residue retention or coverage on the soil surface in no-tillage systems as a suitable management strategy for inhibiting its spread (Tang et al. 2022).

Digitaria sanguinalis is a SS species of high incidence in the study area. Although this species had a similar emergence flux under 0T and CT (Figure 6), it showed higher germination under CT (Figure 4). Seeds dispersed at the end of the summer usually have a high primary dormancy level which is released by low winter temperatures, especially under fluctuating soil temperatures (Oreja et al. 2017). The presence of stubble could reduce the fluctuating temperatures and light remaining reducing seedling emergence under 0T, as also was observed by Nikolić et al (2021). Nevertheless, this species shows a temporal seed bank under 0T where most seeds can survive on the soil surface for less than a year. Thus, to reduce *D. sanguinalis* population in no-till systems, diminishing the re-entry of new seeds in the soil could be a very effective strategy (Oreja et al. 2020).

The greater emergence of *P. oleracea* under CT (Figure 4) coincides with the findings of Tuesca et al. (2001), who argue that the conditions of greater light availability and temperature, typical of cultivated areas, would favor its germination and establishment. However, according to Khakzad et al. 2019, there should be less emergence under CT due to the small size of its seed which, being buried, could go into secondary dormancy or, in the case of germination, not have sufficient reserves to reach the surface.

Considering the fragility of the productive systems in the VIRN region and the need to optimize water resources, the implementation of conservation tillage is imperative, and it is important to know shifts in weed communities under these conditions. On the other hand, by having information on which weeds are expected to emerge, and at what time depending on the tillage system employed, farmers can plan appropriate control practices that can be implemented in a timely manner. This allows for more accurate and efficient management and consequently improving crop yield and quality.

By understanding the critical stages of weed emergence in horticultural crops, farmers can apply herbicides in a selective and targeted manner, thereby reducing the excessive use of chemicals and minimizing environmental impact. In addition, this information can help to schedule labor and machinery more efficiently, saving time and production costs.

CONCLUSIONS

The results concluded that there are important changes in the floristic composition and weed emergence patterns immediately after the implementation of 0T compared to CT, suggesting that the filtering pressures exerted by each

tillage system favor certain weed species over others, depending on their functional traits. Results show that under 0T, anemophilous Asteraceae, such as *Conyza bonariensis* and *Sonchus oleraceus*, increase their presence, whereas under CT, Brassicaceae with indehiscent fruiting such as *Raphanus sativus* and *Rapistrum rugosum,* emerge in abundance.

With this is contributing to improving weed invasion predictive models, developing more sophisticated management strategies and designing new tools and techniques for weed control. This research also provides a scientific basis for better understanding weed-crop interference, which in turn can drive further research in the field of sustainable agriculture.

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Growth and agronomic performance of soybean applied with pre-emergence herbicides

Crecimiento y desempeño agronómico de la soja aplicada con herbicidas pre-emergentes

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Keywords:
Growth regulators
Herbicide selectivity
Injury symptoms
Lodging
Plant height
Yield

ABSTRACT

Among the factors that limit the productive potential of soybean crops, stands out lodging, in addition to competition with weeds. Given this, the importance of soybean pre-emergence herbicides for different purposes is highlighted; however, they have to be evaluated for selectivity. This study aimed to evaluate the growth and agronomic performance of soybean applied with pre-emergence herbicides. Experiment 1 was conducted in the 2017-2018 season at two locations, and experiment 2 in the 2016-2017 season at one location. Treatments consisted of the application of pre-emergence herbicides in soybean in a randomized block design with four replications. Crop injury and chlorophyll indices (experiment 1) and variables related to agronomic performance (experiments 1 and 2) were assessed. Diclosulam and chlorimuron showed potential for application at pre-emergence to reduce plant height and consequently plant lodging. Moreover, diclosulam, chlorimuron, sulfentrazone, flumioxazin, s-metolachlor, pendimethalin, trifluralin, imazethapyr/flumioxazin, and oxyfluorfen did not negatively affect agronomic performance when applied at pre-emergence of soybean. This study evidenced the selectivity of pre-emergence herbicides to soybean.

Palabras clave:
Reguladores del crecimiento
Selectividad de herbicidas
Síntomas de lesiones
Encamado
Altura de planta
Productividad

RESUMEN

Entre los factores que limitan el potencial productivo de los cultivos de soya, se destaca el encamado, además de la competencia con las malas hierbas. Ante esto, se destaca la importancia de usar herbicidas en pre-emergencia de la soya para diferentes propósitos; sin embargo, es necesario evaluar su selectividad. Este estudio tuvo como objetivo evaluar el crecimiento y comportamiento agronómico de la soja bajo la aplicación de herbicidas en pre-emergencia. El experimento 1 se realizó en la cosecha entre 2017-2018 en dos sitios, el experimento 2 en la cosecha entre 2016-2017 en un sitio. Los tratamientos consistieron en la aplicación de herbicidas pre-emergentes en soja en un diseño de bloques al azar con cuatro repeticiones. Se evaluaron índices de daño al cultivo y de clorofila (experimento 1) y variables relacionadas con el comportamiento agronómico (experimentos 1 y 2). El diclosulam y el clorimuron mostraron potencial para su aplicación en soja en pre-emergencia para reducir la altura de las plantas y, en consecuencia, el encamado de las plantas. Además, diclosulam, clorimuron, sulfentrazona, flumioxazin, s-metolacloro, pendimetalina, trifuralina, imazetapir/flumioxazina y oxifluorfeno afectaron negativamente el rendimiento agronómico cuando se aplicaron en la soja de pre-emergencia. Este estudio evidenció la selectividad de los herbicidas de pre-emergencia a la soja.

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Lodging is defined as the process of plant falling or bending. This problem usually occurs from the flowering stage onwards and can extend until the full maturity of the grains, especially in cultivars with greater potential for vegetative growth (Kitabatake et al. 2019; Shan et al. 2022). Lodging can reduce grain yield and quality, favor the development of diseases and pest attacks (Knebel et al. 2006), and make mechanized harvesting difficult (Saitoh et al. 2012; Kitabatake et al. 2019). Thus, it is a limiting factor to the yield potential of soybeans (Hwang and Lee 2019; Shan et al. 2022).

Due to lodging, the proximity of leaves and shading of the upper part of the plant under the lower part is inevitable. In this case, there is a lower incidence of solar radiation on the leaves in the lower parts, reducing the photosynthesis of the shaded parts, the net photosynthesis of the plant community, and the availability of photoassimilates to be used in grain filling (Hussain et al. 2021; Shan et al. 2022). The genotype, high water availability, high soybean fertility, and spatial arrangement of plants, among other factors can contribute to soybean lodging. These factors can lead to massive vegetative growth and, consequently, plant lodging (Hwang and Lee 2019; Li et al. 2022).

Growth regulators or herbicides may be used pre- or post-emergence in soybean to minimize the chance of lodging. These can act to reduce the height or number of branches; however, these products must be selective to soybean plants, i.e., not reduce their yield (Ramesh and Ramprasad 2015). In this way, some can highlight some herbicides that can be applied at the pre-emergence of soybean. Osipe et al. (2014) observed a reduction in soybean plant height after applying chlorimuron, diclosulam, sulfentrazone, and flumetsulam, at pre-emergence, but without reducing yield.

In addition to lodging, another very important factor limiting soybean yield is competition with weeds. For example, in soybean crops, *Digitaria insularis* and *Conyza* spp., stand out, among other weeds. Studies highlight that few plants of these species can already reduce soybean yield by more than 40% (Gazziero et al. 2019). Thus, effective control of these weeds is necessary, but both have cases of glyphosate resistance. The application of pre-emergence herbicides in soybeans also deserves attention for weed control. Because of the possibility of using different modes of action, early management of weeds is one of the most critical aspects in preventing and selecting resistant biotypes and their control (Knezevic et al. 2019). Studies highlight the efficacy of diclosulam (Braz et al. 2017), chlorimuron (Sarangi and Jhala 2019), and sulfentrazone (Sarangi et al. 2017) in weed control and for pre-emergence application in soybean. As mentioned, these herbicides can also reduce the height of soybean plants, so soybean pre-emergence herbicides can be used for different purposes.

However, these herbicides must be selective for soybean plants, i.e., they cannot reduce yield. Some studies report symptoms of injury to soybeans with the application of diclosulam (Neto et al. 2009) and sulfentrazone (Belfry et al. 2016). Thus, the importance of using soybean pre-emergence herbicides for different purposes is highlighted, but they need to be evaluated for crop selectivity. Therefore, this study aimed to evaluate the growth and agronomic performance of soybean applied with pre-emergence herbicides.

MATERIALS AND METHODS

Two experiments (1 and 2) were conducted in a production area in the western region of the state of Paraná (PR), Brazil. Experiment 1 was conducted in the 2017-2018 growing season at two locations in the municipality of Palotina (**A**: 24°18'44" S 53°44'36" W, **B**: 24°20'50" S 53°51'38" W), and experiment 2 in the 2016-2017 growing season at a location in the municipality of Maripá (24°23'21.6"S 53°45'47.3"W). The prevailing climate of the region is Cfa (subtropical, with hot summers), by the Köppen classification. Figure 1 illustrates climatic data during the experimental period.

Experiment 1 (**A**) was carried out on soil with 68.75% clay, 15% silt, 16.25% sand, pH=5.3 ($CaCl_2$), and organic matter (OM) 24.94 g dm^{-3}. Formerly, the area had been planted with maize. Sowing was carried out under no-till on September 7, 2017. Monsoy® 6210 IPRO cultivar (Monsanto Co. Brasil, São Paulo, SP, Brazil) was used, which has an indeterminate growth habit, moderately resistant to lodging, 96 cm average height, 125-day average cycle, and 6.2 maturity group.

Experiment 1 (**B**) was carried out on soil with 69% clay, 15% silt, 16% sand, pH=4.7 ($CaCl_2$), and OM: 28.60 g dm^{-3}. Formerly, the area had been cultivated with wheat.

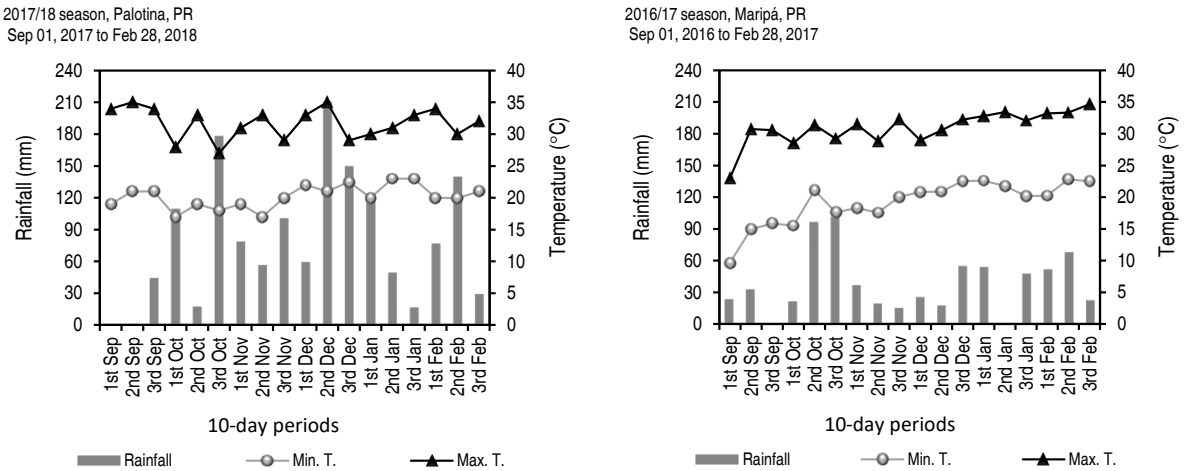

Figure 1. Representation of rainfall, minimum and maximum temperature for the experimental period.

Sowing was done in no-till conditions on October 16, 2017. TMG 7062 IPRO cultivar (Tropical Melhoramento & Genética, Cambé, PR, Brazil) was used. It has a semi-determinate growth habit, 125-day average cycle, and 6.2 maturity group. In both areas of experiment 1, fertilization was performed at sowing with 300 kg ha^{-1} 02-20-20 (N-P-K).

The experiments were a randomized block design with 3x5 m plots, with four replications. The treatments are described in Table 1. Herbicides were applied with a CO_2 pressurized backpack sprayer at a constant pressure of 2 bar, flow of 0.45 L min^{-1} flow rate of 150 L ha^{-1}, with a 3 m spray bar, equipped with six spray nozzles (XR 110.015), at a speed of 1 m s^{-1}.

Table 1. Treatments composed of the application of herbicides at soybean pre-emergence.

	Treatments	Commercial products	Rates[1]
Exp. 1	No application (with weeding)	-	-
	Diclosulam	Spider® 840 WG	33.6
	Chlorimuron	Classic®	37.5
	Sulfentrazone	Boral® 500 SC	600
	Flumioxazin	Flumyzin® 500	60
Exp. 2	No application (with weeding)	-	-
	S-metolachlor	Dual Gold®	1,920
	Pendimethalin	Herbadox® 400 EC	1,500
	Trifluralin	Trifluralina Nortox® Gold	2,250
	Diclosulam	Spider® 840 WG	37.5
	Imazethapyr/flumioxazin	Zethamaxx®	120/60
	Oxyfluorfen	Goal® BR	240

[1]Rates in g active ingredient (a.i.) ha^{-1}, in g acid equivalent (a.e.) ha^{-1} for imazethapyr.

The treatments were applied 1 day after planting for experiment 1 (**A**) on September 8, 2017, at a temperature of 24.4 °C, relative humidity of 49.9%, and wind speed of 2 km h^{-1}, without rain in the 10 days following the application. In experiment 1 (**B**), on October 7, 2017, at a temperature of 20.5 °C, relative humidity of 63.2%, and wind speed of 3 km h^{-1}, with 18 mm rain in the 10 days following the application, without rain on the day of application. For experiment 2, on September 8, 2016, at 25 °C, relative air humidity of 59%, and wind speed of

2 km h^{-1}, with 33 mm of rain in the 10 days following the application, without rain on the day of application.

For experiment 1, assessments were performed for injury symptoms at vegetative stages V_3 and V_6 of soybean plants, using the adaptation of the European Weed Research Council [EWRC] scale, with values from 0 to 9, with 0 for no damage (0% injury) and 9 for plant death (100% injury). Chlorophyll **A**, **B,** and total chlorophyll were determined with an electronic chlorophyll meter (clorofiLOG - CFL1030, Falker Automação Agrícola Ltda., Brazil). This equipment determines the Falker chlorophyll index (FCI). The chlorophyll index was always evaluated on the central leaf of the first fully expanded trifolium.

Plant height, number of pods and branches, and yield were analyzed in all experiments. Plant height was measured using a graduated ruler from the base of the plant to the last node with fully expanded trifolium in five plants per experimental plot at R_2 and $R_{7.2}$ stages. The first pod height was also determined during the height assessment at $R_{7.2}$. On this occasion, the number of branches and the number of pods were also counted in five plants per plot.

Harvesting was performed manually at the R_8 stage in the entire useful area of each plot. Pods were threshed in a thresher for experiments, cleaned, and packed in paper bags for further analysis. Grains produced in each plot were weighed and the moisture was corrected to 13%; from these data was calculated the yield in kg ha^{-1}.

Data were tested by analysis of variance (ANOVA) and F-test ($P \leq 0.05$). When significant, treatment means were grouped by Tukey's test ($P \leq 0.05$). All analyses were run in Sisvar 5.6 (Ferreira 2011).

RESULTS AND DISCUSSION

The results showed soybean injury caused by herbicide application in both areas of experiment 1. In addition, symptoms were found in assessments at V_3 and V_6, with values not exceeding 2.8 (EWRC scale). For chlorophyll indices, differences were detected between treatments only for the evaluation at V_6 in area B, when the application of diclosulam or chlorimuron reduced the index compared to the control treatment (no application) (Table 2).

Table 2. Crop injury and total chlorophyll index of soybean plants under application of herbicides in pre-emergence (experiment 1).

