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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

Effective horticultural production systems in Valle del Cauca

Sistemas de producción hortofrutícolas efectivos en el Valle del Cauca

The agricultural sector constitutes one of the main economic branches of Colombia. However, in the last two decades, this sector has had lower growth than that of the national economy. According to the National Business Association of Colombia (ANDI), between 2001 and 2020, the growth of the agricultural sector was 2.9%, 10% less than that of the national economy, which reached a value of 3.3%. Considering the importance of this sector in the economic and social well-being of the rural communities of the country, as well as the growing demand for food due to the increase in world population, it is important to prioritize policies, programs, and projects that help boost the agricultural sector at a national and regional level.

The recent agricultural development in the country has been deeply influenced by international events, such as the confrontation between Russia and Ukraine, which have significantly affected the price of agricultural supplies, reaching historical highs. Considering that a large part of rural producers in Colombia are classified as small and medium producers with small production plots, these market fluctuations impact their cost structure, putting them in vulnerable situations that affect their economic well-being. Therefore, it is necessary to find solutions that minimize the exposure of producers to market fluctuations without sacrificing their productive yields or their sustainability in the short or long term. This is why we must seek to make efficient use of available resources and implement transition processes from traditional systems towards a rapid substitution of supplies through agroecological principles.

The Valle del Cauca department has the largest extension of areas dedicated to permanent agricultural exploitation in the country, producing, according to information obtained from the Ministry of Agriculture and Rural Development, around 20.5 million tons every year. In this department, several efforts have been made to promote the development of the agricultural sector. Since 2006, the *Plan Frutícola del Valle del Cauca* was adopted, where 16 fruit species were prioritized based on the economic, climatic, technological, environmental, and social conditions of the department. Based on this plan, several projects have been developed with a common objective of transferring technical knowledge to improve agronomic practices, as well as strengthening small-producer associations. The *Programa Integral de Fruticultura* (PIF), executed by the Corporación para el Desarrollo Social y Cultural del Valle del Cauca -Corpovalle-, has been positively impacting the agricultural sector in the department since 2017, especially the aspects of associativity, competitiveness, and productivity of small and medium producers. The results of the different phases of the PIF have brought great progress in the social well-being and economic development of the region.

However, given the global and national conditions already mentioned, it is necessary to go further and look for alternatives to the use of chemical fertilizers in the production processes. An approach that allows for both an efficient and an environmentally friendly production, that protects, preserves, and maintains the department's natural resources through time. Aiming for the sustainability of agriculture as a profitable and long-term business for the local communities, and achieving the rational use of resources, as well as the recognition of farmers as key players in all processes. This last point has been identified as a fundamental aspect to further develop. Through the experience and results obtained in the implementation of the different phases of the PIF in Valle del Cauca, it has been shown that, although strengthening the organizational processes of associations has had significant impacts on them, it is also important to direct efforts towards the associates themselves. While technical and technological knowledge is

important for farmers to optimally develop production activities on their farms, soft skills are necessary for the day-to-day activities of producers and the associations to which they belong. Therefore, a comprehensive strengthening of the agricultural sector in the Valle del Cauca department must be sought to improve the technical and social capacities of producers, as a mechanism for long-term sustainability.

A complete intervention will not only aid with more efficient resource management, which can help improve production levels per hectare, but it can also help consolidate associative schemes through the individual strengthening of the members' capabilities. This can open the door to benefits from mechanisms such as economies of scale, as well as other mechanisms that strengthen their market positioning and their capacity to offer products in agri-food markets. Likewise, including agroecology concepts and achieving the transition from the use of chemical to organic supplies can help improve the sustainability level of productive activities and contribute to the recovery of soils -while avoiding exposure to fluctuations in the agricultural input market-, allowing for a more efficient use of resources.

It is essential to take another step into the consolidation of the agricultural sector by maximizing the efficiency in the use of resources to reduce or stabilize production costs -which are being affected by global dynamics-, and to achieve the maximum production level possible with the available resources, while taking care of the department's natural resources. This requires interventions with precise production processes and a well-founded diagnosis of all the factors involved. This can be complemented with a comprehensive intervention that includes the social aspects that affect the development of farmers as individuals, the consolidation of individual and collective competencies, and the intervention of farmer associations from a social aspect to strengthen the interaction of their members and guarantee that these associative mechanisms reach adequate levels of sustainability. The implementation of these alternative methodologies based on agroecological concepts would allow for promoting the economic, social, and environmental development of the rural areas of the department while impacting the regional and national economy.

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Association between weeds and plant growth-promoting rhizobacteria in the phytoremediation of lead-contaminated soil

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Asociación entre malezas y rizobacterias promotoras del crecimiento vegetal en la fitorremediación de suelo contaminado con plomo

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Keywords:
Bacterial consortia
Echinochloa colona (L.) Link
Lead tolerance
Phytotoxicity
Synergy

ABSTRACT

Lead is a persistent heavy metal in the soil that can accumulate in edible plants, so non-polluting strategies are required for its removal. In this study, the efficiency of weeds with associated rhizobacteria in phytoremediation of soil contaminated with lead (800 ppm) was investigated. Weeds with lead tolerance were selected, as well as rhizobacteria that promote plant growth *in vitro*. Several bacterial consortia (BC) were applied on three weed species, and the weight of the aboveground biomass of the weeds, the phytotoxicity of the soil after phytoremediation, as well as the parameters of the phytoremediation of lead in the soil with lower phytotoxicity, were evaluated. As a result, 20% of the weeds analyzed were tolerant to lead with indices of 0.80 (*Echinochloa colona* (L.) Link), 0.76 (*Cyperus corymbosus* Rottb.), and 0.72 (*Sorghum halepense*). BC solubilized phosphates, produced indole acetic acid, and increased the fresh biomass of plants (4.14-14.32%). Furthermore, the lowest level of phytotoxicity in the soil was detected in the treatment of *E. colona* (L.) Link with *Pseudomonas* spp. and *Acinetobacter* spp. (BC1), as well as a bioaccumulation factor of 0.1650 in the foliage, 1.0250 in the roots, and a translocation factor of 0.1611. Finally, 78.83% lead removal was determined in *E. colona* (L.) Link with rhizobacteria, compared to the 57.58% obtained with *E. colona* (L.) Link without rhizobacteria. The efficiency of the association of weeds and plant growth-promoting rhizobacteria in the phytoremediation of soils contaminated with lead was demonstrated.

Palabras clave:
Consorcios bacterianos
Echinochloa colona (L.) Link
Tolerancia al plomo
Fitotoxicidad
Sinergia

RESUMEN

El plomo es un metal pesado persistente en el suelo que puede acumularse en las plantas comestibles, por lo que se requieren estrategias no contaminantes para su remoción. En este estudio se investigó la eficiencia de malezas con rizobacterias asociadas en la fitorremediación de suelo contaminado con plomo (800 ppm). Se seleccionaron malezas con tolerancia al plomo, así como rizobacterias que promueven el crecimiento vegetal *in vitro*. Se aplicaron diversos consorcios bacterianos (CB) sobre tres especies de malezas, y se evaluó el peso de la biomasa aérea de las malezas, la fitotoxicidad del suelo después de la fitorremediación, así como los parámetros de la fitorremediación del plomo en el suelo con menor fitotoxicidad. Se encontró que el 20% de las malezas analizadas fueron tolerantes al plomo con índices de 0,80 (*Echinochloa colona* (L.) Link), 0,76 (*Cyperus corymbosus* Rottb) y 0,72 (*Sorghum halepense*). Los CB solubilizaron fosfatos, produjeron ácido indol acético y aumentaron la biomasa fresca de las plantas (4,14-14,32%). Además, el menor nivel de fitotoxicidad en el suelo se detectó en el tratamiento de *E. colona* (L.) Link con *Pseudomonas* spp. y *Acinetobacter* spp. (CB1), así como un factor de bioacumulación de 0,1650 en el follaje, 1,0250 en las raíces y un factor de translocación de 0,1611. Finalmente, se determinó 78,83% de remoción de plomo en *E. colona* (L.) Link con rizobacterias, a comparación del 57,58% obtenido con *E. colona* (L.) Link sin rizobacterias. Se demostró la eficiencia de la asociación de malezas y rizobacterias promotoras del crecimiento vegetal en la fitorremediación de suelos contaminados con plomo.

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More than 90% of environmental pollutants, such as heavy metals, are retained in soil particles. Lead contamination is significant because this heavy metal has a long residence time, persisting for 1000–3000 years (Rodríguez et al. 2016). Environmental exposure to lead (1,300–32,260 mg kg^{-1} in soil) affects public health worldwide (Covarrubias and Peña 2017) and is the cause of 143,000 deaths per year (Rodríguez et al. 2016). Lead (0.13-446 ppm) has been quantified in irrigation water contaminated with industrial residues or remains of fertilizers, in vegetables eaten fresh, strawberries, potatoes, cassava, sugar cane juice, and industrial fruit juices and vegetables (Salas-Marcial et al. 2019), a condition that constitutes a potential problem in food safety for humans and animals.

Lead enters the body through the respiratory, digestive, and dermal routes (Covarrubias and Peña 2017). Lead is not essential for plants and does not have any biological function, but it is transported to plant tissues. Once inside, the heavy metal increases the production of reactive oxygen species (ROS) that affect lipid cell membranes and reduce photosynthetic activity, with symptoms such as chlorosis, necrosis, decreased root and foliage growth, as well as changing root branching patterns (Nakbanpote et al. 2016).

Resistant plants present mechanisms such as evasion and tolerance that allow them to survive with high concentrations of heavy metals. In evasion, root cells limit and restrict the uptake and movement of heavy metals in plant tissues through the mechanisms of root uptake, metal precipitation, and exclusion. Exclusion barriers between roots and foliage minimize the accessibility of metals present in the soil to the roots (Sabreena et al. 2022). When the mechanisms are not adequate to reduce toxicity in plants, ROS are formed in the cytoplasm, which causes oxidative stress. In this context, plants activate enzymatic and non-enzymatic antioxidant defense mechanisms to overcome the negative effect of ROS (Sabreena et al. 2022).

Phytoremediation is defined as the use of plants and associated microorganisms to reduce contaminants in water, soil, and air. Also, It does not contaminate the environment and can be used to extract or immobilize metals, metalloids, and radionucleotides, as well as organic xenobiotics (Rigoletto et al. 2020; Sabreena et al. 2022). However, in some cases, the remaining concentrations exceed the maximum permissible limits established by the regulations to consider it as bioremediated soil (MINAM 2017). Phytoremediation can be improved with strategies that favor and accelerate the process, such as the synergy between plants and plant growth-promoting rhizobacteria (PGPR). These plant-associated microorganisms modify the bioavailability of lead through the production of siderophores, organic acids, exopolysaccharides, and solubilization of precipitated phosphates (Shah and Daverey 2020).

Weeds are fast-growing and can be used for phytoremediation of soils contaminated with lead (Contreras-Pinto et al. 2016). However, before its use, it is necessary to investigate its capacity to reduce the contaminant alone, and in combination with rhizosphere microorganisms, with the perspective of overcoming the limitations that affect the process. For this reason, this study aimed to determine the potential of weeds and associated rhizobacteria for soil phytoremediation of a solid waste dump contaminated with lead.

MATERIAL AND METHODS

Sampling location

The municipal solid waste dump, located in Lambayeque, Peru, covers an area of 2.95 km^2 between the parallels 06° 55' 04" South latitude and 79° 44' 07" West longitude. An identification sampling (IS) was carried out, aimed at determining if the soil was contaminated in an area of 1 ha (0-10 cm depth). The sampling pattern was of random distribution with regular grids, for which nine grids or cells (333.5 m^2) were delimited with parallel and perpendicular lines (MINAM 2017). In 1 m^2 squares delimited in the central part of each cell, the 2 cm of surface soil was removed. In each square, 24 kg of soil (10 cm deep) were collected and deposited in polyethylene bags.

The soil from the nine sacks was mixed on a blanket and homogenized using the conning and quartering method (Contreras and Carreño 2018), and two representative samples (1 and 2 kg) were collected. In the 2 kg sample, the physical and chemical analysis was performed, while in the 1 kg sample, the count of total microorganisms was performed using the most probable number (MPN) technique in nutrient broth and the MPN of lead-tolerant microorganisms in nutrient broth with 50 mg of Pb L^{-1}. The

rest of the soil was kept in the greenhouse of the Pedro Ruiz Gallo National University for the experimental phase of this research. The soil had a sandy texture, non-saline condition (EC=0.41 dS m^{-1}), organic matter (0.02%), nitrogen (0.006%), phosphorus (3.7 ppm), potassium (131 ppm), lead (11.91 mg kg^{-1}), cadmium (1.12 mg kg^{-1}) and chromium (16.94 mg kg^{-1}).

Selection of lead-tolerant weeds

In six agricultural fields, six specimens of 15 weeds with similar height and at the beginning of flowering were selected. With a shovel, the plants with their roots and rhizospheric soil were extracted and then transplanted into polypropylene pots (4 kg capacity) with 3 kg of soil from the municipal waste dump, mixed with 0.3 kg of rice husks.

Two specimens of *Echinochloa colona* (L.) Link*, Cyperus corymbosus* Rottb. and *Sorghum halepense* were planted in the pots (three pots per weed) and irrigated with drinking water (stored for 24 h) twice a week. Weeds were classified as adapted to transplanting if their vigor and green color were maintained and they developed leaves or shoots the following 10 days. In the adapted weeds, the tolerance to lead was investigated, for which four of the six plants were irrigated with a solution of lead nitrate Pb $(NO_3)_2$ at a concentration of 10 ppm of Pb once for the first, second, and third weeks. Since the fourth week, irrigation was carried out every 3 days with a Pb $(NO_3)_2$ solution, whose concentration increased geometrically for five weeks: 50, 100, 200, 400, and 800 ppm of Pb. The two remaining specimens of each weed were irrigated with lead-free water and constituted the reference controls of the normal phenotype.

Lead tolerance was qualified by the survival of 100% of the plants, and the physical appearance similar to the controls, in terms of height, vigor, and color of the leaves. After 60 days of the first irrigation with the Pb $(NO_3)_2$ solution, the biomass of the plants was weighed and the stress tolerance index was calculated according to Chaturvedi et al. (2020) method. Weeds tolerant to Pb (800 ppm) were those that presented tolerance indices of 0.5-0.8, while highly tolerant weeds presented indices greater than 0.8 according to Frachia et al. (2022).

Plant growth promotion by rhizospheric bacteria

The rhizospheric soil (10 g) of lead-tolerant weeds was inoculated into 100 mL of nutrient broth and incubated at 30 °C for 5 days (Gupta et al. 2018). The bacteria were isolated using nutrient agar supplemented with 50 mg L^{-1} of Pb, incubated at 30 °C for 5 days. The developed colonies were grouped according to their morphological characteristics (morphotypes) and reaction to Gram staining. A representative of each morphotype was selected, and those that grew (turbidity) in nutrient broth with 100, 200, 400, and 800 mg L^{-1} of Pb were cultured in nutrient agar with and without lead, and incubated at 30 °C for 48 h, constituting the pure cultures of lead-tolerant bacteria (Manzoor et al. 2019).

The characteristics that show the *in vitro* plant growth promotion was investigated in lead-tolerant bacteria. The phosphate-solubilizing activity of bacteria was preliminarily evaluated on Pikovskaya agar by observing the appearance of a translucent halo around the colonies after 96 h of incubation, while the quantification of phosphate solubilization was evaluated in Pikovskaya broth. On the other hand, indole acetic acid (IAA) production was evaluated in Tryptic Soy Broth (TSB) plus the Salkowski reagent, according to Liu et al. (2022).

The soluble phosphorus concentration was calculated with equation 1 using a wavelength of 690 nm, and the IAA concentration with equation 2 using a wavelength of 530 nm.

$$X_1 = \frac{(Y_1 - 0.0002)}{0.07} \quad (1)$$

$$X_2 = \frac{(Y_2 - 0.076)}{0.004} \quad (2)$$

Where X_1 is the soluble phosphorus concentration (ppm), Y_1 is the corrected absorbance of bacterial sample 'n' minus the absorbance of the culture medium control (0.119); X_2 is the concentration of IAA (ppm), Y_2 is the corrected absorbance of bacterial sample 'n' minus the absorbance of the culture medium control (0.080).

Influence of rhizospheric bacterial consortia on the aboveground biomass of weeds and phytotoxicity of the phytoremediated soil

The soil (50 kg) previously collected in the dump was sieved (<5 mm), mixed with 5 kg of rice husk, autoclaved at 121 °C for 20 min (Chaturvedi et al. 2020) and conditioned in 18 pots, at a rate of 3 kg per pot. Next, the soil of nine

pots was artificially contaminated with a $Pb(NO_3)_2$ solution until reaching 0.127% w/w, equivalent to 800 mg kg^{-1} of Pb, considered the minimum concentration for commercial/industrial/extractive soil according to the Environmental Quality Standards (EQS) established by MINAM (2017).

After one month, the three lead-tolerant plant species were transplanted in triplicate into pots with lead-contaminated and non-lead-contaminated soil (two plants per repetition). Before transplanting, in the treatments where the bacterial consortia (BC) were inoculated, the roots of plants with a similar phenotype were immersed in a suspension of the corresponding bacterial consortium for 30 min.

The inoculum of the bacteria was obtained by the large-scale sowing method with two volumes: mother and definitive. The bacteria were grown independently in nutrient broth with 800 ppm of Pb. The mother culture was obtained after inoculating 1.8 mL of cultured broth with each bacterium in 16.2 mL of nutrient broth with 800 ppm of Pb and incubated at 30 °C for 24 h. The definitive culture was obtained after inoculating the mother culture (18 mL) of each bacterium in 162 mL of nutrient broth with 800 ppm of Pb and incubated at 30 °C for 24 h. In this way, 180 mL of the definitive culture of each bacterium were obtained, while for the consortia, the definitive cultures of six bacteria per weed were mixed with a total of 1,080 L (Acosta and Bustamante 2020). Finally, at 90 days after transplanting, the fresh aerial biomass of each plant was weighed.

Phytotoxicity of the phytoremediated soil

The phytotoxicity of the phytoremediated soil was determined in seeds of *Raphanus sativus* L. "rabanito" var. Crimson Giant. In Petri dishes, 10 g of soil from each repetition of the six investigated treatments were deposited in triplicate, moistened with sterilized distilled water, and 25 seeds (in 18 Petri dishes) were conditioned in each plate. Three Petri dishes with uncontaminated soil were included as control. After 120 h at room temperature (20-22 °C), the germinated seeds were selected and quantified, the length of each radicle was measured, and the relative germination percentages (RGP), relative radicle growth (RRG) and the germination index (GI) were calculated according to Lakhal et al. (2017).

The phytotoxicity level was determined by relating the GI to the presence of toxic substances: $GI \geq 80\%$, a very low number of toxic substances with a low level of toxicity, 80%>GI>50%, a moderate number of toxic substances with a moderate level of toxicity, and $GI \leq 50\%$, an abundant number of toxic substances with a severe level of phytotoxicity. The soil and weeds, both with and without bacterial consortia, which exhibited the lowest levels of toxicity in radish seeds, were collected to quantify lead and determine phytoremediation parameters.

Parameters of lead phytoremediation in soil with the lower phytotoxicity

The roots and foliage of the selected weeds were washed, and together with the soil that accompanied them, were dehydrated in an oven at 75 °C for 96 h (Lu et al. 2021). Both were conditioned and sent to the Soil, Plant, Water, and Fertilizer Analysis Laboratory of the National Agrarian University - La Molina to quantify lead by atomic absorption spectrophotometry (Perkin Elmer, Model PIN 500). The quantified lead values in the soil, roots and foliage were used to calculate the bioaccumulation factor (BAC), the translocation factor (TF) and the percentage of lead removal from the soil (Chaturvedi et al. 2020).

Identification of Phytoremediating Bacteria

The identification of the genus of bacteria in the consortium was carried out by several biochemical like catalase, oxidase, motility, production of acidity from glucose on triple sugar iron (TSI) agar, reduction of nitrates to nitrites, hydrolysis of urea and starch, indole production, citrate utilization, lysine decarboxylation on lysine iron agar (LIA) and gelatin and esculin hydrolysis (Silva et al. 1999; Salazar and Nieves 2005; Álvarez-López et al. 2014; Dipak and Sinha 2017).

Statistical analysis

The data analyzed were represented with the mean ± standard deviation (SD) of three repetitions. Statistical analysis of fresh biomass was performed using variance analysis (ANOVA), followed by Tukey's test (P<0.05). For all the analyses, the statistical program SPSS V. 22.0 was used.

RESULTS AND DISCUSSION

Selection of lead-tolerant weeds

A 20% of the selected weeds were considered tolerant to lead (800 ppm), with tolerance indices of 0.80 (*Echinochloa colona* (L.) Link), 0.76 (*Cyperus corymbosus* Rottb.) and

0.72 (*Sorghum halepense*). The tolerance of weeds to lead (800 ppm) coincides with the reports of Amadi and Gbosidom (2022) with *E. colona* (L.) Link, and Sabreena et al. (2022) with *Sorghum* spp. Also, lead decreased the fresh biomass of weeds (Figure 1), a negative effect previously reported by Chaturvedi et al. (2020). The researchers demonstrated that lead (50-100 ppm) decreased plant height (15.38%) and biomass (13.7%), as well as chlorophyll, carotenoids, and nitrogen. On the other hand, Frachia et al. (2022) reported that lead (100-500 ppm) increased the weight of foliage and roots of *Inga uruguensis*, and based on the fresh biomass of the plants, a tolerance index (TI) of 1.21 was reached.

The morphological and physiological changes of plants in lead-contaminated soils vary according to the concentration of the heavy metal and the plant species (Lu et al. 2021). When the concentration of the contaminant is <500 ppm, an increase in growth parameters has been reported (Liu et al. 2018; Frachia et al. 2022), but a decrease in height and biomass in plants developed with 50-100 ppm Pb was also observed (Chaturvedi et al. 2020).

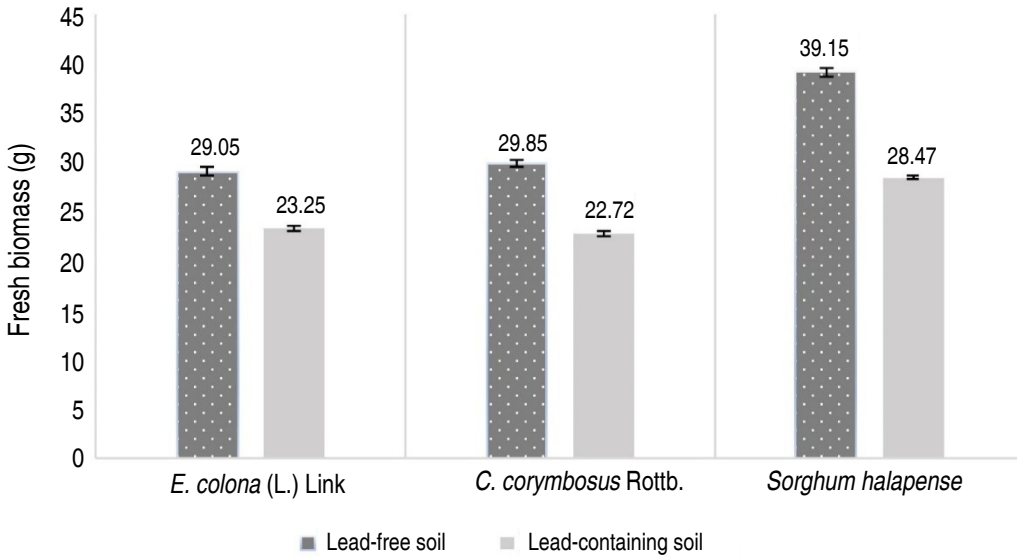

Figure 1. Weight of fresh weed biomass in soil contaminated and not contaminated by lead.

Plant growth promotion by rhizospheric bacteria

In the rhizosphere of three lead-tolerant weeds, 26 colonies of Gram-negative (76.9%) and Gram-positive (23.1%) bacteria were isolated on nutrient agar with 50 mg L^{-1} of Pb, among which 92.3% grew in nutrient broth with 100 and 200 mg L^{-1} of Pb, 76.9% in broth with 400 mg L^{-1} of Pb, and 50% in broth with 800 mg L^{-1} of Pb. In the rhizospheric soil of *E. colona* (L.) Link, 10 cultures of bacteria were obtained, and 60% of Gram-negative bacteria grew with 800 mg L^{-1} of Pb. In the rhizospheric soil of *C. corymbosus* Rottb., seven cultures of bacteria were obtained that grew with 400 mg L^{-1} of Pb, and 71.4% with 800 mg L^{-1} of Pb. In the rhizospheric soil of *S. halepense*, nine cultures of bacteria were obtained, among which 44.4% grew in broth with 400 mg L^{-1} of Pb, and 22.2% in broth with 800 mg L^{-1} of Pb. Similar results were reported by Gorelova et al. (2022) and Manzoor et al. (2019), who isolated microorganisms in the rhizosphere of plants in soil contaminated with lead artificially and by anthropic activity, respectively. Manzoor et al. (2019) determined 62% of lead tolerant Gram-negative bacteria. Moreover, Liu et al. (2018) determined significant changes in the microbial population developed in a lead-contaminated site, an increase in the relative abundance of the bacterial genera *Flavisolibacter, Kaistobacter*, and *Pseudomonas*, as well as a decrease in the genera *Bacillus, Adhaeribacter, Pantibacter*, and *Paenibacillus*. The rhizosphere is the soil close to the roots (5 mm) that is directly influenced by root exudates, and where the largest population of microorganisms that interact with plants is found (Gamalero and Glick 2022).

All the microorganisms from bacterial consortium one (BC1) solubilized tricalcium phosphate and produced AIA (Table 1), characteristics that show *in vitro* growth promotion in plants. Phosphate solubilizing bacteria produce organic acids that destabilize the precipitated mineral and release soluble phosphorus to plants (Gavrilescu 2022). The increase in phosphorus availability favors plant growth and metabolite processes such as photosynthesis, energy transfer, and carbohydrate utilization (Corrales et al. 2017). Likewise, rhizospheric microorganisms produce auxins such as IAA that induce the proliferation of the root system, cytokinins related to cell growth and regulation of hormones such as ethylene through the activity of the enzyme amino 1-carboxylate (ACC) deaminase, in addition to nitrogen fixation, phosphate solubilization, siderophore synthesis and ammonium production (Gavrilescu 2022).

Table 1. Solubilized phosphorus and indole acetic acid produced by lead-tolerant bacterial consortia.

Bacterial consortia	Bacterium code	Isolation source (Weeds)	Soluble phosphorus * (ppm)	AIA* (ppm)
BC1	1	*E. colona* (L.) Link	11.93±0.25	69.00±0.31
	3	*E. colona* (L.) Link	9.09±0.27	51.00±0.29
	4	*E. colona* (L.) Link	7.97±0.32	64.75±0.38
	6	*E. colona* (L.) Link	8.18±0.36	63.50±0.35
	7	*E. colona* (L.) Link	10.55±0.39	65.75±0.41
	9	*E. colona* (L.) Link	7.41±0.24	66.25±0.38
BC2	17	*C. corymbosus* Rottb.	0	37.75±0.28
	11	*C. corymbosus* Rottb.	7.45±0.41	41.75±0.37
	13	*C. corymbosus* Rottb.	7.40±0.34	54.25±0.28
	12	*C. corymbosus* Rottb.	0	42.75±0.27
	14	*C. corymbosus* Rottb.	0	45.5±0.41
	15	*C. corymbosus* Rottb.	0	52.5±0.52
BC3	20	*S. halepense*	0	0
	25	*S. halepense*	6.88±0.39	40.25±0.38
	24	*S. halepense*	0	36.75±0.36
	26	*S. halepense*	7.24±0.33	36.10±0.39
	20	*S. halepense*	0	0
	24	*S. halepense*	0	0

***** Average of three repetitions.

Influence of rhizospheric bacterial consortia on weed growth and phytotoxicity of phytoremediated soil

The consortia of rhizospheric bacteria positively influenced the growth of lead-tolerant weeds, observing increases of 14.32% (*E. colona* (L.) Link + BC1), 4.60% (*Cyperus corymbosus* Rottb. + BC2) and 4.14% (*Sorghum halepense* + BC3). Tukey's mean comparison test showed that the highest value of fresh biomass corresponded to the *E. colonum* plus BC1, with significant differences compared to the other treatments (Figure 2), a similar result to the reported by Manzoor et al. (2019) in the ornamental plants *Pelargonium hortorum* and *Mesembryanthemun criniflorum* grown in soil artificially contaminated with 500-2,000 mg kg^{-1} of Pb. The researchers showed that *Bacillus tequilensis* increased the biomass of both plants in soils with and without lead concentrations. There was an increase of 65.52% in *M. criniflorum* and 64.04% in *P. hortorum* in the soil with 2,000 mg kg^{-1} of Pb.

Liu et al. (2018) showed that 100 ppm of lead did not affect the growth of *T. repens*; however, 500 ppm of lead significantly decreased root length (19.08%), plant height (25.92%), aerial biomass (25.1%) and root biomass (24.3%).

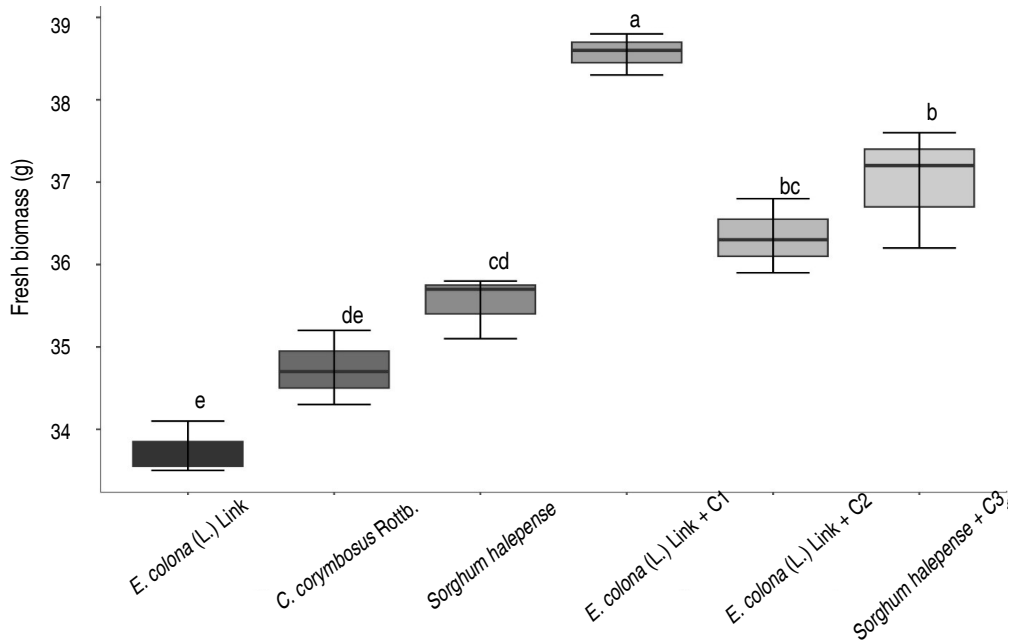

Figure 2. Fresh biomass of weeds at 90 days after inoculation of lead-tolerant bacterial consortia (BC). Different lowercase letters indicate significant differences between treatments (P<0.05).

The level of phytotoxicity was from severe to moderate in the soil where the lead-tolerant weeds were grown for 90 days, and it was from moderate to low in the soil with the lead-tolerant weeds inoculated with the bacterial consortia. The soil where the highest germination rate of radish seeds was reached (89.56%), and therefore the lowest level of phytotoxicity (low) was that corresponding to *E. colona* (L.) Link with BC1 (Table 2).

Table 2. Soil phytotoxicity level in *Raphanus sativus* L. seeds 90 days after sowing weeds with and without lead-tolerant bacteria consortia.

Treatments	Relative percentage of germination (RPG)	Average root elongation (cm)	Relative radicle growth (RRG)	Germination rate GI (%)	Phytotoxicity level
E. colona (L.) Link	79.17	3.22	60.64	48.01	Moderate
C. corymbosus Rottb.	77.08	3.1	58.38	44.99	Severe
Sorghum halepense	76.04	2.97	55.93	42.53	Severe
E. colona (L.) Link + BC1	92.7	5.13	96.61	89.56	Low
C. corymbosus Rottb. + BC2	88.54	4.63	87.19	77.2	Moderate
S. halepense + BC3	86.45	4.8	90.39	78.14	Moderate

Parameters of lead phytoremediation in soil with the lower phytotoxicity

Lead phytoremediation parameters in soil with lower phytotoxicity, planted with *E. colona* (L.) Link increased with the inoculation of bacterial consortia. In the *E. colona* (L.) Link inoculated with BC1, the bioaccumulation factor (BAC) was 0.1651 in the foliage, and 1.0250 in the roots; the translocation factor (TF) was 0.1611 compared to 0.0115, 1.0116 and 0.0114 in plants not inoculated with bacteria. In this context, the percentage of lead removal in the soil was 78.83% with *E. colona* (L.) Link inoculated with BC1, and 57.58% with uninoculated *E. colona* (L.) Link (Table 3).

Table 3. Parameters of phytoremediation of soil contaminated with lead by *Echinochloa colona* (L.) Link with and without bacterial consortium 1 (BC1).

Parameters	Values
Initial lead into the ground (mg kg^{-1})	800.0
Lead in foliage of plants without bacteria (mg kg^{-1})	9.23
Lead in plant roots without bacteria (mg kg^{-1})	808.5
Lead in soil from plants without bacteria (mg kg^{-1})	339.38
Lead in foliage of plants with bacteria (mg kg^{-1})	132.13
Lead in plant roots with bacteria (mg kg^{-1})	820.0
Lead in soil from plants with bacteria (mg kg^{-1})	169.38
BAC of foliage of plants without bacteria	0.0115
BAC of roots without bacteria	1.0106
TF of foliage and roots without bacteria	0.0114
Lead removal in plants without bacteria (%)	57.58
BAC of plant foliage with bacteria	0.1651
BAC of roots with bacteria	1.0250
TF of foliage and roots with bacteria	0.1611
Lead removal in plants with bacteria (%)	78.83

TF<1=Plant that does not translocate metal and is a phytostabilizer. BAC>1 and TF<1=Phytostabilizer plant, and 1 ≤ BAC<10=Bioaccumulator plant.