Treatment	Area A		Area B		Area A		Area B	
	V_3	V_6	V_3	V_6	V_3	V_6	V_3	V_6
	Crop injury (EWRC scale)				Chlorophyll index (FC)			
No application	0.0[a]	0.0[a]	0.0[a]	0.0[a]	36.6	35.3	38.5	41.1[ab]
Diclosulam	2.8[c]	1.5[b]	2.8[c]	2.0[b]	36.2	36.7	37.5	38.9[bc]
Chlorimuron	2.8[c]	2.3[b]	2.8[c]	2.5[b]	38.8	34.8	37.0	38.5[c]
Sulfentrazone	1.0[b]	1.5[b]	1.3[b]	1.5[b]	37.2	36.1	38.0	41.6[a]
Flumioxazin	1.5[b]	1.3[b]	1.0[b]	1.3[ab]	37.7	34.9	37.4	41.1[ab]
CV (%)	20.6	37.8	27.0	38.8	4.0	3.6	2.4	2.5
F	*	*	*	*	ns	ns	ns	*

*Means followed by different letters, in the same column, are significantly different by Tukey's test at the 5% probability level. ns: non-significant, means do not differ from each other by F-test ($P \geq 0.05$).

The application of chlorimuron can cause injuries to soybeans, especially when applied post-emergence of soybeans (Cesco et al. 2018; Silva et al. 2018a). For pre-emergence application, injury in soybeans is less common, but can also be observed, but in general without compromising agronomic performance (Belfry et al. 2015; Priess et al. 2020), as observed in the present study. Also, for the application of diclosulam in pre- or post-emergence of soybeans, symptoms of injury can be observed, in general, up to two weeks after application (Neto et al. 2009; Braz et al. 2017), with subsequent recovery of soybean plants.

Some differences between treatments were found for plant height (experiment 1), mainly for diclosulam, which reduced the height of soybean plants, in the assessments at R_2 (to 31.3 cm) and $R_{7.2}$ (to 46.7 cm). For chlorimuron, at $R_{7.2}$ (to 51.9 cm), these effects were observed in area **B**. For the number of pods and branches per plant, there were no differences in both areas (Table 3). For the first pod height, some differences were verified in area **A**, with a reduction for the application of chlorimuron or sulfentrazone.

The application of diclosulam also reduced soybean plant height by 36% at 30 DAA in another study (Dalazen et al. 2020). Herbicides sulfentrazone and flumioxazin can also reduce the height of soybean plants, as reported by Barbosa et al. (2023), but mainly for post-emergence soybean application. In the present study, herbicides that caused height reduction were applied in soybean pre-emergence.

For 1,000-grain mass and yield, in both areas, no differences were detected between treatments (Table 4). Table 5 lists the results of experiment 2. No differences were registered between treatments for the analyzed variables, an exception to that observed for height at R_2; however, no herbicide reduced the height about the treatment with weeding (no application).

Table 3. Height (H), number of pods and branches of soybean plants under application of herbicides in pre-emergence (experiment 1).

Treatment	Area A		Area B		Area A		Area B	
	H R_2	H $R_{7.2}$	H R_2	H $R_{7.2}$	Pods	Branches	Pods	Branches
	(cm)							
No application	33.0^{ab}	117.7	57.0^{b}	125.6^{b}	86.9	2.2	43.8	1.1
Diclosulam	31.3^{a}	114.2	46.7^{a}	114.6^{a}	81.2	2.0	42.4	1.1
Chlorimuron	31.9^{a}	118.4	51.9^{b}	117.0^{a}	92.3	2.0	46.6	1.1
Sulfentrazone	32.2^{a}	117.7	55.6^{bc}	124.6^{b}	75.4	2.1	42.2	1.0
Flumioxazin	35.3^{b}	118.1	55.9^{bc}	129.7^{b}	87.3	2.1	49.5	1.1
CV (%)	3.6	2.5	3.64	2.50	11.0	5.4	11.2	10.9
F	*	ns	*	*	ns	ns	ns	ns

*Means followed by different letters, in the same column, are significantly different by Tukey's test at the 5% probability level. ns: non-significant, means do not differ from each other by F-test ($P \geq 0.05$).

Table 4. First pod height (FPH), 1,000-grain mass (GM), and yield of soybean plants under application of herbicides in pre-emergence (experiment 1).

Treatment	Area A	Area B	Area A	Area B	Area A	Area B
	FPH		GM		Yield	
	(cm)		(g)		(kg ha^{-1})	
No application	22.1^{b}	28.1	137.6	174.3	4,219	3,955
Diclosulam	20.2^{ab}	25.3	140.8	176.8	3,798	4,281
Chlorimuron	16.1^{a}	25.4	139.0	171.7	3,963	4,376
Sulfentrazone	16.8^{a}	27.2	140.3	173.6	4,215	4,058
Flumioxazin	18.1^{ab}	28.6	139.5	177.9	3,891	4,475
CV (%)	12.2	10.0	30.3	26.7	3.7	11.4
F	*	ns	ns	ns	ns	ns

*Means followed by different letters, in the same column, are significantly different by Tukey's test at the 5% probability level. ns: non-significant, means do not differ from each other by F-test ($P \geq 0.05$).

Table 5. Height (H), first pod height (FPH), number of pods, 1,000-grain mass (GM), and yield of soybean plants under application of herbicides in pre-emergence (experiment 2).

Treatments	H R_2	H $R_{7.2}$	FPH	Pods	GM	Yield
		(cm)			(g)	(kg ha^{-1})
No application	64.4^{ab}	73.2	17.9	45.9	195.6	5,778
S-metolachlor	62.0^{ab}	73.0	17.7	47.8	188.3	5,320
Pendimethalin	63.8^{ab}	74.4	17.9	46.8	182.1	5,509
Trifluralin	62.5^{ab}	71.1	17.5	46.2	192.4	5,564
Diclosulam	59.9^{b}	72.0	17.3	46.6	179.5	5,367
Imazethapyr/flumioxazin	62.8^{ab}	74.1	18.4	47.3	184.5	5,425
Oxyfluorfen	65.9^{a}	76.5	18.1	48.9	185.4	5,938
CV (%)	4.0	5.0	5.4	9.1	7.7	10.1
F	*	ns	ns	ns	ns	ns

*Means followed by different letters, in the same column, are significantly different by Tukey's test at the 5% probability level. ns: non-significant, means do not differ from each other by F-test ($P \geq 0.05$).

Regarding the potential use of pre-emergence herbicides to reduce the height of soybean plants, chlorimuron and diclosulam can be highlighted. In some situations, as in this study, these herbicides have reduced plant height, which makes plants less susceptible to lodging. Cesco et al. (2018) reported a reduction in the height of soybean plants for the post-emergence application of chlorimuron (17.5 g active ingredient [a.i.] ha^{-1}) + glyphosate. Similar to that observed by Dias et al. (2019) for the post-emergence application of chlorimuron in soybean.

Osipe et al. (2014) reported a reduction in soybean plant height for the application of chlorimuron (20 g a.i. ha^{-1}) and diclosulam (25.2 g a.i. ha^{-1}) in pre-emergence of soybean, as found in the present study. This reinforces the potential of these herbicides for this purpose in soybean. Likewise, Neto et al. (2009) with the application of diclosulam + glyphosate, also in the pre-emergence of soybean.

Several factors can contribute to soybean lodging, including genotype, high water availability, high soybean fertility, and spatial arrangement of plants, among others. These factors can lead to incredible vegetative growth and, consequently, plant lodging (Hwang and Lee 2019; Raza et al. 2020). Thus, the decrease in soybean plant height is essential in reducing plant lodging. In this sense, herbicides chlorimuron and diclosulam, as demonstrated in this study, can be used to reduce the height of soybean plants without a negative impact on other yield components.

Although the other herbicides did not reduce the height of soybean plants, the results in this study classify them as selective for soybean, which is very important because these herbicides have great potential for weed management in soybean. In turn, chlorimuron and diclosulam, in addition to their potential to reduce the height of soybean plants, were also selective for the crop. In other studies, diclosulam was also selective for soybean in different combinations (Osipe et al. 2014; Braz et al. 2017). Also, chlorimuron was selective for soybean, as demonstrated by Silva et al. (2018b); Albrecht et al. (2018); Abugho et al. (2019), among others.

Other studies report the selectivity of sulfentrazone (Belfry et al. 2016), flumioxazin (Hay et al. 2019), s-metolachlor (Fornazza et al. 2018), pendimethalin (Yadav et al. 2017), trifluralin (Movahedpour et al. 2010), imazethapyr (Belfry et al. 2015), and oxyfluorfen (Cataneo et al. 2005) for soybean. The use of pre-emergence herbicides with different mechanisms of action and mixtures of these herbicides are fundamental for the management and prevention of selection of herbicide-resistant weed biotypes. The results obtained in this study evidence the selectivity of herbicides for soybean, including for application of imazethapyr/flumioxazin (premix formulation), which combines two different mechanisms of action.

CONCLUSIONS

Diclosulam and chlorimuron showed potential for application at pre-emergence of soybean to reduce plant height and consequently plant lodging. Moreover, diclosulam, chlorimuron, sulfentrazone, flumioxazin, s-metolachlor, pendimethalin, trifluralin, imazethapyr/flumioxazin, and oxyfluorfen did not negatively affect agronomic performance when applied at pre-emergence of soybean. This study evidences the selectivity of pre-emergence herbicides for soybean.

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Influence of drying air temperature on coffee quality during storage

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Influencia de la temperatura del aire de secado sobre la calidad del café durante el almacenamiento

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ABSTRACT

Drying is the most important stage for maintaining coffee quality. The temperature conditions at which drying is performed can affect bean integrity. This research was developed with the aim of determining the effect of mechanical drying air temperature on the quality of coffee during storage and verifying its effect on the generation of bleached beans evaluating two air temperatures at 50 and 40 °C, and solar drying was used as a control, using an experimental design of random blocks with 10 blocks. The response variables were related to beans color and sensory quality. The analysis of repeated measures indicated that there were differences in the initial color of the coffee beans due to the effect of the treatments and the storage time. A greater magnitude of color change was obtained for coffee dried at 50 °C and that dried with solar drying. Germination was lower and different for the 50 °C treatment. This treatment also showed greater fat content since the beginning of the storage; meanwhile, the two other treatments just presented greater fat content at the end of the experiment. Regarding to sample proportion of clean cups, the multiple comparison Tukey–Kramer test was significantly different in terms of favoring solar drying at 40 °C. The effect of the drying conditions on beans has not been appreciated; however, the deterioration generated during this stage occurs during storage and manifests itself in a loss of quality, with an increase in defects.

Keywords:
Coffee arabica
Colorimetry
Germination
Sensory evaluation
Total fat content

RESUMEN

El secado en café es la etapa de conservación más importante para mantener la calidad. Las condiciones de temperatura a las que se realiza pueden afectar la integridad del grano. Esta investigación se desarrolló con el objetivo de determinar el efecto de la temperatura del aire de secado mecánico sobre la calidad del café durante el almacenamiento y comprobar su efecto sobre la generación de granos blanqueados, evaluando dos temperaturas del aire a 50 y 40 °C, utilizando como testigo el secado solar, bajo un diseño experimental de bloques al azar con 10 bloques. Las variables de respuesta fueron las relacionadas con el color de los granos y la calidad sensorial. El análisis de medidas repetidas indicó que hubo diferencias en el color inicial de los granos de café por efecto de los tratamientos y el tiempo de almacenamiento. Se obtuvo mayor magnitud de cambio de color con mayores valores para el café que fue obtenido a 50 °C y secado solar. El porcentaje de germinación fue inferior y diferente para 50 °C. Así mismo, para este tratamiento, el contenido de grasa fue mayor desde el inicio del almacenamiento, mientras que para 40 °C y secado solar se incrementó al final del experimento. La prueba de comparación múltiple de Tukey-Kramer, respecto a la proporción de muestras con taza limpia fue significativamente diferente a favor del secado solar y a 40 °C. El efecto de las condiciones de secado sobre el grano no se aprecia inicialmente; sin embargo, el deterioro generado durante esta etapa se revela durante el almacenamiento y se manifiesta en una pérdida de la calidad, con el aumento de defectos.

Palabras clave:
Café arábica
Colorimetría
Germinación
Evaluación sensorial
Contenido de grasa total

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The drying process for mild-washed coffees is an essential stage for maintaining the coffee quality. A decreased moisture content helps to preserve the coffee beans as this moisture loss reduces both its physiological and the microorganisms' activities, which in turn affects cleanliness, especially during storage (Broissin-Vargas et al. 2018). Washed coffee has a high moisture content (53% w.b.), which, in the shortest possible time must be reduced to preserve the quality of the beans down to levels that allow storage without deterioration, which is in the range between 10 and 12% w.b. Coffee in this state is referred to as dry parchment coffee (dpc). To achieve this stage, the natural energy of the air and solar radiation are used to dry coffee, or hot air is forced through a coffee bed in mechanical drying. In Colombia, the use of each of these systems depends on the size of the farms and coffee production. Solar drying is used more frequently on farms with production less than 3,500 kg dpc year^{-1}, although it is also used as a backup on larger farms, in times of low production (Parra et al. 2017).

Mechanical drying is used by a small number of coffee producers who produce a large amount of coffee. It is estimated that more than 30% of annual production corresponds to approximately 27,650 coffee farms with more than 5 ha that use this type of technology (Parra et al. 2017). The large amount of coffee that is dried by coffee grower cooperatives and drying centers should also be considered; some data have suggested that more than 70% of the coffee produced in Colombia is dried mechanically (Gutiérrez et al. 2012). Given this scenery, in Colombia, recommendations and technological developments have focused on achieving the greatest technical and economic efficiency with respect to the main parameters of the mechanical equipment used to drying coffee (Parra et al. 2017; Parra-Coronado et al. 2008).

Frequently, in comparison to other dynamic dryer types, fixed bed coffee dryers are often used given their economic and maintenance advantages as well as their acceptable thermal efficiency. The dryer type involves placing two or three wet parchment coffee beds that can vary between 20 and 40 cm in thickness, in a series setup. For the fixed-bed coffee dryers, two fundamental conditions are taken into account: the air temperature and the airflow generated by the fan. Regarding the air flow, the research shows that the optimal air flow is 0.1 m^3 min^{-1} kg^{-1} dpc to obtain uniformity in the final moisture of the mass that is drying (Parra et al. 2017). Regarding quality, research results show that variations in flow (0.024, 0.06, and 0.1 m^3 min^{-1} kg^{-1} dpc) have no effect on cup quality (Largo 2020) or on the physiological characteristics of the bean (Alves et al. 2017; Taveira et al. 2012).

In relation to the air temperature, 50 °C is recommended to attain the lowest energy requirement, without dramatic changes in coffee bean structure. Studies reported by Roa et al. (1999) showed that an air temperature of 50 °C generated a loss of viability for 43% of the coffee seeds in the first 3 h of drying, and 95% of the seeds were maintained with germination capacity after drying at 38 °C. Sierra et al. (1990) found that germination began to decrease when the drying temperature was above 40 °C. The same authors found that this phenomenon remained independent of grain moisture and that germination was drastically reduced when the final grain moisture was below 10% w.b.

On the other hand, coffee bean color is the first indicator of physical quality since it can be observed, so it is necessary to maintain this characteristic as long as possible. However, there are some alterations noticeable after storage and transport, such as loss of color or blanching, which result in coffee receiving a lower price in the market, as the alterations are associated with a loss of quality. Thus, several investigations have focused on identifying the cause of this deterioration and exploring the association among drying, packaging, and storage conditions or their combination. Roa et al. (1992) carried out a study on storage conditions, concerned about the loss of coffee quality during this stage when the coffee is packed in fique sacks. The researchers mention that after a year coffee has a blanching defect practically anywhere in the coffee zone, especially in warm places. In the study, they found that the conditions of 15 °C and relative humidity of 65% are adequate so that the coffee in bulk does not deteriorate and they showed that the coffee stored in sacks of fique has an average of 22% discolored grains after 1 year of storage.

Different studies have shown that variations in air temperature have an effect on quality and an inverse relationship with the increase in temperature during

mechanical drying, especially when the temperature exceeds 50 °C (Taveira et al. 2012; Oliveira et al. 2013; Borém et al. 2014; Alves et al. 2017). Therefore, some coffee buyers prefer coffee processed in solar dryers. The exclusive use of solar drying does not have the advantages of mechanical drying in terms of a reduction in processing time, greater volumes of processed coffee, and a decrease in risks to the quality and safety of the coffee. Solar drying is especially risky in harvest peaks because this time is characterized by low solar radiation and high relative humidity due to excessive rain, as drying time can be extended for weeks to obtain coffee within the commercial moisture range.