The concentration of lead accumulated in the roots was higher than the foliage of the *E. colona* (L.) Link plants with and without bacteria, a superiority that coincides with the studies of Gorelova et al. (2022) and Lu et al. (2021). In this regard, Gorelova et al. (2022) quantified 1.57–7.38 mg kg^{-1} of Pb in roots, and 0.14–0.88 mg kg^{-1} of Pb in foliage of *Echinochloa frumentacea*, and concluded that this species is not suitable for remediation, because it accumulates and removes only a small amount of lead (0.18–0.94 g ha^{-1}), compared to other plants such as *Sorghum halepense* with 107–378 g ha^{-1}. After lead enters the soil, it remains mostly in divalent forms (oxides and sulfides). Precipitated forms of lead phosphate [$Pb_3(PO_4)_2$] and lead carbonate ($PbCO_3$) are found in the root, which are difficult to transport to the aerial part (Lu et al. 2021). Plants respond to lead stress in different ways, but higher BAC is common in roots compared to foliage (Lu et al. 2021). These researchers determined a BAC range of 0.931-1.062 in the root of *Plantago asiatica* L. and 0.074-0.087 in the leaves, a result that demonstrated that the root is the main organ for absorption and accumulation of lead.

The bioaccumulation factor (BAC) of lead in the roots of *Echinochloa colona* (L.) Link with and without bacteria was greater than one and the translocation factor (TF) was less than one, a result that demonstrated this plant species is a phytostabilizer of lead, due to plants with a BF>1 and TF<1 are appropriate for phytostabilization (Rigoletto et al. 2020). The bioaccumulation or bioconcentration factor estimates the absorption capacity of metals in plants, and the translocation factor evaluates the transport of metal from the root to the foliage (Rigoletto et al. 2020). The TF values of 0.1611 and 0.0114 in *E. colona* (L.) Link with and without bacteria were lower than the range of 0.061–0.819 reported by Lu et al. (2021) in *Taraxacum mongolicum* and *Plantago asiatica* L. developed with 5% of lead. According to Rigoletto et al. (2020), plants with a translocation factor less than one are not capable of transporting the contaminant from the roots to the foliage and are used for the phytostabilization of the contaminant. In this way, they immobilize lead and avoid the contamination of groundwater.

The lead content decreased in the phytoremediated soil with and without bacteria, a decrease previously reported by Lu et al. (2021) and Gorelova et al. (2022). At the end of the evaluation period of this study, 57.58% removal of the contaminant was reached in the soil of the plants where

bacteria were not applied, and 78.83% when bacteria were applied. The range of percent lead removal is greater than the 13.01–31.89% reported by Lu et al. (2021) in soils contaminated with 2–5% lead and remediated with *Artemisia capillaris* and *Plantago asiatica* L.

Microorganisms favor the phytoremediation of contaminated soil directly by reducing the toxic effect and increasing the metal absorbed in the roots and foliage (Manzoor et al. 2019), and indirectly through the promotion of plant growth (Gavrilescu 2022). Growth-promoting microorganisms increase the solubility of lead because they decrease the pH (Manzoor et al. 2019), adsorb lead on their surface and precipitate (Zhu et al. 2022), capture lead by the biosorption mechanism, modify the bioavailability of lead through the production of siderophores, phosphate solubilization, production of organic acids and exopolysaccharides (Shah and Daverey 2020), and transform lead into inert forms such as lead sulfate ($PbSO_4$) and lead sulfides (PbS) (Rigoletto et al. 2020).

Due to the formation of insoluble precipitates, the availability of lead is very low in the soil. In this context, citric and malic acid are used to improve the accessibility of the heavy metal in the soil and the translocation from the root to the foliage (Rigoletto et al. 2020). Therefore, phosphate-solubilizing bacteria of the genera *Pseudomonas* and *Acinetobacter* favored the availability of lead, as well as greater absorption by the root system of lead-tolerant plants (Gupta et al. 2018).

Identification of the genus of bacteria in the consortium with the greatest influence on phytoremediation

The genera *Pseudomonas* and *Acinetobacter* were identified among the bacteria in consortium one (Table 4). In this regard, it coincides with the microorganisms recovered by Manzoor et al. (2019) (*Pseudomonas*) and Zhu et al. (2022) (*Acinetobacter*). The bacteria reported as tolerant to lead, with potential for phytoremediation of soil contaminated with heavy metal, belong to the genera *Klebsiella, Pseudomonas, Sporosarcina, Microbacterium, Staphylococcus, Bacillus* (Manzoor et al. 2019), *Flavisolibacter, Kaistobacter* (Liu et al. 2018), as well as the species *Acinetobacter calcoaceticus* (Zhu et al. 2022), *Delftia acidovorans* and *Azonexus caeni* (Abdelrahman and Younggy 2022).

Table 4. Characteristics of the *Pseudomonas* and *Acinetobacter* genera identified in bacterial consortium one (BC1) isolated from lead-tolerant *E. colona* (L.) Link.

Biochemical tests	Pseudomonas*	Acinetobacter**
Catalase test	+	+
Oxidase test	+	-
Motility test	+	-
Acid production (TSI)	K/K	A/K
Nitrate reduction	-	-
Urea hydrolysis	-	-
Starch hydrolysis	-	+
Indole production	-	-
Citrate use	+	+
Lysine decarboxylation (LIA)	K/A	K/A
Gelatin hydrolysis	-	-
Aesculin hydrolysis	-	-

*Bacteria code: 1, 3, 7, 9; **Bacteria code: 4, 6. K: Alkalinity, A: Acidity, -: Negative, and +: Positive.

CONCLUSION

The weeds *Echinochloa colona* (L.) Link, *Cyperus corymbosus* Rottb. and *Sorghum halepense* with associated rhizobacteria were efficient in the remediation of soil contaminated with lead. 78.83% of heavy metal removal was achieved with *E. colona* (L.) Link plus *Pseudomonas* spp. and *Acinetobacter* spp. (BC1), compared to 57.80% with *E. colona* (L.) Link without bacteria. *Pseudomonas* spp.

and *Acinetobacter* spp. were favored by the root exudates of lead-tolerant plants, which are used as nutrients. This association between weeds and plant growth-promoting bacteria can be applied as an alternative to achieve environmental sustainability through the remediation of soil impacted by several heavy metals.

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Antifungal evaluation of saponins extracted from quinoa husk (*Chenopodium quinoa* Willd) against *Botrytis cinerea* in strawberry

UNIVERSIDAD NACIONAL DE COLOMBIA

Evaluación antifúngica de saponinas extraídas de cascarilla de quinua (*Chenopodium quinoa* Willd) contra *Botrytis cinerea* en fresa

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Keywords:
Ethanolic extraction
Biopesticides
Minimum inhibitory concentration
Antifungal activity

ABSTRACT

Saponins are widely recognized for their biological properties, which has led to the development of several research projects applicable to the agricultural sector. In this sense, the aim of this study was to evaluate the *in vitro* and *in vivo* antifungal effect of saponins extracted from quinoa husks, using the crude extract, and incorporated in a formulation against *Botrytis cinerea* in strawberries. The extraction of saponins by the maceration method was studied, where the effect of temperature, time, and ethanol concentration on the extraction of saponins was evaluated; the process was optimized using a central composite design (CCD). The *in vitro* antifungal effect of the crude extract of saponins was evaluated by the minimum inhibitory concentration (MIC), while the *in vitro* and *in vivo* antifungal effect of the formulation was determined by the mycelial inhibition percentage and control percentage, respectively. The optimum extraction point of saponins was obtained at 49.8 °C, 0.367 h, and 47.4% ethanol. On the other hand, the antifungal activity against *Botrytis cinerea* was dependent on the concentration of saponins; in the *in vitro* evaluation the MIC of the crude extract was 8.25 mg mL^{-1} of saponins and the mycelial inhibition percentage of the formulation was higher than 90%. In the *in vivo* evaluation of the formulation, the percentage of control ranged from 63.03 to 76.14%, compared to 61.5% control exhibited by the chemical fungicide Carbendazim.

Palabras clave:
Extracción etanólica
Biopesticidas
Concentración mínima inhibitoria
Actividad antifúngica

RESUMEN

Las saponinas son ampliamente conocidas por sus propiedades biológicas, lo que ha llevado al desarrollo de diversas investigaciones aplicadas al sector agrícola. En ese sentido, el objetivo de este estudio fue evaluar el efecto antifúngico *in vitro* e *in vivo* de saponinas extraídas de la cascarilla de quinoa, utilizando el extracto crudo e incorporándolo en una formulación contra *Botrytis cinerea* en fresa. Se estudió la extracción de saponinas por el método de maceración, donde se evaluó el efecto de la temperatura, el tiempo y la concentración de etanol; el proceso se optimizó mediante un diseño central compuesto (DCC). El efecto antifúngico *in vitro* del extracto crudo de saponinas se evaluó mediante la concentración mínima inhibitoria (CMI), por su parte, el efecto antifúngico *in vitro* e *in vivo* de la formulación se determinó a través del porcentaje de inhibición micelial y el porcentaje de control, respectivamente. El punto óptimo de extracción de saponinas se obtuvo a 49,8 °C, 0,367 h y 47,4% de etanol. Por otro lado, la actividad antifúngica contra *Botrytis cinerea* fue dependiente de la concentración de saponinas; en la evaluación *in vitro* la CMI del extracto crudo fue de 8,25 mg mL^{-1} de saponinas y el porcentaje de inhibición micelial de la formulación fue superior al 90%. En la evaluación *in vivo* de la formulación el porcentaje de control osciló entre el 63,03 y el 76,14%, frente al 61,5% de control exhibido por el fungicida químico Carbendazim.

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The use of agrochemicals in the control of pests and diseases in agriculture is a common practice due to the high incidence of various phytopathogens. In strawberry crops, the incidence of *Botrytis cinerea* is considered a major pathogen, exerting significant economic impacts both pre- and post-harvest, resulting in potential losses of up to 50% of production (Petrasch et al. 2019). While agrochemicals exhibit efficacy under certain circumstances, their application under suboptimal management conditions poses detrimental effects on human health, environmental integrity, and non-target organisms. Most chemical synthesis pesticides do not degrade easily, consequently, they accumulate in the environment, which causes contamination of soil, water, and air (Lengai et al. 2020). Moreover, for a pathogen of high risk as *B. cinerea*, the development of resistance to diverse fungicide agents has been reported, for example, benzimidazoles and dicarboximides (Avenot et al. 2020) underscores the inadequacies of synthetic pesticides.

The disadvantages associated with the inappropriate and excessive use of synthetic pesticides have catalyzed the exploration of alternative strategies for pest and disease management. Biopesticides based on plant extracts or essential oils constitute an alternative, due to the presence of bioactive compounds of lower toxicity and easily biodegradable (Avenot et al. 2020). For example, saponins are a group of molecules with antifungal, antibacterial, insecticidal, molluscicide and vermicide properties, synthesized by an important number of plants such as those belonging to the Chenopodiaceae and Amaranthaceae families (Ruiz et al. 2017), represent viable candidates for biopesticide formulations.

The quinoa plant (*Chenopodium quinoa* Willd) is a natural source of triterpenoid saponins, which are found in leaves, stems, flowers, and seeds. Of these structures, the seeds present the highest content of saponins, what are mainly located in the outer layer named the pericarp (Ruiz et al. 2017). However, within the food industry, the presence of saponins in quinoa seeds poses technological challenges, necessitating husk removal through abrasive processes to mitigate saponin content (Jiang et al. 2021), as a consequence, a by-product with an important content of saponins is generated, which is undervalued, leading to the under exploitation of its biological potential.

In this sense, the use of saponins obtained from by-products of the industrial processing of quinoa presents an environmentally sustainable strategy for developing biopesticides effective against diseases such as those associated with *B. cinerea*. The aim of this study was to evaluate the *in vitro* and *in vivo* antifungal effect of saponins extracted from quinoa husks, using the crude extract, and incorporated in a formulation against *B. cinerea* in strawberry.

MATERIALS AND METHODS

Plant material

Quinoa husks: was collected from the Green Line Origin Products S.A.S located in Popayán, Colombia. The material collected came from local quinoa cultivars of sweet varieties, according to the suggested for batches of 50 to 500 kg (Rag et al. 2019). A representative sample was sieved through a number 70 sieve using a FRITSCH Analysette 3 Rotap equipment (Idar-Oberstein, Germany), to obtain a by-product fraction with a particle size less than or equal to 212 µm (Huaman and Shuan 2018). The material obtained in this stage was used in later studies.

Strawberry fruits: were obtained from a commercial crop located in the municipality of Silvia, Colombia. The collection was done randomly, and the quantity was 500 g of fruits of third classification, in stage three of maturity, as established by the Colombian Technical Standard NTC 4103 of 1997 for strawberry variety Chandler.

Physicochemical characterization of quinoa husks

Quinoa husks were characterized by proximal analysis and total content of saponins.

Proximal analysis

The proximal analysis of the quinoa husks was determined in terms of moisture content, ash, protein, fiber, and ethereal extract, following the AOAC method, (1990) (920.151, 942.05, 984.13, 985.29, and 920.39, respectively). Carbohydrates were calculated by difference.

Total content of saponins

The total saponin content of quinoa husk was determined by applying the microwave extraction and maceration methods, according to the methodology described by Le et al. (2018), Villacis (2018) and Guilcapi (2019), with

some modifications. In a flask, 5 g of quinoa husk (particle size ≤212 µm) were weighed then mixed with 100 mL of 47% ethanol and homogenized in a magnetic agitation plate for 30 s, subsequently, the sample was taken to a conventional microwave oven (Samsung AMW831K/XAP, Suwon, South Korea). The sample was subjected to seven irradiation cycles of 20 s each, with a cooling time of 1 min between cycles. Then, the sample was taken to a heating plate and magnetic agitation for 22 min. The temperature was maintained at 50 °C and the agitation at 200 rpm. Once the extraction process was finished, the liquid fraction was separated from the solid fraction by vacuum filtration, then the ethanol was recovered from the liquid fraction by simple distillation. The extract was stored and the solid was subjected to two additional extractions by applying the previously described process. Finally, three extracts were obtained and the concentration of saponins was quantified for each one by UV - VIS spectrophotometry applying the method described by Gianna (2013). The total saponin content was established as the sum of the three concentrations quantified in the extracts and expressed as a percentage dry basis.

Optimization of the extraction process

Quinoa husk was subjected to different extraction conditions by maceration method. The percentage of ethanol in the solvent (50 and 80%), temperature (50 and 60 °C), and extraction time (1 and 3 h) were the variables evaluated. The optimization study of the saponin extraction process was carried out by applying the response surface methodology (RSM), under a central composite design (CCD), for which two levels and three independent factors were defined, including ethanol concentration (X1), time (X2) and temperature (X3) in a factorial arrangement 23. The total number of experimental runs generated by the design was 20, with eight cube points, four central points in the cube, six axial points, and two central points in the axial (Table 1). For the axial points, an α=1.633 was used. The experiments were run randomly. The yield of extraction was calculated for the optimal conditions found by equation 1:

$$\%\text{Yield} = \frac{(\%SE)}{(\%ST)} \times 100 \quad (1)$$

Where SE is percentage (%) of extracted saponins, and ST is percentage (%) of total saponin.

Table 1. Central composite design of the extraction of saponins by the maceration method.

Treatment	X1 (%)	X2 (h)	X3 (°C)
1[b]	65	3.633	55
2[b]	89.495	2	55
3[b]	65	0.367	55
4[b]	40.505	2	55
5[a]	65	2	55
6[b]	65	2	63.165
7[a]	65	2	55
8[b]	65	2	46.835
9	80	3	60
10[a]	65	2	55
11[a]	65	2	55
12[a]	65	2	55
13	80	1	60
14	80	1	50
15	50	1	50
16	80	3	50
17[a]	65	2	55
18	50	3	50
19	50	1	60
20	50	3	60

X1: ethanol concentration, X2: time, X3: temperature.
[a] Central point, [b] Axial points.

Minitab Software version 2019 was used, the statistical analysis of the model was performed through analysis of variance (ANOVA) with a significance level of 95%; to identify the effects of the factors on the response variable and the model fit, which was corroborated based on the *P*-value and R-squared. Confirmation runs were performed in triplicate after response surface analysis to verify the accuracy of the optimal condition predicted by the model. To verify the statistical equality of the predicted and experimental saponin percentage, a t-test was run at a 95% significance level.

Determination of saponins content by LB reaction

The determination of saponins was done as described by Gianna (2013). Briefly, the extract was diluted with 96% ethanol at a ratio of 1:10 (extract: ethanol). 1 mL of this solution was mixed with 3.5 mL of LB reagent, the mixture was shaken for 30 s and allowed to stand for 30 min, with two shakings in between. At the end of the reaction time, the absorbance was measured at 417 nm and the results were expressed as a percentage of saponins on a dry basis.

Isolation and identification of the fungus *Botrytis cinerea*

The methodology described by Apolonio et al. (2017), Marín et al. (2017) and Isaza et al. (2019) was applied. Strawberry fruit infected with *B. cinerea* were harvested from a commercial strawberry crop in Silvia, Colombia. The fruits were disinfected with 1% sodium hypochlorite, washed three times with distilled water and dried with absorbent paper, subsequently small slices of diseased tissue were obtained, and four tissue fragments were seeded in Petri dishes with potato dextrose agar (PDA) and incubated at 25 °C for 3 days. The developed colonies were purified in a PDA solid medium using the hyphae tip technique. The morphological identification of the isolates was performed based on the macroscopic and microscopic characteristics of *B. cinerea*. Macroscopic identification was verified by the appearance, texture, and color of the colonies. For the microscopic description, the isolates were observed in a trinocular microscope through the imprinting method. Additionally, a digital image was taken by scanning electron microscopy equipment (TESCAN VEGA3 LM, Brno, Czech Republic) at an acceleration voltage of 10 kV.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of the saponin extract against the phytopathogen *B. cinerea* was determined by using the agar dilution technique described by Ruiz (2000). Five concentrations of saponin were obtained by serial double dilution: 8.25, 4.125, 2.0625, 1.0312, and 0.5156 mg mL^{-1}. These solutions were prepared from a concentrated extract. Under aseptic conditions, 2 mL of each solution was poured into a Petri dish, then 18 mL of sterilized PDA cooled to 50 °C was added. The dishes were shaken gently until the extract and the culture medium were homogenized. Once the medium had solidified, 10 µl of a spore solution standardized to $1x10^4$ spores mL^{-1} obtained from an 11-day-old culture of *B. cinerea* was inoculated. The spore solution was dispersed over the culture medium. A blank was prepared by mixing 2 mL of distilled water with 18 mL of PDA and inoculated with 10 µL of the spore solution.

Incubation was carried out at 25 °C for 76 h, then the spores that germinated on each plate were counted. The MIC was determined as the concentration at which no spore germination was observed. All assays were performed in quintuplicate.

Preparation of saponins formulation

Three emulsion-type formulations were prepared, whose components and their proportions were defined based on the studies conducted by Campolo et al. (2020). The formulations included saponins extract, vegetable oil, and polysorbate 80 and were standardized at three saponin concentrations: 4.12, 2.062, and 1.0312 mg mL^{-1}. To prepare them, the procedure described by Campolo et al. (2020) was used as a reference.

Evaluation of antifungal activity of formulations against *Botrytis cinerea* under *in vitro* conditions

The determination of the growth inhibition percentage (% GI) of the formulation obtained against *B. cinerea*, was made based on the methodology developed by Apolonio et al. (2017), Saha et al. (2018) and Yang et al. (2020). Under aseptic conditions, 2 mL of the formulation was added in a Petri dish, mixed with 18 mL of sterilized PDA, and cooled to 50 °C, and the Petri dish was gently shaken to homogenize. Once the medium solidified, a 5 mm

diameter disc obtained from the edge of a 10-day *B. cinerea* culture was inoculated. A blank and a control (formulation additives and absolute PDA, respectively) were also included. The Petri dishes were incubated at 25 °C and kept under observation until the blank covered the entire surface of the medium, at which time the diameter of the fungal colony developed in each treatment was measured, the measurements were made with a tape measure and crosswise, averaging the two measurements obtained. All tests were performed in triplicate. The percentage of growth inhibition (% GI) was calculated with equation 2.

$$\% \text{ GI} = \frac{(X - Y)}{X} \times 100 \qquad (2)$$

Where "X" is radial growth (mm) in the blank, "Y" is radial growth (mm) in the treatment.

A completely randomized experimental design (CRD) was applied with three treatments corresponding to the concentration of the formulation, a control treatment, and a blank (additives of the formulation and absolute PDA). The response variable was the percentage of inhibition of pathogen growth. Data were analyzed with Minitab software version 2019, by analysis of variance (ANOVA) (P<0.05) and comparison of means by Tukey's method (P<0.05).

Evaluation of antifungal activity of formulations against *Botrytis cinerea* under *in vivo* conditions

The formulation with saponin extract more effective (EF) in the *in vitro* evaluation was studied, and two additional treatments were included, which corresponded to diluted EF and Carbendazim (methyl benzimidazole-2-ylcarbamate) as chemical control. The concentration of the chemical control was defined as 0.1 mg mL^{-1} and diluted EF was established according to what was reported by Taborda et al. (2015) for the traditional application of commercial products. Therefore, the concentrations of each treatment were defined as: 4.125 and 0.5 mg mL^{-1} of saponins for EF and diluted EF. The procedure described by Taborda et al. (2015) and Yang et al. (2020) was followed, with some modifications. Randomly, four groups of washed and disinfected strawberries of five units each were formed. A hole of 2 mm wide by 2 mm deep was made in the central part of each fruit (equatorial location). Subsequently, the fruits were immersed in their respective treatment for 5 s, then these were placed on a tray and taken to a shaker incubator for drying at 30 °C for 30 min. Later, fruits were inoculated with small agar blocks with mycelium of a 12-day *B. cinerea* culture in the holes made. Afterward, the fruits were placed in glass jars and were left in observation for 96 h at room temperature. The inoculated blocks in each fruit were removed after 24 h (Yang et al. 2020). All assays were done in triplicate. Once the observation time was finished, the measurement of the diameter of the infection caused by *B. cinerea* was done by taking two measurements in a crossed way, which were averaged to determine the diameter of the lesion. To calculate the control percentage of each treatment, equation 3 was used.

$$\% \text{ Control} = \frac{(Bd - Td)}{Bd} \times 100 \qquad (3)$$

Where "Bd" is the diameter in the blank (mm), and "Td" is the diameter in the treatment (mm).

A completely randomized design (CRD) was applied with three treatments corresponding to EF, diluted EF, and chemical control. The response variable was the percentage of control. Data were analyzed with Minitab software version 2019, by analysis of variance (ANOVA) (P<0.05) and comparison of means by Tukey's method (P<0.05).

RESULTS AND DISCUSSION

Physicochemical characterization of quinoa husks

The physicochemical characterization of the quinoa husk is shown in Table 2. The moisture content (6.52% w/w), ethereal extract (9.52% w/w), and fiber (8.81% w/w); were similar to those reported by Suárez-Rivero et al. (2019) and Paniagua et al. (2020). However, the ash (10.01% w/w) and protein (6.87% w/w) contents slightly deviate from those found by Huaman and Shuan (2018) and Paniagua et al. (2020). These results underscore that quinoa husk is a by-product rich in essential nutrients. The composition of carbohydrates, protein, and lipids in the husk may be attributed to the abrasion generated during quinoa seeds processes, where abrasive methods partially reduce the embryo, causing a loss of nutrients reflected in the resultant scarified quinoa seed by-product (Jiang et al. 2021).

The total saponin content in the husk is 3.33% on a dry basis (Table 2). In relation to this result, variable contents

of saponins in quinoa husk have been reported, with values ranging from 1.89 to 22% by weight (Lozano et al. 2012; Huaman and Shuan 2018), which depends on the quinoa variety from which it is obtained; in general, bitter varieties have higher saponin content while sweet varieties have lower levels (Ruiz et al. 2017). The result of the present study corresponds to the previous postulate, since quinoa husks from sweet varieties were used.

Table 2. Chemical composition of quinoa husks (Dry basis).

Component	w/w (%)
Moisture	6.52
Ashes	10.01
Ethereal extract	9.51
Fiber	8.81
Protein	6.87
Carbohydrates	58.28
Saponins	3.33

Evaluation of saponins extraction process

The analysis of variance (ANOVA) of the experimental data of saponin extraction shows that the *P*-value of the quadratic model was less than 0.05 and the R-value was 0.8872 (Table 3), which indicates that the model is significant for the experimental data at 95% confidence level. The results show that only the ethanol percentage is significant for both the linear and quadratic models. Temperature and time were not significant for the regression model. Lack of adjustment measures the reliability of the equation to represent the data of the experimental points that are not included in the regression, if the *P*-values are lower than the significance level, the model does not fit the data well (Ramli et al. 2019). Therefore, the 0.377 value of lack of fit obtained with the quadratic model confirms that the model can give a satisfactory prediction of the percentage of extracted saponins (Table 3). Equation 4 shows the quadratic model generated and used for the surface response analysis, which predicts extractable saponins percentages through varying levels of assessed variables.

Table 3. ANOVA of saponin extraction regression parameters.

Source	GL	Sum of squares	Mean square	*f-value*	*P-value*	Comment
Model		3.68802	0.36880	7.08	0.004	Significant
Blocks	1	2.31031	2.31031	44.33	0.000	-
Linear		0.85568	0.28523	5.47	0.020	Significant
– X_1	1	0.76851	0.76851	14.75	0.004	Significant
– X_2	1	0.03697	0.03697	0.71	0.421	-
– X_3	1	0.05020	0.05020	0.96	0.352	-
Square		0.45834	0.15278	2.93	0.092	-
– $X_1 X_1$	1	0.34663	0.34663	6.65	0.030	Significant
– $X_2 X_2$	1	0.00772	0.00772	0.15	0.709	-
– $X_3 X_3$	1	0.08087	0.08087	1.55	0.244	-
Interaction		0.06369	0.02123	0.41	0.752	-
– $X_1 X_2$	1	0.00039	0.00039	0.01	0.933	-
– $X_1 X_3$	1	0.06322	0.06322	1.21	0.299	-
– $X_2 X_3$	1	0.00008	0.00008	0.00	0.971	-
Error		0.46901	0.05211			-
– Lack of adjustment	5	0.30027	0.06005	1.42	0.377	Not significant
– Pure error		0.16874	0.04218	-	-	-
Total		4.15703	-	-	-	-
Standard deviation	0.2282	-	-	-	-	-
R^2	0.8872	-	-	-	-	-

$$\% \text{ saponins} = -1.43 + 0.0709X_1 + 0.101X_2 - 0.793X_3 - 0.000720X_1 X_1 - 0.00097X_2 X_2 + 0.0783X_3 X_3 - 0.00009X_1 X_2 + 0.00593X_1 X_3 + 0.0006X_2 X_3 \quad (4)$$

The extraction yield was higher when moderate process conditions were applied, which is evident in the contour plots (Figure 1). Between 45 and 65% ethanol and up to 60 °C, the percentage of saponins is higher than 2% (Figure 1A); time versus ethanol percentage shows a saponin yield higher than 2.4% with extraction times and ethanol concentration up to 0.65 h and 60%, respectively (Figure 1B). In Figure 1C, it is observed that between 60 °C and 0.75 h the proportion of extracted saponins is between 2.2 and 2.3%.

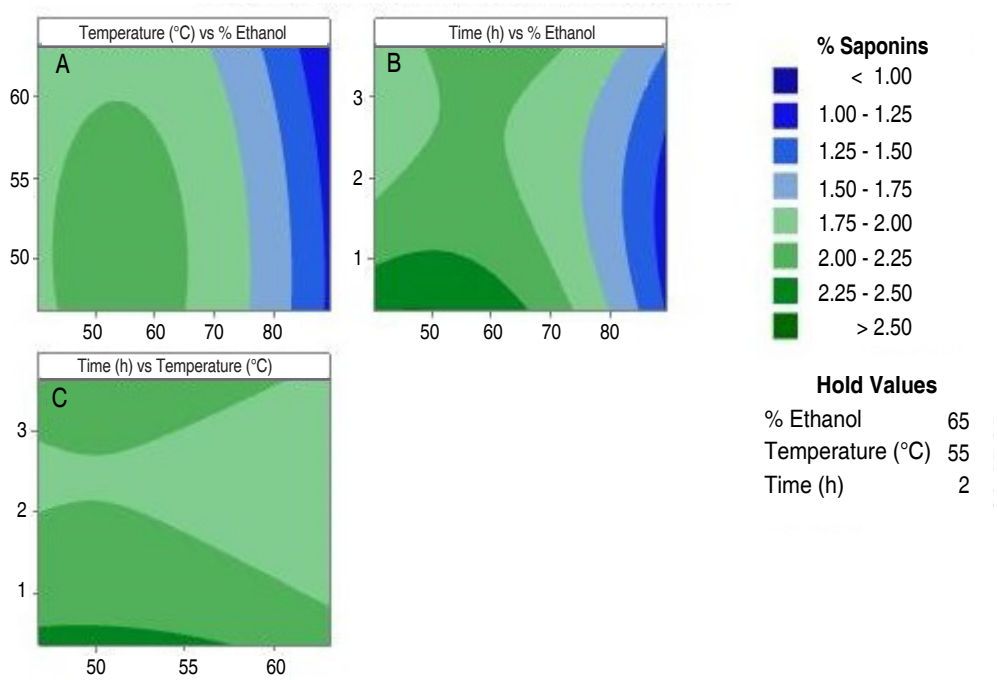

Figure 1. Contour plots of extraction process optimization. A. Temperature vs % ethanol; B. time vs % ethanol and C. time vs temperature.

According to Figure 1, the best extractions are achieved with ethanol percentages between 40 and 60%, temperature lower than 60 °C and time lower than 1 h. To verify this behavior, the optimal point conditions predicted by the software were applied. The prediction was 2.54% of saponins with 47.43% of ethanol, 49.80 °C and 0.367 h. The confirmation runs of the optimal condition, showed an experimental value of 2.47% of saponins, exhibiting no statistical difference (P<0.05). Furthermore, was established that the extraction yield at the optimum point was 74.23%, which represents a significant yield using a simple method and ecological solvent.

In general, the proportion of ethanol in the solvent was the most influential variable in the extraction process, obtaining the highest yield with an ethanol proportion of 47%, this result agrees with the reported by Lozano et al. (2012), Huaman and Shuan (2018), and Villacis (2018), who found the best extraction yields with ethanol proportions between 40 and 50%. However, it is observed that at proportions higher than 80% of ethanol, the extraction is reduced. Consequently, this behavior implies the unfeasibility of anhydrous ethanol as a solvent for the extraction of saponins from quinoa husks, this behavior is probably due to the modification of the degree of polarity of the solvent, since, when increasing the aqueous fraction, the polarity increases and when increasing the ethanolic fraction, the polarity decreases. Therefore, intermediate ethanol concentrations would provide a degree of polarity favorable to the solvent for the solubilization of polar compounds and

therefore better extractions of the compound of interest (Hikmawanti et al. 2021).

Isolation and morphological identification of the fungus *Botrytis cinerea*

The macroscopic and microscopic observation of the isolations allowed to identify the morphological characteristics of the phytopathogen *B. cinerea*. It was found that the observations corresponded to the characteristics reported by Isaza et al. (2019). Thus, in the isolations, branched and septate mycelium was observed, which presented a whitish to grayish coloration. The conidiophores were observed emerging directly from the mycelium, and these were thin and of radial and vertical growth, and irregularly branched in the terminal portion where these presented groups of conidia in the form of clusters. The conidia were smooth and elliptical in shape (Figure 2). In some cultures, the formation of black sclerotia of round and/or irregular shape was observed.

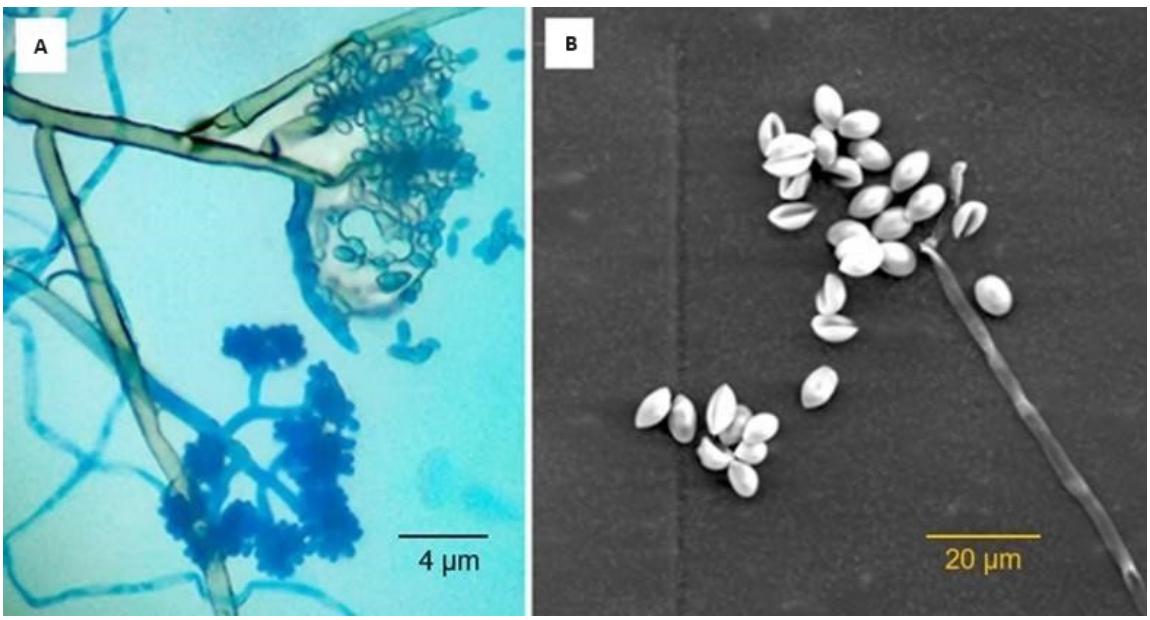

Figure 2. Microscopic characteristics of *B. cinerea*. A. Conidiophores with conidia observed by visible light microscopy and B. Conidia observed by SEM micrographs.