Regarding storage, it has been determined that the least favorable quality occurs when the temperature and the relative humidity of the environment are not controlled (Broissin-Vargas et al. 2018). Similarly, packaging coffee in fique bags generates negative effects on its physical, chemical, and physiological characteristics (Borém et al. 2014, 2019; Rendón et al. 2014). In contrast, the use of hermetic packaging allows atmospheric modification and control to preserve coffee quality, and this system has been used successfully to maintain coffee quality (Donovan et al. 2019; Borém et al. 2013; Selmar 2008).

This study was developed under the hypothesis that a mechanical drying air temperature of 50 °C generates endosperm blanching and deterioration of the quality during storage. For this, the wet coffee process, and the after-drying stages such as packaging and storage were kept constant, in order to determine the effect of drying temperature on bean color and coffee quality.

MATERIALS AND METHODS

Location

This research was conducted at the Cenicafé experimental mill, located in Chinchiná Caldas, Colombia, at an altitude of 1,310 m, with an average annual temperature of 21.2 °C and a relative humidity of 78%. Castillo® variety coffee was used, produced at altitude of 1,321 m.

Experimental design. A complete random blocks design (DRB), with 10 blocks, was used to test three treatments, which consisted of two air temperatures during mechanical drying, a fixed specific airflow, and sun-dried coffee as control:

T1: Drying with forced air at 40 °C and 0.1 $m^3\ min^{-1}\ kg^{-1}$ dpc.
T2: Drying with forced air at 50 °C and 0.1 $m^3\ min^{-1}\ kg^{-1}$ dpc.
T3: Solar drying in a parabolic tunnel dryer.

To maintain the specific airflow constant, a U-shaped manometer was used to measure the air static pressure before passing through the coffee layer, to be included in the Equation (1) (Oliveros and Roa 1986), with which the airflow is obtained.

$$\frac{Q}{A} - (9{,}523 - 0.0476M)\left|\frac{\Delta p}{L}\right|^{0.676} \quad (1)$$

Where Q is the airflow rate in $m^3\ min^{-1}$, A the dryer area in m^2, Δp is the pressure drop occurred between the plenum and the air outlet after passing through the grain layer in cm of water column (by U manometer), M moisture in percentage and L the thickness of the coffee layer in meters. Work units of 65 kg of washed coffee (53% w.b.) were completely randomized among treatments.

Coffee processing. Approximately 500 kg of coffee fruits were processed, and the wetting process was carried out, with classification, pulping, fermenting, and washing to obtain the washed coffee (work units). The obtained washed coffee was placed under the drying conditions of each treatment, for which two fixed-bed mechanical dryers with 37.5 kg of dpc capacity were available, with electric resistances to heat the air; the solar drying treatment used a tunnel-type dryer with an area of 10 m^2. During the drying process, the coffee bean's moisture, air temperature, relative humidity, and beans temperature were monitored. The drying processes ended when the coffee reached a moisture between 10.5 and 11.5% w.b.

Approximately 32.5 kg of dpc were obtained from each treatment, and this amount was stored for 15 days, after which the coffee was husked. The coffee obtained was packed and stored in double-film microperforated plastic bags in a room for 12 months at 15±1 °C and 70±5% relative humidity, according to the best storage conditions for green coffee obtained by Roa et al. (1992). Samples of 500 g were taken out every two months to perform sensory quality tests.

The response variables were the color change of the coffee beans and sensory quality. The other variables were

considered complementary. The analyses performed are described below.

Colorimetry analysis. A Konica Minolta CR 410c colorimeter (Illuminant C, 2° angle) was used. The determination of the color coordinates was performed in a device with controlled illumination, focusing on the coffee sample contained in dishes with a depth of 1.5 cm. The color determination procedure was standardized using 20 g samples of healthy coffee beans without parchment retained by a 14-sieve. The value for each sample over time corresponded to the average of six measurements.

The system was programmed to work in the CIEL*a*b* color space in which there are three readings: Lightness (L*) which measures the intensity of the reflected color with values between 0 and 100, where 0 is black and 100 is white. Variable a* measures the amount of green or red in an image, being negative for green and positive for red, and the variable b* measures the amount of blue and yellow in the image, being negative for blue and positive for yellow. Since the handling of color intensities in computers is between 1 and 255 the values for a* and b* vary between -128 and 127.

To determine the color difference during storage, the magnitude of the color change was determined and defined as the numerical comparison of a sample with the standard or the initial color of the coffee. This comparison indicates the differences in each of the delta color coordinates (Δ). To estimate the total difference, delta E (ΔE*), the following Equation (2) was used:

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad (2)$$

Sensory quality analysis [SCA points]. An expert panel in coffee cupping, composed of at least three tasters with Q-Grader certificates, performed the analysis of the samples using the Specialty Coffee Association - SCA protocol, and the results showed the individual scores of each attribute and the total score received.

Coffee moisture [% w.b.]. Coffee moisture was determined by the gravimetric method in an oven, according to ISO 6673 (2003), both at the beginning and at the end of storage.

$$M = \frac{m_i - m_o}{m_i} \qquad (3)$$

Where M is the decimal moisture on a wet basis, m_i is the mass with which it enters the stove in grams, and m_0 is the mass with which it reaches constant weight in grams.

Water activity - a_w. This thermodynamic variable measures the susceptibility of a material to be damaged by microorganisms and is given by the following Equation 4:

$$a_w = \frac{p_v}{p_v^*} \qquad (4)$$

Where a_w is the decimal water activity, p_v is the vapor pressure in the coffee beans, and p_v^* is the vapor pressure of pure water under the same temperature conditions. Water activity was determined using a LabMaster-aw neo (Novasina) water activity analyzer with temperature compensation.

Germination [%]. To indicate the physiological quality, 400 coffee beans were taken from the samples of each treatment, and these were divided into subsamples of 50 beans, from which the parchment or endocarp were manually removed, providing eight repetitions. The beans were moistened in excess and then placed in butter boxes for 25 days, after which the number of beans that had germinated was counted to estimate the percentage, in numbers, of germinated beans.

Fat content [% db]. The total fat content in coffee beans was determined at the beginning and end of storage by the Soxhlet method (AOAC 920.39).

Statistical analysis. The analysis was performed using the statistical program SAS version 9.4 TS Level 1M7. To analyze the color of the beans during storage, an analysis of repeated measurements was performed for each of the color coordinates, using the three drying conditions and the storage time as variation factors. From the analysis of mixed models, the best model was the one in which the covariances were adjusted under a first-order autoregressive structure. The main effect obtained was analyzed from the analysis of orthogonal polynomials to establish the behavior of the variable.

For cup quality, a 5% analysis of variance was performed taking into account the treatment or drying condition. An analysis of multiple comparisons between treatments was performed using the Tukey–Kramer test for the percentage of samples with a cup without defect. For the other variables, averages, and variations were obtained using the 5% confidence interval.

RESULTS AND DISCUSSION

The drying time for each treatment was between 16 and 21 h for 50 °C, between 41 and 49 h for 40 °C, and between 6 and 14 days for solar drying. For solar drying, the temperature recorded inside the dryer was an average of 23.8 °C, with a range from 15 to 38 °C. Similarly, the temperature of the bean in the treatments was approximately 2 °C below the drying temperature, and the smallest difference was observed at the end of the drying. The coffee moisture was between 10.8 and 11.0% w.b., as established in the methodology, corresponding to water activity values below 0.59. After 12 months of storage, the moisture of the beans remained within the commercial range (Table 1).

Table 1. Average and standard deviation (SD) for moisture (w.b.) and water activity (a_w) of dry parchment coffee obtained under different drying conditions.

Type of drying	Air temperature (°C)	Moisture at the beginning of storage (% w.b.)		Moisture after storage (% w.b.)		Water activity (a_w)	
		Average	SD	Average	SD	Average	SD
Mechanical	50	10.8	0.2	11.0	1.1	0.588	0.024
	40	10.9	0.2	11.7	0.2	0.586	0.023
Solar	Variable	11.0	0.4	11.5	0.2	0.588	0.041

Both the water activity and bean moisture-maintained product stability throughout storage. This was achieved mainly due to the combination of two factors: temperature and relative humidity control and the type of packaging used, which allowed the hygroscopic equilibrium conditions of the green coffee bean to be maintained, as determined by Trejos-Rodríguez et al. (1989). Studies conducted by Donovan et al. (2019) with different types of packaging demonstrated that in comparison to jute packaging, double film packing helps to protect the coffee, preventing changes in moisture during storage. Jute packaging in combination with storage under variable conditions of humidity and temperature that occur naturally in the environment generates color change, an increase in the populations of filamentous fungi, and a loss of general quality (Borém et al. 2019; Broissin-Vargas et al. 2018).

Coffee bean color analysis

After drying, a coffee bean has a characteristic bluish-green color when it has a moisture close to 11% w.b. This color tends to be lighter, turning green–yellow when it has a moisture lower than 10%, because this color indicates the beans are over dry and is considered a physical defect of coffee that is easily detected after drying. This scenario did not occur in this study since the coffee obtained by the different drying methods presented moisture levels close to 11% and were comparable. The differences in color resulting from the treatments occurred mainly in the beans obtained by solar drying. The a* and b* values indicated that this type of bean presented a more green and yellow coloration from the beginning of storage, probably due to the degradation of coffee pigments generated during drying with exposure to solar radiation, which does not occur when the coffee is mechanically dried as it is confined in a space without light exposure.

Tables 2, 3, and 4 show the coordinates L*, a* and b* color values, with respect to the storage time. The analysis of repeated measurements performed for each of the color coordinates showed a significant effect for the luminosity coordinate (L*) due to the effect of storage time (P<0.001), indicating a lighter color after 12 months with respect to the initial color. For coordinates a* and b*, there was a significant effect for the treatment and storage time factors (P<0.001, for both cases) but not for the interaction P=0.6410 and P=0.9688 for a* and b*, respectively. This result indicates that the changes in

Table 2. Average and standard deviation (SD) for variable L* according to storage time and type of drying.

Storage time (months)	Type of drying (treatment)						General time	
	40 °C		50 °C		Solar			
	Average	SD	Average	SD	Average	SD	Average	SD
0	75.12	3.17	75.61	3.33	76.73	2.83	75.82	3.08
2	76.33	2.93	77.43	2.23	77.50	2.20	77.08	2.45
4	78.06	2.47	78.93	2.66	79.78	1.77	78.89	2.37
6	77.83	2.07	78.70	2.32	79.81	2.01	7879	2.22
8	78.38	1.23	79.79	1.54	80.28	1.72	79.48	1.67
10	77.93	2.07	79.99	2.73	80.62	1.64	79.59	2.33
12	77.30	3.52	78.73	4.31	79.59	3.14	78.64	3.57
General treatment	77.25	2.67	78.33	2.97	79.14	2.55	78.25	2.83

Table 3. Average and standard deviation (SD) for variable a* according to storage time and type of drying.

Storage time (months)	Type of drying (treatment)						General time	
	40 °C		50 °C		Solar			
	Average	SD	Average	SD	Average	SD	Average	SD
0	-2.67	0.48	-2.60	0.33	-1.55	0.28	-2.27	0.63
2	-2.56	0.46	-2.69	0.25	-1.62	0.15	-2.29	0.57
4	-2.84	0.38	-2.94	0.34	-1.74	0.18	-2.53	0.62
6	-2.89	0.37	-2.95	0.39	-1.77	0.26	-2.52	0.65
8	-3.00	0.52	-3.10	0.33	-1.81	0.22	-2.63	0.70
10	-3.20	0.37	-3.33	0.20	-1.97	0.18	-2.73	0.70
12	-3.08	0.31	-3.19	0.25	-1.95	0.26	-2.66	0.65
General treatment	-2.86	0.46	-2.93	0.38	-1.76	0.26	-2.50	0.66

Table 4. Average and standard deviation (SD) for variable b* according to storage time and type of drying.

Storage time (months)	Type of drying (treatment)						General time	
	40 °C		50 °C		Solar			
	Average	SD	Average	SD	Average	SD	Average	SD
0	12.66	0.99	13.06	0.75	11.26	0.59	12.33	1.10
2	12.90	0.70	13.25	0.67	11.65	0.83	12.60	1.00
4	13.47	0.67	13.59	0.72	12.06	0.73	13.07	0.98
6	13.38	0.59	13.37	0.65	12.19	0.78	12.97	0.87
8	13.42	0.57	13.63	0.44	12.16	0.98	13.07	0.94
10	13.40	0.71	13.86	0.80	12.47	0.86	13.14	0.97
12	13.12	0.90	13.35	1.15	12.16	1.10	12.81	1.14
General treatment	13.19	0.76	13.43	0.73	11.98	0.89	12.84	1.02

these color coordinates occurred by the effects of drying or storage, and not by their combination.

Discoloration in beans or bleaching was perceived, in all the treatments, after several months of storage (Figure 1). Previous studies have shown that changes in a* and b* coordinates values, from green–yellow to very light yellow, indicate oxidative processes and biochemical degradation. These changes cause differences in flavor and aroma precursor compounds composition that are reflected in a loss of final quality, related to effects on the bean that occurred during processing, drying, packaging, and storing under different conditions (Borém et al. 2019; Broissin-Vargas et al. 2018; Abreu et al. 2015; Borém 2014).

Figure 1. Color of coffee beans at the beginning (A) and at the end of storage (B).

Complementary to the above, the magnitude of the color change encompassed the three coordinates (**ΔE**), which increased with storage time, but this increase was lower for coffee obtained at 40 °C (Figure 2). The values obtained for the first two months were similar for the three treatments, which indicates that color changes were more easily perceived after four months, especially for coffee obtained by solar drying; however, the color changed during the last two evaluation periods, with the solar drying treatment presenting the greatest color change with respect to the initial color. A maximum color change was also observed after eight months of storage for coffee at 50 °C, which may indicate maximum deterioration of the bean, while for the other treatments, the color change continued.

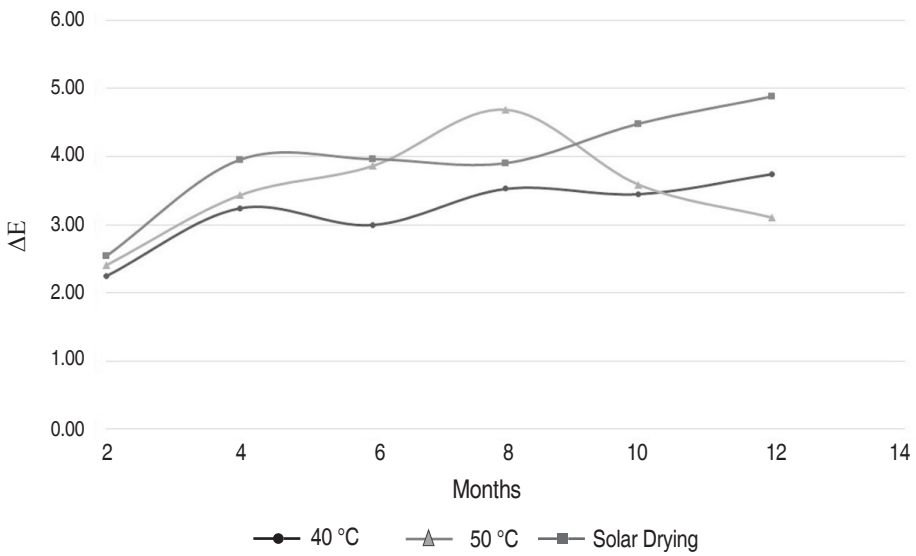

Figure 2. Magnitude of color change (ΔE), for color coordinates L*a*b* for the coffee obtained in the different drying conditions during storage.

Sensory quality

From each treatment and rep, samples were taken at 0, 2, 4, 6, 8, 10 and 12 months to evaluate sensory quality based on the SCA score. Additionally, the proportion of clean cups was calculated for each treatment.

The coffee obtained by drying conditions was comparable quality since no significant differences were found due to the effect of the treatments (*P*=0.3042), as the quality value was close to 81 SCA points (Table 5). This result indicates that the differences in drying did not allow to easily perceive differences in quality, at least at the beginning of storage.

During storage, 186 samples were obtained for sensory analyses. 65% of the samples had no defects in the cup, maintaining a score with an average higher than 80 SCA points (Table 5). However, all the samples of the 50 °C treatment showed defects after 8 months. Therefore, there were not samples to send for cup quality analyses for the months 10 and 12.

Table 5. Average and confidence interval for the sensory quality analysis (SCA points) for the coffee obtained under different drying conditions during storage.

Type of drying	Months						
	0	2	4	6	8	10	12
Mechanical (50 °C)	81.0±0.7	81.9±1.9	81.0±1.1	80.9±1.8	80.5±1.7	-	-
Mechanical (40 °C)	80.9±1.1	81.6±1.2	81.9±0.7	81.4±1.2	81.1±0.5	80.8±0.4	80.8±0.7
Solar	80.6±0.7	81.5±0.8	81.0±0.9	81.2±0.8	81.3±0.7	80.6±0.7	80.7±0.7

A generalized linear model was used to evaluate the proportion of clean cups related to the type of drying and storage. This model was conducted by assuming a response variable with binomial distribution and logit as a link function. As a result, type of drying was significant (*P*=0.0006).

The multiple comparison Tukey–Kramer performed on the proportion of treatment samples with a clean cup showed a significant effect (*P*=0.0098) between the two temperatures used for mechanical drying, with the probability of obtaining a clean cup during storage being significantly higher when the coffee was dried at 40 °C. Similarly, the comparison between 50 °C coffee and solar drying in terms of a clean cup was significant, favoring the latter (*P*=0.0005). The difference between 40 °C and solar drying was not significant (*P*=0.1141).

These results are consistent with the results obtained by Largo (2020), in which the coffee quality from mechanical drying at 40 °C and by a solar dryer did not present significant differences in terms of clean cups. However, Suárez et al. (2018) obtained the best coffee quality with drying at 50 °C compared to that obtained with solar drying and different temperatures and pre-drying times. This result can be explained by the bean cellular structure. Studies carried out by Borém et al. (2008), in which the plasma membrane integrity was observed in the endosperm cells in coffee beans that were dried at 40 °C, while the beans dried at 50 °C disorganized bean membrane system was identified. Consequently, could have been related to the lower quality and damage caused by the temperature during drying.

The photographs (Scanning Electron Microscope, SEM) show the bean structure taken with a zoom of 100 µm (Figure 3). The more defined porous structure of the coffee bean can be observed and corresponds to solar drying. This structure was lost with mechanical drying, especially with the higher drying temperature.

A relationship was also found between the coffee fruits maturity and 50 °C drying air temperature. When were present more than 70% of overripe fruits, color 7 in Cromacafé® (Peñuela-Martínez et al. 2022) there was a greater probability of presenting a fermented defect that had become more evident with storage advancement. In studies conducted by Velásquez et al. (2021), some physical characteristics of coffee beans from different ripeness states were found to influence the thermodynamic properties, which affected the drying behavior, especially when the drying rate was

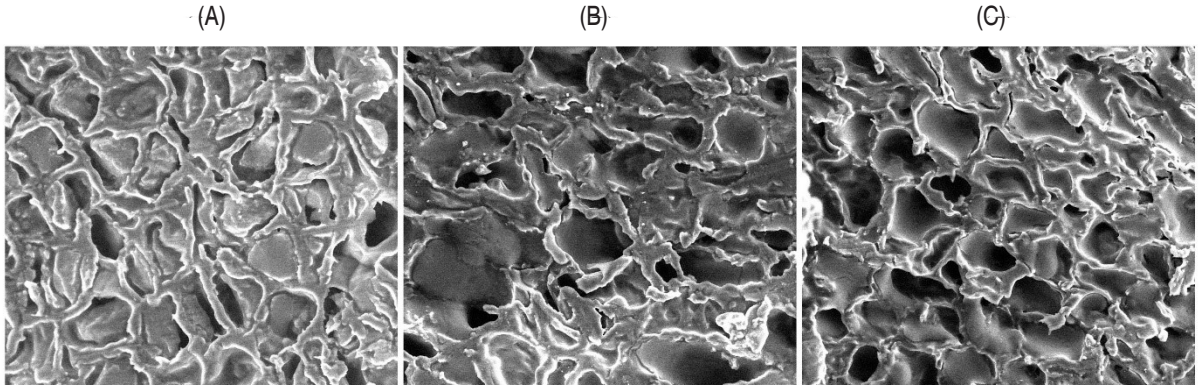

Figure 3. Scanning electron microscopy of coffee beans obtained under different drying conditions. (A) Mechanical drying at 50 °C, (B) mechanical drying at 40 °C, and (C) solar drying.

constant. In this study, it was shown that color 7, which corresponds to the overripe coffee fruit, presented the greatest differences, which was related to the greater susceptibility of the bean to moisture loss under certain drying conditions, such as high temperatures. These differences were associated with damage of endosperm cell's structure (Borém et al. 2008, 2013) and releasing the lipids. The lipids are unsaturated fatty acids approximately 50% that are easily oxidized and alter the coffee composition (Rendón et al. 2014) then perceived as a fermented defect in coffee beverage.

Additionally, the drying conditions generated differences in bean germination (Figure 4A), which was lower with 50 °C. These beans showed greater susceptibility to contamination by filamentous fungi during the germination tests, probably due to deterioration of the embryo and the loss of the cell wall under this condition. In a coffee bean, the heat generated during drying affects the embryo; therefore, this is indirect evidence that the seed physiological quality can be related to the quality of the coffee, especially when washed coffees are processed (Alves et al. 2017; Oliveira et al. 2013).

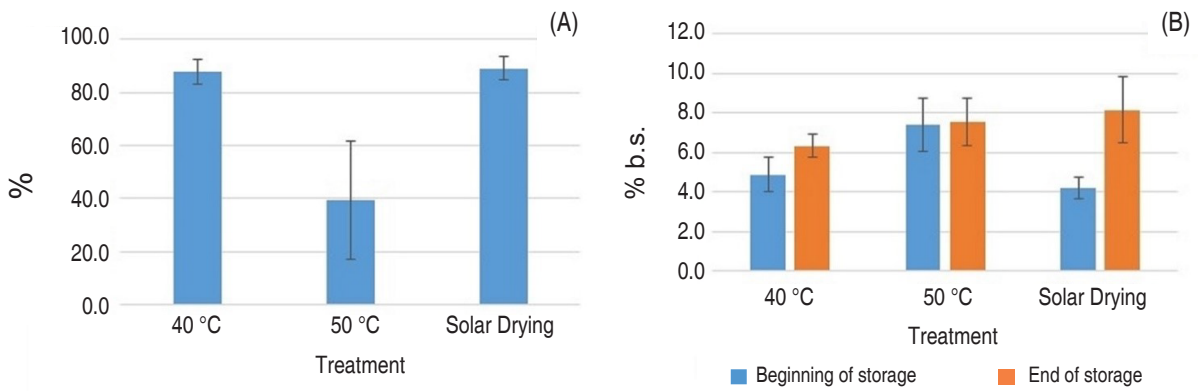

Figure 4. Averages and confidence intervals for the variables related to physiological quality of the beans. (A) Percent germination and (B) total fat content at the beginning and end of storage.

Complementary to the above, the fat content in coffee can also indicate deterioration of bean cellular structure since an inverse relationship has been identified between this variable and sensory quality (Rendón et al. 2014; Oliveira et al. 2013). In Figure 4B, coffee dried at 50 °C had the highest average total fat content from the beginning of storage. This behavior was stable until the storage end. In addition, the other treatments resulted in an increase in the total fat content, which was especially noticeable for the solar-dried coffee for which the amount equaled the

levels obtained for the coffee dried at 50 °C. These results can be related to the magnitude of the color change, which reached the highest levels for solar-dried coffee at the end of storage (Figure 2). Similarly, coffee dried at 40 °C showed the lowest change in fat content during storage.

CONCLUSIONS

The aim of storage is to maintain the quality of the coffee until the end of the marketing and distribution chain. The conditions under which the coffee was stored during this research work allowed the beans to be maintained at hygroscopic equilibrium humidity, due to the type of packaging used.

Some physical changes were observed in the beans stored in this study, the most notable being color. Changes were observed in the L* coordinate, which represents the clarity of the grains, with a positive rate of about 3 units year^{-1} difference in the time that corresponded to this research work, for the three treatments. Therefore, there was no association of drying temperature or type of drying with the change in clarity during storage. This led to the conclusion that color degradation processes are normal and difficult to control.

Regarding the mechanical drying process at a temperature of 50 °C, it was observed that the coffee suffered deterioration during storage, resulting in a change in color and loss of quality, corroborating the hypothesis of bleaching in the endosperm generated at a temperature of 50 °C, in addition to the degradation of quality during storage. In addition, the coffee obtained by solar drying presented the greatest color degradation, noticeable at the beginning of storage; however, this change was not related to the quality, since it was comparable to that obtained by coffee dried at 50 °C, in addition to the deterioration of quality during storage.

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Development of a functional beverage based on fermented whey, goldenberry (*Physalis peruviana* L.), and tumbo (*Passiflora mollissima*)

Desarrollo de una bebida funcional a base de lactosuero fermentado, Aguaymanto (*Physalis peruviana* L.), y tumbo (*Passiflora mollissima*)

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Keywords:
Andean fruits
Bioactive compounds
Dairy by-product
Nutritional properties
Sensorial properties

ABSTRACT

This study aimed to develop a beverage with functional and nutritional properties based on fermented whey and Andean fruit juices by using a simple-lattice mixture desing. The used proportions varied from 0.5 to 0.8 fermented whey, and from 0.1 to 0.4 for goldenberry (*Physalis peruviana* L.) and tumbo (*Passiflora mollissima*) juices, respectively. The influence of the mixture was analyzed on physicochemical and sensorial properties of beverages. It was evaluated that beverages contained protein, fat and acidity up to 1.92, 0.25, and 2.15%, respectively. Furthermore, the maximum bioactive compounds content was $343.54x10^{-5}$ kg(GA) kg^{-1} (total phenolics), $0.52x10^{-3}$ kg (CE) kg^{-1} (total flavonoids), $65.41x10^{-5}$ kg(AA) L^{-1} (Vitamin C), and $105x10^{-3}$ kg(Trolox) kg^{-1} (antioxidant capacity). Regarding sensorial evaluation results, the beverage with 55% fermented whey, 30% goldenberry juice, and 15% tumbo juice showed the best scores in taste regarding taste, colour and overall acceptability. It was observed that an increase in the percentage of fermented whey above 60% has negative effects, on the contrary, an increase in the percentage of goldenberry juice has a positive effect on sensorial properties. Finally, by optimization of both protein content and overall acceptability, the formulation with 50% fermented whey, 40% goldenberry juice, and 10% tumbo juice was the optimal mixture. Therefore, fermented whey, goldenberry, and tumbo juices can be used to obtain a beverage with high nutritional and functional value.

Palabras clave:
Frutas andinas
Compuestos bioactivos
Subproducto lácteo
Propiedades nutricionales
Propiedades sensoriales

RESUMEN

El objetivo de este estudio fue desarrollar una bebida con propiedades funcionales y nutricionales a base de lactosuero fermentado y jugos de frutas andinas utilizando un diseño de mezcla simplex-lattice. Las proporciones utilizadas variaron de 0,5 a 0,8 para lactosuero fermentado, y 0,1 a 0,4 para jugo de aguaymanto (*Physalis peruviana* L.) y tumbo (*Passiflora mollissima*), respectivamente. Se analizó la influencia de la mezcla sobre las propiedades fisicoquímicas y sensoriales de las bebidas. Se evaluó que las bebidas contenían proteína, grasa y acidez hasta 1,92, 0,25 y 2,15%, respectivamente. Además, el contenido máximo de compuestos bioactivos fue de $343,54x10^{-5}$ kg (GA) kg^{-1} (fenólicos totales), $0,52x10^{-3}$ kg (CE) kg^{-1} (flavonoides totales), $65,41x10^{-5}$ kg (AA) L^{-1} (Vitamina C), y $105x10^{-3}$ kg (Trolox) kg^{-1} (capacidad antioxidante). En cuanto a los resultados de los atributos sensoriales, la bebida con 55% de lactosuero fermentado, 30% de jugo de aguaymanto y 15% de jugo de tumbo presentó los mejores puntajes en cuanto a sabor, color y aceptabilidad general. Se observó que un aumento en el porcentaje de lactosuero fermentado por encima del 60% tiene efectos negativos sobre los atributos sensoriales y la aceptabilidad general, por el contrario, un aumento en el porcentaje de jugo de aguaymanto tiene un efecto positivo sobre las propiedades sensoriales. Finalmente, mediante la optimización tanto del contenido de proteína como de la aceptabilidad general, la formulación con 50% de lactosuero fermentado, 40% de jugo de aguaymanto y 10% de jugo de tumbo fue la mezcla óptima. Por lo tanto, los residuos de lactosuero fermentado y los jugos de frutas de aguaymanto y tumbo pueden utilizarse para obtener una bebida con alto valor nutricional y funcional.

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During these last decades, to counteract the consumption of ultra-processed and caloric products, a growing trend towards the consumption of healthy foods based on fruits and vegetables has been reported. This behaviour is due to the presence of bioactive compounds of high nutritional value and excellent health benefits such as the possibility of avoiding some chronic diseases (Ruiz Rodríguez et al. 2021). The nutritional importance of fruit juices is that they provided an excellent carrier to add probiotics to dairy products and may offer a synergy of health benefits over the fruit components such as natural antioxidants, vitamins, minerals, enzymes, and fiber (Žuntar et al. 2020). However, some fruits have not been properly valued for food processing. In Latin American countries, the *Physalis peruviana* L. (Goldenberry*)* is called: aguaymanto, uchuva, capulí, and uvilla. These fruits are juicy orange-yellow berries and contain numerous seeds, are rich in nutrients such as ascorbic acid and β-carotene, which could provide benefits with antioxidant potential (Kasali et al. 2021; Chasquibol and Yácono 2015). The principal bioactive components of goldenberry fruits are phytosterols (campesterol, β-sitosterol and stigmasterol), phenolic compounds, physalines, and withanolides, which could provide anti-inflammatory, antimicrobial, and anticancer properties (Puente et al. 2011). Another little explored fruit is the *Passiflora mollissima*, commonly known as poro poro, curuba or tumbo, which is a tropical fruit native to the southern Andes, whose fruit is generally consumed as fresh food (Conde-Martínez et al. 2014). *Passiflora mollissima* has potential bioactive metabolites with excellent antioxidants, phenolic components, a wide variety of flavonoids, and abundant proanthocyanidins (Ballesteros-Vivas et al. 2019, 2020).

On the other hand, the importance given to the by-products of an agro-industrial process is minimal. For example, approximately 90% of the total milk used in the cheese industry is eliminated as whey. It is important to note that this by product retains about 55% of the total ingredients of milk such as lactose, soluble proteins, lipids and mineral salts (Remón et al. 2016). Some possibilities for using this residue have been proposed, but statistics indicate that an important portion of this residue is still discarded as efluent, which creates a serious environmental problem in soil and water because it physically and chemically affects the structure of the soil and reduces aquatic life by depleting dissolved oxygen (Yadav et al. 2015). The industrial potential of whey is high, with emphasis on its agri-food technological processes, isolating a variety of nutrients such as vitamins, minerals, proteins, lipids, carbohydrates, and other components of high functional value for obtaining functional food products such as beverages (Illanes 2011; Rama et al. 2019; Mudgil and Barak 2019). Apart from the contribution of nutrients, functional beverages also have biologically active compounds that contribute to the prevention and treatment of chronic and degenerative diseases, generating benefits for improving health (Silveira Rodríguez et al. 2003; Minj and Anand 2020). For example, Montaño et al. (2011) reported that this type of beverage significantly reduces diastolic blood pressure, generating a positive effect in the adult population with heart failure, thus demonstrating cardioprotective properties, with endothelial function and blood pressure reducer.

Therefore, this work aimed to evaluate a functional beverage elaborated by taking advantage of fermented whey, *Physalis peruviana* L. (goldenberry) juice, and *Passiflora mollissima* (tumbo) juice. Their physicochemical, nutritional, and sensorial characteristics were evaluated.