Determination of Minimum Inhibitory Concentration (MIC)

According to the results (Table 4), no spore germination was observed at 8.25 mg mL^{-1} of saponins, which corresponds to a final concentration in the Petri dish of 0.825 mg mL^{-1}, so this was determined as the MIC of the extract of saponins against the phytopathogen evaluated. There are few studies in which the determination of the MIC of quinoa saponins against fungal phytopathogens has been carried out. The study carried out by Stuardo and San Martín (2008) report the antifungal activity against *B. cinerea* of crude and hydrolyzed saponin extracts obtained from quinoa peel. It is indicated that MIC was not found in the range of concentrations evaluated (1-7 mg mL^{-1}) with the extract of saponins in its crude form. On the other hand, triterpenoid saponins obtained from diverse vegetable sources have also been studied in the control of *B. cinerea*. The MIC of commercial saponin from *Quillaja saponaria* and the fruit of *Sapindus mukorossi* was 30 and $\geq$250 mg mL^{-1}, respectively (Fischer et al. 2011; Porsche et al. 2018), which quite distant from the results found in the present investigation.

***In vitro* evaluation of formulation including saponin extract**

After 9 days of incubation at 25 °C, the formulations inhibited mycelial growth of *B. cinerea* according to saponin concentrations. The inhibitions of the products containing saponins were higher than 90% (Figure 3). At the highest concentration (4.125 mg mL^{-1}) an inhibition of 99.22%

Table 4. Minimum inhibitory concentration (mg mL^{-1}) of saponin extract against the germination of *B. cinerea* spores, after 72 h of incubation at 25 °C.

Concentration (mg mL^{-1})	CFU count	MIC (mg mL^{-1})
8.25	0	0.825
4.125	6.2±0.8	-
2.0625	10.2±1.3	-
1.0325	12.2±1.5	-
0.515	13.6±1.5	-
Blank treatment	14.0±1.9	-

was achieved. The percentage of control was significantly different (P<0.05) among the evaluated treatments. Inhibition was also observed with the formulation without saponins (FWS), which reduced mycelial growth of *B. cinerea* by 60% (Figure 3).

Antifungal inhibition tests were carried out on the formulation additives, where it was found that the surfactant polysorbate 80 inhibited the mycelial growth of *B. cinerea*, against this behavior, it has been reported that emulsions based on polysorbate 80 present antagonism against spore-forming fungi such as *Aspergillus niger* (Kaur and Mehta 2017). From the above, it can be deduced that the percentage of *in vitro* inhibition of mycelial growth of *B. cinerea* exhibited by the formulated product is caused by a combined effect of quinoa saponins and polysorbate 80. For instance, for the treatment with the highest saponin concentration, the inhibition attributable to saponins is 39.22%, considering the 60% inhibition generated by the formulation without saponins.

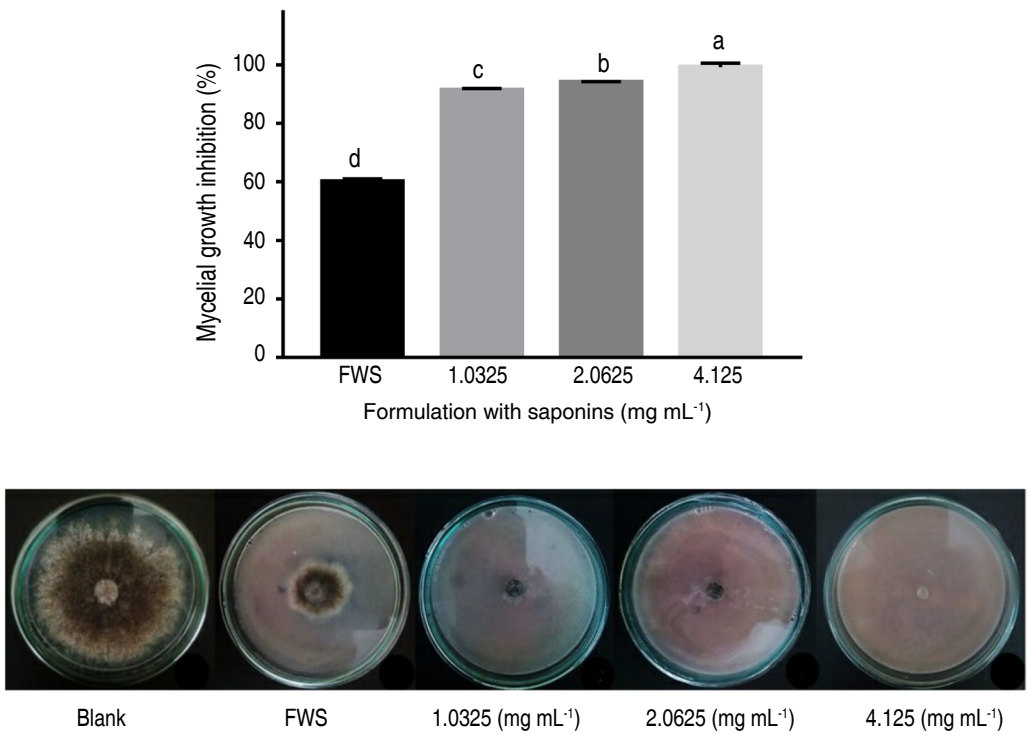

Figure 3. *In vitro* antifungal activities of the biopesticide formulation with saponin extract against mycelial growth of *B. cinerea.*

No research is reported in which saponins are incorporated into a formulation and their antifungal effect is evaluated; instead, inhibition of *B. cinerea* has been studied using saponin extracts from quinoa and other plant sources, in their crude form. For instance, Stuardo and San Martín (2008) and Mccartney et al. (2019) found that inhibitions of 32 and 43.7% were achieved with 5 mg mL^{-1} of saponins, respectively. Similarly, Fischer et al. (2011) reported an inhibition of 36% with 4 mg mL^{-1} of *Quillaja saponin*, while saponins obtained from the pericarp of the fruit of *Sapindus mukorossi* inhibited the growth of *B. cinerea* by 36% with a concentration of 5 mg mL^{-1} of saponin (Porsche et al. 2018). These findings indicate consistency between the results of this study and those reported in existing literature.

The toxic effect of saponins against fungi is due to the formation of pores and loss of cell membrane integrity. In the case of triterpenoid saponins, the group to which quinoa saponins belong, the mechanism involves the insertion of aglycones into the membrane, binding to sterols, followed by the association of sugar residues and the formation of the sterol-saponin complex, ending with pore formation and cell lysis (Zaynab et al. 2021).

In vivo evaluation of formulation including saponin extract

Figure 4 shows that the EF treatment presented a significantly different percentage of control compared to the diluted EF and the chemical control. The development of the infection caused by *B. cinerea* was reduced by 76.14±2.2% with the formulation containing 4.125 mg mL^{-1} of saponins (EF), while its diluted form (0.5 mg mL^{-1} of saponins) reached 63.03±6.3% of control, showing no significant difference to the control expressed by the chemical treatment (0.1 mg mL^{-1} of methyl benzimidazole-2-ylcarbamate). Observation throughout the experiment revealed the aggressiveness of the infection caused by *B. cinerea* on fruits without application of the controllers. The reduction in disease severity after application of the formulation is evident, and despite the slight reduction in efficacy after dissolution, the product continues to show inhibition over the pathogen.

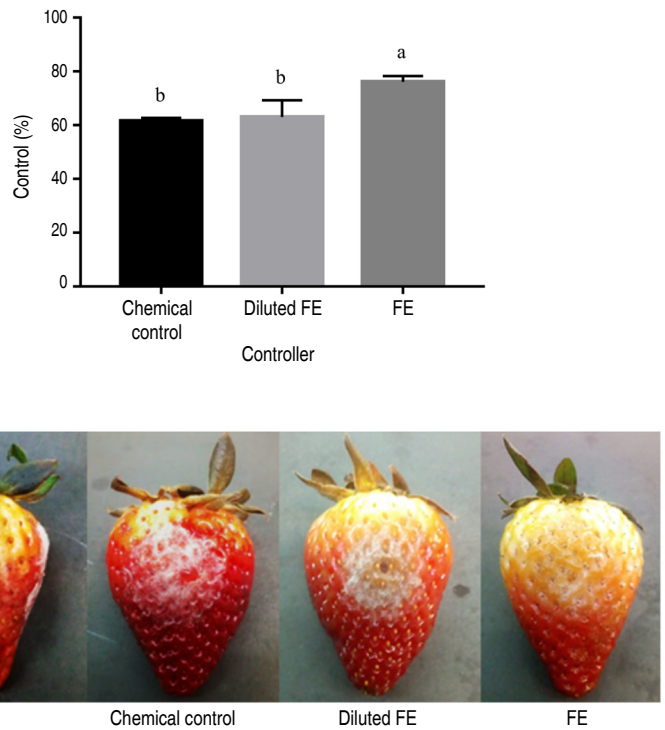

Figure 4. Efficacy of the biopesticide formulation at 4.125 mg mL^{-1} saponins (FE) and diluted to 0.5 mg mL^{-1} saponins (diluted FE), compared to the commercial fungicide Carbendazim (0.1 mg mL^{-1}); on the severity of disease caused by *B. cinerea* in strawberry fruits.

The results were consistent with those obtained in the *in vitro* evaluation. In both experiments the percentage inhibition of the formulations on *B. cinerea* was significant; however, there was a reduction in the in the *in vivo* evaluation. These results are attributable to the influence of environmental factors on the development of gray mold caused by *B. cinerea* and in the case of infections in strawberry, there is a dependence on the maturity of the fruit (Petrasch et al. 2019). In effect, conditions such as temperature and humidity change in the *in vivo* evaluation, and the strawberry acts as culture medium, where the pathogen can have its nutrients for its development. Therefore, contribute to the observed fluctuation in antifungal efficacy across experiments.

There are no reports in the literature of research evaluating a formulation with saponins as an active ingredient against phytopathogens *in vivo*. In contrast, there have been studies evaluating other plant extracts and essential oils, crude or formulated, against *B. cinerea*, for instance, Yang et al. (2020) reported a 71.9% control of *B. cinerea* development on strawberries by the effect of tobacco cembratrien-diols (CBT). It should be noted that CBTs are diterpene compounds, molecules that bear some similarity to triterpene saponins.

Hence, the potential of bioactive compounds of plant origin for the control of phytopathogenic fungi of high impact in agriculture is evident. Saponins obtained from quinoa processing by-products are positioned as a viable alternative for the control of fungal diseases that affect crops and agricultural products.

CONCLUSION

The study of saponin extraction allowed obtaining a yield of 74.23% using an ecological solvent and a simple method, a process that was a key step, since it allowed obtaining extracts with a high concentration of saponins for the antifungal evaluation. The biocontrol capacity of quinoa saponins against *Botrytis cinerea* was determined through *in vitro* and *in vivo* tests; the minimum inhibitory concentration (MIC) was 8.25 mg mL^{-1} and the *in vitro* evaluation of the formulation reached inhibitions higher than 90%; however, it was observed that polysorbate 80, used in the formulation, also presented an antifungal effect, so that the inhibition attributable to the saponins of the most effective formulation was 39.22%. On the other hand, *in vivo* evaluation of the antifungal effect of the formulation exhibited higher and equal control percentages to the chemically synthesized fungicide Carbendazim: at 4.125 and 0.5 mg mL^{-1} of saponins the control percentage was 76.14 and 63.03%, respectively, while Carbendazim fungicide achieved 61.5% control. The results of this study show that quinoa saponins can be a viable option for the control of fungal diseases affecting crops and agricultural products, as well as an alternative for the development of new formulations based on bioactive compounds, which allows for the reduction of environmental impacts and the revaluation of quinoa agroindustry waste.

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Evaluation of the physiological quality of lettuce (*Lactuca sativa* L., var. *Longifolia*) grown using silvoagroaquaculture waste

Evaluación de la calidad fisiológica de lechuga (*Lactuca sativa* L., var. *Longifolia*) cultivada usando residuos silvoagroacuícolas

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Keywords:
Acid soil
Lettuce leaves
Natural fertilizer
Vitamin C

ABSTRACT

Soil acidity poses a challenge to crop production by limiting the availability of nutrients for plants. The aim of this study was to assess the efficacy of a natural waste-based fertilizer composed of *Mytilus chilensis* seashells, coffee bean wastes, banana peels, and wood ashes on lettuce growth and physiological quality. The seashells were used with organic matter (W-OM), without organic matter (Wo-OM), and a mixture of equal parts of W-OM and Wo-OM (50:50). The coffee bean wastes, banana peels, and wood ashes were used as ingredients. The soil pH, chlorophyll index in the lettuce leaves, and nitrogen level (in the soil and leaf) were measured for a period of 66 days. Vitamin C content in the harvested leaves was measured. The W-OM fertilizer allowed for an increase the soil pH from less than 6.5 to 7.0±0.5. The nitrogen provided by the coffee bean waste was partially available to the plant root, which decreased the chlorophyll index in the lettuce leaves. The studied fertilizer (W-OM, Wo-OM, and 50:50) allowed to increase of vitamin C content in the lettuce leaves. In conclusion, the natural waste-based fertilizer showed a promising effect in alleviating soil acidity and enhancing the nutritional quality of lettuce plants.

Palabras clave:
Suelo ácido
Hojas de lechuga
Fertilizante natural
Vitamina C
.

RESUMEN

La acidez del suelo plantea un desafío para la producción de cultivos al limitar la disponibilidad de nutrientes para las plantas. Este estudio tuvo como objetivo evaluar la eficacia de un fertilizante a base de residuos naturales compuesto de conchas de *Mytilus chilensis*, residuos de granos de café, cáscaras de plátano y cenizas de madera sobre el crecimiento y la calidad fisiológica de la lechuga. Las conchas se utilizaron con materia orgánica (W-OM), sin materia orgánica (Wo-OM), y una mezcla a partes iguales de W-OM y Wo-OM (50:50). Residuos de granos de café, cáscaras de plátano y cenizas de madera fueron usados como ingredientes. El pH del suelo, el índice de clorofila en las hojas de lechuga y el nivel de nitrógeno (en el suelo y en las hojas) fueron medidos durante 66 días. El contenido de vitamina C fue medido en las hojas cosechadas. El fertilizante W-OM permitió aumentar el pH del suelo desde menos de 6,5 a 7,0±0,5. El nitrógeno proporcionado por los residuos del grano de café estuvo parcialmente disponible para la raíz de la planta, lo cual disminuyó el índice de clorofila en las hojas de lechuga. Los fertilizantes estudiados (W-OM, Wo-OM y 50:50) permitieron aumentar el contenido de vitamina C en las hojas de lechuga. En conclusión, el fertilizante a base de residuos naturales mostró un efecto prometedor para aliviar la acidez del suelo y mejorar la calidad nutricional de las plantas de lechuga.

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Soil acidity is one of the main limitations to agricultural production worldwide, affecting many chemical and biological reactions that control nutrient availability to plants. Plant root growth is reduced in acid soils, which increases the vulnerability of the crop to water stress and nutrient deficiency and reduces yields (Fageria and Nascente 2014). The acid soils represent approximately 30% of the total surface of the planet and more than 50% of potentially arable lands (Golla 2019). In southern Chile, from the Ñuble Region to the Los Lagos Region, soil pH is within the acid range of 4.5 to 5.5 (Bernier and Alfaro 2006), which means a significant degree of acidity in the soil.

Agricultural activity could accelerate soil acidification through increased leaching due to water movement in the soil, accumulation of organic matter, and soil amendment (fertilizers and other chemicals). Ammonium, which is the main nitrogen compound found in fertilizers, has the greatest potential for soil acidification, whereas nitrate-based nitrogen fertilizers have a weaker acidifying potential (Wang et al. 2021). For soil pH neutralization, lime can also be used. However, industrial and organic wastes can represent a great opportunity to produce organic-mineral fertilizers by using the three key principles (recycling, reusing, and reducing) for a circular economy with numerous agricultural and environmental advantages, such as improvement and contribution to the neutralization of soil acidity. In addition, the organic and industrial wastes also decrease trace elements availability to the plants due to their alkalinity (Muscolo et al. 2022).

The lettuce (*Lactuca sativa* L., var. *Longifolia*) is widely consumed as a salad because it contains several nutritional benefits, such as low levels of calories, fat, and sodium, while it contains an adequate source of fiber, iron, folic acid, and vitamin C (Vargas-Arcila et al. 2017). It has a short growth period which makes its production fast and economically viable (Ahmed et al. 2021a). The productive quality of lettuce leaves is based on the average leaf area and number of leaves, while total soluble solids and vitamin C content are used for physiological quality (Zapata-Vahos et al. 2020; Alneyadi et al. 2024). The measurement of vitamin C content in vegetables is based on the measurement of an antioxidant compound, ascorbic acid. Its derivatives have been tested on cancer cells and revealed anticancerous activity (Medina-Lozano et al. 2021). Ekinci et al. (2020) reported an increased chlorophyll value and vitamin C content when organic fertilizers are used in growing lettuce. Zhang et al. (2015) demonstrated that nitrogen (N), phosphorus (P), and potassium (K) when combined with organic fertilizer could increase vitamin C content in tomatoes. Consequently, the changes in the soil pH have an important impact on the quality of vegetables and represent an important agroecological factor.

Currently, there is a high availability of industrial and organic waste, such as seashells, food waste, and wood ashes that can be used as natural fertilizers (Ahmed et al. 2021b). In this context, seashells (75% $CaCO_3$) and coffee beans with banana peels as food waste, together with wood ashes could contribute to neutralizing soil acidity, improving the physiological quality of the crop, and playing a key role as a source of NPK.

Therefore, the current study aimed to assess the impact of a natural waste-based fertilizer, composed of *Mytilus chilensis* seashells, coffee bean waste, banana peels, and wood ashes, on the growth of lettuce and its physiological quality.

MATERIALS AND METHODS

Preparation of mussel seashells

Mussel seashells (*Mytilus chilensis*) from the shellfish industry located in Dalcahue (Chiloé Island, Chile) were classified as seashells with organic matter (W-OM) and without organic matter (Wo-OM) waste products after removal of their meat. The seashells were cleaned by washing with potable water and, subsequently, dried in a tray dryer (Model 30-1060, Memmert GmbH + Co. KG, Schwabach, DEU) for 12 to 20 hours at 50 °C, until a moisture content of 1 to 20% w/w was attained. A hammer mill (Model M0LTN0H11, Plaspak, Santiago, Chile) with a fine sieve (1.7 mm opening) was used to grind seashells. The dried and ground seashells were packed in hermetically sealed bags.

Preparation of fertilizer

Food waste (coffee bean (CB), banana peels (BP), and wood ashes (WA)) were prepared. The N, P, and K sources were CB, WA, and BP, respectively. The CB and BP were dried in a tray dryer (Model 30-1060, Memmert GmbH + Co. KG, Schwabach, DEU) from 12 to 20 h at 50 °C. The BP was ground with a hammer mill (Model M0LTN0H11, Plaspak, Santiago, CL) using a fine sieve (1.7 mm opening).

The WA was sieved (mesh opening of 2 mm) to remove impurities. Subsequently, CB, BP, and WA were packed in hermetically sealed bags. The fertilizer preparation considered the incorporation of seashells, CB, BP, and WA, as part of its ingredients. The seashell type was evaluated in three treatments: *i*) W-OM, *ii*) Wo-OM, and *iii*) a mixture of equal mass of W-OM and Wo-OM (50:50). The CB, BP, and WA ingredients were fixed variables. For each treatment, 10 g seashells, 10 g CB, 14 g BP, and 3 g WA constituted the applied dose, which allowed adjusting N (19 mg kg^{-1}), P (98.7 mg kg^{-1}), and K (61.6 mg kg^{-1}) levels by mass balance. The NPK dose adjusted coincides with the average fertility level (N, <108 mg kg^{-1}; P, 9 mg kg^{-1}; K, from 45 to 112 mg kg^{-1}) for crop soils recommended by El-Seedy and Saeed (2019).

Collection and cleaning of soil

The studied soil was of alluvial type from Buchupureo, Commune of Cobquecura, Ñuble Region, Chile, and was collected at a depth of 20 cm and transported in plastic containers according to Carvajal-Mena et al. (2023). Stones and impurities were extracted by sieving soil with a 2 mm aperture sieve. The soil sample was considered a control (alluvial soil without added fertilizer) and a blank (leaf soil formulated from plant and forestry waste).

Experimental planting conditions

Lettuce plants (*Lactuca sativa* L., var. *Longifolia*) also known as romaine lettuce were grown in pots (n=12). The plants were sown in October 2022, transplanted and applied the previously specified fertilizer dose at a surface level around the stem in November 2022, and then lettuce leaves were harvested at the end of February 2023. Water was applied every two days around the stem to moisten and infiltrate the applied fertilizer. The average air temperature was ranged between 7.3±1.8 and 19.1±2.6 °C. The average air humidity was 77.6±2.7%. The average daylight was approximately 14.1±0.6 h. The lettuce plants had a control (lettuce plant in alluvial soil without added fertilizer) and a blank (lettuce plant in leaf soil).

pH and nitrogen level in alluvial soil

Soil pH was measured directly with a digital soil meter (PH328, SMART SENSOR, Fujian, CN).

The N level (mg kg^{-1}) was measured using a soil nutrient sensor (Chengdu Sentec Technology Co., Sichuan, CN). The digital soil meter and soil nutrient sensor showed an accuracy of ±0.2 pH and ±2%, respectively. The pH and N levels were based on the electrical conductivity measurement of steel electrodes, which were immersed in wet soil (69.2±6.2% wet basis), and the variability in electrical conductivity is expressed as pH value or N level (Carvajal-Mena et al. 2023). Measurements were performed in triplicate.

Chlorophyll and nitrogen level on lettuce leaves

The leaf chlorophyll and N levels were measured using a ZYS-4N Portable Plant Nutrition Test Analyzer Machine (WANT Balance Instrument Co., Changzhou, CN). The chlorophyll measurement principle was based on the quantitative evaluation of the intensity of the green color of the leaf from 650 to 940 nm, where accuracy was ±1 SPAD unit (Cunha et al. 2015). According to the equipment manufacturer, the determination of nitrogen level was based on a statistical model due to the high linear correlation between chlorophyll and N level, with an accuracy of ±5%. Measurements were performed in triplicate.

Determination of vitamin C content in lettuce leaves

The procedure for measuring vitamin C content in lettuce leaves was based on the spectrophotometric method using the reagent 2,6-dichlorophenolindophenol (Anal Parimal and Shuchi Desai 2019). A 2 g lettuce sample was crushed in a mortar with 20 mL of 0.4% w/v oxalic acid. The homogenate was filtered two times with Wathman grade 41 filter paper. 9 mL of distilled water (blank sample) was added to the filtered sample (1 mL). Then, 9 mL of 0.0012% w/v 2,6-dichlorophenolindophenol was added to 1 mL of the filtered sample. The absorbance readings were obtained on a GENESYS 10S UV-Vis spectrophotometer (Thermo Scientific, Shanghai, CN) at 520 nm and 22 °C at room temperature. Measurements were performed in triplicate and expressed as mg ascorbic acid per 100 g fresh weight of lettuce leaves.

Statistical analysis

All results were analyzed with Statgraphics Centurion XVI statistical software (Statistical Graphics Corp., Herdon, VA, USA). The results were calculated as the mean value ± standard deviation and reported as plots displaying the estimated trends. Significant differences between the mean values ($P \leq 0.05$) were determined using Tukey´s test.

RESULTS AND DISCUSSION

pH trends in alluvial soil

The lettuce is grown in soil with a pH range from 6.0 to 6.5, for ideal growth and to decrease root infections (Nkosi and Msimango 2022). However, many agricultural soils in Chile and specifically on the Ñuble Coast have pH values lower than 6.0 (Carvajal-Mena et al. 2023), which limits their use for growing vegetables.

Figure 1 shows the effect of seashell type (W-OM, Wo-OM, and 50:50 treatments) on soil pH. The W-OM and 50:50 treatments showed an increasing trend of soil pH from pH values less than 6.5 (acidic range) to close to a neutral pH range (7.0±0.5) during the period analyzed. Similarly, other studies have reported higher pH values in paddy soils with organic matter content and/or calcium carbonate (Zhao et al. 2014). In general, the decomposition of organic matter leads to the production of more organic acids, thus lowering pH (Hong et al. 2019). However, organic acids produce calcium salts when it reacts with calcium carbonate (Luai et al. 2020), and, such salts, as calcium lactate, act as an antacid agent contributing to the pH upturn. The Wo-OM treatment showed a constant trend, probably due to the buffering capacity of the calcium carbonate contained in the seashells and the absence of organic matter (Ng et al. 2022). On the other hand, the blank and control treatments exhibited a decreasing trend in soil pH; in the first case, as leaf soil is an organic medium, the soil pH could decrease when it comes into contact with irrigation water, due to the soluble organic acids it contains (Adeleke et al. 2017), and, in the second case, the alluvial soil of the area under study was made up of rocks with acid reaction, such as, granites and siliceous sandstones, which give soils with pH between 4.8 and 5.6 (Li and Shi 2020).

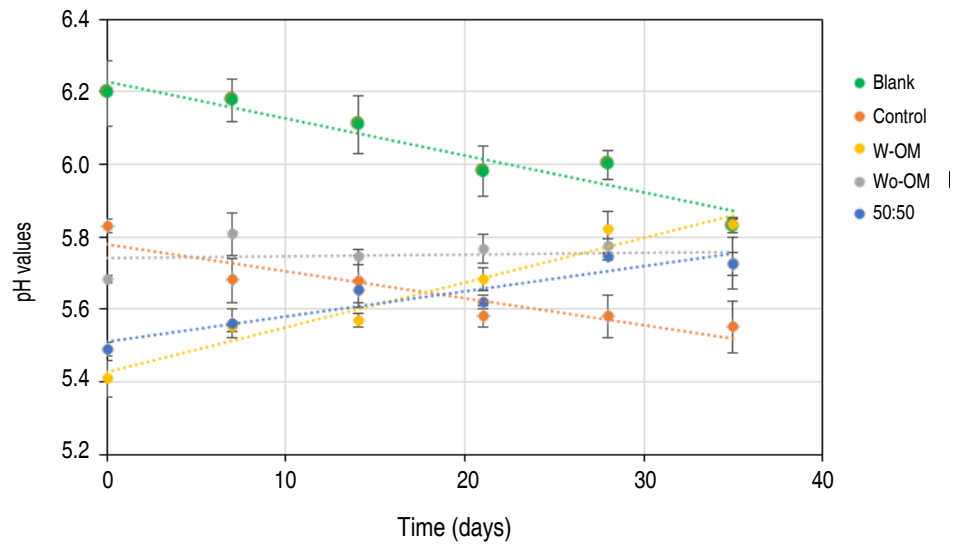

Figure 1. Soil pH. Blank: lettuce plant in leaf soil; Control: lettuce plant in alluvial soil; W-OM: lettuce plant in alluvial soil more seashells with organic matter; Wo-OM: lettuce plant in alluvial soil more seashells without organic matter, and 50:50: lettuce plant in alluvial soil more a mixture of equal masses of W-OM and Wo-OM.

Nitrogen trends in alluvial soil

Soil nitrogen is a nutrient with great impact on natural ecosystems and its decrease can cause acidification, nutrient imbalance, and loss of biodiversity, affecting the quality and yield of cultivated vegetables (Blanco and Martínez 2019).

Figure 2 shows the effect of W-OM, Wo-OM and 50:50 treatments on soil N level, which was approximately 9 mg kg^{-1}, during the 35 days analyzed. Considering that during fertilizer preparation, the N level incorporated into soil was adjusted to 19 mg kg^{-1} and, given that the coffee bean (CB) waste had a severe heat treatment applied during its house preparation, therefore, it could be suggested that not all the nitrogen was available for the plant, probably, due to a protein denaturation in CB (Mazzafera et al. 2019). Additionally, given the low absorption of N, root differentiation and expression of

nitrate transporters could have been affected, altering plant quality (Singh et al. 2022). Visually, this was verified because a lower number of shortened plants was found (n=3), that is, a greater leaf opening was observed in the plants (n=9), which promoted leaf dehydration (Blanco and Martínez 2019). For its part, the control treatment showed a low N level (5.8±0.1 mg kg^{-1}), which allowed the improvement of N in the soil with the W-OM, Wo-OM, and 50:50 treatments. The blank treatment showed the highest N level, 102.6±9.2 mg kg^{-1}, which can be explained because the leaf soil was formulated by plant and forestry waste, which represent the largest nitrogen reserve in the soil and most of this nitrogen can be available to the plant.

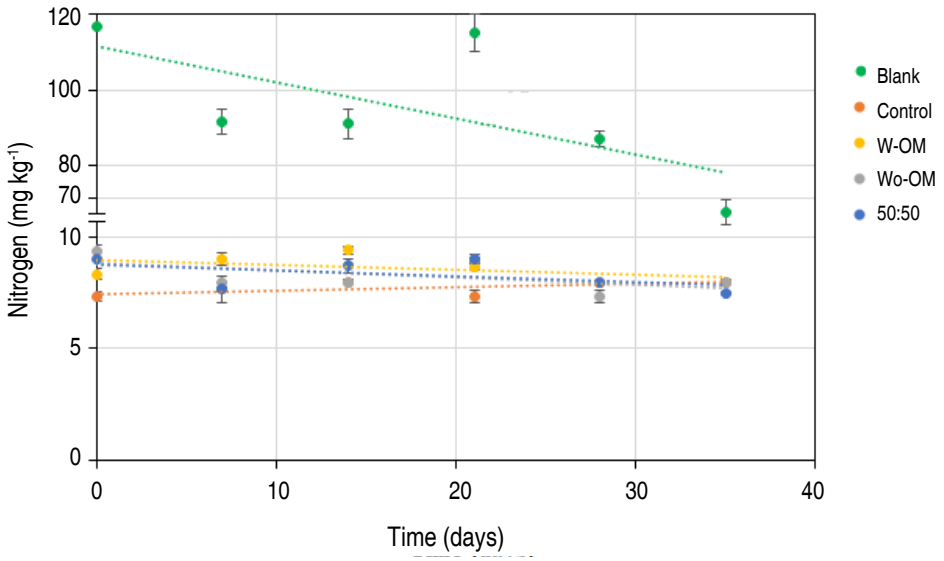

Figure 2. Nitrogen level in soil. Blank: lettuce plant in leaf soil; Control: lettuce plant in alluvial soil; W-OM: lettuce plant in alluvial soil more seashells with organic matter; Wo-OM: lettuce plant in alluvial soil more seashells without organic matter, and 50:50: lettuce plant in alluvial soil more a mixture of equal masses of W-OM and Wo-OM.

Nitrogen trends in lettuce leaves

Nitrogen is the nutrient responsible for the green pigmentation of vegetables and plays a key role in the leaf´s growth, size, and quality (Ncama and Sithole 2022; Albadwawi et al. 2022).

Figure 3 shows the effect of W-OM, Wo-OM, and 50:50 treatments on leaves N level, which exhibited an increase with time for all treatments, although the highest N level was observed in the leaves of blank treatment. Karam et al. (2002) suggest that the N level in the leaf for an ideal chlorophyll content, is the one that avoids toxicity or lack of nutrients to the plants. Therefore, the lettuce leaf plants could have exhibited a lower sensitivity to the nitrogen source incorporated into the soil because a weak appearance was observed in their leaves.

Chlorophyll index trends in lettuce leaves

Chlorophyll is a natural and active pigment responsible for transforming light energy into chemical energy, used in plant growth (Chowdhury et al. 2021).

Figure 4 shows the effect of W-OM, Wo-OM, and 50:50 treatments on leaves chlorophyll content. An increase in chlorophyll was observed in the lettuce leaves for all treatments during the study period. These findings were consistent with a study carried out by Santos et al. (2016), which reported similar trends of chlorophyll production in lettuce leaves cultivated with a compost of natural origin. Bassi et al. (2018) reported that total chlorophyll content increases under high nitrogen supply. However, except for the blank treatment, the low nitrogen levels reported in Figure 4, could be related to the degree of nitrogen availability in the soil from coffee bean waste sources.

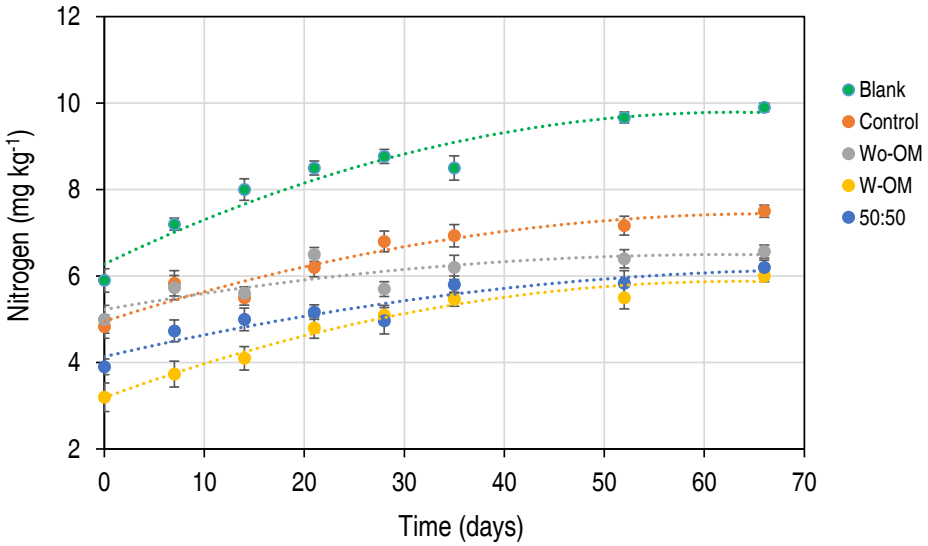

Figure 3. Nitrogen level in lettuce leaves. Blank: lettuce plant in leaf soil; Control: lettuce plant in alluvial soil; W-OM: lettuce plant in alluvial soil more seashells with organic matter; Wo-OM: lettuce plant in alluvial soil more seashells without organic matter, and 50:50: lettuce plant in alluvial soil more a mixture of equal masses of W-OM and Wo-OM.