MATERIALS AND METHODS

Raw material description

Physalis peruviana L. (goldenberry) and *Passiflora mollissima* (tumbo) fruits and whey were obtained from the city of Tarma (Junín, Peru). The fruits were selected at optimum commercial maturity with uniform yellow colour, and they had a mean weight and diameter of $6.2\pm0.2x10^{-3}$ kg and $1.7\pm0.1x10^{-2}$ m, and $170.5\pm3.8x10^{-3}$ kg, and $4.5\pm0.4x10^{-2}$ m, for goldenberry and tumbo fruits, respectively. On the other hand, the utilized fermented whey showed optimum characteristics of taste, smell, and yellowish colour, and it was without salt. All raw materials were characterised, and their physicochemical composition is shown in Table 1.

Experimental design

The experiment was designed using a simplex-lattice mixture design, described in Table 2. The independent variables were the different proportions of the factors: fermented whey, *Physalis peruviana L.* (goldenberry), and *Passiflora mollissima* (tumbo) juices. The proportions varied from 0.5 to 0.8 (whey), 0.1 to 0.4 (goldenberry)

Table 1. Physicochemical characteristics of raw materials.

Physicochemical characteristics	Whey	Goldenberry	Tumbo
Moisture (%)	91.2±0.18	79.90±0.13	85.60±0.55
Protein (%)	2.65±0.05	1.87±0.11	1.94±0.09
Fat (%)	0.30±0.06	0.01±0.01	0.12±0.03
Acidity (% citric acid)	0.35±0.04	4.60±0.11	2.90±0.05
Soluble solids (°Brix)	14.2±0.10	13.3±0.10	12.9±0.40
pH	5.45±0.04	3.90±0.05	2.90±0.17
Total phenolic content ($x10^{-5}$ kg (GA) kg^{-1})	11.12±0.08	343.54±1.80	198.32±1.42
Antioxidant capacity ($x10^{-3}$ kg (Trolox) kg^{-1})	0.46±0.02	4.87±0.28	105.15±0.49
Total flavonoid ($x10^{-3}$ kg (CE) kg^{-1})	0.09±0.03	0.75±0.10	0.61±0.13
Vitamin C ($x10^{-5}$ kg (AA) L^{-1})	0.75±0.05	42.90±1.08	148.50±1.62

Mean values±SD (n=3). GA: Gallic acid; CE: Catechin equivalent; AA: Ascorbic acid.

and 0.1 to 0.4 (tumbo). With these proportions, the functional beverage was elaborated and the influence of the factors on the physicochemical and sensory properties of the beverage was evaluated.

Beverage development

To prepare the beverage, firstly, the whey was pasteurized at 70 °C for 30 min and fermented at 42 °C for 4 h, for which acidophilic milk culture was used: *Streptococcus thermophilus* & *Lactococcus bulgaricus* (Sacco Lyofast, Italy). The fermented whey was filtered through No. 4 filter paper.

Goldenberry fruits were blanched at 85 °C for 1 min and then pulped, obtaining pulp free of seeds and shell, then filtered through a 60-mesh sieve to reduce the particle size of the pulp and obtain a homogeneous juice. Tumbo juice was obtained by pulping, followed by filtering through a 60-mesh sieve to separate particles, obtaining a homogeneous juice. Subsequently, the obtained juices were mixed with the fermented whey, according to the corresponding proportions of each one, as established in Table 2. The mixture was immediately homogenized, quickly pasteurized at 80 °C for 30 s, packaged, and subjected to instant cooling, obtaining the functional beverage.

Table 2. Treatments and factor levels were obtained by the simplex-lattice mixture design.

Treatment	Design levels			Factor levels		
	A	B	C	Whey (W)	Goldenberry (G)	Tumbo (T)
1	0.000	1.000	0.000	0.50	0.40	0.10
2	0.333	0.333	0.333	0.60	0.20	0.20
3	0.167	0.167	0.667	0.55	0.15	0.30
4	0.000	0.000	1.000	0.50	0.10	0.40
5	0.167	0.667	0.167	0.55	0.30	0.15
6	0.500	0.000	0.500	0.65	0.10	0.25
7	0.667	0.167	0.167	0.70	0.15	0.15
8	1.000	0.000	0.000	0.80	0.10	0.10
9	0.000	0.500	0.500	0.50	0.25	0.25
10	0.500	0.500	0.000	0.65	0.25	0.10

Physicochemical analysis

Antioxidant capacity (AC). The antioxidant capacity of beverage samples was determined using the method proposed by Onyeoziri et al. (2016). For the procedure, 600 µL of DPPH (1,1-diphenyl-2-picryl-hydracyl) (Sigma-Aldrich, USA), was used and the initial absorbance at 517 nm was read, then 200 µL of each of the previously conditioned samples were added, the absorbance was taken every 30 s for 10 min, using a UV-Visible spectrophotometer (Shimadzu UV-1900, Japan). A calibration curve was constructed under the same conditions by replacing the sample with different Trolox concentrations and then used to express the results were expressed in kg of Trolox per kg of sample.

Total phenolic content (TPC). Total phenolic content was measured using the methodology proposed by Singh et al. (2016). For the analysis, the Folin-Ciocalteau reagent, sodium carbonate 7.5%, sodium fluoride (NaF), gallic acid, methanol and distilled water were used. For the procedure, the sample in a 1:2 ratio was placed in a test tube, and NaF 2 mM was added to inactivate the enzyme polyphenol oxidase and prevent the degradation of polyphenols during the test. The mixture was homogenized and centrifugated at 10,000 rpm for 15 min at 10 °C, then the supernatant was recovered and used to perform the reaction. For this, Folin-Ciocalteau reagent was added to the supernatant and mixed, after 5 min sodium carbonate was added and it was left to react for 1 h in the dark. Finally, the absorbance at 765 nm is measured using a UV-Visible spectrophotometer (Shimadzu UV-1900, Japan). A calibration curve was constructed under the same conditions by replacing the sample for different gallic acid concentrations and then used to express the TPC in kg of gallic acid equivalents (GAE) per kg of sample.

Flavonoid content (TF). Total flavonoid content was determined using the methodology proposed by Giura et al. (2019), with modifications. For the analytical procedure, a mixture of 0.5 mL of the sample with 40 mL of sulfuric acid solution (10% w/v) and 40 mL of ethanol (50% v/v) was refluxed for 2 h, then cooled and vacuum filtered, the residue was washed with 60 mL of ethanol (50% v/v), subsequently, the absorbance was read at 258 nm using a UV-Visible spectrophotometer (Shimadzu UV-1900, Japan). Flavonoid concentration was estimated using a calibration curve of catechin and the results were expressed in kg of catechin equivalent (CE) per kg of sample.

Vitamin C. Vitamin C content was determined using the methodology proposed by the AOAC (1990). For the analysis, 1 mL of filtered sample was mixed with 4-methoxy-2-nitro aniline acid reagent and then the absorbance was measured at 570 nm using a UV-Visible spectrophotometer (Shimadzu UV-1900, Japan). The results were expressed in kg of ascorbic acid (AA) per kg of sample.

Protein content. To determine the protein content, the Kjeldahl method was described by the FAO (1986) and Goyal et al. (2022). For the analysis, 1 g of sample was taken, placed in the flasks and sulfuric acid was added with the catalyst, after digestion, it was distilled to recover the amount of nitrogen, it was titrated and finally, the protein was quantified and expressed as a percentage concerning the sample.

Fat content. The fat content was determined by using the soxhlet method described by the FAO (1986) and Nyakang'i et al. (2023). For the analysis, 1 g of dehydrated extract was placed in the thimble, it was placed inside the soxhlet balloon and petroleum ether was added, the extraction process was performed for 16 h and finally, the fat content was quantified and expressed as a percentage concerning to the sample.

Soluble solids, pH, and solid content. Furthermore, it was evaluated the sample acidity by titration with NaOH (0.1 N), and soluble solids (°Brix) by direct reading with a refractometer, both according to the method described by the NTP (2017). In addition, pH was evaluated by direct reading with a pH-meter according to the method described by the A.O.A.C. 981.12 (AOAC 2012), and the solid content (dry matter) was determined according to the method described by FAO (1986).

Sensorial analyses. The samples were delivered to consumers in the morning in random order, the evaluation was carried out by 40 untrained consumers (males and females) with ages in the range of 20-50 years. For all treatments, the overall acceptability and the organoleptic attributes of appearance, taste, smell, and colour, were evaluated. For this, a consent form and a 7-point hedonic scale were used.

Statistical analysis. Results were processed with a confidence level of 95% by analysis of variance (ANOVA)

and the least significant difference (LSD) test to evaluate differences among means of formulations by using the Statgraphics Centurion XVI software (Statgraphics Technologies Inc.; USA). The results of sensorial analyses were analysed by the Kruskal Wallis test to evaluate differences among medians of formulations. Subsequently, both physicochemical and sensorial results were evaluated using a simplex-lattice mixture design (Table 2) by using Statistica 7 (StatSoft. Inc., USA) software.

RESULTS AND DISCUSSION

Physicochemical characteristics and composition of beverages

Table 3 shows the results of the physicochemical composition and characteristics of beverages prepared for each treatment. Regarding the physicochemical content, the dry matter content ranged from 10.10 to 21.17%, acidity from 0.45 to 2.15%, protein from 1.13 to 1.92%, and fat from 0.11 to 0.18%. It is observed that the proportions of fermented whey or fruit juices influence the physicochemical composition, it is because as shown in Table 1, whey contributes mainly to protein and fat content. In fact, whey has been used to increase the protein value of fruit-based beverages such as *Theobroma grandiflorum* (copoazú) (Basantes et al. 2020) and *passiflora edulis* (passion fruit) (Valencia et al. 2002), where similar results of pH, dry matter and protein were reported.

Table 3. Physicochemical characteristics and composition of functional beverages.

Treatment	Dry matter (%)	Acidity (%)	°Brix	pH	Protein (%)	Fat (%)
1	10.10±0.30^{a}	0.45±0.07^{a}	11.9±0.2^{ab}	5.00±0.20^{de}	1.90±0.16^{c}	0.25±0.05^{c}
2	10.79±0.50^{b}	0.85±0.11^{b}	12.1±0.2^{b}	5.10±0.13^{e}	1.56±0.14^{b}	0.11±0.04^{a}
3	12.88±0.66^{c}	0.91±0.08^{bc}	12.0±0.2^{ab}	4.20±0.15^{a}	1.44±0.10^{b}	0.12±0.06^{ab}
4	17.77±0.56^{d}	1.05±0.06^{c}	11.8±0.2^{a}	4.40±0.10^{ab}	1.89±0.07^{c}	0.20±0.07^{bc}
5	20.01±0.45^{ef}	1.45±0.06^{de}	12.8±0.1^{d}	4.60±0.08^{bc}	1.41±0.08^{b}	0.24±0.05^{c}
6	19.46±0.34^{e}	1.82±0.10^{f}	12.9±0.2^{d}	5.20±0.11^{e}	1.13±0.09^{a}	0.11±0.06^{a}
7	21.17±0.22^{h}	1.65±0.15^{ef}	12.5±0.1^{c}	4.80±0.15^{cd}	1.18±0.07^{a}	0.24±0.04^{c}
8	20.45±0.24^{fg}	1.35±0.17^{d}	11.8±0.1^{a}	5.20±0.10^{e}	1.38±0.11^{b}	0.18±0.06^{abc}
9	21.05±0.18^{gh}	1.75±0.20^{f}	11.9±0.1^{ab}	4.80±0.12^{cd}	1.89±0.12^{c}	0.15±0.04^{ab}
10	20.10±0.09^{ef}	2.15±0.10^{g}	12.1±0.1^{b}	4.80±0.10^{cd}	1.92±0.12^{c}	0.18±0.04^{abc}

Mean values±SD (n=3). Different superscript letters indicate significant differences among treatments by LSD Test (P<0.05).

Bioactive compounds in beverages

The incorporation of fruit juices will contribute to increasing bioactive compounds such as the phenolic content, antioxidant capacity and Vitamin C in beverages. Table 4 shows the content of total phenols (TPC), antioxidant capacity (AC), total flavonoid (TF), and Vitamin C. The treatment T1 showed the lowest values for all the parameters evaluated. This sample contains the lowest proportions of whey (50%) and tumbo (10%), of these two ingredients an increase in tumbo juice proportion provided a greater amount of bioactive compounds since as observed in Table 1, tumbo juice presents higher amounts of bioactive compounds compared to whey. Total phenolic content, antioxidant capacity, and flavonoid content were in the range reported in a fermented whey beverage with *Passiflora mollissima Bailey* extracts (Sánchez et al. 2013). However, it is important to consider a possible interaction between phenols of fruit juices and macromolecules such as proteins and lipids from whey could occur (Bansal et al. 2019). Therefore, this may explain the non-linear behaviour between the amounts of biocompounds (Table 4) and the proportions of the ingredients used in the formulations (Table 2).

Similar functional beverages with high bioactive compounds content have been studied, which include the use of whey (reconstituted whey powder or fresh whey) and fruits, such as guava-flavoured whey beverages (Silveira et al. 2019), strawberry-flavoured whey beverage (Souza et al. 2019). The presence of phenols gives fermented milk beverages excellent antioxidant potential. The presence of phenols gives fermented milk beverages excellent antioxidant potential. In addition, whey-based beverages made from

Table 4. Bioactive composition of functional beverages.

Treatment	TPC (x10^{-5} kg(CA) kg^{-1})	AC (x10^{-3} kg(Trolox) kg^{-1})	TF (x10^{-3} kg(CE) kg^{-1})	Vitamin C (x10^{-2} kg L^{-1})
1	12.22±0.39^{a}	0.46±0.02^{a}	0.11±0.04^{a}	1.90±0.11^{a}
2	343.54±1.00^{j}	4.87±0.09^{d}	0.75±0.06^{d}	10.40±0.08^{b}
3	198.32±1.23^{f}	105.15±0.19^{j}	0.16±0.05^{a}	43.80±0.15^{c}
4	121.82±0.20^{c}	1.93±0.06^{b}	0.26±0.06^{b}	52.10±0.23^{d}
5	73.46±0.22^{b}	35.32±0.05^{e}	0.13±0.04^{a}	60.40±0.27^{e}
6	232.84±0.16^{g}	3.4±0.02^{c}	0.50±0.06^{c}	65.41±0.17^{g}
7	184.34±0.21^{e}	36.9±0.04^{f}	0.27±0.07^{b}	60.20±0.20^{e}
8	135.98±0.24^{d}	70.29±0.04^{h}	0.14±0.06^{a}	65.20±0.20^{g}
9	295.18±0.18^{i}	38.26±0.03^{g}	0.52±0.06^{c}	62.40±0.27^{f}
10	246.68±0.19^{h}	71.76±0.03^{i}	0.29±0.05^{b}	60.40±0.18^{e}

Mean values±SD (n=3). Different superscript letters indicate significant differences among treatments by LSD Test (P<0.05).

fruits and whey provide significant amounts of vitamins, as was reported by Tranjan et al. (2009).