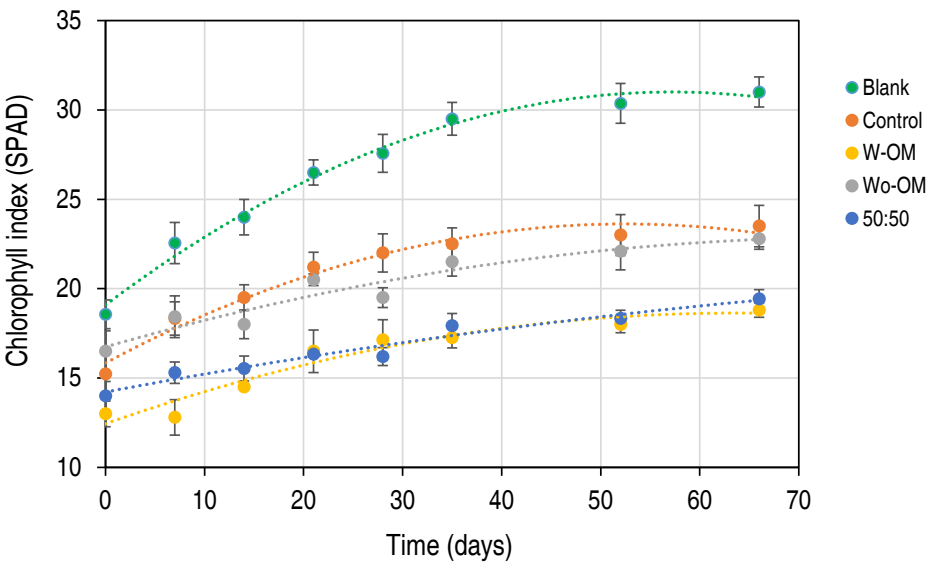

Figure 4. Chlorophyll index in lettuce leaves. Blank: lettuce plant in leaf soil; Control: lettuce plant in alluvial soil; W-OM: lettuce plant in alluvial soil more seashells with organic matter; Wo-OM: lettuce plant in alluvial soil more seashells without organic matter, and 50:50: lettuce plant in alluvial soil more a mixture of equal masses of W-OM and Wo-OM.

Vitamin C content in lettuce leaves

The vitamin C is an indicator of the nutritional value of vegetables (Medina-Lozano et al. 2021). Figure 5 shows the effect of W-OM, Wo-OM, and 50:50 treatments on the vitamin C content of lettuce leaves. It was observed that the addition of seashells improved the vitamin C content when compared to the blank treatment based on leaf soil. Specifically, the plants grown in the soil without organic matter (Wo-OM) showed a high vitamin C content (51.0±3.6 mg 100 g^{-1}). It was observed that the organic

matter content of seashells (W-OM) contributed significantly to reducing the vitamin C content in the lettuce leaves. Although, the vitamin C content in the plants from the W-OM (25.8±1.0 mg 100 g^{-1}) and 50:50 (37.0±3.0 mg 100 g^{-1}) soils were that of the plants from leaf soil (24.6±1.7 mg 100 g^{-1}). These findings suggest that the addition of seashells without organic matter (Wo-OM) to the lettuce plants has a positive effect on the vitamin C content (51.0±3.6 mg 100 g^{-1}), but the presence of organic matter in the seashells could reduce the vitamin C content. Lee and Kader (2000) confirm these findings and indicate that the organic waste derived from meat (as the organic matter of seashells) represents an additional nitrogen source, whose excess could partially inhibit vitamin C production in plants because the excess nitrogen increases the concentration of NO_3, a known inhibitor of vitamin C production in vegetables.

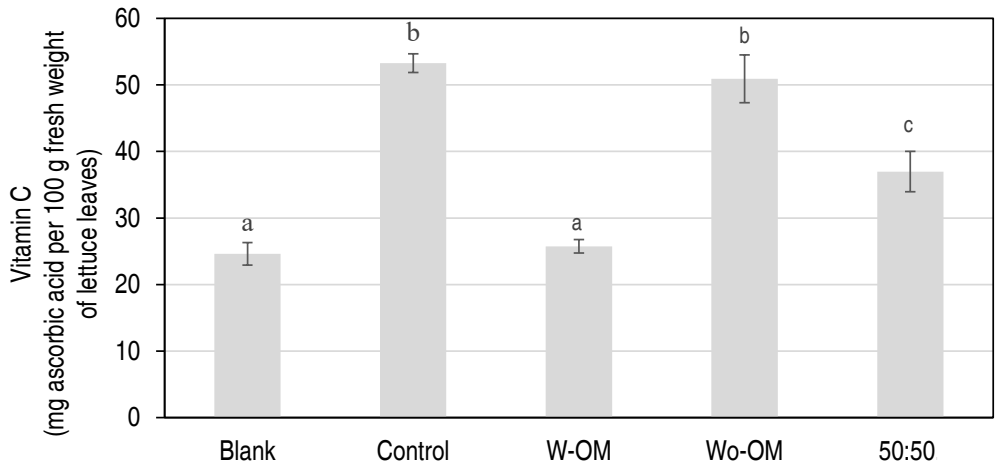

Figure 5. The vitamin C content in lettuce leaves. Blank: lettuce plant in leaf soil; Control: lettuce plant in alluvial soil; W-OM: lettuce plant in alluvial soil more seashells with organic matter; Wo-OM: lettuce plant in alluvial soil more seashells without organic matter, and 50:50: lettuce plant in alluvial soil more a mixture of equal masses of W-OM and Wo-OM.

CONCLUSION

The utilization of *Mytilus chilensis* seashells, combined with coffee beans waste, banana peels, and wood ashes, in the formulation of natural waste-based fertilizer, proved beneficial for lettuce cultivation and improving its physiological quality: *i*) the alluvial soil from Ñuble Coast (Ñuble Region, Chile) increased its pH value with the incorporation of seashells, *ii*) the coffee bean waste, as nitrogen source incorporated into the soil, were partially available for the lettuce plant, *iii*) the low nitrogen levels measured in the lettuce leaves could have been the cause of a low chlorophyll index on the lettuce leaves, and (iv) the seashells contributed to improving the physiological quality in the lettuce leaves expressed as vitamin C. Additionally, it is established that some improvements in fertilizer composition are required to act as an effective pH control and nitrogen supply agent. Therefore, it is recommended to identify other sources of natural wastes that provide available nitrogen for the plant´s needs.

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Effect of warm temperature and water shortages on early growth of *Lepidium meyenii* Walpers

Efecto de la temperatura cálida y el déficit hídrico en el crecimiento temprano de *Lepidium meyenii* Walpers

https://doi.org/10.15446/rfnam.v77n2.108243

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ABSTRACT

Increasing water deficits and warming temperatures due to climate change threaten agricultural systems in the Peruvian Andes, where environmental conditions are themselves challenging. *Lepidium meyenii* Walpers also known as "maca" is an endemic root crop that can tolerate adverse conditions such as low temperatures and high UV radiation, but little is known about its response to drought. This study aimed to test the effect on maca germination and early seedling growth of water restriction under two maximum temperatures: 15 °C (current scenario) and 20 °C (warming scenario). Water restriction had either a direct or a temperature-dependent effect on germination and above-ground seedling growth, which was greater at 15 °C. By contrast, its effects on seedlings growing at 20 °C were completely overcome by faster germination, initial growth, and biomass acquisition. The results are consistent with those from other crops and contribute to the understanding of how climate change is affecting high-mountain agriculture.

Keywords:
Andean crop
Climate change
Maca
Seedlings
Temperature

RESUMEN

El aumento de los déficits de agua y las temperaturas más cálidas debido al cambio climático amenazan los sistemas agrícolas en los Andes peruanos, donde las condiciones ambientales son desafiantes. *Lepidium meyenii* Walpers, también conocida como “maca”, es un cultivo de raíz endémico que puede tolerar condiciones adversas como bajas temperaturas y alta radiación ultravioleta, pero se sabe poco sobre su respuesta a la sequía. El objetivo de este estudio fue evaluar el efecto de la restricción hídrica sobre la germinación de maca y el crecimiento temprano de las plántulas en dos temperaturas máximas: 15 °C (escenario actual) y 20 °C (escenario de calentamiento). Se encontró que la restricción hídrica tuvo un efecto directo o dependiente de la temperatura sobre la germinación y el crecimiento foliar de las plántulas, que fue mayor a 15 °C. Por el contrario, sus efectos sobre las plántulas que crecieron a 20 °C fueron completamente superados por una germinación más rápida, crecimiento inicial y adquisición de biomasa. Los resultados son consistentes con los de otros cultivos y contribuyen a la comprensión de cómo el cambio climático está afectando a la agricultura de alta montaña.

Palabras clave:
Cultivo andino
Cambio climático
Maca
Plántulas
Temperatura

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In the Tropical High Andes [3,500-4,500 meters above sea level (masl)] only a few crops and highland grasses can grow under extremely harsh environmental conditions characterized by frosts and high levels of UV radiation (Zhang et al. 2016). One such crop is *Lepidium meyenii* Walpers (Brassicaceae), also known as maca, traditionally grown in the Puna of central Peruvian Andes. The latter is an area characterized by plateaus and intermountain basins, as well as high spatial and temporal climatic variability (Rolando et al. 2017). Because maca hypocotyls are high in nutrients and secondary metabolites with useful pharmacological properties (Wang et al. 2007), their cultivation has been intensified in the last 20 years to meet consumer demand in both local and export markets. Climate change, though, might compromise maca production and have a negative economic impact on this predominantly family farming activity.

Among abiotic challenges associated with climate change, water restriction and high temperatures have the most negative impact on crop productivity and the availability of arable land (Fahad et al. 2017; Restrepo-Díaz et al. 2021). Climate change manifests in the Tropical High Andes through an increase in temperature (Vuille et al. 2015) and a delayed and shortened rainy season, with less frequent but more intense precipitation (Thibeault et al. 2010; Giráldez et al. 2020). Delay and shortening of the rainy season are likely to impose water restrictions on crops, either at the beginning of the growth season, if sowing takes place at the regular dates, or later, if sowing is deferred.

Seed germination and early seedling growth are the most sensitive stages to factors such as temperature and soil moisture in several crop species (Farooq et al. 2009). Interaction between temperature and water availability strongly affects these early stages of plant development, ultimately influencing crop productivity (Mahalingam 2015). Noteworthy, below ground crops of the Andes may have reduced yields when exposed to drought during early plant growth, as has already been shown in specific potato cultivars (Tourneux et al. 2003).

As for maca, aside from recent efforts to understand its tolerance to the adverse conditions under which it grow (Shi et al. 2019; Huarancca-Reyes et al. 2020; Wang et al. 2020), little is known about the effects of drought on crop cycle, in particular on early growth stages. It is possible that the water restriction present in the germination and emergence stages could be generating changes in the characteristics of the seedlings that may be influenced by other factors such as temperature. Therefore, the purpose of this study is to evaluate the effects of water restriction on germination and seedling establishment of *L. meyenii* in the context of warmer temperatures, as an approach to possible climate change scenarios for this crop in the currently cultivated areas. The results of this study are important because they will not only contribute to the understanding of maca's response to water restriction but also to have a better idea of how climate change would be affecting high mountain agriculture.

MATERIALS AND METHODS

Plant material

Six seed accessions, three from Junín district (coordinates: -11.0604, -75.9739; elevation: 4,215 masl) and three from Chupaca district (coordinates -12.2011, -75.3870; elevation 3,990 masl), were obtained from local maca producers. These accessions were selected from a bigger collection due to their best performance in pilot germination trials, in addition to being representative of the districts of Junin and Chupaca, two main maca production areas. Seeds were duly cleaned, weighed, and stored at 4 °C until use.

Experimental design

An experiment was conducted *in vitro* in a growth chamber (BINDER KBW-400, Germany), with a three-way factorial design (seed accession, water restriction treatment and temperature). To establish regimes that compose a reasonable scenario of short-term temperature changes in the maca-producing areas of the Junín region, the temperature records of the period 1954 to 2014 were examined (SENAMHI 2020). The maximum mean temperature above 4,000 masl rose from 16 to 18 °C, whereas at lower altitudes (3,000 to 3,600 masl) it went up from 18.5 to 20 °C. As for the minimum temperature, it rose from 2.5 to 3.7 °C on average at both altitudes. Based on these observations, two temperature regimes were set concerning maximum temperatures: a "current scenario", at 15 °C; and a "warmer scenario", at 20 °C, keeping the same minimum temperature (4 °C). All experimental groups were subjected to both temperature conditions under a 14:10 hour scheme (cold:warm), corresponding also to the dark:light periods. Daylight fluorescent tubes

Color 865 (Philips) provided light during the 10-hour high-temperature period.

Germination conditions

Seeds were surface sterilized in 70% ethanol for 5 min and in 2% sodium hypochlorite with 0.05% Tween™ (Thermo Scientific) for 15 min, and finally washed three times with distilled water.

They were transferred to sterile 25x200 mm culture tubes (5 seeds/tube) containing 20 mL of solid Murashige and Skoog medium [34.3 g L^{-1} and Phytagel (4 g L^{-1})], with polyethylene glycol (PEG) 8000 added as a water retention agent (Michel 1983). Three osmotic potentials were tested: -0.25 MPa (0 g L^{-1} PEG as control), -0.3 MPa (150 g L^{-1} PEG), and -0.5 MPa (250 g L^{-1} PEG) (Verslues et al. 2006). These PEG levels were selected based on a pilot germination trial with PEG concentrations of up to 500 g L^{-1}, where it was observed that at 250 g L^{-1} of PEG, seed germination was reduced to below 50%. At least six replicates per accession were used for each osmotic treatment. The tubes were tightly sealed with Parafilm M® to avoid water losses and incubated for 52 days (where seedlings reached the 5-leaf stage) in a growth chamber under the two temperature regimes.

Germination indexes

A visual record of seed germination was kept every 4 days using radicle extrusion ($\geq$2 mm) as a criterion. The final germination percentage (%G) was scored and the mean germination time (MGT) was calculated as follows equation 1 (Kader 2005):

$$MGT(days) = \frac{\sum(NiTi)}{\sum Ni} \qquad (1)$$

Where N is the number of seeds germinated on an ith day, and T is the number of days from the start of the experiment to the ith observation.

Seedling traits

Physiological and morphological traits were measured on day 52 after sowing as follows:

Physiological traits

Two seedlings per tube were randomly selected and used for the fluorometric quantification of Fv/Fm (OS-30P, Opti-Sciences) as an indicator of photosynthetic efficiency, and to estimate the chlorophyll content (CCM-300, Opti-Sciences).

Morphological traits

The number of leaves of two seedlings per tube was recorded. For seedling height and root length measurements, two seedlings were carefully cleaned and photographed, and their image was analyzed using ImageJ software (Schneider et al. 2012). Root:shoot ratio was calculated using the values of seedling height and root length.

Statistical analyses

A mixed-model ANOVA considering water restriction treatment with PEG (P) and temperature regime (T) as fixed effects and seed accession (A) as a random effect was used to analyze the data. When testing for the fixed factors P and T, the error term (denominator) for the F-ratio was the Mean Squares (MS) of A x P and A x T, respectively; and when testing for the P x T interaction, the error term was the MS of the A x P x T interaction. The differences between specific groups were verified by Tukey HSD tests. The analyses were performed with JMP (SAS Institute Inc.), and the graphs were created with SigmaPlot software (Systat Software, Inc.).

RESULTS AND DISCUSSION

Regarding the main effects, temperature (T) affected all variables, except Fv/Fm; and water restriction treatment with PEG (P) affected the mean germination time, the number of leaves, and the seedling height. As for seed accession, it only influenced root length significantly (Table 1). In addition to the main effects, significant A x T interactions were detected for all variables except for germination percentage and root length, and significant P x T interactions for chlorophyll content. It is worth noting that PEG treatment was significant either as a main effect or when interacting with temperature (Table 1); no A x P interactions were detected among the evaluated variables, which means that water restriction affected all accessions similarly.

Among the water restriction effects on germination indexes and seedling traits, only the mean germination time was affected in a similar way (negatively, Figure 1B) at both temperature regimes. Maca accessions grown at 15

°C showed germination percentages below 60%, with a tendency to decrease by about 5 units with each increment in PEG concentration (Figure 1A). On the other hand, the mean germination time required to reach these percentages was more than 25 days, with a clear trend to increase at higher PEG concentrations (Figure 1B). By contrast, maca accessions that grew at 20 °C showed higher and faster germination. Their germination percentages stood above 70% regardless of PEG treatment, and despite increasing at higher PEG concentrations, the mean germination time was never longer than 22 days (Figures 1A, 1B). As for seedling traits, the water restriction effect was negative on the number of leaves at 15 °C (current scenario, Figure 1C), while negative on the seedling height and positive on the chlorophyll content at 20 °C (warmer scenario, Figure 1E, 1H). However, the effects of a higher temperature on these variables completely overcame in magnitude those caused by water restriction, with the sole exception of the photosynthetic efficiency (Fv/Fm, Figure 1C-H) that remained within optimal values (around 0.78) under all treatments. In general, the changes induced by a warmer scenario minimized the influence of water restriction and favored plant development and life cycle completion (Lin et al. 2010).

Table 1. Mixed-Model ANOVA for the effects of PEG treatment and temperature as fixed effects and seed accession as a random effect.

	Df	%G	MGT	NL	SH	RL	R:S	Chl total	Fv/Fm
Fixed effects									
PEG (P)	2	2.13	39.21***	5.56*	8.64**	0.44	3.98	1.67	0.83
Temperature (T)	1	18.63**	25.06**	34.34**	56.21***	196.23***	29.81**	54.18***	0.11
P x T	2	2.13	1.64	3.83	0.42	0.42	1.11	4.35*	1.09
Random effects									
Accession (A)	5	6.16	0.68	1.49	2.16	6.84*	2.34	0.75	1.17
A x P	10	0.28	0.64	2.58	0.64	1.69	1.70	1.01	0.49
A x T	5	3.27	8.98**	7.12**	5.02*	2.27	6.08**	3.67*	3.43*
A x P x T	10	1.91*	1.05	0.60	1.41	0.73	0.65	1.36	1.44
R^2 – full model		62.05%	66.39%	56.54%	74.11%	69.78%	53.55%	70.33%	31.01%

Germination percentage (%G), Mean Germination Time (MGT), Number of Leaves (NL), Seedling Height (SH), Root Length (RL), Root:Shoot ratio (R:S), Total Chlorophyll content (Chl total) and photosynthetic efficiency (Fv/Fm). F values are shown.* $P<0.05$; ** $P<0.01$; *** $P<0.001$.

PEG treatments also influenced other variables under specific conditions. Although root length was not affected, a significant reduction in seedling height was observed with 250 g L^{-1} PEG under both temperature regimes. While statistical significance was not reached in the linear mixed model or with individual regimes, the root:shoot ratio showed a notorious trend to increase as water restriction became more severe, regardless of the temperature conditions (Figure 1G). These results imply that when maca seedlings are exposed to moderate water deficit, they prioritize maintaining their roots' attributes over those of the aerial structures (Poorter and Nagel 2000). Similar responses have been reported in alfalfa (Zeid and Shedeed 2006; Zhang et al. 2018) and potato (Lahlou and Ledent 2005), in the latter case with positive consequences for tuber yield.

Two observations with physiological implications deserve further comment. First, the results show that all the treatments applied were mild at most, as no indication of stress was evident from the Fv/Fm measurements. No substantial variations of the photosynthetic efficiency accompanied any of the changes in the other variables, thus suggesting that metabolic adjustments occurred in the seedlings of all accessions to keep the Fv/Fm values within the optimal range. This supports the idea that the photosynthetic machinery of maca is tolerant to moderate water restriction, despite the latter having a significant effect on plant growth. Similar results have been previously reported for peanuts (Celikkol et al. 2010). Whether the increase in chlorophyll content of maca seedlings facing moderate drought challenges in a warmer scenario

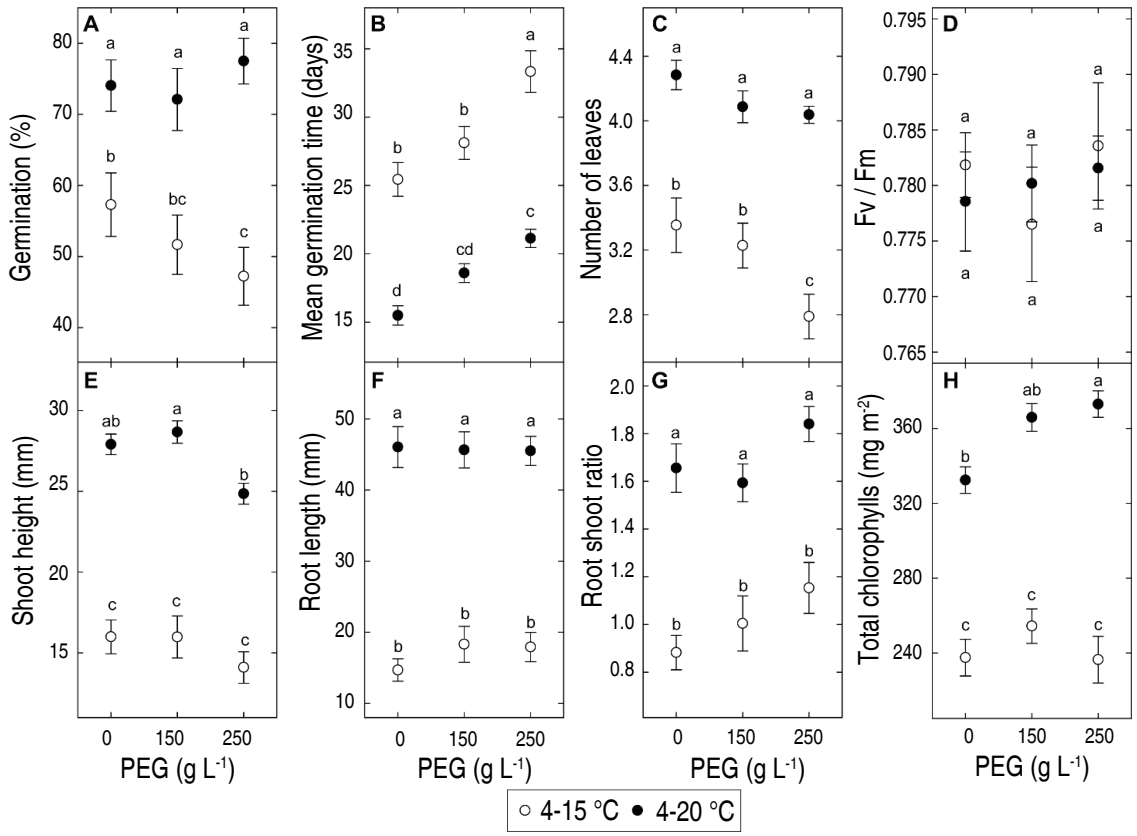

Figure 1. Effect of PEG treatments and temperature on germination percentage (%G), mean germination time (MGT), number of leaves (NL), seedling height (SH), root length (RL), root:shoot ratio (R:S), total chlorophyll content (Chl total) and photosynthetic efficiency (Fv/Fm). Black dots: 4-20 °C, white dots: 4-15 °C. Means of all accessions ± standard error are shown. Means with different letters are significantly different (P<0.05, Tukey HSD test).

contributes to preserving photosynthetic capabilities remains to be established. Sample measurements of carbon dioxide assimilation in a steady-state system shall answer this question.

Second, within the maximum temperatures evaluated and with the variables measured, maca appeared to be more responsive to temperature than to water availability. Because the values of the germination indexes and seedling traits were so overwhelmingly favorable in the warmer scenario, likely, the negative effects of water restriction at these temperatures did not have the biological constraints of those at lower temperatures. Previous studies have revealed that the effects of water restriction are more severe as the temperature moves away from the optimal range for the species' growth and development (Mokhberdoran et al. 2009). These findings are consistent with models that predict the influence of water availability and temperature on germination and initial seedling growth (Shah et al. 2021; Khan et al. 2022). Taken together, these observations strongly suggest that the temperatures at which maca is cultivated nowadays (current scenario, 15 °C) are below the optimal range for the species' growth and development. This notion is further supported by previous results (Valqui-Peña et al. 2021) and implies that the plant must grow at sub-optimal temperatures to yield a hypocotyl with the desired attributes. Interestingly, an earlier work by González et al. (2009) has reported an analogous phenomenon in carrots, where a sub-optimal temperature produced plants with reduced general size while simultaneously favoring growth and active allocation of carbon to the storage organ.

CONCLUSION

This study showed for the first time the effects of water restriction on maca in the context of climate change.

In general, water restriction significantly affects the germination of maca seeds, having a later impact on seedling development. However, an increase in the maximum temperature could have a compensatory effect by overcoming the negative effect of water restriction. The consequences of these changes for hypocotyl development remain to be investigated, but the assumption is that under a warmer scenario, it will be compromised. In the same sense, all seed accessions evaluated behaved similarly, suggesting that the development of new maca varieties that are resistant to future environmental conditions would be necessary. Finally, its findings stress the relevance of further studying the phenological responses of Andean crops to anticipate the possible impact of climate change on their cultivation and management.

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Use of processed grape pomace and whey bio ferment to improve the agronomic performance of radish (*Raphanus sativus* L.) in arid soils

Uso de orujo de uva procesado y biofermento de suero de leche para mejorar el comportamiento agronómico del rabanito (*Raphanus sativus* L.) en suelos áridos

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ABSTRACT

Keywords:
Agroindustrial-wastes
Biofertilizer
Root vegetable
Vegetable crops

Agro-industrial wastes representing a significant problem can be revalued as biofertilizers. The present paper aims to determine the effect of processed grapevine pomace (PGP) and whey bio-ferment (WB) on radish cultivation under conditions of arid zone soil. A 3x3 factorial arrangement of completely randomized design was used, with three levels of PGP (0, 1.25, and 2.50 g kg^{-1} soil applied in total dose before planting) and three levels of WB (0, 50, and 100 mL L^{-1} applied in irrigation water). Radish leaves were evaluated for length and chlorophyll; root dry matter content, length, diameter, weight, and total soluble solids (TSS) were measured; organic matter (OM), N, P, K, pH, electrical conductivity (EC) and cation exchange capacity (CEC) was determined in the soil. Specific differences in means were determined by the LSD-Fisher method applied after analysis of variance (ANOVA); the significance of differences was defined at $P<0.05$. The most remarkable result to emerge from the data is that using PGP and WB improved soil attributes and promoted crop development. Specifically, the higher the PGP dose, the greater the benefits; in the case of WB, the dose of 50 mL L^{-1} showed the best results.

RESUMEN

Palabras clave:
Residuos agroindustriales
Biofertilizantes
Tubérculos
Cultivo de hortalizas

Los residuos generados en procesos agroindustriales que representan un problema importante pueden revalorizarse como biofertilizantes. El objetivo de la investigación fue determinar el efecto del orujo de vid procesado (PGP) y bio-fermento de lactosuero (WB) en el cultivo de rabanito bajo condiciones de suelo de zona árida. Se utilizó un diseño completamente aleatorizado con arreglo factorial de 3x3, con tres niveles de PGP (0, 1,25 y 2,50 g kg^{-1} de suelo aplicado en dosis total antes de la siembra) y tres niveles de WB (0, 50 y 100 mL L^{-1} aplicado en el agua de riego). En las hojas de rabanito se evaluó el tamaño (largo) y porcentaje de clorofila, en la raíz se midió contenido de materia seca, largo, diámetro, peso y sólidos solubles totales (TSS); en el suelo se determinó la materia orgánica (OM), N, P, K, pH, conductividad eléctrica (EC) y capacidad de intercambio catiónico (CEC). Las diferencias específicas de las medias se determinaron por el método de la diferencia mínima significativa LSD-Fisher aplicado tras el análisis de la varianza (ANOVA); la significación de las diferencias se definió a $P<0,05$. Se encontró que la utilización de PGP y WB mejoraron los atributos del suelo y promovieron el desarrollo del cultivo. De manera específica, a mayor dosis de PGP, mayores fueron los beneficios; en el caso del WB, la dosis 50 mL L^{-1} presentó los mejores resultados.

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Environmental pollution resulting from inadequate management of agro-industrial wastes is a pressing environmental issue, particularly in developing countries (Álvarez-Palomino et al. 2018; Nayak and Bhushan 2019; Leite et al. 2021) and arid climates (Gravuer et al. 2019; Liu et al. 2021). Most of these residues contain lignocellulosic biomass, with cellulose and hemicellulose polymers accounting for approximately 75 to 80%, which hinders their degradation rate (Cotacallapa-Sucapuca et al. 2020; Gurgenidze et al. 2022). Consequently, these materials require prior processing to be converted into raw materials that offer environmental, social, or economic benefits (Nayak and Bhushan 2019).

The viticulture industry produces significant amounts of by-products that, if not properly managed, can have a significant environmental impact (Troilo et al. 2021). Grapevine pomace, which is composed of skins, seeds, and any other solids remaining after grape pressing, is the main solid by-product of the winemaking process. Grapevine pomace is primarily comprised of cellulose, lignin, hemicellulose, and pectin (Saval 2012; Gurgenidze et al. 2022). and it is commonly used for producing functional foods (Gurgenidze et al. 2022), distillates, ethanol, animal feed, compost, and other biofertilizers (Cotacallapa-Sucapuca et al. 2020).

Whey is a by-product of milk processing for cheese making and is generated through the enzymatic breakdown of the colloidal system of milk into liquid and solid components (Pais et al. 2017). It contains a high concentration of nutrients that are of agricultural interest, including calcium, phosphorus, potassium, and iron (Mazorra-Manzano and Moreno-Hernández 2019; Williams and Dueñas 2021). Currently, whey is used in the production of animal feed, food, beverages (Williams and Dueñas 2021), energy (País et al. 2017), fertilizer (Felli et al. 2012) and even fungicides (Liu et al. 2021); however, due to the large volume of production, a significant portion of it is discarded into rivers and soils, leading to environmental pollution issues (Liu et al. 2021; Felli et al. 2012; Osorio et al. 2018; López-Barreto et al. 2018). In this context, the revalorization of waste from the viticulture and dairy agroindustry presents itself as a sustainable option. One crop that could greatly benefit from applying processed whey and grapevine pomace is radish (*Raphanus sativus* L.). Radish is characterized by its short phenological period and the development of its edible organ in direct contact with the substrate (Ramírez and Pérez 2006). From a nutritional standpoint, radish has high calcium and potassium requirements (Mendivil-Lugo et al. 2020; Liriano et al. 2020), aligning with the nutrients these biofertilizers provide. This study aim was to determine the impact of using processed grape pomace (PGP) and whey bio-ferment (WB) on the cultivation of radish under arid soil conditions.

MATERIALS AND METHODS

The experiment was developed in the agricultural plot B4-60 of the Majes Irrigation, Arequipa, Peru (16°21'11"' S, 72°11'27" W), 1,410 meters above sea level (masl) during May and June 2022. The average temperature was 18.3 °C (maximum 27.2 °C and minimum 9.4 °C), with a relative humidity of 29% and no precipitation. The area's climate is classified as desert, characterized by extreme aridity. The soils in the area are classified as Entisols (MINAG 1975; Wei et al. 2021), with low organic matter (OM) (1.12%) and sandy-loam texture (88.14% sand, 6.61% silt and 5.25% clay).

Experimental design and treatments

A completely randomized design with a factorial arrangement of treatments was used. The study involved two factors: processed grapevine pomace (PGP) with three levels (0, 12.5, and 25 g kg^{-1} of soil) and whey bio-ferment (WB) with three levels (0, 50, and 100 mL L^{-1}). This resulted in nine treatments, with three replicates per treatment. The experimental unit consisted of rectangular containers measuring 45 cm in length, 20 cm in width, and 15 cm in height. Each container contained 15 kg of agricultural soil obtained from a 13 cm undisturbed soil profile. The PGP was thoroughly mixed with the soil before planting. The WB was applied through the irrigation system 7, 14, and 21 days after seeding.

Preparation and characterization of biofertilizers

On a black plastic base, dry and fragmented pomace (10 kg), molasses (5 L), ash (2 kg), yeast (100 g), and cow dung (2 kg) were mixed. The pomace was obtained as a residue from the production of wine (var. Moscatel), after an alcoholic fermentation process for 10 days at 25°C. Water was sprayed onto the mixture until it reached 70% humidity. Subsequently, the mixture was covered with black plastic to increase the temperature and

accelerate the activity of microorganisms. Daily turning was performed to facilitate the processing. After 30 days, processed grapevine pomace was obtained, which was sieved using a 2 mm mesh. The chemical analysis of the obtained product revealed the following nutrient levels: OM 26.15%, carbon (C) 15.17%, nitrogen (N) 1.25%, phosphorus (P) 0.32%, potassium (K) 0.67%, calcium (Ca) 0.85%, magnesium (Mg) 0.11%, C/N ratio 12.15, pH=7.82, electrical conductivity (EC) 2.15 dS m^{-1}.