Sensorial acceptability of functional beverages.
It was performed the sensorial evaluation of beverages (10 treatments) and the results were analysed through multiple comparisons by the Kruskal Wallis test. On the one hand, regarding the taste and colour (Figure 1A and C), compared to other formulations, the formulation T5 showed the best score (P<0.05), which contained 55% whey, 30% goldenberry juice, and 15% tumbo juice, while the least preferred was the beverage corresponding

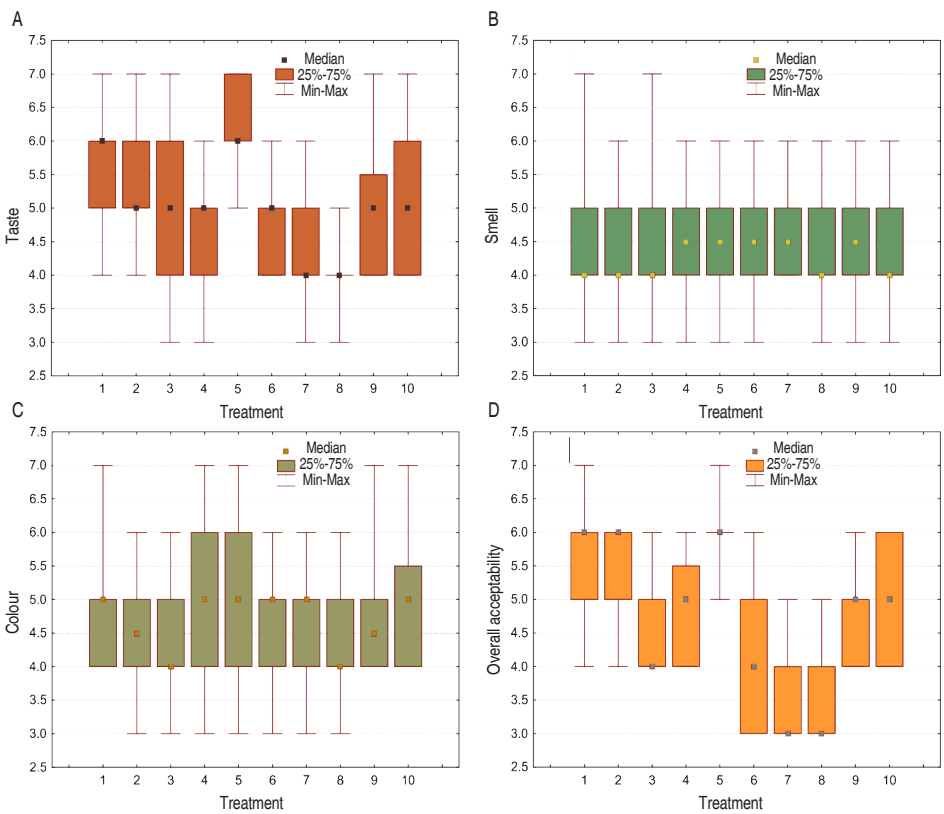

Figure 1. Boxplots of sensorial attributes (taste, smell, colour) and overall acceptability of functional beverages.

to formulation eight (T8) containing 80% whey, 10% goldenberry juice and 10% tumbo juice. Lower scores may be due to too much cheese flavour in the beverage. This was demonstrated years ago by Branger et al. (1999), who found that with an increasing percentage of fermented whey in the beverage, cheese flavour increased, while acidity, astringency, sweetness, and fruit flavour decreased. In contrast, regarding the smell (Figure 1B), there were no significant differences (P>0.05) among formulations.

On the other hand, the overall acceptability (Figure 1D) reflects the observed when individual sensorial attributes were evaluated, where T5 and T8 obtained the highest and lowest scores, respectively. However, regarding the overall acceptability, there was no significant difference (P>0.05) among T1, T2, and T5 (most preferred) while the less preferred were formulations T7 and T8. Therefore, by considering the composition of these formulations (Table 2), an increase in the percentage of whey above 60% in the formulations has negative effects on sensory attributes and general acceptability, on the contrary, an increase in the percentage of goldenberry juice has a positive effect on sensory acceptability. In fact, it has been reported that the incorporation of whey and fruits in the formulation of beverages influences flavour, mouthfeel, aftertaste, viscosity, soluble solids and sedimentation (Saxena et al. 2015). Showing that the development of functional beverages based on whey requires the addition of fruits, plants, cereals, and others, which positively contribute to the taste, smell, colour, homogeneity, sweetness, acidity, and viscosity (Rodríguez-Villacis and Hernández-Monzón 2017; Basantes et al. 2020). Therefore, more optimization studies are necessary for this type of product so that, in addition to the potential functional properties, they also have organoleptic quality.

Simplex-lattice mixture design analysis

The simplex-lattice mixture design analysis was applied to physicochemical and sensorial experimental data. Three models were applied, of which the special cubic model had the best fit for all variables. However, the values of the coefficient of determination (R^2) were low, especially for the variables: fat, TF, and AC (Table 5) which means that, for these response variables, the three models adjusted do

Table 5. *P*-value, fit criteria, and coefficients of the models fitted to experimental data for each variable response.

Variable response	Model type	R^2	Coefficients of fitted model						
			W	G	T	W*G	W*T	G*T	W*G*T
Protein (%)	L	0.40	0.85	3.08	2.22	-	-	-	-
	Q	0.78	2.00	-1.15	15.63	6.45	-26.62	-5.57	-
	Sc	0.82	0.51	-13.13	3.64	32.69	-0.38	109.25	-221.44
Fat (%)	L	0.27	0.16	0.37	0.04	-	-	-	-
	Q	0.57	0.33	0.74	1.80	-0.48	-3.09	-3.19	-
	Sc	0.59	0.47	1.87	2.94	-2.96	-5.56	-14.03	20.92
TPC ($x10^{-5}$ kg(CA) kg^{-1})	L	0.12	2.43	-0.86	2.81	-	-	-	-
	Q	0.69	-2.22	-37.05	-20.12	62.29	37.42	87.15	-
	Sc	0.70	-5.03	-59.70	-42.76	111.87	86.99	304.05	-418.32
Vitamin C ($x10^{-2}$ kg L^{-1})	L	0.30	86.01	-57.79	40.88	-	-	-	-
	Q	0.41	106.28	-427.44	260.67	505.50	-599.70	808.22	-
	Sc	0.73	-209.80	-2972.60	-2284.50	6078.30	4973.10	25189.10	-47020.30
TF ($x10^{-3}$ kg(CE) kg^{-1})	L	0.06	0.27	0.08	0.68	-	-	-	-
	Q	0.43	-0.67	-3.67	-5.97	6.75	12.19	12.97	-
	Sc	0.43	-0.58	-2.92	-5.22	5.11	10.54	5.79	13.86
AC ($x10^{-8}$ mol(Trolox) kg^{-1}	L	0.10	3.32	-1.40	-1.20	-	-	-	-
	Q	0.33	5.24	-34.29	19.44	44.91	-55.46	70.88	-
	Sc	0.34	1.51	-64.37	-10.64	110.77	10.40	359.01	-555.69
Overall acceptability	L	0.74	2.16	11.22	6.05	-	-	-	-
	Q	0.81	0.05	-2.81	10.93	33.01	-2.44	-12.14	-
	Sc	0.87	5.20	38.69	52.43	-57.85	-93.31	-409.67	766.67

Where: L=linear; Q=quadratic; Sc=special cubic; W=fermented whey; G=goldenberry; T=tumbo.

not adequately describe their behaviour. This could be due to a possible complex interaction among components of fermented whey, goldenberry, and tumbo in formulations. Therefore, the interaction between components of the raw materials used here and other models could be better explored in future research. Therefore, only for the variables of protein, TPC, Vitamin C, and OA, in which the model had a value of $R^2 \geq 0.70$, the contour plots (fitted response) were shown and analyzed. From the contour plots, it is possible to establish ranges of the levels of the factors that allow obtaining the highest content of compounds or the highest acceptability.

Figure 2A shows that a higher level of TPC could be found in the level ranges of 0–0.333 for fermented whey, 0.167–0.583 for goldenberry, and 0.417 and 0.667 for tumbo juices, which correspond to proportions from 50 to 60% for whey, from 15 to 27.5% for goldenberry juice and from 22.5 to 66.7% for tumbo juice. On the other hand, Figure 2B shows that values higher than $50x10^{-2}$ kg L^{-1} of Vitamin C can be obtained at whey levels between 0 to 0.083, goldenberry juice from 0.167 to 0.583, and tumbo juice from 0.333 to 0.833, which correspond to ranges of proportions between 50 to 52.5; 15 to 27.5 and 20 to 35% for fermented whey, goldenberry and tumbo juices, respectively.

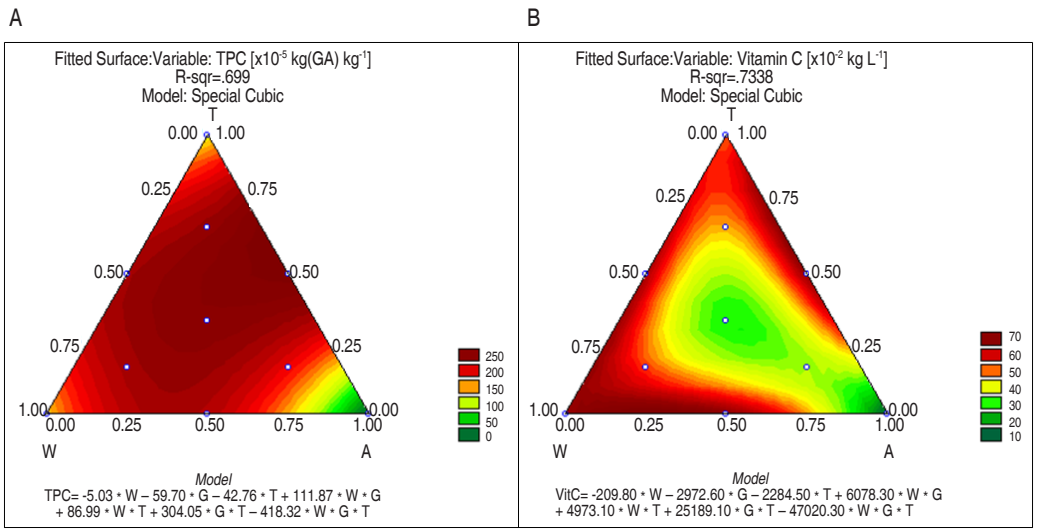

Figure 2. The Contour graph and the special cubic model fitted to the data of TPC (A) and Vitamin C (B) depending on the whey (W), goldenberry (G), and tumbo (T) juice proportions.

The contour graph of Figure 3 shows that higher protein content could be found at levels between 0 and 0.167 for whey and between 0 and 1 for both goldenberry and tumbo juices, which correspond to proportions of 0 to 55% for fermented whey, 10 to 40% for each juice, respectively. However, it is not the only region where higher values could be found in terms of protein content. It is observed that, if the fermented whey level is increased to 0.333 (60%), the goldenberry juice level must be greater than 0.667 (>30%) and the tumbo juice level must be lower than 0.167 (<15%). On the other hand, Figure 4 shows that the greater overall acceptability of beverages can be obtained at levels lower than 0.33 for whey (<60%) and levels higher than 0.5 for goldenberry (>0.25%), and levels lower than 0.25 for tumbo (<17.5%).

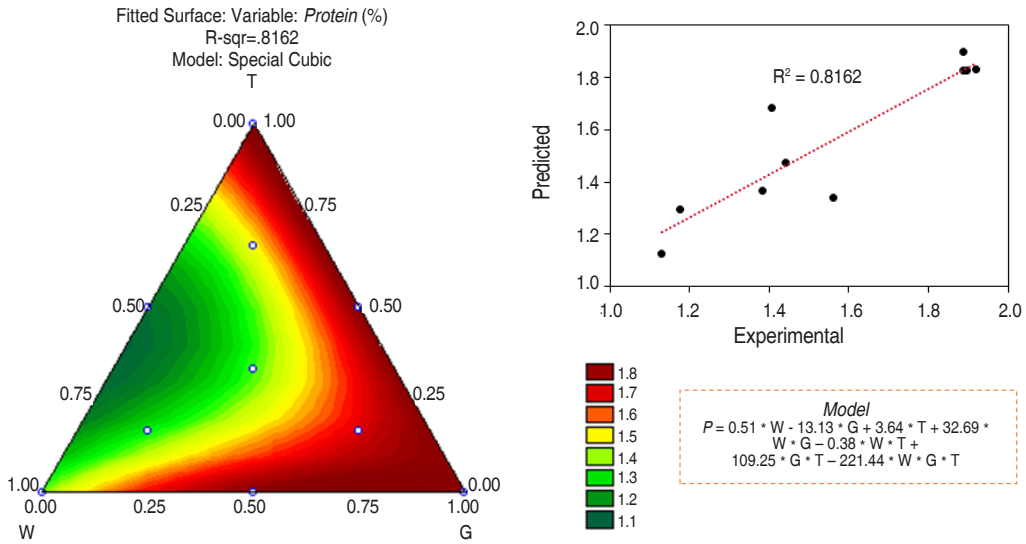

Figure 3. The Contour graph obtained with the special cubic model fitted to the data of protein content depending on whey (W), goldenberry (G), and tumbo (T) juice proportions. In detail, the fit value R^2 of the model is shown.

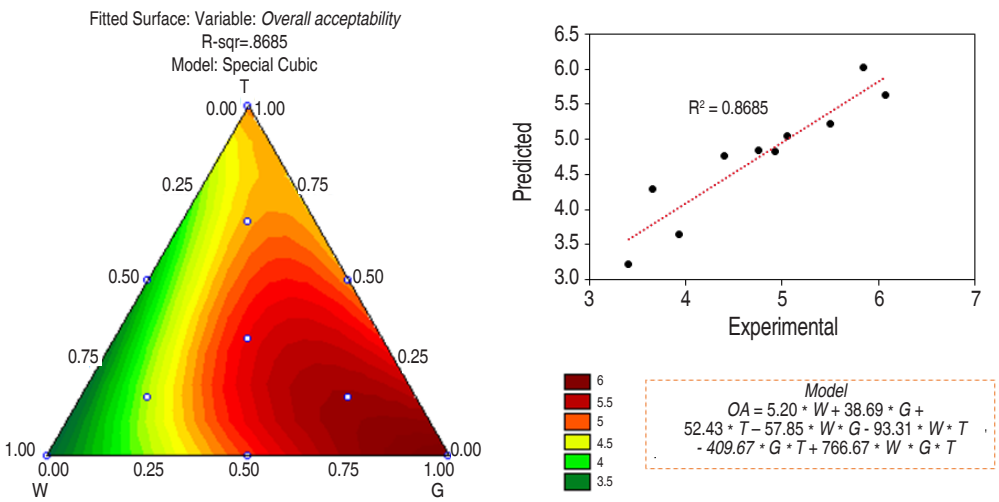

Figure 4. The Contour graph obtained with the special cubic model fitted to the data of overall acceptability depending on whey (W), goldenberry (G), and tumbo (T) juice proportions. In detail, the fit value R^2 of the model is shown.

The content of protein, TPC and Vitamin C in the beverage was optimized using the desirability function. Therefore, it was obtained that to simultaneously obtain the best values in these three variables, it is necessary to use 50% of whey, 21.7% of goldenberry juice, and 28.3% of tumbo juice. However, the ranges of the proportions of goldenberry and tumbo juice are not within the range of optimal values for general acceptability observed in Figure 4.

Therefore, to ensure that the beverage has good composition and sensory properties, it was selected the variables with the best fit of the model (protein and general acceptability) to perform the optimization. Figure 5 shows the optimization to obtain the highest values of protein and overall acceptability, being necessary with the use of a proportion of 50% whey, 40% goldenberry juice, and 10% tumbo juice.

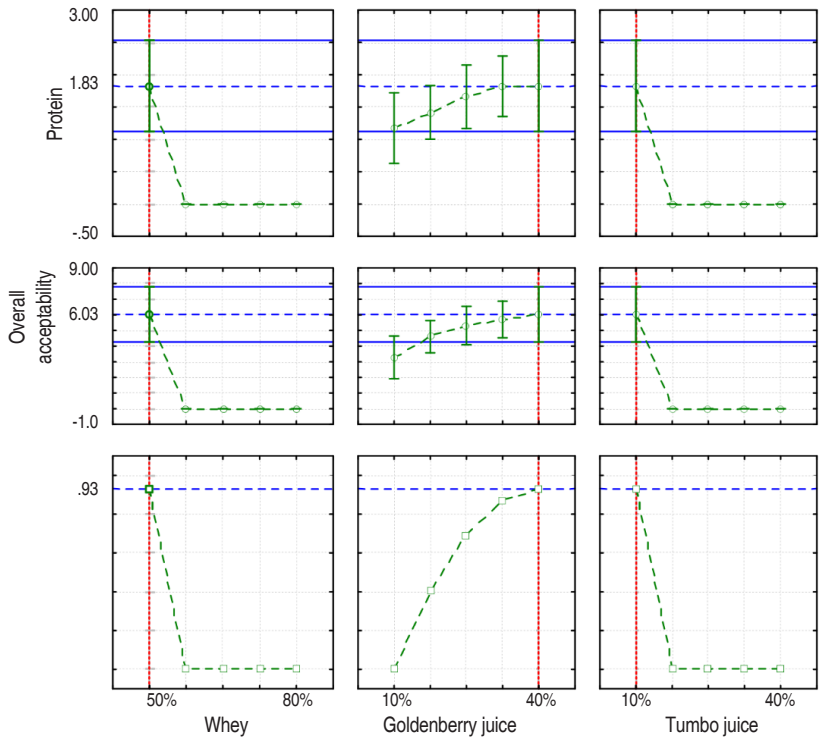

Figure 5. Optimization results for the values of protein and overall acceptability obtained through the desirability function.

CONCLUSIONS

The proportions of fermented whey, *Physalis peruviana* L., and *Passiflora mollissima* affected the protein, fat, and acidity, total phenolics content, total flavonoids, ascorbic acid, and antioxidant capacity of beverages. Regarding sensorial attributes, it was observed that an increase in the percentage of fermented whey above 60% in the formulations has negative effects on sensory attributes and general acceptability, on the contrary, an increase in the percentage of goldenberry juice has a positive effect on sensorial properties. Finally, by optimization of both protein content and overall acceptability, the formulation with 50% fermented whey, 40% *Physalis peruviana,* and 10% *Passiflora mollissima* was the optimal mixture. It is recommended to evaluate lower serum concentrations (<50%) to avoid residual cheese flavour and thus improve the organoleptic properties.

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Effect of low-temperature storage time on rejected green banana for flour production

Efecto del tiempo de almacenamiento a baja temperatura en banano verde de rechazo para la producción de harina

UNIVERSIDAD NACIONAL DE COLOMBIA

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Chilling injury
Enzymatic browning
Musa cavendish

ABSTRACT

Banana (*Musa* sp.) crops have one of the greatest economic impacts in Colombia, with an estimated production of 2.2 million tons in 2019. The aim of this study was to evaluate the effect of three anti-browning solutions: S1 (citric acid), S2 (citric acid + ascorbic acid), and S3 (citric acid + ascorbic acid + sodium metabisulfite) on the color, moisture, aw, pH, and acidity characteristics of flour from rejected green bananas. No significant differences were found (P>0.05). The values of a* and b* in all samples were in the grey zone. L* and WI presented values close to 50, which could be defined as a flour in a medium range of clarity. The citric acid anti-browning solution was selected based on criteria such as cost and availability. The second part of the study assessed the effect of the storage time (1, 3, 5, 7, 9 and 11 days) at 7 °C on the color and texture of fresh bananas; and pH, instrumental, and sensory color of banana flour. There were differences noticeable for the human eye in the color (ΔE) of the peel from day 3 compared to day 1; while in the pulp, these changes were observed from day 7. Statistically significant differences in instrumental and sensory color properties of banana flour were observed after day 7 (P<0.05). The maximum storage time at 7 °C of fresh green bananas to produce banana flour should not exceed 7 days because color may be affected.

Palabras clave:
Harina de banano
Daño por frío
Pardeamiento enzimático
Musa cavendish

RESUMEN

El banano (*Musa* sp.) es uno de los cultivos de mayor impacto económico en Colombia, se estima que para el 2019 se produjeron 2,2 millones de toneladas. El objetivo de este estudio fue evaluar el efecto de tres soluciones antipardeantes S1 (ácido cítrico), S2 (ácido cítrico + ácido ascórbico) y S3 (ácido cítrico + ácido ascórbico + metabisulfito de sodio) en las características de color, humedad, aw, pH, y la acidez de la harina de banana verde de rechazo. No se presentaron diferencias significativas (P>0,05). Los valores de a* y b* de todas las muestras se ubicaron en la zona gris. L* y WI presentaron valores cercanos a 50, lo que podría definirse como una harina en un rango medio de claridad. La solución antipardeante con ácido cítrico fue seleccionada basándose en criterios como el costo y la facilidad de acceso. La segunda parte del estudio consistió en evaluar el efecto del tiempo (1, 3, 5, 7, 9 y 11 días) de almacenamiento a 7 °C de los bananos frescos sobre las propiedades de color y textura en la fruta, el pH; además del color instrumental y sensorial de la harina de banano. Se observaron diferencias evidentes para el ojo humano en el color (ΔE) de la cáscara a partir del día 3 con respecto al día 1; mientras que, en la pulpa estos cambios tan evidentes se observaron a partir del día 7. Las diferencias estadísticamente significativas de propiedades de color instrumental y sensorial en la harina de banano se observaron después del día 7 (P<0,05). El tiempo máximo de almacenamiento a 7 °C de los bananos verdes en fresco para la producción de harina de banano no debe ser superior a los 7 días porque se puede afectar su color.

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Bananas are one of the most consumed fruits in the world. In Colombia, this crop has one of the greatest impacts on the economy and is one of the most exported agricultural products, along with coffee and flowers (Minagricultura 2022). It is estimated that 2.2 million tons were produced in 2019, a figure that placed the country as the ninth largest producer in the world and the fifth largest exporter of this fruit after Ecuador, the Philippines, Guatemala, and Costa Rica, thus supplying one tenth of the export market (Andersen et al. 2020; Minagricultura 2020). According to the Ministry of Agriculture and Rural Development (MADR), the departments of Antioquia, Magdalena, and La Guajira are the main producers of banana, and 86% of their production is exported (Minagricultura 2020). Andersen et al. (2020) estimate that 1.75 million tons were exported, and it generated approximately 155,000 direct and indirect jobs.

The cavendish variety is one of the most produced and marketed in the world. It is also an important product of international trade (Kuan et al. 2015; Padhi and Dwivedi 2022). In nutritional terms, bananas stand out as a source of minerals, vitamins, carbohydrates, flavonoids, phenolic compounds, among others (Singh et al. 2018). However, this fruit is climacteric, thus perishable, and prone to post-harvest loss (Odetayo et al. 2022). Different investigations have been developed to mitigate these losses, so that green bananas can be used, specifically rejected ones, i.e., those that do not meet the standards of international markets such as variety, number, and size of fingers per bunch, color, appearance, diameter, packaging, and phytosanitary conditions (Sartori and Menegalli 2016; Stanley 2017).

One way to delay the biochemical processes of biological material—like the production of ethylene in climacteric fruits—is storage at low temperatures, although it can damage the structure of the plant material, which is known as chilling injury (Tian et al. 2022). In the case of bananas, the physiological damage occurs below 12 °C and worsens as the temperature decreases. Among the undesirable effects, color changes in the shell (discoloration and brown spots) caused by polyphenol oxidase (PPO)—an enzyme responsible for browning—and an impediment in the normal maturation process have been reported (Tian et al. 2022). Moreover, other possible damages during storage due to mechanical and low moisture effects should be considered (Kader 2022).

Different by-products have been developed from rejected bananas to give them an added value, e.g., syrup, precooked products, dried banana, flour, and powder obtained by different technologies for food and pharmaceutical applications (Singh et al. 2018). Furthermore, green banana flour has gained greater interest due to its functional properties. However, some unfavorable reactions such as enzymatic browning occur during the flour production process. It is a biochemical reaction that occurs naturally in foods and affects especially fruits because the color of their pulp becomes dark. This leads to unwanted quality, sensory, and nutritional defects in the final product (Anyasi et al. 2017). The PPO enzyme, responsible for that reaction, acts on the phenolic compounds and oxidizes them to quinones. Subsequently, the latter are polymerized to form a relatively insoluble brown pigment known as melanin (Moon et al. 2020). Precisely, one of the challenges faced by the banana processing industry is to avoid the enzymatic browning that affects the pulp once the shell is removed (Anyasi et al. 2015).

Currently, there are various methods to control, reduce, or inhibit enzymatic browning. The most used physical methods are oxygen reduction and modified atmospheres, coating films, and inactivation of enzymes by heating, e.g., blanching (Dávila et al. 2016). Chemical methods have specialized in the use of substances that, according to their active ingredient, focus on the inhibition of the enzyme, oxygen, or formed products: acidulants reduce the pH of the food, therefore, the enzymatic action; sulfites delay the rate of melanin formation, thus reducing quinones to diphenols; antioxidants react with oxygen to prevent the oxidation of phenolic compounds; and chelating agents form complexes with the copper oxide present in PPO (Moon et al. 2020; Yupangui Tenesaca 2016).

The advantages of physical methods to avoid enzymatic browning are the homogenization of food treatment and the possibility of modulating process conditions. Some disadvantages are alteration of the consistency of the treated product, sometimes giving a cooked flavor, generating losses of nutrients, and resulting in a decreased weight of the product. Moreover, the effectiveness of

chemical methods depends on environmental factors such as pH, water activity (aw), temperature, light, and composition of the atmosphere, and are limited by the presence of the cuticle. The advantages of these methods are quickly and effectively inhibiting enzymatic browning, they can be applied to a variety of fruits, are often readily available and relatively inexpensive compared to other methods, and do not cause noticeable changes in the taste, aroma, or texture of fruits when used in suitable concentrations. Despite these advantages, it is important to note that the use of chemical inhibitors should be regulated to ensure food safety and minimize any potential adverse effects (Moon et al. 2020).

Ascorbic acid and citric acid are among the most common agents to inhibit enzymatic browning, alone or in combination. They are Generally Recognized as Safe (GRAS), and their use is less restricted (Anyasi et al. 2015; Wang et al. 2014). It has also been determined that the use of sulphites prevents banana pulp from browning and enables obtaining a clearer flour with greater acceptability by the consumer (Salazar et al. 2022). However, their use is more limited due to their toxicity (Ojeda et al. 2020). The aim of this study was to evaluate the influence of pretreatments with anti-browning solutions based on ascorbic acid, citric acid, and sodium metabisulfite, and the storage time of rejected green banana fruits at 7 °C on the color, firmness, moisture, aw, pH, acidity, and sensory color of banana flour.

MATERIALS AND METHODS

Location

The plant material used to produce the flour was rejected green banana (*Musa cavendish*) from the department of Magdalena (Colombia), municipalities of Aracataca, Ciénaga, and Zona Bananera, specifically from the Gran Vía area (Latitude 10° 50'47.336''N, Longitude 74° 8' 30.477''W) and Santa Rosalía (Latitude 10° 49'45.672'' N, Longitude 74° 7' 26.297''W). Their maturity stages were 1 and 2 (green) (Kader 2022).

Preparation of plant material

The bunches were harvested between 10 and 12 weeks, taking into account the harvest age criteria for banana exports. Bunches older than 12 weeks cannot be exported because by the time this product reaches the destination country, they will be more ripe than normal and could even have black spots (Alonso-Ugaglia et al. 2022). Furthermore, rejected bananas are not suitable for export due to the high-quality standards required by importers. The most outstanding characteristics of rejected bananas are low weight, presence of spots, physical damage and scars higher than 1%, fruit length less than 22 cm, and fruit diameter less than 3.5 cm (Vásquez-Castillo et al. 2019). The cutting process was carried out with the help of tapes placed during the taping process. When the order to cut a certain color of tape was given at first, only 25% is cut; the following week, 50%; and the last week, the remaining 25% (Alonso-Ugaglia et al. 2022).

Food-grade detergent and organic acid-based disinfectant (1% Acid-tech solution) were used for cleaning and disinfection. The following substances were used for pretreatment with anti-browning solutions: citric acid and ascorbic acid powder (Bell Chem, Medellín, Colombia), sodium metabisulfite powder (Químicos JM, Medellín, Colombia), reactive-grade phenolphthalein (Chemi, Milán, Italy), and sodium hydroxide (NaOH) pearls (Panreac, Darmstadt, Germany).

Rejected green banana fruits were transported from Santa Marta to Medellín by air at room temperature, packed in cardboard boxes, and properly arranged inside the box to be processed at Universidad Nacional de Colombia. The transportation time of the raw material was less than 12 h. Upon arrival, they were washed and disinfected. Subsequently, they were stored at 7 °C to delay the ripening process until they were used in flour preparation. During the pre-climacteric period, mature green fruit has a low basal respiration rate and ethylene production is almost undetectable. This period is also called "Green Life" (GL). The high sensitivity of the banana fruit to the duration and intensity of low temperatures could provoke chilling injury. The suitable storage GL is 35 days at 13 °C (Brat et al. 2020). However, this study was intended to evaluate the lowest temperature possible to verify its effect on the quality traits of banana flour; it was set at 7 °C. Studies have been carried out previously storing banana fruit at 7 and 9 °C (Chen et al. 2022; Othman et al. 2021).

Preparation of immersion solutions (Pretreatment)

For banana pre-treatment, anti-browning solutions (S1, S2, and S3) were prepared using citric acid, ascorbic acid, and sodium metabisulfite, as specified in Table 1. The

methodology employed to prepare them was taken from Cortés et al. (2013). Each experimental unit consisted of 288 g of 5 mm-thick slices of green banana, which were immersed for 10 min in 1 L of anti-browning solution (S1, S2, and S3).

Obtaining green banana flour

The rejected green banana was processed 3 days after the banana was harvested. Bananas were manually peeled and then chopped into 5 mm-thick slices, using manual mandolins. The slices were immediately immersed in the anti-browning solution for 10 min. Subsequently, they were dried at 55 °C for 15 h in a forced convection oven (Memmert 750, Germany) and then ground in a blade mill (IKA MF 10 Basic, USA) to obtain the flour, which was packed in No. 12 metalized pet resealable bags (Alico, Colombia). They were stored at room temperature in a cool, dry place for further analysis.

Table 1. Anti-browning solutions.

Immersion solution	S1	S2	S3
Citric acid	50 mg 100 g^{-1} sample	50 mg 100 g^{-1} sample	50 mg 100 g^{-1} sample
Ascorbic acid		90 mg 100 g^{-1} sample	90 mg 100 g^{-1} sample
Sodium metabisulfite			500 ppm of the solution

Physicochemical characterization of green banana flour

The color was determined by instrumental measurement with a colorimeter (Chroma Meter CR-400/410, Japan) using the CIELAB color scale with the parameters L* (brightness), a* (red/green), and b* (yellow/blue) (Vélez-Uribe et al. 2023). Measurements were made in triplicate using Illuminant D65. The chromaticity parameters (C), hue angle (°H), and whiteness index (WI) were calculated. The pH was measured following the methodology described by AOAC 943.02 (AOAC 1997); titratable acidity (Padhi and Dwivedi 2022) with some modifications; water activity (aw) by modified AOAC 978.18 (AOAC 1997), moisture by AOAC 925.10 (AOAC 1997). The properties of the banana flours used in these treatments were compared to commercial banana flour (Harina de Cambur verde, Artesanos fit, Trujillo, Venezuela).

Evaluation of storage

The rejected green bananas' storage was evaluated on days 1, 3, 5, 7, 9 and 11. Color and firmness tests were carried out on both the shell and the pulp of the fresh fruits. In addition to the flour obtained, the color and pH parameters were evaluated under the aforementioned methodologies. Mechanical characterization was performed using the firmness test (Sanaeifar et al. 2016). It was determined in triplicate on a texture analyzer (Stable Micro System, TA-Xt2i) at three points. A firmness test was performed on bananas' shells and pulp. The firmness of the samples was determined by a one-way penetration test, using a 2 mm diameter stainless steel cylindrical shaft and a load cell 50 kg_f. The operating parameters for the firmness test were pre-test speed, 2 mm s^{-1}; test speed, 2 mm s^{-1}; post-test speed, 2 mm s^{-1}; and penetration distance, 20 mm. The color sensory analysis was carried out by a triangular test for sensory differentiation according to ISO 10399:2017 (ISO - International Standards Organization 2017) to a group of 40 selected panelists. They were presented with three samples—two from the same treatment and one different—and asked to identify the sample that had a different color among the three samples.

Statistical analysis

The effect of the anti-browning solution (S1, S2, and S3) on the physicochemical and color properties of processed banana flour, and the effect of storage time of the rejected green bananas (days 1, 3, 5, 7, 9, and 11) on the flour produced from it were analyzed by one-way factorial design. Means were assessed by a Tukey multiple comparison test with a significance level α=0.05 using the R statistical software (R Core Team 2022).

RESULTS AND DISCUSSION

Anti-browning solution

The effect of anti-browning solutions on physicochemical properties and CIELAB color coordinates (L*, a*, and b*) are shown in Table 2. The physicochemical and color properties of banana flour were not affected by pretreatment with S1,

S2, and S3 solutions. The parameters in the chromatic plane a* and b* of all samples are in the grey zone. L* is a parameter associated with the clarity of the sample; in general, the results obtained in this research show values close to 50, which could be defined as a flour in a medium range of clarity. The WI is a parameter derived from the combination of luminosity and yellow/blue (b*) in a single term (Pathare et al. 2013). Therefore, when obtaining low values of L* and b*, the WI was not high. However, when comparing these results with a commercial flour, more relevant differences are observed in parameters such as L* and WI, which are lower.