For the preparation of the WB, a mixture of whey (15 L), water (25 L), fresh cattle manure (5 kg), and ash (1 kg) was blended in a 50 L capacity plastic cylinder. The container was then tightly sealed for anaerobic digestion over 20 days. Following the process, an irrigation system was applied to the mixture after filtering it through a canvas cloth. The chemical characterization of the obtained product indicated the following nutrient contents: OM 8.42%, N 0.27%, P 0.11%, K 0.31%, Ca 0.17%, magnesium Mg 0.02%, pH=5.85, EC of 5.42 dS m^{-1}.

Crop management

The crop was grown in rectangular containers arranged in an open field, with a density of 15 plants per container. Before seeding, the seeds were applied with a mixture of thiamethoxam (insecticide) + difenoconazole (fungicide) + fludioxonil (fungicide) (1.5 mL kg^{-1} seed). Additional fertilization included two applications of 100 kg ha^{-1} at 10 and 20 days after seeding, totaling 200 kg ha^{-1}. Manual weed control was performed before each fertilization. Abamectin (insecticide) (0.75 mL L^{-1}), chlorpyrifos (insecticide) (2.5 mL L^{-1}), and fosetyl aluminum (fungicide) (1.5 kg ha^{-1}) were used for pest and disease control. Irrigation was carried out through a drip system with a flow rate of 2 L h^{-1}, applying an irrigation depth of 3.2 mm and an irrigation duration of approximately 30 min every other day. Manual harvesting was conducted 32 days after seeding.

Characteristics evaluated in the soil

The soil was analyzed before crop establishment and at the end of the growing season (32 days after seeding) in three representative samples for each treatment. OM (%), total N (%), available P (mg kg^{-1}), K (mg kg^{-1}), pH, EC (dS m^{-1}), and cation exchange capacity (CEC) (cmol kg^{-1}) were determined by Walkley-Blac method, micro-Kjeldahl, modified Olsen, spectrophotometry with ammonium acetate extraction, potentiometer (Hanna, HI9126, USA) in soil/water ratio 1:1, conductivity meter (Hanna, HI993310, USA) in saturated soil extract, and saturation with ammonium acetate, respectively. The methodologies for each determination correspond to those described by Bazán (2017).

Characteristics evaluated on the crop

Radish samples were collected at the end of the vegetative period (32 days after seeding). Three plants were selected from each experimental unit, and the roots and leaves were separated to obtain average results. Measurements were taken for leaf length (cm), fresh weight (g), diameter, root length (cm), percentage of dry matter (DM), and total soluble solids (TSS) of the roots. The leaf chlorophyll index was recorded directly before harvest using a chlorophyll meter (Minolta - SPAD - Soil Plant Analyzer Device, 502, New Jersey, U.S.A.). The root dry matter content was determined by placing a fresh subsample of 200 g in an oven (Binder, ED115, Germany) at 65 °C until a constant weight was achieved. The results were calculated using the equation 1. TSS was measured using a digital refractometer (Hanna, HI96801, USA) with 1 mL of previously filtered tissue extract.

$$DM(\%) = \frac{DW}{FW} \times 100 \quad (1)$$

Where DM(%) represents the percentage of dry matter, DW constant dry weight, and FW is the fresh weight.

Statistical analysis

Statistical analysis was performed by using the Infostat software in its 2011 release (National University of Cordoba, Argentina). The means-specific differences were determined by the least significant difference – LSD method applied following the analysis of variance (ANOVA); the significance of differences was defined at $P<0.05$.

RESULTS AND DISCUSSION

Agro-industrial waste is currently perceived as an opportunity to reuse nutrients (Cantão et al. 2021), with benefits that were evidenced in this study. Analysis of the evaluated soil characteristics at the main effects and interaction levels revealed that the differences were significant for all the variables evaluated (Table 1). At the level of main effects, PGP applications significantly favored

the content of OM, N, P, K, and CEC, increased pH, and significantly decreased EC compared to the absolute control. It has been described that in arid and semi-arid climates, the incorporation of biofertilizers improves soil physical, chemical and biological properties (Celestina et al. 2019; Lu et al. 2020; Liu et al. 2021) with environmental benefits, such as increased soil carbon, soil water holding capacity, net primary productivity and N content in plant tissues and decreased amount of surface runoff (Gupta et al. 2018; Gravuer et al. 2019; Hafez et al. 2020). In this trial, the initial OM content is typical of an arid zone, probably due to limited plant cover, lack of moisture and microorganisms (Wei et al. 2021); this initial condition made it possible for soil improvements to be significant despite the fact that bio-fertilizer applications were spotty.

Regarding WB, the application of 100 mL L^{-1} showed the highest values of EC with a significant difference from 50 mL L^{-1} and the absolute control (Table 2), evidencing one of the most important limitations of the use of bio-ferments and organic fertilizers in arid and semi-arid zones: the high salt content (Lu et al. 2020).

Table 1. Results of the *P* value of the main effect of processed grapevine pomace, whey bio-ferment and their interaction on arid zone soil characteristics (analysis of variance - ANOVA).

Factor	OM	N	P	K	pH	EC	CEC
PGP - Processed grapevine pomace	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
WB - whey bio-ferment	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
PGP:WB	<0.0001	<0.0001	0.0044	<0.0001	<0.0001	<0.0001	0.0004
CV(%)	0.58	2.28	0.64	0.1	0.69	3.84	0.69

CV: coefficient of variability; OM: organic matter; EC: electrical conductivity; CEC: cation exchange capacity.

Table 2. Effect of the application of processed grapevine pomace and whey bio-ferment on the chemical characteristics of the soil at crop harvest.

		OM (%)	N (%)	P (mg kg^{-1})	K (mg kg^{-1})	pH	EC (dS m^{-1})	CEC (cmol kg^{-1})
Initial value		1.12	0.048	21.661	320.452	7.85	1.22	10.321
Processed grapevine pomace	0 g kg^{-1}	1.31^{c}	0.05^{c}	22.74^{c}	321.00^{c}	6.23^{c}	2.41^{a}	10.08^{c}
	12.5 g kg^{-1}	1.66^{b}	0.29^{b}	27.24^{b}	343.25^{b}	6.55^{b}	2.15^{b}	12.98^{b}
	25 g kg^{-1}	1.81^{a}	0.37^{a}	30.39^{a}	370.28^{a}	6.82^{a}	1.83^{c}	14.26^{a}
Whey bio-ferment	0 mL L^{-1}	1.38^{c}	0.27^{b}	23.97^{c}	326.79^{c}	6.31^{c}	2.06^{b}	10.82^{c}
	50 mL L^{-1}	1.76^{a}	0.37^{a}	29.31^{a}	363.34^{a}	6.74^{a}	1.77^{c}	13.81^{a}
	100 mL L^{-1}	1.64^{b}	0.07^{c}	27.1^{b}	344.4^{b}	6.54^{b}	2.56^{a}	12.68^{b}

Means with equal letters vertically within each group are not statistically different (P<0.05). CV: coefficient of variation. OM: organic matter. EC: electrical conductivity. CEC: cation exchange capacity.

At the interaction level, it was observed that combined applications of PGP and WB decreased EC by 0.99 dS m^{-1} concerning individual applications. The negative relationship of K with EC (R^2 -0.45; P=0.02) (Table 3) could explain why joint applications of PGP and WB caused less salinity than applying separately; the K contained in PGP may have a buffering effect on the elevated salinity of WB. Hafez et al. (2020) reported a similar result when applying vermicompost and biochar, which generated a combined effect by decreasing the detrimental effects of soil salinity and water stress in wheat plants.

The results obtained in the crop evaluations showed significant differences in the effects of the treatments applied, both at the level of the main effects and interaction levels (Table 4). To analyze the effects of PGP and WB,

Table 3. Pearson correlation matrix for quantitative plant and soil variables.

Variables	Leaf length	Root length	Root diameter	Root weight	DM	Chlorophyll	TSS	OM	Total (N)	P	K	pH	EC	CEC	WB	PGP
Leaf length	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Root length	0.94	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Root diameter	0.96	0.95	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Root weight	0.92	0.91	0.89	1	-	-	-	-	-	-	-	-	-	-	-	-
DM	0.97	0.96	0.97	0.93	1	-	-	-	-	-	-	-	-	-	-	-
Chlorophyll	0.95	0.94	0.92	0.99	0.95	1	-	-	-	-	-	-	-	-	-	-
TSS	0.97	0.94	0.94	0.92	0.97	0.94	1	-	-	-	-	-	-	-	-	-
OM	0.87	0.86	0.87	0.91	0.91	0.93	0.86	1	-	-	-	-	-	-	-	-
Total N	0.5	0.52	0.59	0.44	0.6	0.43	0.51	0.49	1	-	-	-	-	-	-	-
P	0.97	0.95	0.97	0.95	0.99	0.97	0.95	0.95	0.55	1	-	-	-	-	-	-
K	0.99	0.93	0.96	0.89	0.97	0.92	0.96	0.86	0.58	0.96	1	-	-	-	-	-
pH	0.98	0.93	0.95	0.92	0.98	0.94	0.96	0.89	0.58	0.98	0.99	1	-	-	-	-
EC	-0.46	-0.42	-0.4	-0.38	-0.37	**-0.37**	-0.44	-0.06	**-0.21**	-0.33	-0.45	-0.43	1	-	-	-
CEC	0.93	0.93	0.93	0.95	0.96	0.97	0.92	0.98	0.5	0.98	0.91	0.94	-0.21	1	-	-
WB	**0.32**	**0.31**	**0.25**	**0.25**	**0.29**	**0.32**	**0.31**	0.38	**-0.25**	**0.33**	**0.27**	**0.29**	**0.27**	**0.35**	1	-
PGP	0.76	0.74	0.76	0.87	0.79	0.82	0.75	0.74	0.41	0.79	0.75	0.76	**-0.32**	0.78	**0**	1

Bold highlighted values have no significant difference.

the results are shown at the main effects level. The application of biofertilizers was found to improve crop features in comparison to absolute control, based on the benefits identified in the soil characteristics. (0 g PGP, 0 mL WB) (Figure 1). Regarding PGP doses, a strong directly proportional relationship was found with the variables leaf length (P<0.0001), chlorophyll (P<0.05), length (P<0.05), diameter (P<0.05), weight (P<0.05), DM (P<0.05), and TSS (P<0.05) of the root. In the case of WB, although correlations were positive in all cases (minimum R^2 0.25 and maximum 0.38), they were not significant (Table 3). These results correlate fairly well with Ramírez and Pérez (2006) and Mali et al. (2018), who concluded that applying organic fertilizers and biofertilizers affects crop growth directly, development, and production.

Table 4. Results of the *P* value of the main effect of processed grapevine pomace, whey bio-ferment and their interaction on characteristics in radish crop (analysis of variance – ANOVA).

Factor	Leaf		Root				
	Length	Chlorophyll	Length	Diameter	Fresh weigh	Dry matter	TSS
PGP - Processed grapevine pomace	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
WB - whey bio-ferment	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
PGP:WB	<0.0001	<0.0001	0.0044	<0.0001	<0.0001	<0.0001	0.0004
CV (%)	19.48	19.60	18.56	18.85	20.88	7.39	5.84

CV: coefficient of variability; TSS: total soluble solids.

Plant size was significantly increased by the application of PGP (25 g kg^{-1}), with a difference of 6.53 cm in leaf length (Figure 1A), 24.45 g in fresh weight (Figure 1E), 1.77 cm in root length and 1.6 cm in root diameter (Figure 1D) over the control. Chlorophyll and TSS measurements showed the same trend: higher PGP concentration, higher chlorophyll (Figure 1B), and TSS (Figure 1F). These improvements would be related to the nutritional supply of MO, N, P, K, Ca, and Mg from the PGP.

For WB, the highest values were obtained with the 50 mL L^{-1} dose, but when increasing to 100 mL L^{-1}, the leaf length (Figure 1A), chlorophyll index (Figure 1B), DM (Figure 1C), root length (Figure 1D), root weight (Figure 1E) and root TSS content (Figure 1F) decreased significantly (P<0.05). This could be attributed to the high salinity (EC) of WB impacting nutrient imbalance, uptake blockage, and even phytotoxicity (Lu et al. 2020), which is reflected in the negative correlation of crop variables and soil EC (Table 3). Ghosh et al. (2014) found that salinity adversely affected the growth of three radish varieties, an EC of 12 dS m^{-1} limited root growth relative to an EC of 4 dS m^{-1}.

Plant growth had a strong positive correlation with the final edaphic content of OM (average R^2 0.89; P<0.0001), P (average R^2 0.96; P<0.0001), K (average R^2 0.95; P<0.0001), pH (average R^2 0.95; P<0.0001) and CEC (average R^2 0.94; P<0.0001), characteristics that are beneficial to achieve better yields (Table 3). Fertilizers from agro-industrial waste are characterized by their contribution of OM, microorganisms, amino acids, macro and micronutrients, structural compounds, and biostimulants that accelerate plant metabolism (Saval 2012; López-Barreto et al. 2018; Lu et al. 2020), increasing photosynthetic rate, leaf area development, fresh and dry biomass, TSS (Ramírez and Pérez 2006; Liriano et al. 2020). In the case of PGP, it mainly provides N, which acts directly on growth, production of reserve substances, and their maturation; on the other hand, WB mainly provides K, which participates in protein synthesis, assimilation, and transport of substances from the leaves to the radish reserve organs (De Sousa et al. 2018; Rattin et al. 2022).

WB applications have been reported to range from 26,000 L ha^{-1} (Canada) to 60,000 L ha^{-1} (Wisconsin, USA) (Felli et al. 2012); however, under the arid climate conditions of this study, the 50 mL WB L^{-1} dose presented the best results at the soil level and on radish characteristics. In the case of PGP, the results indicate that the higher the dose, the greater the benefits on the soil and on the agronomic yield of the crop (Table 2).

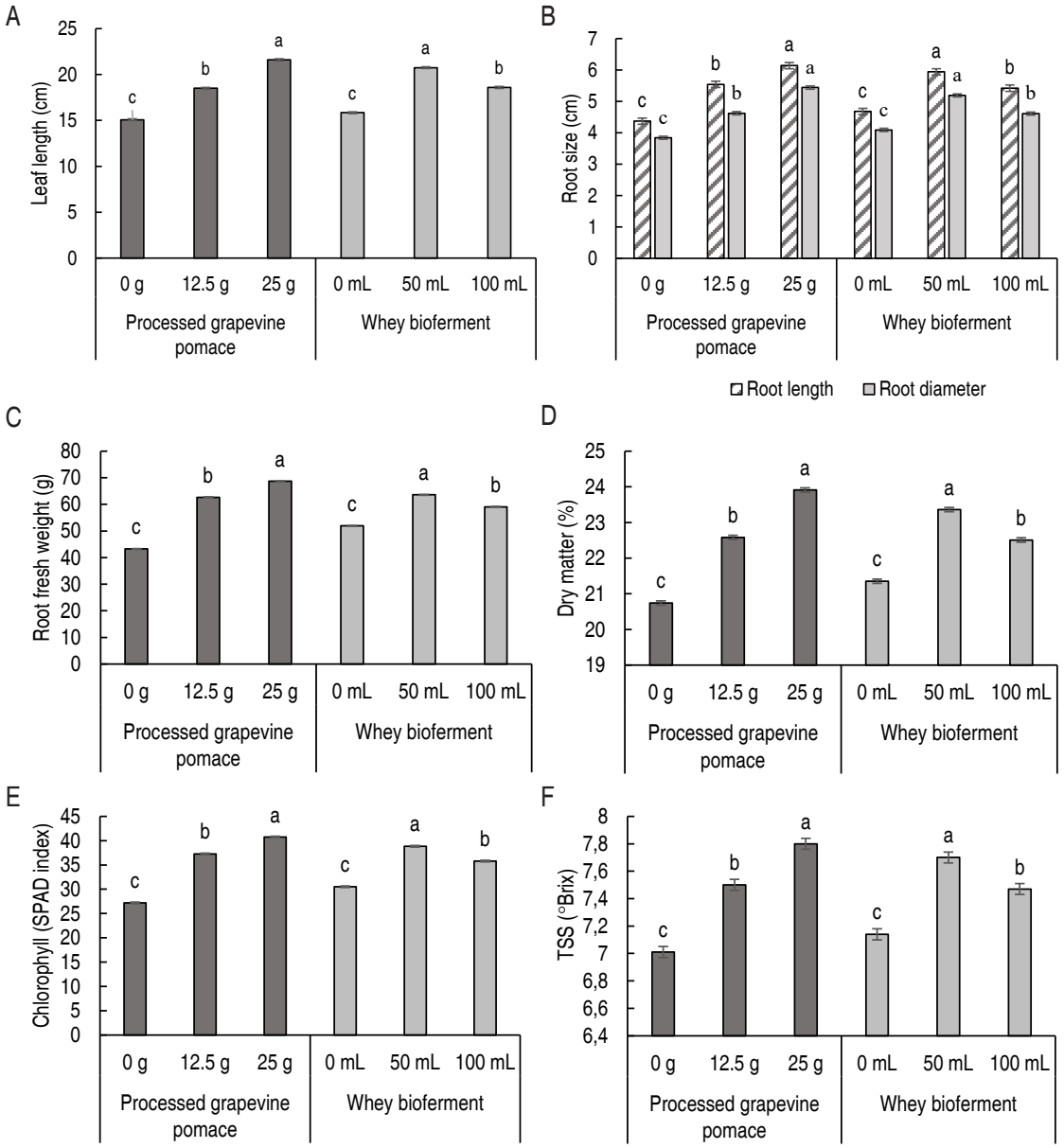

Figure 1. Main effects of the application of processed grapevine pomace (g kg^{-1}) and whey bio-ferment (mL L^{-1}) on A. leaf length, B. leaf chlorophyll (SPAD index), C. root dry matter (%), D. root length and diameter, E. root fresh weight, and F. root total soluble solids TSS. Means with equal letters between columns of the same color are not statistically different (P<0.05).

CONCLUSION

The results of this study underscore the potential for enhancing the agronomic performance of radish crops through the utilization of processed grape pomace and whey bio ferment. These have demonstrated efficacy in augmenting the availability of crucial macronutrients, including nitrogen, potassium, phosphorus, calcium, and organic matter. Such innovative approaches hold promise for optimizing crop productivity and sustainability in agricultural systems.

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Revista Facultad Nacional de Agronomía

Assessment of agro-physiological traits for identifying drought-tolerant durum wheat (*Triticum durum* Desf.) genotypes under rainfed conditions

UNIVERSIDAD NACIONAL DE COLOMBIA

Evaluación de rasgos agrofisiológicos para la identificación de genotipos de trigo duro (*Triticum durum* Desf.) tolerantes a la sequía en condiciones de secano

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ABSTRACT

Breeders are focused on developing high-yielding genotypes that can grow in semi-arid regions under water stress. A field experiment was conducted during the 2020 to 2021 cropping season at the experimental field of ITGC, Setif. The aim of this study was to assess the performance of durum wheat genotypes for agronomic traits growing under semi-arid conditions. The 10 genotypes evaluated were grown in a randomized block with three replications. Analysis of variance showed that the genotype effect was significant for most parameters studied. The best grain yield was recorded for genotypes G3 (3.52 t ha^{-1}) G2 (3.48 t ha^{-1}), and G5 (2.89 t ha^{-1}); thus, they maintained the highest water content (81.09, 84.95, and 84.34%, respectively) and lower temperatures under these conditions. Simple linear regression showed that grain yield correlated positively with the number of spikes, and the number of grains per spike. The principal component (PC) analysis classified both genotypes G2 and G3 as high grain-yielding genotypes; by contrast, genotypes Jupare C 2001, Bouatleb, and G1 were low-yielding.

Keywords:
Grain yield
Genotype
Semi-arid
Water
Performance

RESUMEN

Los mejoradores se centran en desarrollar genotipos de alto rendimiento que puedan crecer en regiones semiáridas sometidas a estrés hídrico. Se realizó un experimento de campo durante la temporada agrícola de 2020 a 2021 en el campo experimental del ITGC, Sétif. El objetivo de este estudio fue evaluar el desempeño de los genotipos de trigo duro en cuanto a características agronómicas en condiciones semiáridas. Los 10 genotipos evaluados fueron cultivados en bloques al azar con tres repeticiones. El análisis de varianza mostró que el efecto del genotipo fue significativo para la mayoría de los parámetros estudiados. El mejor rendimiento de grano se registró para los genotipos G3 (3,52 t ha^{-1}), G2 (3,48 t ha^{-1}) y G5 (2,89 t ha^{-1}); así mantuvieron el mayor contenido de agua (81,09; 84,95 y 84,34%, respectivamente) y temperaturas más bajas en estas condiciones. La regresión lineal simple mostró que el rendimiento de grano se correlacionaba positivamente con el número de espigas y el número de granos por espiga. El análisis de componentes principales (PC) clasificó ambos genotipos G2 y G3 como genotipos de alto rendimiento de grano; por el contrario, los genotipos Jupare C 2001, Bouatleb y G1 fueron de bajo rendimiento.

Palabras clave:
Rendimiento de grano
Genotipo
Semiárido
Agua
Desempeño

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Cereal cultivation is an ancient activity in the Algerian agricultural environment, practiced in all regions, including the Saharan zone, with a predominance of durum wheat cultivation (Chourghal et al. 2023). Durum wheat (*Triticum durum* Desf.) is an important cereal species and is cultivated worldwide over almost 17 million hectares (Xynias et al. 2020). It is a central crop grown in Algeria, and its production is based on the adoption of modern varieties derived from plant material from CIMMYT (the International Center for the Improvement of Maize and Wheat), ICARDA (the International Center for Agricultural Research in the Dry Areas), and traditional cultivars derived from local heritage varieties. Though durum wheat production covers only 24 to 55% of the country's annual consumption (ITGC 2022; Djoudi et al. 2024), it is insufficient to meet the country's needs, estimated at 8.5 million metric tons per year (Hannachi and Fellahi 2023). This low production is often explained by unpredictable weather, long dry seasons, inconsistent rainfall, and soils that are poor in nutrients, which especially characterize the semi-arid regions (Krishnamurthy et al. 2011). Thus, Hussain et al. (2019) stated that several abiotic stresses, such as drought, chilling, high temperature, and salinity, are strongly affecting plant growth, development, and yield. Indeed, drought is one of the most important abiotic factors that reduces yield under rainfed conditions. Durum wheat varieties grown in a dry area must be able to tolerate water and thermal stress to improve their grain yield potential (Mekaoussi et al. 2021). According to Bendjama and Ramdani (2021), water stress is the main constraint, reducing yield and potential production. Mamrutha et al. (2022) mentioned drought as one of the critical factors that reduce wheat yield at the worldwide level. It can occur at each stage of plant growth and induce a series of morphological, physiological, biochemical, and molecular changes in plants (Bendada 2021). Drought also negatively affects relative water content, gas exchange, and chlorophyll content (Othmani et al. 2021). They also observed that drought stress reduced stomatal conductance, which results in increased leaf temperature by limiting transpiration (Melandri et al. 2020). Further, Bali and Sidhu (2019) cited that relative leaf water content is the primary factor that decreased the growth of wheat in response to drought stress. Drought not only reduced water content but also chlorophyll content (Keyvan 2010). The stress effect depends on its degree, duration, and stage of development. During the early stages of growth, stress involves multiple morphological and physiological alterations during germination (Jian et al. 2016). While, during flowering and grain-filling periods, drought can decrease the number of fertile tillers, ear fertility, grain weight, and aboveground biomass (Pour-Aboughadareh et al. 2020). Improving grain yield has been a primary goal of most breeding programs. Then, developing drought-tolerant cultivars with high grain yields has been the principal goal of wheat breeders (Mohammadi et al. 2014; Mao et al. 2022). Various physiological traits, such as relative water content, electrolyte leakage, chlorophyll content, and canopy temperature, have been used to select desirable genotypes with high yield and stress tolerance. Likewise, the selection of genotypes using yield is assisted by morphological and physiological characteristics related to yield under drought conditions (González-Ribot et al. 2017). This research was conducted to assess the variability of 10 durum wheat genotypes in response to drought conditions based on agro-physiological traits and to select desirable genotypes under these conditions.

MATERIALS AND METHODS

Site, Plant materials and Experiment design

The experiment was carried out at the experimental site of the Technical Institute of Field Crops (ITGC) of Setif 36°09" N; 05°22" E; 981 meters above sea level (masl) during the 2021 to 2022 agricultural season. The experiment was set up on 14th December 2022, in a randomized complete block design (RCBD) with three replications. Each plot consisted of six lines 5 m long, spaced 0.2 m apart, which made up 6 m^2 of plot dimension. The plant materials used consisted of ten durum wheat genotypes shown in (Table 1).

The soil is calcareous (Calcisol) with a silty clay texture, and organic matter content is 1.4% on the surface. The amount of monthly rainfall, temperature (min, max, mean) are presented in (Table 2).

Physiological traits

The chlorophyll content index (CCI) of each flag leaf was measured using a digital Chlorophyll Content Meter Model CCM-200 Plus. The relative water content (RWC) was determined at the heading stage according to Pask et al. (2012) method, five fresh leaves were weighted to record fresh mass (FM). The leaves were placed in distilled water for 24 h and weighed to get a turgid

Table 1. The pedigrees of the genotypes tested.

Genotypes	Pedigrees
G1	RASCON_37/GREEN_2/9/USDA595/3/D67.3/RABI//CRA/4/ALO/5/…
G2	MINIMUS_6/PLATA_16//IMMER/3/SOOTY_9/RASCON_37/9/…
G3	CMH77.774/CORM//SOOTY-9/RASCON-37/3/SOMAT-4
G4	CNDO/PRIMADUR//HAI-OU-17/3/SNITAN/4/SOMAT-3/
G5	CNDO/VEE//CELTA/3/PATA_2/6/ARAM_7//CREX/ALLA/5/ENTE/...
G6	SILVER 14/MOEWE//BISU_I/PATKA_3/3/PORRON_4/YUAN_I/9/...
Jupare C 2001	STINKPOT//ALTAR-84/ALONDRA
Bousselam	Heider/Martes/Huevos de Oro. ICD-414
Boutaleb	GTA dur /Ofanto
Oued El Bared	Hedba3/Ofanto

Table 2. The climatic characteristics of the 2021–2022 agricultural season.

Month	Rainfall (mm)	Temperature (°C)		
		Max	Min	Mean
September	41.42	30.1	17.5	23.8
October	8.13	20.6	9.1	14.7
November	84.6	11.9	5	8.5
December	21.1	12	1.5	6.4
January	10.91	10.4	-1.4	4.3
February	28.69	13.6	1	7.2
March	46.98	12.8	4.4	8.6
April	82.29	17.2	5.9	11.7
May	6.09	25	10.5	17.8
June	0.25	35.3	18.2	27.1

mass (TM). Samples were oven-dried at 65 °C for 24 h to record dry mass (DM). Relative water content was calculated as follows the equation 1.

$$RWC = \frac{(FM - DM)}{(TM - DM)} \times 100 \quad (1)$$

The relative electrolyte leakage (REL%) of leaf tissues was measured using the method developed by Bajji et al. (2001), two leaves were collected, washed with tap water then with distilled water, and cut into 1 cm length segments. The samples were placed in tubes with 10 mL of distilled water and incubated for 24 h at room temperature in the laboratory. Subsequently, the first reading (EC1) was carried out. The final conductivity (EC2) was measured after placing tubes in a boiling water bath at 100 °C for 1 h. The relative electrolyte leakage (REL%) was calculated as follows the equation 2.

$$REL(\%) = \frac{EC1}{EC2} \times 100 \quad (2)$$

The Canopy temperature (CT) measurements were taken on a sunny day using a portable infrared thermometer (Fluke Corporation. Everett.WA. USA). Readings were taken on sunny days between 11:00 to 14:00 hours.

Flag leaf area (FLA) was determined according to Spagnoletti-Zeuli and Qualset (1990). Five fresh leaves were collected, Leaf length (L) and wide (l) were measured and the area was calculated as follows the equation 3.

$$FLA\ (cm^2) = 0.607\ (L \times l) \quad (3)$$

Agronomic traits

At maturity, data were collected on grain yield (GY) (t ha^{-1}), thousand kernel weight (TKW, g), number of spikes per m^2 (NSm^{-2}, spike), and number of grains per spike (NGS, grain).

Statistical analysis

An analysis of variance was performed for a measured trait at the 5% probability level to test the differences among genotypes, and the linear correlations were done to study the different associations among variables using Costat software (6.400, 1998). A principal component analysis (PCA) was done using the R Core Team.

RESULTS AND DISCUSSION

Physiological variation among genotypes

Crop yield is mainly dependent on the different biochemical and physiological traits of genotypes, as well as the impact of environmental conditions. The results of the variance analysis showed significant differences among genotypes tested for all physiological parameters (Table 3). The differences between the physiological traits of genotypes depend on the distribution of genetics and environmental conditions.

The mean chlorophyll content index was significantly higher in variety Boutaleb (51.3 cci), while it was significantly the lowest in genotype G5 (32.6 cci) with an overall mean of 42.83 cci. Leaf chlorophyll content is a major indicator of the photosynthetic ability of plant tissues. It can directly regulate the photosynthetic rate and reflect photosynthetic potential and primary production (Liu et al. 2015). The change in chlorophyll contents is a useful indicator to evaluate the influence of environmental stress on plant growth and yield (Kohila and Gomathi 2018). Thus, several studies reported that the amount of chlorophyll in flag leaves was significantly affected by many environmental factors (Kaya et al. 2015; Atar et al. 2020). According to Yang et al. (2022), genotypes tolerant to various stresses display higher chlorophyll content and thus maintain stronger photosynthetic efficiency. It was also reported that stay-green bread genotypes have also shown higher grain yield and total biomass in field conditions (Del Pozo et al. 2016). Also, the study of Mansouri et al. (2018) proved that drought conditions accelerate chlorophyll degradation, reducing leaf area and photosynthesis; thus, genotypes that stay green with delayed senescence can improve their performance under drought conditions. Naveed et al. (2014) mentioned that wheat genotypes were negatively influenced by severe drought stress at several growth stages, which reduced CO_2 assimilation, stomatal conductance, transpiration rate, and chlorophyll content and later inhibited grain yield at both tillering and flowering stages. Besides, the flag leaf area played an important role in improving the grain yield of wheat. The mean values were changed from 18.85 (G2) to 26.74 (G5), with an average of 22.74 cm^2. The flag leaf area is a very important metric for assessing crop growth and is closely related to above-ground biomass and yield (Singh et al. 2023). Larger flag leaf sizes tend to produce more grain per spike in wheat (Tshikunde et al. 2019) and barley (Alqudah and Thorsten 2015). Guendouz et al. (2016) stated that water stress greatly reduces leaf area; it may also decrease turgor pressure and cell expansion, which result in approximately the same dry mass being contained within a smaller leaf area thus raising density.

The minimum, maximum, and average values for relative water content were 70.19% (Oued El Bared), 91.24% (G4), and 82.82%, respectively. The relative water content of flag leaves is often used to assess the response of a plant to stress; it is a reliable index of leaf water deficit status at the time of sampling (Kohila and Gomathi 2018). Optimal plant water status is important for maintaining normal cell activity under water stress conditions (Mamrutha et al. 2022); thus, genotypes that maintain a higher relative water content, ensuring better hydration and more favorable internal water, showed better drought tolerance capacity (Kardile et al. 2018). In addition, the finding of Bayoumi et al. (2015) stated that wheat genotypes that maintained higher relative water content under stress conditions were supposed to be drought-tolerant and show high grain yield. Thus, Bali and Sidhu (2019) mentioned that reduced leaf water potential and relative water content about increased drought stress. Recently, Chaouachi et al. (2023) mentioned that under water stress, plant species lose water mostly through transpiration, and then they tend to control their stomatal closure. Indeed, plants that can maintain relative water content under water stress are the most resistant.

The main relative electrolyte leakage was significantly higher in G3 (90.93%), which was the sensitive one, though genotype variety Oued El Bared was the most susceptible

one with the lowest value (48.34%). The measurement of electrolyte leakage was considered a typical criterion to assess membrane integrity in response to environmental stresses (Slama et al. 2018). According to Chowdhury et al. (2017), maintaining the integrity and stability of membranes under water stress is a major element of drought resistance in plants. Indeed, membrane protection ensures cellular structures remain intact, enabling plants to ensure their survival and productivity in various environmental conditions. Ramadan et al. (2022) stated that relative electrolyte leakage increased with increasing levels of water deficit. Cell membrane stability is considered a possible selection criterion for grain yield since it has a reasonable relationship with plant performance under stressed environments (Anzer et al. 2017). Similarly, the finding of Rehman et al. (2016) described that wheat genotypes with high cell membrane stability produced a high grain yield. According to the results of Slama et al. (2018), increased electrolyte leakage under stress conditions is attributed to the disturbance of cell membranes, which probably induces protein degradation.

Table 3. Means values, maximum and minimum and statistical significance of physiological traits measured.

Genotypes	CCI (cci units)	FLA (cm²)	RWC (%)	REL (%)	CT (° C)
G1	50.03^{a}	21^{d}	86.41^{ab}	71.36^{cd}	26.33^{abc}
G2	39.40^{d}	18.85^{e}	81.09^{b}	84.48^{abc}	22^{d}
G3	42.70^{c}	26.74^{a}	84.05^{ab}	90.93^{a}	22.43^{cd}
G4	43.26^{c}	20.93^{d}	91.24^{a}	70.29^{d}	24.96^{bcd}
G5	32.36^{e}	26.63^{a}	84.34^{ab}	88.50^{ab}	24.63^{bcd}
G6	46.40^{b}	21.08^{d}	81.07^{b}	76.03^{bcd}	25.76^{bcd}
Jupare c 2001	51.20^{a}	22.43^{cd}	88.35^{ab}	79.60^{abcd}	25.66^{bcd}
Boussalem	37.26^{d}	24.08^{b}	81.73^{ab}	85.60^{ab}	27.46^{ab}
Boutaleb	51.30^{a}	22.20^{cd}	79.69^{bc}	85.47^{ab}	30.33^{a}
Oued El Bared	34.43^{e}	23.23^{bc}	70.19^{c}	48.34^{e}	26.20^{abcd}
Mean	42.83	22.72	82.82	78.06	25.58
Max	51.30	26.74	91.24	90.93	30.33
Min	32.36	18.85	70.19	48.34	22
Genotype Effect	***	***	*	***	*
LSD5 (%)	2.64	1.62	9.81	13.56	4.30

CCI: Chlorophyll content index; **RWC (%):** Relative water content; **REL (%)**: Relative electrolyte leakage; **CT (° C):** Canopy temperature, **FLA (cm²):** flAg leaf area. ns; *, **, and *** non-significant, significant and highly significant effects at 5, 1, and 0.1% probability respectively.