Table 2. Effect of the anti-browning solution on color parameters and physicochemical properties of banana flour.

Solution	Variable				
	L*	a*	b*	C	°H
S1	44.77±0.18[a]	1.56±0.34[a]	8.69±0.21[a]	8.83±0.26[a]	79.86±1.92[a]
S2	45.60±0.80[a]	1.50±0.21[a]	9.26±0.50[a]	9.38±0.46[a]	80.78±1.75[a]
S3	49.50±2.99[a]	0.94±0.43[a]	9.51±0.29[a]	9.56±0.24[a]	84.30±2.73[a]
Commercial flour	36.85±2.40	1.46±0.14	7.70±0.17	7.84±0.19	79.30±0.87
	Variable				
Solution	WI	Moisture %	a_w	pH	Acidity**
S1	44.07±0.14[a]	6.29±1.12[a]	0.166±0.045[a]	5.4±0.0[a]	0.16±0.05[a]
S2	44.80±0.72[a]	6.24±1.14[a]	0.168±0.037[a]	5.3±0.0[a]	0.20±0.00[a]
S3	48.60±2.98[a]	6.26±1.07[a]	0.175±0.035[a]	5.2±0.1[a]	0.20±0.00[a]
Commercial flour	36.36±2.37	10.56±0.15	0.459±0.06	5.9±0.0	0.13±0.03

The means of pretreatments with a common letter in a column do not differ significantly at a significance level α=0.05, according to Tukey's DSH test. **Acidity expressed as malic acid.

The moisture content and water activity (aw) properties in powdered products are critical, as they can affect other physical and chemical properties of food. In addition, they are key for shelf life and stability (Savlak et al. 2016). The moisture content found in green banana flours from Magdalena is within the percentages allowed in NTC 2799 (moisture<10%) (ICONTEC 2020). The results obtained are higher than those reported by Khoozani et al. (2019), who showed a moisture content in dried banana flour of 5.09, 4.56, and 4.46% at temperatures of 50, 80, and, 110 °C, respectively, and aw values of 0.25, 0.34, and 0.39 for the same temperatures—higher than those obtained in this experiment. Campuzano et al. (2018) evaluated the moisture content in cavendish banana flour (*Musa acuminata* AAA) from Ecuador at maturation stage one and obtained a higher moisture value of 10.88±0.17 g 100 g^{-1}. Meanwhile, Savlak et al. (2016) evaluated moisture, aw, and pH in "Dwarf cavendish" green banana flour (*Musa* spp. AAA) from Turkey and reported a moisture value of 9.07±0.35%, aw of 0.42±0.02, and pH of 5.66±0.01—higher than those presented in this study and similar to those of commercial flour. Solutions with only organic acids (S1 and S2) in some cases tend to be slightly more acidic because of the nature of the substances employed in the pretreatment; however, no statistically significant differences are reported between samples. The literature reports data that tend towards neutrality with pH=6.12 and 0.11% for acidity (Padhi and Dwivedi 2022).

As there were no significant differences among the anti-browning solutions to produce banana flour in the proposed design, the solution was selected considering other criteria such as the most used anti-browning agent, its adverse effects on health, and costs. Although all the agents used are considered GRAS, a positive effect of anti-browning agents has been observed when obtaining banana flours, e.g., citric acid used at different concentrations (Chang et al. 2022; Savlak et al. 2016). In addition, Sarpong et al. (2018) report the positive effect of citric acid on the inhibition of enzymes such as polyphenol oxidase. Ascorbic acid, in addition to inhibiting polyphenol oxidase, can repress the yield of o-quinones in diphenols (Homaida et al. 2017). Ali et al. (2015) also observed that a lower concentration of ascorbic acid acted as an inhibitor of enzymes, but a higher concentration only reduced the formation of quinones.

Sulfur dioxides or their salts, such as sodium metabisulfite, have also been applied due to their positive effect as anti-browning agents when obtaining banana flours (Padhi and Dwivedi 2022; Salazar et al. 2022). Although sodium metabisulfite has been widely used in the food industry due to its ability to prevent enzymatic and non-enzymatic browning and microbial growth, and as a bleaching agent that helps preserve flavor, texture, and color in food, it is regulated by INVIMA (maximum 1,500 mg kg^{-1}) in Colombia and there are some doubts about the use of sulphites in food products due to possible health effects (Ojeda et al. 2020).

Citric acid was selected as the best anti-browning agent considering that approximately 15,000, 1,500, and 204 t of citric acid, ascorbic acid, and sulfites were imported in 2021, which indicates increased availability of the first one, and their import costs were approximately 1.29, 641, and 2.13 USD kg^{-1}, respectively (MinCIT 2022); citric acid having the lower import cost.

Storage time

Once the anti-browning solution (S1) was selected, the fruit's storage time at low temperatures to obtain flour was evaluated using the color and firmness parameters of fresh green banana with the peel and in the pulp. Results are shown in Table 3. There are no significant changes in the peel firmness during storage with an average value of 13 to 14.5 N. Moreover, an increase in the pulp firmness was observed on the 3rd day of storage, and then it remained with values close to 6 N. The texture changes in the pulp can be due to several factors like the contributions of starch hydrolysis, the enzymatic disruption of the cell wall structure, the water migration from the banana peel to the pulp, fruit mechanical damage, fruit physiological problems, or poor post-harvest handling (Sinanoglou et al. 2023).

The hue angle or tone (°H) in vegetables expresses the color variation, which takes 0° for red, 90° for yellow, and 180° for green. Although this parameter gradually changes over time from green to yellow in bananas (Jaiswal et al. 2014), in this experiment, it remained green due to the low storage temperature that delayed ethylene production (Facundo et al. 2015). Values close to 90° in the pulp

Table 3. Effect of fresh banana storage time on color and firmness parameters.

Parameter	Part of the banana	DAY					
		1	3	5	7	9	11
L*	Peel	54.01±1.92^{a}	53.62±1.83^{a}	54.08±3.61^{a}	53.22±1.71^{a}	54.00±4.73^{a}	55.48±3.51^{a}
	Pulp	83.31±1.15^{b}	82.91±0.83^{b}	84.54±1.38^{b}	88.21±1.37^{a}	86.91±1.40^{a}	87.96±1.41^{a}
a*	Peel	-15.99±0.88^{c}	-17.1±0.75^{c}	-9.25±2.75^{a}	-11.94±1.25^{b}	-11.93±2.01^{b}	-13.32±0.92^{b}
	Pulp	-1.21±0.17^{ab}	-1.21±0.12^{ab}	-1.05±0.25^{a}	-1.24±0.12^{ab}	-1.54±0.29^{c}	-1.36±0.21^{bc}
b*	Peel	30.07±1.54^{b}	34.31±1.29^{a}	29.64±1.76^{b}	30.46±1.69^{b}	30.30±3.35^{b}	33.24±1.20^{a}
	Pulp	19.56±1.09^{bc}	18.17±0.92^{c}	19.79±1.10^{bc}	19.54±1.93^{bc}	22.13±1.23^{a}	20.97±0.95^{ab}
C	Peel	34.06±1.64^{bc}	38.33±1.45^{a}	31.12±2.43^{c}	32.73±1.94^{bc}	32.58±3.76^{c}	35.81±1.39^{ab}
	Pulp	19.59±1.08^{bc}	18.21±0.92^{c}	19.82±1.10^{bc}	19.58±1.93^{bc}	22.18±1.22^{a}	21.02±0.95^{ab}
°H	Peel	118.01±1.11^{a}	116.49±0.52^{a}	107.07±4.09^{c}	111.37±1.45^{b}	111.42±1.80^{b}	111.82±0.96^{b}
	Pulp	93.56±0.64^{ab}	93.81±0.50^{ab}	93.05±0.77^{b}	93.67±0.44^{ab}	94.00±0.80^{a}	93.70±0.57^{ab}
WI	Peel	42.73±1.32^{a}	39.81±1.75^{b}	44.41±2.19^{a}	42.86±0.57^{a}	43.38±2.21^{a}	42.83±3.08^{a}
	Pulp	74.24±1.13^{b}	75.02±1.00^{ab}	74.85±1.53^{b}	77.14±2.30^{a}	74.22±1.40^{b}	75.76±1.46^{ab}
ΔE	Peel	-	4.40	6.75	4.15	4.06	4.40
	Pulp	-	1.45	1.26	4.90	4.44	4.86
Firmness (N)	Peel	14.32±1.34^{a}	13.07±1.31^{a}	14.57±3.01^{a}	13.47±2.22^{a}	13.26±2.40^{a}	13.65±1.55^{a}
	Pulp	5.22±0.40^{b}	6.28±0.52^{a}	5.66±0.97^{ab}	5.51±0.44^{ab}	5.79±0.45^{ab}	5.90±0.60^{ab}

The means of properties with a common letter in a row do not differ significantly at a significance level α=0.05, according to Tukey's DSH test. Parameter ΔE was calculated based on day 1.

show a tendency to yellow. According to the ΔE in the banana peel, it was observed that the color difference on days 3, 5, 7, 9, and 11 is obvious to the human eye. Moreover, in banana pulp, the color difference in days 3 and 5 is smaller and could be appreciated by the human eye depending on the tone with respect to day 1. From day 7 of storage, changes in color with respect to day 1 are easily perceived by the human eye (Goswami et al. 2015).

Facundo et al. (2015) studied the influence of low-temperature storage on skin color and carotenoid content in two banana cultivars (cv. Prata and cv. Nanicao). They found that bananas stored at temperatures of 10 and 13 °C did not show changes in color and it was maintained until 15 days. Figure 1 shows that the color in the banana peel remained green during the 11 days of storage, although at day 5 some brown colorations began to appear. These results are similar to those reported by Wang et al. (2021), who observed some brown spots from day 4 of storage at 7 °C that may be associated with cold damage. It affects the parameters L*, C, and °H. During the 11 days, it was also found that the luminosity (L*) of the banana peel did not present significant differences, while significant changes were observed in the pulp (P<0.05) from day 7. The color changes of the banana pulp could be attributed to increases in the moisture content and oxygen penetration to the pulp during the storage period. Moreover, the redness (a*) fluctuations of the banana pulp could be associated with a differentiation of the total carotenoid-content during ripening and the brown spots on the peel (Sinanoglou et al. 2023).

Figure 1. Evolution of bananas in cold storage over time.

The effect of the storage time (1, 3, 5, 7, 9, and 11 day) on the fresh banana fruit to produce banana flour is shown in Table 4. In products such as flour, color is one of the sensory attributes that impacts the evaluation and acquisition of food for both the consumer and the food industries. Thus, whiter flour improves acceptance and interest, since when incorporated as an ingredient in products, it will cause few changes in the final color. When the yellow component (b*) or the green component (a*) predominates on the values of the coordinates a* and b*,

Table 4. Parameters of pH and color of banana flour.

Day	Parameter							
	pH	L*	a*	b*	C	°H	WI	ΔE'
1	5.57±0.01^{e}	52.46±1.48^{c}	1.45±0.06^{cd}	8.52±0.42^{b}	8.65±0.42^{b}	80.32±0.13^{cd}	51.68±1.38^{c}	-
3	5.68±0.0^{a}	52.97±0.88^{c}	1.58±0.05^{bc}	8.83±0.21^{b}	8.97±0.21^{b}	79.87±0.14^{d}	52.12±0.82^{c}	0.60
5	5.64±0.00^{bc}	51.83±1.26^{c}	1.48±0.02^{bc}	8.89±0.22^{b}	9.01±0.22^{b}	80.52±0.12^{c}	50.99±1.20^{c}	0.73
7	5.63±0.01^{cd}	53.53±1.12^{c}	1.36±0.04^{d}	9.42±0.20^{ab}	9.51±0.20^{ab}	81.78±0.14^{a}	52.57±1.05^{c}	1.40
9	5.62±0.01^{d}	64.69±2.70^{a}	1.62±0.08^{b}	10.37±0.60^{a}	10.49±0.61^{a}	81.12±0.08^{b}	63.14±2.42^{a}	12.37
11	5.65±0.01^{b}	59.11±2.64^{b}	1.94±0.03^{a}	9.35±0.34^{b}	9.55±0.34^{ab}	78.27±0.34^{e}	58.00±2.50^{b}	6.72

The means of pretreatments with a common letter in a column do not differ significantly at a significance level α=0.05, according to Tukey's DSH test. Parameter ΔE' was calculated based on day 1.

flour with tendencies to a darker or lighter yellow color is obtained (Bezerra et al. 2013).

The L* and WI values showed no statistically significant differences (P>0.05) until day 7 of storage with respect to day 1. However, although they are slightly higher than those found in the first part of this experiment, in the chromatic plane, the samples continue to be in the gray area with medium luminosity and whiteness. Lower L* values can be attributed to the oven drying process (Savlak et al. 2016). Additionally, the browning process is known to occur due to the presence of four components: oxygen, oxidizing enzyme (polyphenol oxidase), a metal ion (copper), and a suitable substrate (phenolic substrate) (Moon et al. 2020). Consequently, it would not be possible to attribute browning in flour only to the action of polyphenol oxidase. Regarding the color differences with respect to day 1, it is observed that the flours processed on days 3 and 5 are classified as non-perceptible ($\Delta E'$<1). On day 7 the changes are minor and could be perceived by the human eye depending on the tone of the sample on day 1 (1<$\Delta E'$<3). From day 9 of storage, there would be obvious changes for the human eye ($\Delta E'$>3) (Goswami et al. 2015).

Sensory color

Sensory analysis encompasses a set of techniques for correct judgment of human responses to food and tracks the potential bias effects of fraud, brand identity, and quality that influence consumer perception. Since it is a measurement science, it seeks—like other instrumental techniques—the accuracy, sensitivity, and prevention of false-positive results (Drake et al. 2023). Consumers generally evaluate the quality of a product based on its appearance and color; then, through this additional evaluation of color by instrumental methods, it intended to find out if a group of 40 panelists could determine the color differences between banana flours made on days 3, 5, 7, 9, and 11 with respect to day 1. Therefore, a triangular test was carried out in which a series of correct choices, beyond what is expected, is considered evidence of a discernible difference between samples (Mihafu et al. 2020). The results obtained for each day are presented in Table 5. The data were taken from a binomial statistical table, which establishes that when more than 19 panelists out of 40 respond correctly, the samples present statistically significant differences (P<0.05) (International Standards Organization- ISO 2017). The sensory panel of the triangular test showed a significant difference (P<0.05) between the banana flour made on days 9 and 11 of storage and that made on day 1, with a total of 20 and 24 correct answers, respectively. The color results of the flour at the sensory level confirmed what was obtained at the instrumental level, i.e., the color differences are more obvious for the human eye from day 9 of fresh green banana storage.

Table 5. Sensory color.

Day	Correct answers	*P*
3	10	0.9034
5	14	0.4703
7	9	0.9517
9	20	0.0214
11	24	0.0005

CONCLUSIONS

The contribution of this research indicates the anti-browning solutions used in this study do not have a significant effect on the color, moisture, aw, pH, and acidity of banana flour. Therefore, it is beneficial for the industry to select them appropriately based on their availability and cost. The use of citric acid is recommended as an anti-browning solution to produce banana flour due to its wide application in this field, low cost compared to the other two solutions evaluated, availability in the local market, and it is generally recognized as safe. Furthermore, this research corroborates the findings of other previous studies that have employed these chemical agents, obtaining similar results in line with the standards established by the corresponding regulations. Moreover, it is crucial to have knowledge of the maximum storage time of the fruit in the food industrial field to ensure

flour production that does not exhibit alterations in terms of taste, color, or poor texture, while avoiding the loss of essential nutrients. These factors can directly impact production costs and process profitability. According to the literature, the suitable storage temperature for green bananas should be close to 13 °C; however, they were stored at 7 °C to evaluate a limited storage temperature for this climacteric fruit. The maximum storage time of rejected fresh green banana fruits at 7 °C to produce banana flour should not exceed 7 days because their color may be affected. It is suggested that future research delve further into the combination of physical treatments along with the use of anti-browning agents, or even the use of natural extracts as alternatives to these chemical agents. Furthermore, the effect of pretreatment with citric acid should be evaluated in pasting, thermal, and techno-functional properties of banana flour. Thus, innovative approaches can be identified to optimize the quality and preservation of banana flour, and both the industry and consumers could profit from it.

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