The mean canopy temperature was significantly higher in both genotypes G2 and G3 with 22 and 22.43 °C, respectively while it was significantly the lowest in the variety Boutaleb (30.33 °C). Canopy temperature is an indirect measure of transpiration rate and stomatal conductance that may be useful in determining genotypic differences in drought response (Guendouz et al. 2021). This indicator is associated with plant water stress since the evaporative cooling involved in transpiration may cool leaves under ambient air temperature (Bazzaz et al. 2015). Wheat genotypes that have a cooler canopy during the heading stage and grain filling in the same environment can be an important indication of drought stress tolerance (Thapa et al. 2018). Canopy temperature was used by Singh et al. (2022) as an important screening criterion to identify potential heat-tolerant genotypes along with a heat susceptibility index based on grain below optimum and stress environments. Sohail et al. (2020) revealed that genotypes maintain a low canopy temperature under rainfall conditions due to their ability to extract water through a better root system and greater stomatal conductance. According to the results of Bazzaz et al. (2015), in water stress conditions, the foliar temperature of wheat genotypes increased due to an increase in breathing and a decrease in transpiration. Also, it was noticed that plants with a suitable supply of

water maintained their canopy temperature below the air temperature, while plants with an insufficient supply of water showed a canopy temperature above the air temperature.

Agronomic traits for the assessed durum wheat genotypes

Numerous agronomic characters that have been widely explored in wheat improvement programs influence grain yield. The data presented in Table 4 shows the genotype effect was significant for grain yield and the number of spikes per m². The highest-yielding genotypes were G3, G2, and G5 (3.52, 3.48, and 2.89 t ha^{-1}, respectively), with an overall mean of 2.54 t ha^{-1}. For thousand kernel weight was significantly higher in Boutaleb, G2, and G4 (34, 32.72, and 31.8 g, respectively) though it was significantly lowest in genotype Jupare C 2001 (28.4 g). The number of spikes for the genotypes evaluated was recorded from 320 to 556.66 spikes per m²; the genotype G2 recorded the highest value, while the genotypes G1, G3, G4, and G5 exhibited the lowest values with 346. 66, 358.33, 346.66, and 320 spikes. The mean values for NG/S varied from 21 grains for the introduced genotype Jupare C 2001 to 43 grains for genotype G5, with a mean of 30.48 grains overall for all genotypes. Grain yield is a complex characteristic determined by three components: the number of spikes per area, grain number per spike, and grain weight. MajidiMehr et al. (2024) stated that water stress is a crucial environmental factor that decreases grain yield in bread wheat. Liu et al. (2015) proved that durum wheat genotypes were better adapted to water deficits and were able to maintain their grain numbers in unfavorable environments, which contributed to a smaller decrease in grain yield. Under stressful conditions in arid and semi-arid regions, the major purpose of wheat breeding programs is to develop durum wheat cultivars with high grain yields. It has been reported that wheat yield improvements are principally due to increases in grain weight and grain number per spike (Feng et al. 2018; Hu et al. 2022). Nouri et al. (2011) mentioned that the relative yield performance of genotypes in drought-stressed and favorable conditions helps to select the desirable genotypes. Hence, the development of high-yielding genotypes with acceptable stability and adaptability is a suitable method for improving durum wheat yield in drought conditions (Pour-Aboughadareh et al. 2020).

Table 4. Means values, maximum and minimum and statistical significance of agronomic characters measured.

Genotypes	GY (t ha$^{-1)}$	TKW (g)	NS m^{-2} (spike)	NG S^{-1} (grain)
G1	2.18^{bc}	29.40^{ab}	346.66^{b}	29^{bcd}
G2	3.48^{a}	32.73^{ab}	556.66^{a}	26.66^{cd}
G3	3.52^{a}	29.03^{ab}	358.3^{3}b	35^{abc}
G4	2.23^{bc}	31.80^{ab}	346.6^{6}b	30.66^{bcd}
G5	2.89^{ab}	30.22^{ab}	320^{b}	43^{a}
G6	2.46^{bc}	29.14^{ab}	448.33^{ab}	38^{ab}
Jupare c 2001	2.28^{bc}	28.40^{b}	400^{ab}	21^{d}
Boussalem	2.74^{ab}	29.60^{ab}	553.33^{a}	28.66^{bcd}
Boutaleb	1.73^{c}	34^{a}	376.66^{b}	24.66^{cd}
Oued El Bared	1.85^{c}	30.40^{ab}	451.66^{ab}	29.66^{bcd}
Mean	2.54	30.47	415.83	30.48
Max	3.52	34	556.66	43
Min	1.73	28.4	320	21
Genotype Effect	**	ns	ns	*
LSD5 (%)	0.86	5.17	173.78	13.87

GY (t ha^{-1}): Grain yield; **TKW (g)**: Thousand-kernel weight; **NS m^{-2} (spike)**: Number of spikes; **NG S^{-1} (grains)**: Number of grains per spike. Ns; *, **, non-significant, significant, and highly significant effects at 5, 1, and 0.1% probability respectively.

Correlation among assessed traits

Table 5 shows the correlations between different traits and grain yield. Grain yield had positive and significant correlation with number of grains per spike (0.43*) and a non-significant association with number of spikes NS (0.27^{ns}). Fellahi et al. (2019) supports this finding, and several researchers agree, suggesting that higher numbers of grains per spike and number of spikelets increase grain yield (Würschum et al. 2018; Wolde et al. 2019). The number of kernels per spike has been suggested as a useful trait for improving wheat grain yield, especially under drought conditions (Bogale and Tesfaye 2016). By contrast, the findings of Iqbal et al. (2017) revealed a non-significant relationship with these traits. Grain yield also had a significant positive correlation with thousand kernel weights (r=0.44 ns), while the study of Ullah et al. (2021) suggested a significant association between grain yield and thousand kernel weights. On the other hand, Boudersa et al. (2021) suggested that all yield components, such as grain weight, number of grains per spike, and biomass, have a considerable contribution to grain yield and that any direct or indirect disturbance affecting any of the yield components inevitably affects the grain yield. Hence, grain yield improvement has been significantly associated with increased thousand-kernel weight; it expresses the grain size and considerably enhances the final yield of wheat (Iqbal et al. 2015). Ullah et al. (2021) conclude that for bread wheat, increased grain weight directly contributed to improved grain yield. Canopy temperature showed significant negative relationships with grain yield; Singh et al. (2022) stated similar findings. Low canopy temperatures in durum wheat lines were associated with higher grain yields (Sohail et al. 2020). Furthermore, Oulmi et al. (2020) noticed that a high canopy temperature causes a decrease in grain yield. Chlorophyll content did not correlate significantly with grain yield while showing a negative correlation with the number of grains per spike (r=-0.38). Similar findings were reported by Mohammadi et al. (2018). The flag leaf area exhibited a significant relationship with the number of grains per spike (r=0.43*) and chlorophyll content (r=-0.37*). This result agrees with the findings of Nor et al. (2015), who found that leaf area showed a significant positive correlation with the number of grains per spike. Wang et al. (2022) also stated similar results and observed that wheat genotypes with a larger flag leaf tend to produce more kernels per spike.

Table 5. Correlations among different traits measured.

Traits	CCI	RWC	REL (%)	FLA	CT	GY	TKW	NS	NG S^{-1}
CCI	1	-	-	-	-	-	-	-	-
RWC	0.31^{ns}	1	-	-	-	-	-	-	-
REL	0.12^{ns}	0.14^{ns}	1	-	-	-	-	-	-
FLA	-0.37^{*}	-0.09^{ns}	0.26^{ns}	1	-	-	-	-	-
CT	0.31^{ns}	-0.02^{ns}	0.18^{ns}	-0.05^{ns}	1	-	-	-	-
GY	-0.26^{ns}	0.14^{ns}	0.30^{ns}	0.22^{ns}	-0.47^{**}	1	-	-	-
TKW	-0.26^{ns}	-0.17^{ns}	-0.02^{ns}	-0.26^{ns}	-0.25^{ns}	0.44^{ns}	1	-	-
NS	-0.26^{ns}	-0.43^{ns}	-0.025^{ns}	-0.37^{ns}	0.37^{ns}	0.27^{ns}	0.33^{ns}	1	-
NG S^{-1}	-0.38^{*}	0.02^{ns}	0.11^{ns}	0.43^{*}	-0.1^{ns}	0.43^{*}	-0.1^{ns}	0.1^{ns}	1

CCI: Chlorophyll content index; **RWC (%)**: Relative water content; **REL (%)**: Relative electrolyte leakage; **CT (° C):** Canopy temperature; **FLA (cm^2)**: Flag leaf area; **GY (t ha^{-1})**: Grain yield; **TKW (g)**: Thousand-kernel weight; **NS m^{-2} (spike)**: Number of spikes; **NG S^{-1} (grains)**: Number of grains per spike.

Principal component analysis

The principal component analysis (PCA), one of the methods of multivariate analysis, elucidates among a set of traits which ones are decisive in genotypic differentiation and selection (Ara et al. 2018). The data shown in Table 6 revealed that three components exhibited an eigenvalue near or higher than one. These three PCs accounted for 72.36% of the total variation. Furthermore, an increase in the number of PCs was correlated with a decrease in Eigenvalues. Based on the results shown in Figure 1, PC1 was highly correlated with grain yield and thousand kernel weights. genotypes G2 and G3 were positively correlated with PC1, suggesting that they had high productivity. While genotypes Jupare C 2001, Bouatleb, and G1 were

negatively correlated with PC1, which was described as a low-yielding genotype, this result is in accordance with the previous results of Frih et al. (2021) and Guendouz et al. (2021), who stated that grain yield and thousand kernel weights were associated with the two first components. The second component was a physiological axis, with relative electrolyte leakage and relative water content as the major contributors. The genotype G4 was positively connected with this axis and consequently was classified as a drought-tolerant line. By contract varieties, Oued El Bared and Boussallem were negatively correlated with this axis, suggesting that they were the most susceptible to drought conditions. PC3 was negatively connected with flag leaf area; both genotypes G5 and G6 were negatively associated with this axis, which was characterized by a large flag leaf area.

Table 6. Eigenvalues, % proportion variance and % cumulative variance of three first components.

	PC1	PC2	PC3
Variance	2.849	2.026	1.638
% of variance	31.65	22.51	18.20
Cumulative	31.65	54.16	72.36

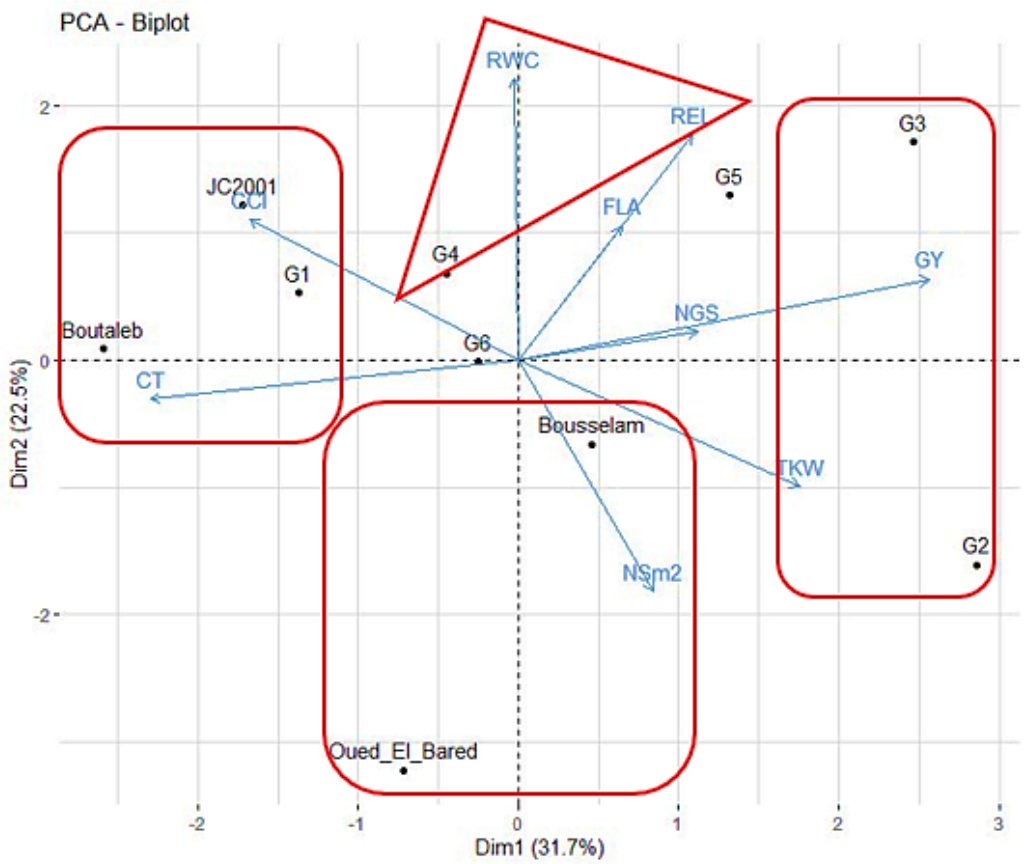

Figure 1. Biplot of genotypes and measured parameters with the first three components of PCA.

CONCLUSION

Drought is one of the most important abiotic stresses that reduce grain yield in rainfed regions. This study allowed the evaluation of the different durum wheat genotypes based on their agro-physiological characteristics. The results obtained provide insights to facilitate the selection and cultivation of these genotypes under semi-arid conditions. An analysis of variance demonstrated a significant difference among genotypes for the majority of traits studied. The genotypes G2, G3, and G5 recorded the highest yield (3.52, 3.48, and 2.89 t ha^{-1}, respectively) with a moderate water content and low values of temperature with G (22 °C), G3 (22.43 °C), and G3 (24.63 °C). Correlation among assed characters revealed that grain yield showed a positive and significant association with the number of grins per spike and a non-significant relationship with the number of spikes per m^2 and the thousand kernel weights. However, a non-significant association was found between all physiological traits. Moreover, the principal component analysis displayed three components. The first component related to GY and TKW genotypes associated with this axis exhibited high values of these traits. The second was the physiological axis; their genotypes were the most tolerant to semi-arid conditions. The results of the mean performance revealed that the genotypes Bousselam, G2, and G5 were the appropriate genotypes for growing under semi-arid conditions.

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Poultry meat preservation with citric acid obtained from the fermentation of wheat straw by *Aspergillus niger*

Conservación de carne de ave con ácido cítrico obtenido de la fermentación de paja de trigo por *Aspergillus niger*

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Keywords:
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Shelf-life

ABSTRACT

Wheat straw is a highly abundant waste material that can be utilized as a carbon source in various fermentation processes. This study aimed to generate citric acid using *Aspergillus niger* from wheat straw and to evaluate its preservative potential in fresh poultry meat samples. Wheat straw samples were dried, pulverized, and chemically pretreated. The obtained wheat straw slurry (100 g L^{-1}) was saccharified at 50 °C using cellulases obtained from *Trichoderma viride*. The hydrolyzed substrate was then subjected to fermentation by *Aspergillus niger* at 35 °C, 180 rpm, and pH=5 for 7 days. The citric acid generated was determined via the HPLC technique. Poultry meat was obtained and treated by soaking in different concentrations (1, 2, and 3%) of citric acid (n=4). The treated samples were then stored in sterile plastic bags for 14 days at 4 °C. Total Bacterial Count (TBC), Total Coliform Count (TCC), TVB-N, and TBARS were determined as storage progressed, and pH, TTA, and sensory evaluation were carried out. The highest citric acid obtained was 14.15 g L^{-1} which resulted in a percent yield of 26.18%. Treatment of meat with 3% citric acid had the lowest TBC and TCC of 2.55 and 0.34 Log_{10} CFU g^{-1} after 7 days of storage respectively. There were significant differences in the TBC and TCC observed within the treatments (P<0.05) as observed. T-VBN and TBARS reduction during storage was most evident in meat samples treated with 3% citric acid, retaining acceptability of 31.22 mg 100 g^{-1} and 0.74 mg kg^{-1}, respectively at day 10. The 2% citric acid treatment had the best sensory attributes (16) on day 7. Findings from this study show that treatment with 2% citric acid and above showed promising results in extending the shelf-life of fresh poultry meat samples.

Palabras clave:
Fermentación
Residuos lignocelulósicos
Conservación de carne
Ácidos orgánicos
Vida útil

RESUMEN

La paja de trigo es un material de desecho muy abundante que puede servir como fuente de carbono en diversos procesos de fermentación. El estudio tuvo como objetivo generar ácido cítrico a partir de la paja de trigo utilizando *Aspergillus niger* y evaluar su potencial conservante en muestras de carne fresca de ave. Las muestras de paja de trigo se secaron, pulverizaron y pretrataron químicamente. La suspensión de paja de trigo obtenida (100 g L^{-1}) se sacarificó a 50 °C usando celulasas obtenidas de *Trichoderma viride*. El sustrato hidrolizado luego se sometió a fermentación por *Aspergillus niger* a 35 °C, 180 rpm y pH=5 durante 7 días. El ácido cítrico generado se determinó mediante la técnica de HPLC. La carne de ave se obtuvo y se trató mediante remojo en diferentes concentraciones (1, 2 y 3%) de ácido cítrico (n=4). Las muestras tratadas se almacenaron en bolsas de plástico estériles durante 14 días a 4 °C. Se determinaron el recuento total de bacterias (TBC), el recuento Total de Coliformes (TCC), el TVB-N y el TBARS a medida que avanzaba el almacenamiento, y se realizó el pH, el TTA y la evaluación sensorial. El ácido cítrico obtenido fue de 14,15 g L^{-1}, lo que resultó en un rendimiento porcentual del 26,18%. El tratamiento de la carne con un 3% de ácido cítrico presentó los valores más bajos de TBC y TCC de 2,55 y 0,34 Log_{10} UFC g^{-1} después de 7 días de almacenamiento, respectivamente. Se observaron diferencias significativas (P<0,05) en el TBC y el TCC dentro de los tratamientos. La reducción de T-VBN y TBARS durante el almacenamiento fue más evidente en las muestras de carne tratadas con ácido cítrico al 3%, conservando una aceptabilidad de 31,22 mg 100 g^{-1} y 0,74 mg kg^{-1}, respectivamente, en el día 10. El tratamiento con ácido cítrico al 2% tuvo los mejores atributos sensoriales (16) en el día 7. Los resultados de este estudio indican que el tratamiento con ácido cítrico al 2% mostró resultados promisorios en la prolongación de la vida útil de las muestras de carne fresca de ave.

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Lignocellulosic materials such as straws, stalks, and shells, offer a plentiful and renewable carbon resource for diverse fermentation processes, ensuring long-term sustainability (Singh et al. 2012). Growing interest in harnessing lignocellulosic materials from agricultural and domestic sources has led to significant economic and environmental benefits. These include reducing land usage for waste disposal and valorizing these wastes by utilizing them as raw materials for various bio-production processes (Singh et al. 2012).

Wheat straw, comprising over 80% of total domestic agricultural residues, is widely regarded as an ideal biomass feedstock because of its relatively low cost and the high volume of lignocellulose present in the biomass (Kadam and McMillan 2003). The current availability of wheat straw is estimated at 80 million dry tons per year (USDA 2003), a majority of which could be available to bio-production plants in the near term. Its exploitation and transformation have been inadequate (Steiner et al. 2015). Several applications of wheat straw in biotechnological processes have been reported, and this has been achievable on an industrial scale by adopting solid-state fermentation (SFF) or submerged fermentation (SMF) since wheat straw contains basic nutrients required for microbial growth (Mussatto 2014).

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) is the most important commercial product, which is found in almost all plant and animal tissues. It exists widely in nature and is present as a kind of fruit acid in lemon, orange, pineapple, plum, peas, and peach and animal bones, muscles, and blood. It has many applications in the food, pharmaceutical, and cosmetic industries as an acidulant, flavor enhancer, preservative, antioxidant, emulsifier, and chelating agent (Książek 2023). In recent years, citric acid has been commercially produced by fungal fermentation mainly by *Aspergillus niger* (Książek 2023). Poultry meat, which is believed to be a perishable product is highly susceptible to spoilage in the form of discoloration, off odours/taste, and altered viscosity during storage at ambient conditions. Foodborne illnesses resulting from poultry meat contamination have also become a major source of global concern. *Salmonella* and *Campylobacter* cause more foodborne illnesses in poultry than any other bacteria (Hafez and El-Adawy 2019). It was estimated that one in every 25 packages of chicken at the grocery store is contaminated with *Salmonella* (CDC 2022). Verotoxin-producing *Escherichia coli* O157:H7 (VTEC), *Listeria,* and *Yarsinia* have become prominent in some areas as additional foodborne pathogens. A number of other toxigenic pathogens such as *Clostridium perfringes, Bacillus cereus* and *Staphylococcus aureus* can also enter the food chain via contaminated poultry products. Several regulations have been passed by the European Parliament Council Regulation (EPCR) on the control of several foodborne zoonotic agents, which covers the adoption of certain regulations aimed at reducing the prevalence of specified zoonosis in food animals at the level of primary production. These infections are distributed worldwide and result in severe economic losses when no effort is made towards their control. It is therefore paramount to employ proper and adequate methods in preservation to prevent such changes and hence prolong its storage time (Hafez and El-Adawy 2019).

The application of organic acids in food preservation has since been considered. Researchers have investigated the efficacy of applying organic acids on meat surfaces during storage (Da Costa et al. 2019; Barcenilla et al. 2022). Various studies have also been carried out to evaluate the antimicrobial effects of certain acids-producing bacteria on the surfaces of meat products (Casas et al. 2021). Microbial proliferation and chemical spoilage are the two major causes of reduced shelf-life in fresh poultry meat during refrigeration storage, therefore the employment of adequate preservative agents in the treatment of meat surfaces could go a long way in inhibiting microbial growth. Owing to their ability to alter the proton motive force (PMF) generated on the cell surfaces of microorganism's organic acids have the potential to be highly effective in meat preservation if applied optimally in meat treatments (Van Ba et al. 2018). Hence citric acid produced from lignocellulosic waste using *A. niger* in submerged fermentation can be used as a preservative agent, which will not only help to reduce environmental wastes but will also preserve meat from post-slaughter spoilage.

Therefore, this study aims to assess the ability of citric acid produced by wheat straw fermentation with *A. niger,* in extending the shelf life of fresh poultry meat.

MATERIALS AND METHODS

Materials and reagents

The wheat straw was collected from farms within Ondo and Osun state in southwestern Nigeria. The collected samples were cut into pieces, milled (Jinsong, China), and sieved to obtain 40-60 mesh fractions. The samples were then homogenized and stored in plastic bags for further use. Poultry meat samples were obtained from freshly slaughtered chickens at a local poultry farm in Akure, Ondo state, Nigeria. NaOH, HCl, distilled water, plate count agar, violet, red bile glucose agar (VRBGA), glucose, urea. All reagents used were of Sigma brand, Darmstard, Germany.

Microorganism

Aspergillus niger (OQ607797) and *Trichoderma viride* (OQ686701) used in this study were cultured in the Department of Microbiology, Federal University of Technology, Akure, Nigeria. The microorganisms were maintained on PDA (Potato-Dextrose agar).

Sample preparation

Poultry meat samples were obtained from freshly slaughtered broiler chickens (pH=6), the meat samples were aseptically deboned, defatted, and cut into strips, using sterilized utensils. Prepared meat strips were then packed into sterile polyethylene bags, sealed, and rapidly transferred to the laboratory in ice packs for immediate treatment.

Inoculum preparation

Aspergillus niger was grown in Erlenmeyer flasks with 100 mL of liquid media containing; glucose, 20 g L^{-1}; $(NH_4)_2SO_4$, 2 g L^{-1}; $ZnSO_4 \cdot 7H_2O$, 0.05 g L^{-1}; $FeSO_4$, 0.018 g L^{-1}; KH_2PO_4, 0.3 g L^{-1}; and $MgSO_4$, 0.3 g L^{-1}. The flasks were incubated on an incubator shaker (MRC Laboratory Instruments, Israel) continuously at 160 rpm and 35 °C for 18 hours before use (Kou et al. 2013).

Dilute acid pretreatment

Dilute acid pretreatment of wheat straw was carried out using a modified method by Mood et al. (2013). Previously milled samples straw was deacetylated using a dilute NaOH (0.4% w/v) at 80 °C for 2 h, after which solids were washed with water and then dilute H_2SO_4 solution was added to achieve a 0.8% (w/w) acid concentration for dilute acid pretreatment. The slurry was vigorously stirred for 2 h at room temperature, dewatered to approximately 40% solids, and then incubated in a horizontal pretreatment reactor at 140 °C with a residence time of 10 min. After pretreatment, the material was then separated into the slurry stream with high solid content and a volatile flash vent stream. Pretreated deacetylated dry slurry was then neutralized using a 50% NaOH solution.

Enzyme extraction and assay

The fungi specie *Trichoderma viride*, was used as a source of cellulases. For cellulases production, 150 mL liquid medium containing: $(NH_4)_2SO_4$ (1.4 g L^{-1}); Urea (0.3 g L^{-1}); KH_2PO_4 (2.0 g L^{-1}); $MgSO_4.7H_2O$ (0.3 g L^{-1}); $CaCl_2$ (0.3 g); Tween 80 (0.2%); wheat straw powder (20 g); cellulose powder (8 g); and 1 mL trace element solution (Alrumman 2016), was added in 250 mL conical flask. Each flask was inoculated with $2x10^8$ *Trichoderma viride* spore suspension. Enzyme production was carried out at 30 °C and pH=7 in an incubator shaker with a speed of 130 rpm for 96 h. The culture medium was then harvested by centrifugation at 8,000 rpm for 10 min at 4 °C (MRC laboratory instruments, Israel). The supernatant was then used as the source of cellulose and enzyme activity was then determined (Zhao et al. 2012).

Enzymatic hydrolysis

Enzymatic hydrolysis of pretreated slurry was carried out using the method of Holtzapple (2003). 100 g of slurry was diluted by the addition of process water to 10% total solids. The diluted slurry was mixed with an appropriate amount of the clarified enzyme (30 FPU g^{-1} of pretreated substrate slurry) in a sterile fermenter containing 0.05 M acetate buffer (pH=5). Hydrolysis of the substrates was carried out at 50 °C for 72 h and agitation speed of 100 rpm (Zhang et al. 2012).

Submerged fermentation

The fermentation medium used for this study was composed of carbon source (wheat straw hydrolysate) supplemented with KH_2PO_4 (0.5 g L^{-1}); $ZnSO_4 \cdot 7H_2O$ (0.05 g L^{-1}); $MgSO_4.7H_2O$ (0.3 g L^{-1}); $CaCO_3$ (30 g L^{-1}); NH_4NO_3 (2 g L^{-1}) (Huang et al. 2006). The agitation speed was maintained at 180 rpm (Ngouénam et al. 2021).

Fermentation procedure

Wheat straw hydrolysate from above was supplemented with the required nutrients. The pH was adjusted using HCl

(1 N) and NaOH (2 M, pH=5). Thereafter, 10% inoculum size was aseptically added, and the medium was covered. The medium was then incubated at 35 °C for 7 days (Azaizeh et al. 2020). The fermentation medium was then centrifuged, and the supernatant was analyzed for citric acid.

Determination of reducing sugar and citric acid content
The reducing sugar content was quantified using the DNS assay method, while citric acid levels were determined via High-Performance Liquid Chromatography (HPLC) (Shimadzu, Japan) with a C_{18} column and IR detector. Sulfuric acid at 0.7 mL min^{-1} was used as the mobile phase. The detection was carried out at 210 nm (Wang et al. 2017).

Acid-soaking of fresh poultry meat
Citric acid was diluted using distilled water to achieve desired concentrations (1, 2 and 3%). The solutions were then used to soak the previously prepared meat samples, meat samples were also soaked in distilled water under similar conditions and used as a control. Treated samples were packed in HDPE film and stored at 4 °C for 14 days (Kang et al. 2003).

Variation of meat soaking parameters
Meat soaking parameters were varied according to the method of Xiaowei et al. (2015). Different acid soaking times (5, 10, 15, 20, and 25 min) and acid-soaking temperatures (10, 20, 30, 40, and 50 °C) were evaluated for their effect on the pH of treated meat before and during storage.

Meat acid activity determination
Treated meat samples (10 g) were homogenized in 90 mL of distilled water. Subsequently, the mixture underwent centrifuging, and the resulting supernatant was collected for titration using a standard NaOH solution (0.001 mol L^{-1}) (Hatcher et al. 2004).

Microbiological quality of treated meat samples
The microbiological quality of both treated and untreated poultry meat samples was assessed using culture-dependent methods involving plate counts. Total viable counts (TVC) and Coliform counts were conducted daily over a span of 14 days, following the protocols outlined by Yang et al. (2016). Specifically, 10 g of meat sample was aseptically plated onto appropriate agar medium and incubated at 37 °C. Plate count agar and violet, red bile glucose agar (VRBGA) were utilized for TVC and TCC determination respectively.

Determination of total volatile basic nitrogen (TVB-N) and thiobarbituric acid reactive substances (TBARS)
TVB-N was determined according to the procedures described by FAO (1986). Meat samples were distilled into a 2% boric acid solution and titrated with 0.1 N H_2SO_4 (titer). TVB-N (mg N 100 g^{-1} flesh) was then calculated using the equation 1:

$$TVB\text{-}N = 14 \times (titer\text{-}blank) \quad (1)$$

TBARS was determined according to the method of Schmedes and Hølmer (1989).

Meat samples were mixed with 25 mL of trichloroacetic acid (20% w/v) and filtered. The filtrate was then incubated with aqueous thiobarbituric acid at boiling temperature for 30 min, after which, the absorbance was measured at 532 nm using a UV– spectrophotometer. TBARS estimates were expressed as mg malondialdehyde (MDA) kg^{-1} of broiler fillet sample.

Sensory evaluation of treated poultry meat samples
Sensory evaluation of the treated meat samples was carried out at the Department of Microbiology, Federal University of Technology, Akure Ondo State, Nigeria. The sensory attributes evaluated include the appearance, viscosity, texture, color, and odour of the meat samples. The evaluation was carried out on a total of four treatments, by a 12-member semi-trained panel, using a 5-point hedonic scale (Chen et al. 2019). The scale utilized ranged from 1 to 5, with details provided in Table 1 below. The 12-member panelists were carefully drawn from members of the university community comprising of students and staff. The panelists were made up of seven females and five males all between the ages of 20 and 50. Before the sensory evaluation, the panelist was tasked with evaluating each sample independently, without comparison to others. A minimum total score of 17 was set as the threshold for considering the sample fresh, while a score of 12 was deemed the lowest acceptable threshold.

Table 1. Five-point hedonic scale and range of scores.

Scale	Ranges of score	Level of acceptability
1	1.00–1.49	Not Acceptable (NA)
2	1.50–2.49	Slightly Acceptable (SA)
3	2.50–3.49	Moderately acceptable (MA)
4	3.50–4.49	Acceptable (A)
5	4.50–5.00	Highly Acceptable (HA)

Statistical analyses

All analyses were performed in triplicates after which the results were presented by means with standard deviation. Data were displayed as mean values attached to the standard deviation (One-way ANOVA). Duncan's new multiple range test (P<0.05) was employed for the determination of significant differences between means, using the SPSS 20 statistics software (2020, IBM, Chicago, Ill., U.S.A.).

RESULTS AND DISCUSSION

Properties and yield of all fermentation parameters involved in citric acid production

Table 2 shows the cellulase activity of the crude enzyme obtained from *T. viride,* FPase was 6.25 U mL^{-1}, endoglucanase activity was 8.5 U mL^{-1}, and β-glucosidase activity was 5.0 U mL^{-1}. The On-site cellulose produced by *T. viride* was found to effectively hydrolyze available cellulose fractions in wheat straw. This result agreed with the findings of Zhao et al. (2012).

Figure 1 shows the retention time and peak representing the products generated (citric and oxalic acids) from *A. niger* fermentation. Citric acid was identified at 11.921 min with a peak area of 1120.827, while oxalic acid, which is a by-product of citric acid fermentation was also identified. The citric acid yield obtained as shown in Table 3 was 14.15 g L^{-1} which cumulated to 26.18% (w/w). These results contrast with the findings of Ramesh and Kalaiselvam (2009), who reported citric acid from *A. niger* with a value of 50.0 g L^{-1} and a percent yield of 70.4%. Auta et al. (2014) reported a value of 1.15 g L^{-1} with a higher yield of 22.5% from *Parkia biglobosa*. The higher yield of citric acid in their study could be attributed to the use of better-adapted strains of *A. niger* which facilitated higher sugar-to-acid conversion rates. Ozen and Ozilgen (1992) also noted that low enzymatic activity as recorded in this study, is capable of limiting the saccharification process which in turn could affect the overall yield of citric acid. The high pKA of citric acid, its potential as a pH regulator, and its antibacterial activity make it a good preservative agent (Thangavelu and Murugaiyan 2011).

Over the years, low-cost agro-residues have been effectively utilized in the production of organic acids by fungi through submerged fermentation (Gao et al. 2013).

Table 2. Cellulase activity of crude enzyme obtained from *Trichoderma viride.*

Parameter	Activity (U mL^{-1})
Filter paper activity	6.5
Endoglucanase activity	8.5
β-glucosidase activity	5.0

Table 3. Fermentation yield for citric acid production from wheat straw.

Parameter	Yield
Reducing sugar Yield	54.04 g L^{-1}
Citric acid Yield	14.15 g L^{-1}
Yield	26.18%

Wheat straw is an interesting biomass with an abundance of cellulose and hemicellulose which can be converted to citric acid with different organisms through co-fermentation strategies (Ogidi et al. 2020).

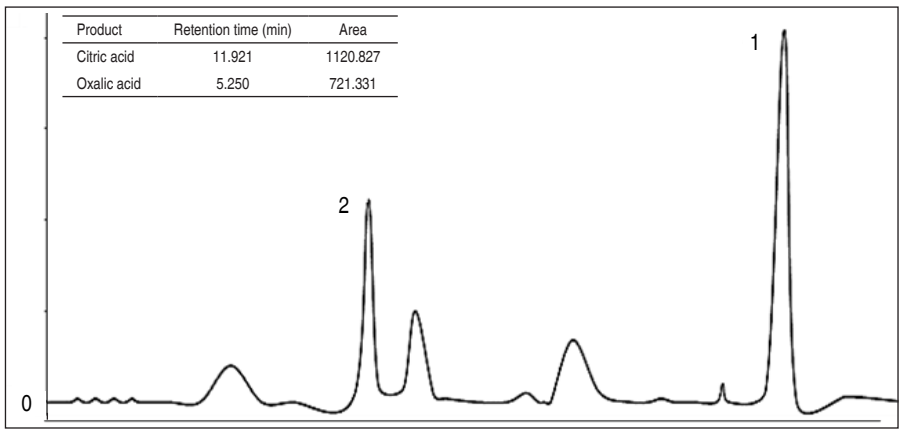

Figure 1. HPLC chromatogram for *Aspergillus niger* production of citric acids from wheat straw. (1 = Citric acid and 2 = Oxalic acid).

Effect of acid-soaking time and temperature

The effect of soaking time and temperature on the initial pH of the meat samples was estimated using varied acid treatment concentrations (1, 2, and 3%) (Figures 2 and 3). The acid-soaking time was positively related to the initial meat pH. It was observed that an increase in the acid-soaking time resulted in a corresponding decrease in the initial pH of the meat samples. The lowest pH of 5.2±0.20, 5.4±0.20, and 5.6±0.10 was observed after 20 min of soaking with 3, 2, and 1% citric acid solution, respectively. This was regarded as the optimum soaking time. The acid-soaking temperature was also positively related to the initial pH of the meat samples up to 30 °C. Further increases in temperature beyond 30 °C led to a rise in the meat pH and hence reduced acidity, because the organic acids used were highly volatile at temperatures above 30 °C, hence drastically reducing their effectiveness (Ren et al. 2012). The lowest pH of 5.2±0.10, 5.3±0.20, and 5.5±0.10 were observed at 30 °C soaking temperature with 3, 2 and 1% citric acid solution, respectively. This was also regarded as optimum. Pre-storage conditions of meat have been identified as one of the major factors that influence the keeping quality of the meat samples. Food processors have resulted in salting, drying, etc., in a bid to achieve optimal pre-storage conditions in meat (Kang et al. 2003). Varying the acid-soaking time and temperature of the

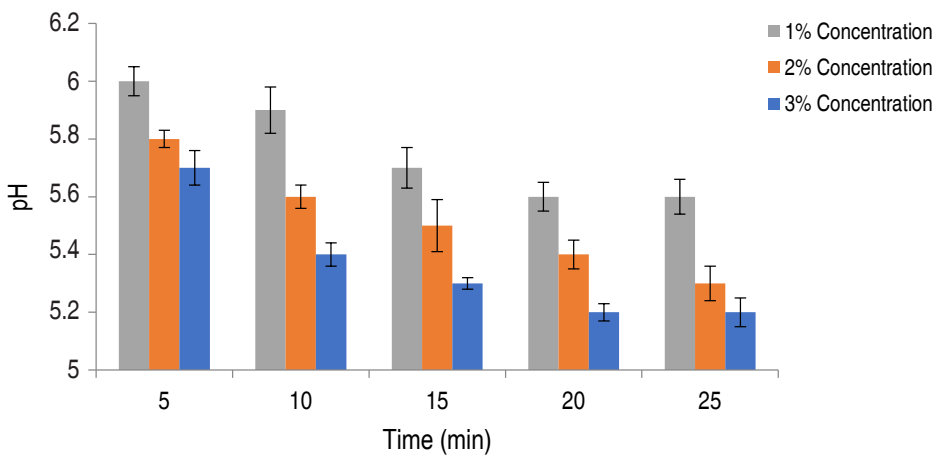

Figure 2 . Effect of citric acid soaking time on the initial pH of poultry meat. Significance (P<0.01).

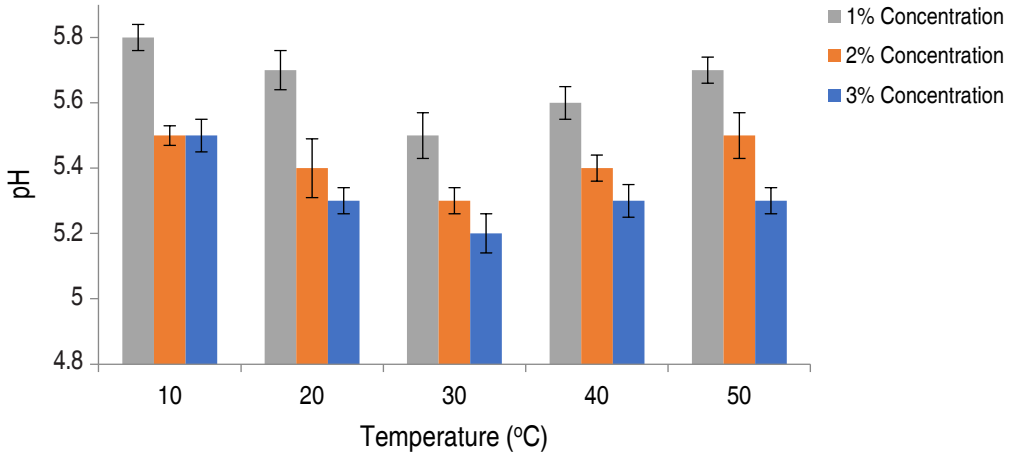

Figure 3. Effect of citric acid soaking temperature on the initial pH of poultry meat. Significance (P<0.01).

meat treatment, had a significant effect on the initial pH of the meat samples (P<0.05). The observed decrease in pH at optimal soaking parameters of 25 min and 30 °C, respectively, enabled the establishment of ideal pre-storage conditions in the meat samples. Reduction in pH (which is a major factor influencing microbial growth) to unfavorable levels have been found to directly improve the keeping quality of most food substances (Sánchez-Clemente et al. 2018). It was noted that meat treatment with 3% citric acid concentration had the greatest effect on the pH reduction of meat prior to storage.

Microbiological quality of meat

The shelf-life and safety of the preserved poultry meat were evaluated by estimating the TVC and TCC of the meat treated with different acid concentrations (1, 2, and 3%) at 25 min acid soaking time and 30 °C acid soaking temperature for 14 days (Figure 4 and 5). Reduction in TVC was directly proportional to the acid treatment concentration used. The highest counts were observed in the control samples, in which the TVC was observed to increase during storage. The lowest TVC of 2.55±0.25 Log_{10} CFU g^{-1} was observed on day 8 of storage, using a 3% concentration. However, TVC in all samples was observed to increase after day 8 of storage. There were significant differences in the TVC of the meat samples treated with different acid concentrations (P<0.05). TCC was also lowest when a 3% citric acid concentration was used. The highest coliform counts were recorded in the control samples, which showed a steady increase in coliform bacteria during storage with the highest TCC of 3.11±0.05 Log_{10} CFU g^{-1} on day 14. Citric acid treatment resulted in considerable reduction in TCC during storage, lowest TCC of 0.34±0.04 Log_{10} CFU g^{-1} was observed on day 7 using 3% acid concentration. This was similar to the work of Tian et al. (2022), who employed lactic acid in the treatment of beef. An increase in TCC in all samples was observed after day 10. There were significant differences in the TCC of the meat samples during storage with different treatments (P<0.05). TVC and TCC are often regarded as direct quality indicators in food samples and have been proven to have a positive correlation to the food spoilage process and food safety respectively (Zhang et al. 2021). The initial bacterial count in meat samples were within the acceptable range (6.0 Log_{10} CFU g^{-1}), indicative of proper meat handling/hygiene (Santos et al. 2018). TVC and TCC values of 6.0 and 2.0 Log_{10} CFU g^{-1} are regarded as the threshold for fresh meat acceptability by the International Commission on Microbiological Specifications for Foods (ICMSF 2022), hence, rendering the control samples completely unacceptable beyond day three. The observed reduction in the rise in TVC and TCC within the acid-treated samples as storage progressed was significantly influenced by acid treatment concentration. This reduction in pH can severely affect the growth and survival of non-acidophilic bacteria, which are the group predominantly responsible for meat spoilage and infection (Tian et al. 2022). This effect can be attributed to a disruption in pH homeostasis, which is highly critical in microbial metabolism; due to its role in maintaining the proper function of biological

macromolecules as well as maintaining the kinetic and thermodynamic force of chemical reactions involving protons as metabolites (Sánchez-Clemente et al. 2018). Although citric acid exhibited good bacteriostatic and bactericidal effects on treated meat samples, 3% citric acid treatment was; however, most effective. Odu et al. (2020), suggested that certain organic acids such as lactic and citric had high bacteriostatic effect due to their pKA value (3.1 for citric acid), displaying less dissociation than others, hence making them more lethal to bacteria.

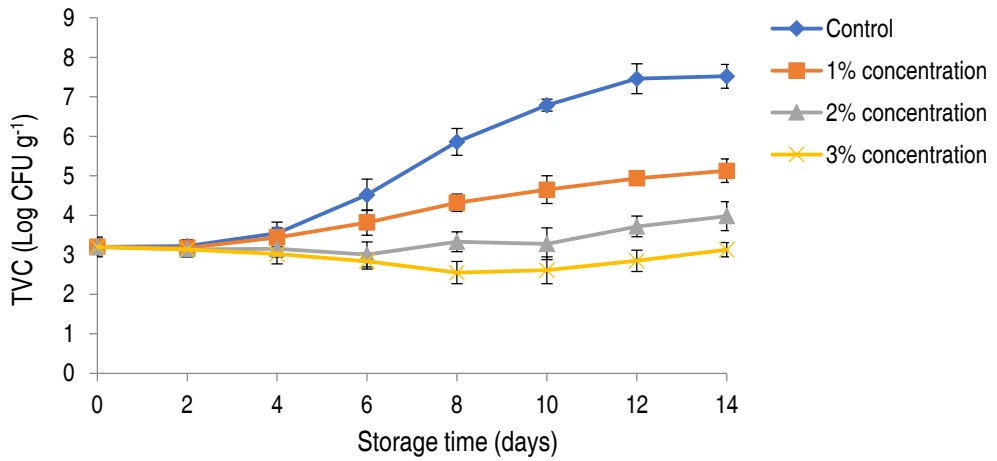

Figure 4. The total viable count of poultry meat treated with different concentrations of citric acid and stored for 14 days. Significance (P<0.05).

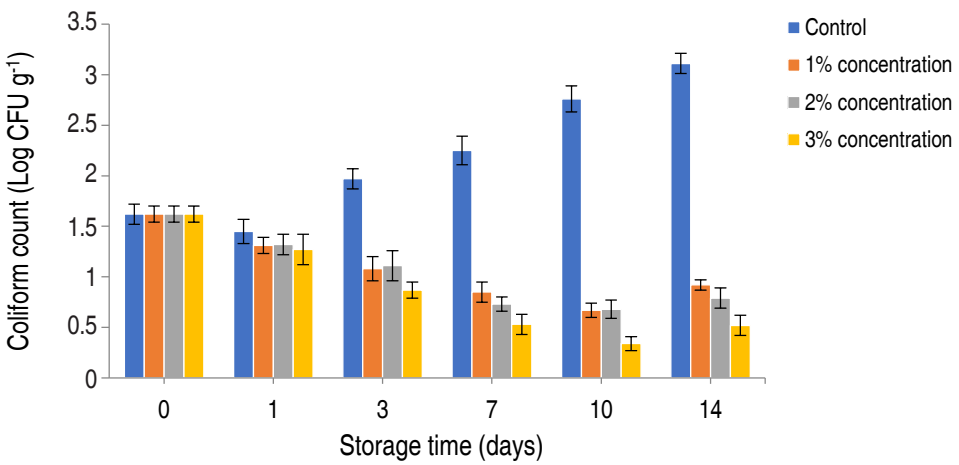

Figure 5. Total coliform count of poultry meat treated with different concentrations of citric acid and stored for 14 days. Significance (P<0.05).

Changes in pH and acidity activity of samples during storage

Figure 6 shows the changes in pH of poultry meat stored for 14 days using different concentrations of citric acid at 25 min soaking time and 30 °C soaking temperature. The enduring effect of the acid treatment process on the meat samples during storage was further confirmed by the observed changes in pH in both the treated and untreated meat samples. A general decrease in the pH of treated meat samples was observed up until day 8, this was contrary to the findings of Han et al. (2020) who recorded fluctuations in the pH of broiler meat spread with lactic acid. The lowest pH of 5.0±0.10 was observed using a 3% citric acid concentration on day 12. The pH of the control sample on the other hand was observed to increase as storage progressed beyond day 12 and this

can be attributed to the observed increase in microbial growth at this stage of storage (Gao et al. 2013). There were significant differences in the pH of the meat samples treated with varying concentrations of acid as storage progressed (P<0.05). Higher acid treatment concentrations led to lower meat pH during storage. The pH of meat plays a vital role in its quality and shelf-life during storage, and any deviations from the acceptable range can result in adverse effects on the color/appearance and water-holding capacity of fresh meat (Han et al. 2020). pH fluctuations of broiler meat during storage have been found to greatly affect the production of sulfur-containing and carbonyl volatiles. Extreme alkaline and acidic conditions in meat during storage greatly increase the production of these volatiles. At these extreme conditions, darkening tends to occur resulting in meat discoloration and loss of flavor (Haščík et al. 2013). The optimal pH for the preservation of poultry meat was between 4.5 and 5.5, it was however evident that meat treatment with 2 to 3% citric acid solution led to delayed glycolysis and effectively prevented rapid meat acidification at the early stages of storage; these treatments were also observed to efficiently prevent undesirable pH fluctuations in the treated meat samples at the mid and late stages of storage (Holman et al. 2016).

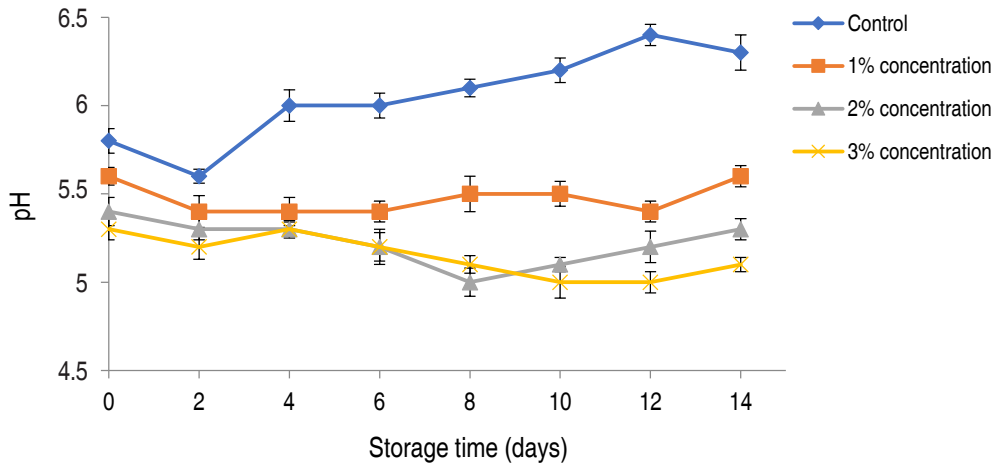

Figure 6 . pH changes of poultry meat preserved with different concentrations of citric acid for 14 days**.** Significance (P<0.02).

The correlation between the meat acid activity, TVC, and TCC is shown in Figure 7. The higher the meat acid activity, the lower the TVC and TCC, the lowest TVC of 1.95±0.08 Log_{10} CFU g^{-1} was observed at an acid activity of 0.4%, while the lowest TCC of 0.03±0.01 Log_{10} CFU g^{-1} was observed at same acid activity. Reduction in TVC and TCC during storage showed significant differences in comparison with the acid activity of the treated meat samples (P<0.05). Since the acid activity of the meat samples was a cumulative effect of all treatment parameters, it was conceivable that the observed increase in antimicrobial activity in the form of TVC and TCC reduction was a direct effect of the use of optimal treatment conditions (in relation to treatment time and temperature) (Zhang et al. 2021).

Anti-oxidative effect of citric acid treatment on poultry meat samples

Table 4 shows the effect of different concentrations of citric acid treatments on the formation of Total Volatile Basic Nitrogen (TVB-N) and Thiobarbituric Acid Reactive Substances (TBARS) in poultry meat samples during storage. TVB-N and TBARS content in chicken as an important reference index has been used to evaluate its freshness (Castro et al. 2006). TVB-N compounds in chicken contain ammonia, trimethylamine (TMA), and dimethhylamine (DMA), and the level of TVB-N compounds increases with spoilage by either bacteria or enzymatic degradation. TBARS is an index of lipid oxidation, measuring malondialdehyde (MDA) content, which is one of the degradation products of lipid hydroperoxides formed through the oxidation of unsaturated fatty acids (Gatellier et al. 2009).

It was clear that there were no significant differences in both TVB-N and TBARS between treatments (P<0.05) on day zero. A significant increase in storage time (P<0.05)

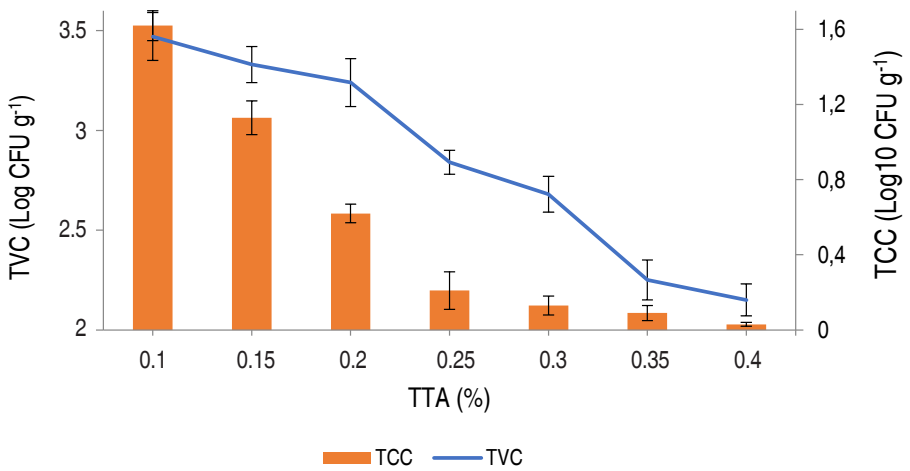

Figure 7. Relationship between acid activity, TVC, and TCC of poultry meat during preservation. Significance (P<0.05).

in both parameters was observed in all treatment groups. This agreed with Alasnier et al. (2000), Rukchon et al. (2014) and Rahman et al. (2012) who recorded similar differences as storage progressed. Significant differences (P<0.05) were again observed between the control and citric acid treatment groups. The control groups showed the overall highest values with a maximum of 59.37±2.92 mg 100 g^{-1} TVB-N and 2.01±0.02 mg kg^{-1} TBARS on day 14. An overall reduction in the formation of both TVB-N and TBARS was observed in the treated meat samples during storage as compared to the control (untreated) samples. 3% citric acid treatment had the highest effect in TVB-N and TBARS reduction with 41.84±0.10 mg 100 g^{-1} and 0.97±0.01 mg kg^{-1} of meat samples on day 14, respectively. A clear relationship was observed between the microbiological quality of the treated meat samples and the levels of TVB-N and TBARS formation, this was again in agreement with Smaoui et al. (2012) who noted that reductions in TVC resulted in similar reductions in the formation of TVB-N. Since TVB-N is a function of protein breakdown, the observed increase may be attributed to the formation of ammonia, which could be a result of residual microbial activity in the meat samples during storage (Khalafalla et al. 2016). TVB-N values of all treated groups were above the limit of 40 mg 100 g^{-1} recommended by FAO (1986) at day 14 of storage. Likewise, all treatment groups had TBARS values above the permissible limit of 0.9 mg MDA kg^{-1} recommended by the United States Department of Agriculture (FAO 1986) at day 14 of storage.

Table 4. Changes in TVB-N and TBARS content of poultry meat treated with different concentrations of citric acid and stored for 14 days.

Test	Treatment	Day 0	Day 3	Day 7	Day 10	Day 14
TVB-N (mg 100 g^{-1})	Control	9.73±1.41^{a}	19.21±1.82^{b}	31.82±1.02^{d}	45.59±1.61^{f}	59.37±2.92^{g}
	1%	9.73±0.41^{a}	15.88±0.11^{a}	29.74±0.96^{b}	38.23±1.34^{c}	47.90±1.22^{ab}
	2%	9.73±0.41^{a}	15.41±0.11^{a}	25.32±0.96^{b}	35.98±1.34^{c}	46.82±1.22^{ab}
	3%	9.73±0.41^{a}	14.26±0.52^{a}	22.37±0.61^{a}	31.22±1.00^{b}	41.84±0.10^{b}
TBARS (mg kg^{-1})	Control	0.18±0.03^{a}	0.49±0.02^{b}	0.92±0.09^{c}	1.53±0.07^{d}	2.01±0.02^{d}
	1%	0.18±0.03^{a}	0.49±0.07^{a}	0.99±0.08^{c}	1.27±0.06^{c}	1.55±0.05^{c}
	2%	0.18±0.03^{a}	0.45±0.04^{a}	0.94±0.02^{bc}	1.00±0.03^{c}	1.32±0.09^{c}
	3%	0.18±0.03^{a}	0.41±0.03^{a}	0.80±0.08^{b}	0.94±0.05^{b}	1.07±0.01^{b}

Data are represented as mean ± standard deviation, n=3 with the same superscript down the column are not significantly different (P=0.05).

Effect of acid treatment on sensory parameters

The sensory parameters of untreated and treated poultry meat with different concentrations of citric acid (1, 2, and 3%) at 25 min soaking time and 30 °C soaking temperature for 14 days are shown in Table 5. There were significant differences in sensory parameters of meat samples treated with varying acid concentrations ($P<0.05$). Citric acid treatment had positive effects on the sensory parameters of poultry meat when compared with the control (untreated) samples. There were no significant differences between the treatments and control samples at day 3 ($P<0.05$); all samples-maintained freshness, and this was an indication of the ability of citric acid treatments to extend poultry meat shelf-life, without adversely affecting its sensory quality (Maaya and Al-Abdullah 2016). On the other hand, significant differences ($P \leq 0.01$) were observed in the sensory parameters of treated and untreated meat samples, as well as between different treatment concentrations from day 7. Only samples treated with 2% acid concentration and above were able to maintain freshness at day 7, (Chen et al. 2019). On day 14, all samples had sensory scores below the acceptable limit of 12, making them unacceptable. 2% citric acid-treated groups were observed to be the most effective in maintaining desirable sensory parameters (16.08 as of day 7). The maintenance of sensory parameters observed in the acid-treated groups were probable due to one or more carboxylic acid or acid phenolic groups present in the treatment acids such as amides, esters, and peptides (Carpes et al. 2009). These carboxylic groups play a functional role in lipids and protein metabolism and acid-base balance, thereby positively influencing the sensory parameters of poultry meat (Haščík et al. 2013). These findings were in accordance with Bobko et al. (2012), who found a significant positive influence of different plant supplements containing organic acids on the sensory quality of poultry meat.

Table 5. Sensory parameters of poultry meat preserved with different concentrations of citric acid for 14 days.

Treatments	Day	Appearance	Viscosity	Texture	Odour	Color	Total
Control	3	4.50±0.05^{a}	3.64±0.02^{b}	4.05±0.01^{ab}	4.12±0.03^{a}	4.00±0.00^{ab}	**20.31**
	7	2.36±0.03^{c}	2.05±0.01^{cd}	1.81±0.01^{d}	1.28±0.04^{d}	2.35±0.10^{c}	**9.85**
	10	1.93±0.01^{dc}	1.26±0.06^{d}	1.28±0.04^{d}	1.30±0.05^{d}	2.04±0.02^{cd}	**7.81**
	14	1.49±0.02^{d}	1.02±0.02^{d}	1.05±0.01^{d}	1.10±0.10^{d}	1.86±0.03^{d}	**6.52**
1% citric acid	3	4.47±0.13^{a}	3.65±0.13^{b}	4.01±0.11^{ab}	4.18±0.03^{a}	4.02±0.10^{ab}	**20.33**
	7	2.46±0.09^{c}	2.55±0.15^{cd}	2.17±0.09^{d}	1.79±0.13^{d}	2.80±0.25^{c}	**11.77**
	10	2.25±0.10^{dc}	1.60±0.10^{d}	1.65±0.07^{d}	1.58±0.14^{d}	2.34±0.30^{cd}	**9.42**
	14	1.79±0.07^{d}	1.17±0.12^{d}	1.10±0.10^{d}	1.20±0.10^{d}	1.97±0.15^{d}	**6.23**
2% citric acid	3	4.40±0.00^{a}	3.90±0.03^{b}	3.96±0.04^{ba}	4.27±0.10^{a}	4.21±0.02^{a}	**20.74**
	7	3.58±0.05^{b}	3.19±0.02^{b}	3.12±0.06^{b}	2.94±0.01^{c}	3.25±0.08^{b}	**16.08**
	10	3.21±0.10^{b}	2.20±0.10^{c}	1.90±0.04^{d}	2.09±0.03^{c}	2.51±0.07^{c}	**11.91**
	14	2.22±0.07^{c}	1.30±0.10^{d}	1.62±0.01^{d}	1.73±0.03^{d}	1.95±0.10^{d}	**8.82**
3% citric acid	3	3.90±0.10^{a}	4.02±0.08^{ab}	4.01±0.01^{ab}	4.19±0.10^{a}	3.62±0.14^{a}	**19.74**
	7	3.55±0.15^{a}	3.46±0.06^{b}	2.28±0.12^{b}	3.10±0.10^{b}	2.72±0.12^{b}	**15.11**
	10	2.68±0.09^{b}	2.32±0.09^{c}	2.12±0.02^{c}	2.10±0.14^{c}	1.76±0.14^{c}	**10.98**
	14	1.95±0.13^{c}	1.48±0.04^{d}	1.58±0.06^{d}	2.03±0.05^{cd}	1.10±0.20^{d}	**8.14**

Data are represented as ± standard deviation, data with the same superscript down the column are not significantly different ($P<0.01$).

CONCLUSION

Citric acid produced from the fermentation of wheat straw with *A. niger* significantly inhibited the proliferation of spoilage organisms as well as the rate of protein and lipid oxidation in treated meat samples. It was found that untreated poultry meat had a maximum shelf-life of 4 days

at 4 °C, while poultry meat treated with 2% citric acid and above was preserved for up to 10 days. The presence of one or more carboxylic acid or acid phenolic groups present in citric acid such as amides, esters, and peptides make it an efficient meat preservative agent. This study gives insight into the further industrial application of lignocellulosic biomass with an emphasis on food preservation. However, less expensive conversion and purification techniques should be explored to make the whole process more feasible.

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Chemical composition and biological evaluation of tea tree (*Melaleuca alternifolia* L.) leaves essential oils

Composición química y evaluación biológica de los aceites esenciales de las hojas del árbol del té (*Melaleuca alternifolia* L.)

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ABSTRACT

Melaleuca alternifolia L. essential oils (MaEO), extracted through steam, distillation of its leaves, offer a multitude of benefits. The aim of the study was to determine the physicochemical and biological properties, such as relative density, absolute density, acid value, saponification value, ester value, freezing point, fragrance retention, antioxidant and antibacterial activity of MaEO. Gas chromatography-mass spectrometry (GC-MS) analysis was used to analyze the chemical composition of essential oils (EO), and the obtained results displayed those 45 compounds were identified and quantified; among them, the main component was Terpinen-4-ol (44.55%). In addition, the antioxidant capacity (AC) was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay with the half maximal inhibitory concentration (IC_{50}) of 360 mg mL^{-1}. Moreover, MaEO also had excellent antibacterial activity (AA) against certain types of bacteria, using the paper disc diffusion method for antibiotic susceptibility testing. The diameter of the inhibitory zones were 12.33, 14, 15.67, and 24.33 mm for *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 25923), *Salmonella enterica* (ATCC 13076), and *Escherichia coli* (ATCC 25922), respectively.

Keywords:
Antioxidant capacity
Antibacterial activity
Essential oils
Herbs
GC-MS

RESUMEN

Los aceites esenciales de *Melaleuca alternifolia* L. (MaEO), extraídos mediante destilación al vapor de sus hojas, ofrecen múltiples beneficios. El objetivo del estudio fue determinar las propiedades fisicoquímicas y biológicas, como la densidad relativa, la densidad absoluta, el índice de acidez, el índice de saponificación, el índice de éster, el punto de congelación, la retención de fragancia, la actividad antioxidante y antibacteriana del MaEO. Se utilizó análisis de cromatografía de gases-espectrometría de masas (GC-MS) para analizar la composición química de los aceites esenciales (AE), y los resultados obtenidos mostraron que se identificaron y cuantificaron 45 compuestos; entre ellos, el componente principal fue terpinen-4-ol (44,55%). Además, la capacidad antioxidante (CA) se evaluó mediante el ensayo de 2,2-difenil-1-picrilhidrazilo (DPPH) con la concentración inhibidora media máxima (IC_{50}) de 360 mg mL^{-1}. Asimismo, MaEO también tuvo una excelente actividad antibacteriana (AA) contra ciertos tipos de bacterias, utilizando el método de difusión en disco de papel para pruebas de susceptibilidad a los antibióticos. El diámetro de las zonas inhibidoras fueron 12,33, 14, 15,67 y 24,33 mm para *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 25923), *Salmonella enterica* (ATCC 13076) y *Escherichia coli* (ATCC 25922), respectivamente.

Palabras clave:
Capacidad antioxidante
Actividad antibacteriana
Aceites esenciales
Hierbas
GC-MS

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Melaleuca alternifolia L., commonly known as the tea tree, belongs to the family Myrtaceae, native to Australia (Rodney et al. 2015). Currently, it is widely distributed globally, mainly in Australia, tropical America, South Asia, and Indonesia (Yasin et al. 2021). Tea tree is a white-barked shrub up to 7 m tall, with narrow leaves scattered into cymbals, 10–35 mm long and 1 mm wide, and the leaves are especially rich in essential oils. Tea tree flowers are white, each flower solitary in a bract, with petals 2–3 mm long. The fruit is a woody, cup-shaped capsule 2–3 mm in diameter and has many seeds (Rodney et al. 2015).

The leaves of this plant can help fight wound infections and are used in traditional medicine (Rodney et al. 2015; Yasin et al. 2021). *Melaleuca alternifolia* leaves essential oil (MaEO) has many applications in various fields, such as agriculture, pharmaceuticals, cosmetics, veterinary products, and food. Currently, tea tree growth is quite popular in Vietnam. Essential oils (EO) comprise terpene hydrocarbons, mainly monoterpenes, sesquiterpenes, and their related alcohols (Carson et al. 2006). According to ISO 4730 (2017), Terpinen-4-ol is the main ingredient in tea tree oil, with concentrations ranging from 35 to 48%. The ingredients' content significantly differs depending on geographical location, extraction method, harvesting season, and storage conditions (Borotová et al. 2022). MaEO has high biological activity, including antibacterial, antifungal, and antiviral activities, which has been studied on many bacteria and fungi (Carson et al. 2006). The application of MaEO in food preservation has been studied quite a lot in recent years. Typically, chitosan films obtained in malic acid with MaEO have the highest antioxidant activity, color, and insignificant transparency change (Cázon et al. 2021). In addition, MaEO is used to inhibit molds isolated from meat products (Sevik et al. 2021).

It knows that the same plant material distributed in different regions will have different characteristics. Until now, many studies have been related to the tea tree and its essential oil. However, this is the first time *M. alternifolia* has been grown in Lam Dong province (Vietnam) and the MaEO may differ from that in other regions regarding volatile chemical composition and biological activities. Therefore, this study aimed to evaluate the physicochemical properties, chemical profile, and biological activities of MaEO. The results of this study could contribute significantly to understanding this essential oil in various regions. With its distinctive chemical composition and biological properties, this material holds potential applications in food technology, medicine, and cosmetics.

MATERIALS AND METHODS

Materials

The leaves of *M. alternifolia* were harvested after about 9 months of age in Lam Dong province, Vietnam (Coordinates: 11°40'1.20"N, 107°19'58.80"E). The plant specimen (coded MA151022VST) has been archived at the Plant Biotechnology Laboratory of the Institute of Biotechnology and Food Technology, Ho Chi Minh City University of Industry. On average, the per-batch yield was approximately 50 kg leaves/batch and MaEO was extracted using steam distillation for 3 h at 100 °C, with the yield obtained at about 1.5% (v/w), and the EO was stored in a dark sealed bottle at 4 °C before analysis.

Bacterial strains used in this study include *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 25922), and *Salmonella enterica* (ATCC 10376).

Determination of physicochemical properties of MaEO

The freezing point (FP), relative density (RD), absolute density (AD), acid value (AV), esters value (EV), and saponification value (SV) were evaluated according to International Organization for Standardization including ISO 1041 (1973), 279 (1998), 1242 (2023), and 7660 (1983), respectively.

Determination of fragrance retention (FR) of MaEO

According to the procedure described by Mahajan (2022), fragrance retention (FR) is determined by the concentration of the flavoring ingredient and its retention time, with some minor corrections. EO was mixed into concentrations (5, 10, 15, 20, and 25%, v/v) in 96% ethanol. Next, three drops of EO were placed on the odor test paper, and a few seconds were allotted for the EO to penetrate the paper. Finally, the time until the smell of EOs disappeared was calculated and the results recorded.

Gas chromatography-mass spectrometry (GC-MS)

The chemical volatile composition of EO was analyzed using the GC–MS method. 1 µL of EO was injected into a gas chromatograph (Shimadzu Nexis GC-2030, Japan)

with a versatile capillary column (Rtx-5sil-MS, 30 m×0.25 mm×0.25 µm, Restek Technologies, USA) equipped with a quadrupole mass analyzer (Shimadzu GC-MS-QP2020 NX, Japan). Helium was used as a carrier gas at a constant flow rate of 3 mL min^{-1}, and a split ratio of 10:1. The injection temperature was 250 °C and the temperature program was set as follows: initial temperature of 50 °C, held for 2 min, increased until 250 °C at a rate of 10 °C min^{-1}, and held for 5 min; finally, increased to 280 °C at a rate of 10 °C min^{-1}, and held for 3 min. Mass spectra were recorded at the ionization energy of 70 eV in EI mode (Hao and Quoc 2024).

Determination of antioxidant capacity (AC) of MaEO

The AC of MaEO was determined using DPPH assay according to the procedure described by Rahman et al. (2015) with some minor corrections. EOs were diluted into concentrations (100, 200, 300, 400, 500, and 600 mg mL^{-1}) in 96% ethanol. 0.3 mL of the obtained EO and 3.7 mL DPPH 0.1 M in 96% ethanol solution were mixed. The mixture was kept in dark conditions for 30 min at room temperature. DPPH radical scavenging capacity ($DPPH_{RSC}$) of MaEO was expressed by the degree of color reduction of the DPPH solution, as determined by measuring the absorbance at a wavelength of 517 nm, and antioxidant capacity was calculated following equation 1, while vitamin C was used as a control. Percent inhibition plotted against EO concentration to estimate concentration gives 50% inhibition (IC_{50}).

$$\%\ DPPH_{RSC} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \qquad (1)$$

Determination of the antibacterial activity (AA) of MaEO

The AA was determined by susceptibility testing by the agar plate diffusion method described by Quyen and Quoc (2023) with minor adjustments. First, a sterile spreader spread 100 µL of bacterial suspension (0.5 McFarland standard, approximately 1.5×10^8 CFU mL^{-1}) onto Muller-Hinton agar medium (MHA). Then, paper plates with a diameter of 6 mm were impregnated with essential oils (5 µL) put on MHA media, while ampicillin (10 µg $disc^{-1}$) and 5% dimethyl sulfoxide (DMSO) solution (5 µL $disc^{-1}$) were used as a positive and negative control. Petri dishes were incubated for 24 h at 37 °C, and the diameter of the inhibition zone was measured in mm.

Statistical data analysis

All the experimental results were analyzed using Statgraphics Centurion software (Version 15.1.02). Every assay was done in triplicates. Analysis of variance (ANOVA) with Fisher's least significant difference procedure was used to determine the significant differences (P<0.05) between means.

RESULTS AND DISCUSSION

Determination of physicochemical properties of *Melaleuca alternifolia* leaf essential oils

MaEO is a light-yellow liquid with a characteristic odor and a bitter taste. The physicochemical properties of EO are shown in Table 1. The pH value of MaEO is about 4.94 and this result is higher than that in some EOs from other materials, such as *Melaleuca cajuputi* leaves (pH=4.46) (Quoc 2021) and *Ceratonia siliqua* pulp (pH=4.3) (Ouis and Hariri 2018). The chemical component of EO affects pH strongly, leading to a significant difference in pH in materials.

The FP of EO remains undetermined at -40 °C; the oil still exists in liquid form, with no freezing phenomenon. The results show that the FP of MaEO must be below -40 °C; compared to other materials, FPs of the EO of *M. cajuputi* leaves and *Ocimum gratissimum* were -45 and -38 °C (Quoc 2021; Hao and Quoc 2024). This indicated that FP depends on the chemical composition of the EO significantly. In addition, the RD and AD of the oil were also analyzed; both values are lower than one, 0.9064 and 0.9048 g mL^{-1}, respectively. These findings agree with the ISO 4730 (2017), and they did not differ significantly from those of *M. cajuputi* (RD: 0.9102 and AD: 0.9086 g mL^{-1}) (Quoc 2021) or *M. arvensis* (RD: 0.8987 and AD: 0.8959 g mL^{-1}) leaves' EO (Quoc 2022). The obtained values also revealed that the MaEO belongs to the EO group lighter than water.

The AV, SV, and EV of MaEO were determined to be 2.10, 7.34, and 5.24 mg KOH g^{-1} EO, respectively. In general, the AV is agreement with that of previous studies for other materials, such as *C. siliqua* pulp and seeds (AV:

3.82 and 2.2 mg g^{-1} EO) (Ouis and Hariri 2018), basil EO (AV: 3.95 mg KOH g^{-1} EO), and lemongrass (AV: 4.09 mg KOH g^{-1} EO) (Mustapha 2018), while the obtained SV is significantly lower than that of *M. cajuputi* leaves EO (SV: 28.05 mg KOH g^{-1} EO) (Quoc 2021), basil EO (SV: 198 mg KOH g^{-1} EO), and lemongrass (SV: 143 mg KOH g^{-1} EO) (Mustapha 2018). The AV, SV, and EV are indicators used to assess the quality of the EO and they are influenced by distillation technique, cultivar, climatic conditions, harvest period, and chemical composition of the initial materials.

Table 1. Physicochemical properties of *Melaleuca alternifolia* leave essential oils.

No.	Physicochemical properties	Value
1	pH	4.94±0.09
2	Freezing point (FP, °C)	<-40
3	Relative density (RD)	0.9064±0.0015
4	Absolute density (AD, g mL^{-1})	0.9048±0.0015
5	Acid value (AV, mg KOH g^{-1} EO)	2.10±0.1354
6	Saponification value (SV, mg KOH g^{-1} EO)	7.34±0.8609
7	Ester value (EV, mg KOH g^{-1} EO)	5.24±0.8068
8	Fragrance retention (FR, h):	
	5% EO	1.25
	10% EO	2.17
	15% EO	5.42
	20% EO	6.17
	25% EO	10.42
	100% EO	25.25

The staying power is also the critical index to evaluate the quality of EO. In this study, FR of MaEO can reach a range of 6–10 h at 20–25%, and this shows that FR is quite long, similar to perfume at a fragrance concentration of 20–30%, and it can last for more than 6–8 h (Mahajan 2022), while pure MaEO can last more than 24 h. This finding proves that MaEO has potential applications in cosmetics and the food industry.

Chemical composition of *Melaleuca alternifolia* leaves essential oils

The volatile compounds of MaEO were analyzed by GC-MS. The results are displayed in Table 2. A total of 45 compounds were identified and quantified in the EO isolated from the *M. alternifolia* leaves; they account for 99.35% of the oil and are tested by retention times ranging from 7 to 22 min. Compounds comprising the highest content in MaEO, include Terpinen-4-ol (44.55%), γ-Terpinene (19.42%), p-Cymene (8.75%), α-Terpinene (6.73%), and Terpineol (3.33%). These components play an important role in the quality of MaEO and there is variation in the chemical composition of EO from tea tree leaves collected in different regions. In previous studies, authors also used the distillation method to extract EOs from the initial material. For example, MaEO isolated from Tien Giang province (Vietnam) possess 19 volatile components: Terpinen-4-ol (36%), γ-Terpinene (17.8%), 1,8-Cineole (10%), etc. (Hòa et al. 2016), while MaEO from Slovakia has 47 identified components, including Terpinen-4-ol (40.3%), γ-Terpinene (11.7%), 1,8-Cineole (7%), p-Cymene (6.2%), etc. (Borotová et al. 2022), and while MaEO collected in Thailand has 24 components, including Terpinen-4-ol (30.42–34.76%), γ-Terpinene (25.08–26.23%), α-Terpinene (12.31–12.43%) and 1,8-Cineole (5.99–9.08%), etc. (Sukatta et al. 2011). In this study, the main component of MaEO is Terpinen-4-ol and its content is higher than that in MaEO from other places. The MaEO from the Lam Dong province complied with the ISO 4730 (2017) standard analysis with few variations in the contents of major constituents. This difference in the chemical composition of MaEO can be explained due to different extraction methods, climatic conditions, harvesting periods, genes, etc.

Table 2. Chemical composition of *M. alternifolia* leaves essential oils.

No.	Compounds	Molecular formula	RT. (min)	Content (%)
1	Bicyclo[3.1.0]hex-2-ene,2-methyl-5-(1-methylethyl)-	$C_{10}H_{16}$	6.998	0.65
2	α-Pinene	$C_{10}H_{16}$	7.146	2.35
3	Camphene	$C_{10}H_{16}$	7.484	0.02
4	Ethanone	$C_8H_{14}O$	7.828	0.01
5	Bicyclo[3.1.0]hexane,4-methylene-1-(1-methylethyl)-	$C_{10}H_{16}$	7.956	0.14
6	Bicyclo[3.1.1]heptane,6,6-dimethyl-2-methylene-, (1S)-	$C_{10}H_{16}$	8.051	0.51
7	3-Methyl-3-cyclohexen-1-one	$C_7H_{10}O$	8.196	0.05
8	β-Myrcene	$C_{10}H_{16}$	8.283	0.52
9	Dodecane	$C_{12}H_{26}$	8.502	0.05
10	α-Phellandrene	$C_{10}H_{16}$	8.612	0.29
11	**α-Terpinene**	**$C_{10}H_{16}$**	**8.821**	**6.73**
12	**p-Cymene**	**$C_{10}H_{14}$**	**8.960**	**8.75**
13	Bornylene	$C_{10}H_{16}$	9.059	1.02
14	β-Phellandrene	$C_{10}H_{16}$	9.085	0.41
15	1,8-Cineole	$C_{10}H_{18}O$	9.115	0.91
16	**γ-Terpinene**	**$C_{10}H_{16}$**	**9.599**	**19.42**
17	Cyclohexene	$C_{10}H_{16}$	10.101	3.2
18	Benzene, (2-methyl-1-propenyl)-	$C_{10}H_{12}$	10.169	0.15
19	Linalool	$C_{10}H_{18}O$	10.320	0.05
20	p-Mentha-1,5,8-triene	$C_{10}H_{14}$	10.577	0.04
21	2-Cyclohexen-1-ol,1-methyl-4-(1-methylethyl)-, cis-	$C_{10}H_{18}O$	10.780	0.18
22	β-Ocimene	$C_{10}H_{16}$	10.971	0.13
23	2-Cyclohexen-1-ol,1-methyl-4-(1-methylethyl)-, trans-	$C_{10}H_{18}O$	11.094	0.14
24	Benzene, (methyl(1-methylethyl)-	$C_{10}H_{16}O$	11.623	0.07
25	**Terpinen-4-ol**	**$C_{10}H_{18}O$**	**11.745**	**44.55**
26	p-Cymen-8-ol	$C_{10}H_{14}O$	11.815	0.04
27	**Terpineol**	**$C_{10}H_{18}O$**	**11.968**	**3.33**
28	p-Menth-2-en-1,4-diol	$C_{10}H_{18}O_2$	13.224	0.3
29	cis,cis-Photocitral A	$C_{10}H_{16}O$	14.038	0.35
30	2-Cyclohexyl-hex-5-en-2-ol	$C_{12}H_{22}O$	14.845	0.03
31	α-Copaene	$C_{15}H_{24}$	15.254	0.15
32	α-Gurjunene	$C_{15}H_{24}$	16.037	0.31
33	Caryophyllene	$C_{15}H_{24}$	16.363	0.25
34	Aromandendrene	$C_{15}H_{24}$	16.886	0.97
35	Alloaromadendrene	$C_{15}H_{24}$	17.529	0.31
36	δ-Cadinene	$C_{15}H_{24}$	17.869	1.33
37	β-Selinene	$C_{15}H_{24}$	18.435	0.1
38	1H-Xycloprop[e]azulen, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,4,7-tetrametyl-	$C_{15}H_{24}$	18.503	0.89
39	Naphtalen, 1,2,3,4-tetrahydro-1,6-dimetyl-4-(1-metyletyl)-, (1S-cis)-	$C_{15}H_{22}$	19.305	0.21
40	Isoledene	$C_{15}H_{24}$	19.365	0.23
41	Naphtalen,1,2,3,4,4a,7-hexahydro-1,6-dimetyl-4-(1-metyletyl)-	$C_{15}H_{24}$	19.607	0.18
42	(-)-Globulol	$C_{15}H_{26}O$	20.874	0.21
43	Guaiol	$C_{15}H_{26}O$	21.103	0.23
44	Di-epi-1,10-cubenol	$C_{15}H_{26}O$	21.725	0.18
45	Epicubenol	$C_{15}H_{26}O$	22.018	0.06

Antioxidant capacity (AC) of *Melaleuca alternifolia* leaves essential oils

Figures 1 and 2 show the efficiency of the antioxidant process of EO and control (vitamin C). An increase in MaEO concentration leads to an increase in AC. Based on the obtained curve, the IC_{50} of MaEO was determined at 360 mg mL^{-1} (Figure 1), while the IC_{50} of vitamin C was only about 26 µg mL^{-1} (Figure 2). The results show that the IC_{50} of MaEO is much higher than that of vitamin C, showing that the AC of EO is very weak. These results are consistent with those of MaEO from Thailand (IC_{50}: 29.34–38.68 mg mL^{-1}) (Sukatta et al. 2011) (Their AC is extremely low compared to the control). Besides, compared to some EOs from other materials, such as *C. siliqua* pulp and seeds (IC50: 7.8 and 31.25 µg mL^{-1}) (Ouis and Hariri 2018), *M. arvensis* (IC_{50}: 330 mg mL^{-1}) (Quoc 2022), the AC of MaEO is also weaker. This is a disadvantage when applying this EO in the cosmetic and food industry. Differences in AC of various EOs may be due to differences in their chemical composition.

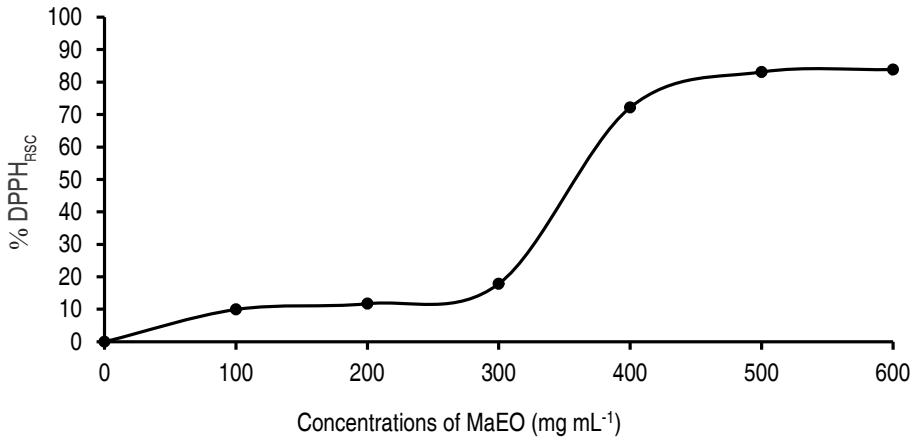

Figure 1. Antioxidant capacity of the *Melaleuca alternifornia* leaves EO.

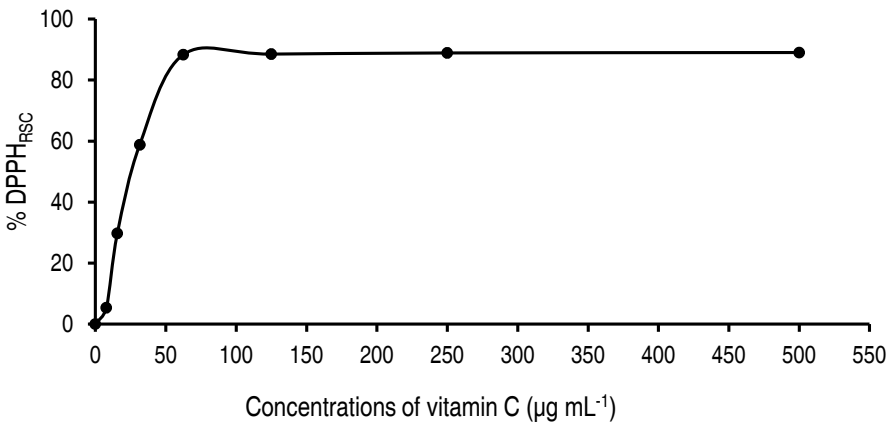

Figure 2. Antioxidant capacity of vitamin C.

Antibacterial activity (AA) of *Melaleuca alternifornia* leaves essential oils

Table 3 shows that MaEO exhibited to AA against four bacterial strains, and ampicillin was used as an inhibitory control against the bacteria used in the study. AA of ampicillin are arranged in order of susceptibility: *E. coli*> *S. enterica*>*S.aureus*/*B. cereus*. Similarly, AA of MaEO is arranged in order of susceptibility: *E. coli*>*S. enterica*/*S. aureus*/*B. cereus*. The AA of MaEO was 1.5 times stronger than that of ampicillin for *S. aureus* and *B. cereus*, while

the AA of MaEO was much lower than that of ampicillin for *S. enterica* and *E. coli*. These obtained results are also different from those of MaEO collected from Thailand (Sukatta et al. 2011) and other materials (*C. siliqua* pulp and seeds, *M. cajuputi* leaves, and *O. gratissimum* EO) (Ouis and Hariri 2018; Quoc 2021; Hao and Quoc 2024). The AA of MaEO was observed in the presence of a high content of Terpinen-4-ol; according to Halcón and Milkus (2004), Terpinen-4-ol strongly influences the bacterial cell wall and compromises the cytoplasmic membrane of bacteria (*S. aureus*), giving it a bacteriostatic and bactericidal effect. In addition, combining other bioactive ingredients, such as γ-Terpinene, 1,8-Cineole, α-Terpineol, etc., can also increase the ability to inhibit bacteria.

Table 3. Antibacterial zones of *M. alternifornia* leaves essential oils.

No.	Microorganisms	Diameter of the inhibitory zones of ampicillin (mm)	Diameter of the inhibitory zones of EO (mm)
1	*S. aureus*	9.33^{Aa}±1.53	14^{Ba}±1
2	*B. cereus*	8.33^{Aa}±1.53	12.33^{Ba}±2.31
3	*S. enterica*	22.67^{Bb}±1.16	15.67^{Aa}±1.16
4	*E. coli*	29.33^{Bc}±4.04	24.33^{Ab}±3.51

Within a row (A–B) or a column (a–c), different letters denote significant differences (P<0.05) between samples or microorganisms, respectively.

CONCLUSION

The essential oil distilled from *M. alternifornia* leaves in Lam Dong province, Vietnam, exhibits unique physicochemical properties, notably exceptional fragrance retention lasting approximately 25 h for pure EO. It has weak AC compared to the control. However, this material also possesses high AA and strongly inhibits four pathogenic bacterial strains in food, including *E. coli*, *S. enterica*, *S. aureus*, and *B. cereus*. By the GC-MS method, 45 major volatile components were determined in tea tree leaf EO. This is a natural, precious, and rich source of phytochemicals, especially Terpinen-4-ol (44.55%), γ-Terpinene (19.42%), etc. Therefore, the oil from *M. alternifornia* leaves is ideal for use in the pharmaceutical, food, and cosmetic industries.

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Effect of drying parameters on the physicochemical, microbiological, and sensory properties of lemon balm (*Melissa officinalis* L.)

Efecto de los parámetros de secado sobre las propiedades fisicoquímicas, microbiológicas y sensoriales del toronjil (*Melissa officinalis* L.)

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Keywords:
Drying kinetics
Forced air drying
Melissa
Overall quality
Sensory analysis

ABSTRACT

Lemon balm (*Melissa officinalis* L.) has been recognized for multiple health benefits due to bioactive compounds. Dehydration is usually the most widely used method to preserve and concentrate these elements. However, it can also affect and totally or partially degrade the quality of the product under incorrect processing conditions. This research aimed to evaluate the effect of drying parameters on *Melissa's* physicochemical, microbiological, and sensory properties. In total, four treatments were analyzed according to the experimental design (T1: 25 °C and 1.5 m s^{-1}, T2: 25 °C and 3.0 m s^{-1}, T3: 45 °C and 1.5 m s^{-1}, and T4: 45 °C and 3.0 m s^{-1}). Drying kinetics were determined using a vertical airflow dryer and a continuous weighing system. The results were compared with fresh leaves. The findings obtained show that increasing temperature and varying drying speed reduces moisture content and a_w but increases enzymatic activity and essential oil content. In the drying process, temperature has a greater effect in the initial stages of the process, while drying speed on the internal structure of the raw material. By optimizing the drying conditions, it is possible to reduce the drying time by 44%. Page's model showed excellent ability to predict drying kinetics under various drying conditions (RMSE <0.04 and R^2 >0.98). In terms of color, lightness decreased because of temperature, while a* and b* values were affected by non-enzymatic browning. Treatment T4 was the product with the highest acceptability. The findings obtained provide a theoretical basis to optimize the lemon balm drying process. Drying *Melissa* at 45 °C and 3.0 m s^{-1} can improve the quality and composition of the final product.

Palabras clave:
Cinética de secado
Secado por aire forzado
Toronjil
Calidad
Análisis sensorial

RESUMEN

El toronjil (*Melissa officinalis* L.) ha sido reconocido por múltiples beneficios para la salud, en gran parte gracias a su composición nutricional y compuestos bioactivos. La deshidratación suele ser el método más utilizado para conservar y concentrar estos elementos. Sin embargo, también puede afectar y degradar total o parcialmente la calidad del producto en condiciones de procesamiento incorrectas. El objetivo de este estudio fue evaluar el efecto que tienen los parámetros de secado en las propiedades fisicoquímicas, microbiológicas y sensoriales del toronjil. En total, se analizaron cuatro tratamientos según el diseño experimental (T1: 25 °C y 1,5 m s^{-1}; T2: 25 °C y 3,0 m s^{-1}; T3: 45 °C y 1,5 m s^{-1}; y T4: 45 °C y 3,0 m s^{-1}). La cinética de secado se determinó utilizando un secador de flujo de aire vertical y un sistema de pesaje continuo. Los resultados fueron comparados con las hojas frescas. Los hallazgos obtenidos muestran que aumentar la temperatura y variar la velocidad de secado reduce el contenido de humedad y a_w, pero aumenta la actividad enzimática y el contenido de aceite esencial. En el proceso de secado, se observó que la temperatura tiene un mayor efecto en etapas iniciales del proceso, mientras que la velocidad de secado en la estructura interna de la materia prima. Optimizar las condiciones de secado puede disminuir en un 44% el tiempo de secado. El modelo de Page demostró una excelente capacidad para predecir la cinética de secado en diversas condiciones de secado (RMSE <0,04 y R^2 >0,98). En términos de color, la luminosidad disminuyó por efecto de la temperatura, mientras que a* y b* se vieron afectados por el pardeamiento no enzimático. El tratamiento T4 fue el producto con mayor aceptabilidad. Los hallazgos obtenidos proporcionan una base teórica para optimizar el proceso de secado del toronjil. Secar la *Melissa* a 45 °C y 3,0 m s^{-1} puede mejorar la calidad y composición del producto final.

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Lemon balm (*Melissa officinalis* L.) is an essential and valued medicinal plant in national and international trade for its significant applications in the pharmaceutical and food industries. The genus *Melissa* is native to Europe and Asia, although it has also been found in the Mediterranean region (Abdellatif et al. 2021). It is a perennial plant belonging to the Lamiaceae family, measuring between 30 to 80 cm and presenting simple, quadrangular stems. Petiolate leaves characterize it, and when it reaches maturity, it exhibits a bloom with a color that can vary between white, pink, or blue (Waheed et al. 2019). This herb does not require much care and can proliferate, even in the wild, which allows it to spread worldwide (Kanatas et al. 2020). In Ecuador, as in Latin America, the practice of this crop occurs under altitudinal conditions from 1,800 to 3,356 meters above sea level (masl), tolerating an extensive range of conditions (low and high temperatures, low irrigation conditions, pests, among others), and adapting to humid temperate zones, warm and even in cold climates (Waheed et al. 2019).

On the other hand, *Melissa* is characterized by several therapeutic properties, which depend on the content and physicochemical compounds found in the final product. The therapeutic characteristics of this plant are in its leaves and are related to antioxidant, antimicrobial, anxiolytic, antidepressant, and anti-inflammatory properties (Sarkar et al. 2016). Fresh lemon balm herbs have been reported to contain phenolic compounds, l-ascorbic acid, carotenoids, flavonoids (rutin, luteolin, quercetin, cymaroside, and others), and terpenoids (Shamizi et al. 2022), which are responsible for such mechanisms. However, these depend on the post-harvest treatment given to the raw material.

With the above, lemon balm can be harvested anytime during its growth cycle. However, it is recommended to do so just before flowering for higher yield and operational quality (Avci and Giachino 2016). After harvesting, *Melissa* must be appropriately processed to ensure its quality and durability. Drying is one of the most common techniques for preserving aromatic and medicinal plants. The drying aim is to reduce the water content, decrease water activity, and prevent the growth of microorganisms and the oxidation of volatile components (Bonazzi and Dumoulin 2011; Shamizi et al. 2022). Conventional warm air drying typically involves low temperatures between 30 and 50 °C to protect active heat-sensitive ingredients (Argyropoulos and Müllera 2011). However, incorrect application of the method affects the quality of the dried product. For example, high drying temperature or prolonged drying time (DT) can lead to color deterioration and significant losses of essential oil (bioactive compounds) or other compounds (Arabhosseini et al. 2011). For this reason, it is common for producers to dry these plants in the shade to preserve the plant's chemical composition. However, at the industrial level, the drying process is affected by many factors, such as the size and composition of the feed, thermodynamic properties of the air, temperature, moisture, and dry air velocity (DAV) (Oyinloye and Yoon 2020). In the latter, temperature and DAV are the main parameters of this process, as they influence the drying kinetics and the dried product's physicochemical, microbiological, and sensory properties (Levate et al. 2020).

In the scientific literature, the application of drying in different medicinal plants has recently been studied (Ahmed and Langthasa 2022; Torki-Harchegani et al. 2016), but of these, a few studies deal with *Melissa* with applications on antioxidant activity, physicochemical, and microstructural properties (Argyropoulos and Müllera 2011; Shamekhi-Amiri et al. 2018; Shamizi et al. 2022). Therefore, the influence of tray drying on lemon balm and the quality properties of the product obtained is not well known, especially in developing countries. As a result, this has led to non-compliance with market requirements and, consequently, a lack of development in the tea industry. For this reason, this study aimed to evaluate the effect of tray drying parameters on the physicochemical, microbiological, and sensory properties of lemon balm to determine the best drying condition for bulk tea production. In the latter, different gradients of time, temperature, and drying air were tested through forced convection conditions. It's expected that the results obtained will allow a better understanding of the lemon balm drying process, which will be especially useful for the dehydrated industry.

MATERIALS AND METHODS

Raw Materials

Lemon balm plants were collected before flowering at a farm in Otavalo, Ecuador. The leaves were separated from the stems, and visual impurities were removed. The plant material was stored at 2 °C in a refrigerator for subsequent drying trials. Before conducting the experiments, the lemon balm plants underwent a selection, classification,

and disinfection process (sanitization with distilled water and white acetic acid). Finally, the lemon balm leaves went through a standardization process according to the method proposed by Buitrago-Zapata et al. (2018) to subsequently proceed to perform the drying process.

Drying method and construction of the drying curves

Lemon balm leaves were dehydrated in an electric convective tray dryer. The dryer has a design with a fan and four electric heaters and is equipped with temperature and air velocity controls. The dehydration process was carried out under the criteria of Tinebra et al. (2022), under the forced air-drying method. Initially, fresh lemon balm leaves had a moisture content of about 80%, but after the drying process, they reached a final moisture content of around 12%, which is the value recommended by NTE INEN 2392:2017 for storing dried *Melissa*. To elaborate on the drying kinetics, the Page model was used (equation 1), which is the most widely used statistical model to describe the drying process in plants.

$$MR = \exp^{(-\kappa.t^{n})} \quad (1)$$

Where κ is the drying constant (min^{-1}), η the function coefficient and t is the drying time in minutes. In the model, MR represents the relative water content as a function of time and is calculated from equation 2.

$$MR = \frac{M_t - M_e}{M_o - M_e} \quad (2)$$

In the equation 2, M_t represents the moisture content at time t, Mo is the initial moisture content, and M_e is the equilibrium moisture (kg water. kg dry $matter^{-1}$). However, since the values of M_e are relatively low and do not affect the calculation, the equation can be simplified as equation 3:

$$MR = \frac{M_t}{M_o} \quad (3)$$

Drying data were recorded at 0.5 h intervals until equilibrium moisture content was achieved for each drying condition, measured by constant product mass. This process was conducted to describe the thermodynamic effect of the drying process on the moisture content, which is responsible for the loss of volatile compounds and essential oils (loss of quality) of the raw material.

Experimental Desing

The operating parameters were selected according to the guidelines of Fiestas et al. (2014) as well as NTE INEN 2392:2017. The experimental phase was developed based on the research by Tinebra et al. (2022) through a randomized factorial design. The design was delimited and constructed, referencing the guidelines of Casler (2015) and Trujillo-Echeverria et al. (2020). The study variables were selected based on the scientific studies by Badmus et al. (2019), which report that the drying method and parameters significantly influence the quality of the raw material (as the final product). In particular, evidence suggests that *i*) the same drying technique can produce different color changes according to the time-temperature-moisture gradient used for drying (Petikirige et al. 2022), *ii*) quality parameters (e.g., physicochemical, sensory, nutritional, microbiological, functional, and other characteristics) may vary according to the characteristics of each product, and *iii*) microstructural profiles may be modified as a result of enzymatic reactions, structural alterations and other reactions inherent to the operation (Bonazzi and Dumoulin 2011; Oyinloye and Yoon 2020). For this reason, physicochemical (including lead, cadmium, moisture, ash insoluble, and essential oil [EO] yield), microbiological (*Escherichia Coli*, *Salmonella*, *Bacillus cereus*, and *Clostridium Perfringens*), and sensory characteristics of lemon balm were taken as response parameters. The same treatments were used to construct the drying curves (Table 1). For the sensory analysis, the design was developed under nonparametric statistics given, the measurement characteristics, and assumptions of the model (normality and homoscedasticity). The experimental model and levels of the independent factors are given in Table 1.

The experimental design suggested 12 experimental units for two factors at four levels in triplicate. All the response data from the proposed experiments were expressed in a generalized polynomial equation, as shown in equation 4:

$$\gamma_{ijk} = \mu + \alpha_i + \beta_j + (\alpha_i\beta_j) + \varepsilon_{ijk} \quad (4)$$

Where γ_{IJK} represents the response variable obtained from the k-th (k=1,..., n) replicate receiving the i-th level of A and the j-th level of B; n is the number of replicates for each of the AxB treatment groups; μ is the overall grand mean of γ; α_i is the effect of the i-th level of factor A($\sum_i$ i =1, ..., a); β_j is the effect of the j-th level of factor B($\sum_j$ j=1, ..., b); $\alpha_i\beta_j$ is the effect of the interaction between factors A and B; ε_{ijk} is the random effect error attributable to the ijk-th individual observation.

Table 1. Independent factors and levels used in tray drying of Lemon balm.

Treatments	Factor levels	
	A: Drying temperature (°C)	B: Dry air velocity (m s^{-1})
T1	a1:35	b1:1.5
T2	a1:35	b0:3.0
T3	a0:45	b1:1.5
T4	a0:45	b0:3.0

Determination of response variables

Determination of physicochemical and microbiological composition

The physicochemical and microbiological properties of lemon balm were evaluated in the raw material and the finished product. After drying, the samples were ground for further analysis. For the physicochemical analyses, samples of 0.5–5 g were weighed for each test. For microbiological analysis, the guidelines by Passafiume et al. (2021) were followed, preparing solutions 1:10. The solutions had 10 g of Lemon balm and 90 mL of sodium chloride solution. The samples were homogenized to identify the main microbial groups belonging to decomposition and pathogenic populations. Quantification was performed by plate count following ISO standards for each microorganism. The technical standard used for this process was NTE INEN 2392:2017. The methods used to quantify these components are detailed in Table 2. All the results of the physicochemical analyses were expressed on a dry weight basis (db), unlike moisture, which was expressed on a wet basis (wb).

Table 2. Physicochemical and microbiological variables.

Analysis	Requirements	Testing methods
Physicochemical	Moisture	AOAC, 930.15
	Ash Insoluble	ISO 1577
Microbiological	*Escherichia Coli*	NTE INEN-ISO 16649-1, 2, and 3
	Salmonella spp.	NTE INEN-ISO 6579 and ISO 16140
	Bacillus cereus	NTE INEN-ISO 7932
	Clostridium Perfringens	NTE INEN-ISO 7937

On the other hand, lemon balm EO was obtained by hydrodistillation following the approach proposed by Trujillo-Echeverria et al. (2020). The particle size for the extraction process was 0.3 mm, and the solute-solvent ratio was 1:15 (w v^{-1}). The EO yield was calculated from equation 5:

$$\text{EO yield (\%)} = \frac{\text{mL EO obtained}}{\text{g plant material}} \quad (5)$$

Colorimetric analysis was performed to determine the color values of dried lemon balm. A Minolta model Chroma-400 digital colorimeter was used. Calibration of the colorimeter was performed with black and white mosaics before each measurement. The color parameters L, a*, and b* (brightness: L*, red/greenish: a*, yellowish/bluish: b*) were evaluated under the CIELAB system.

Sensorial evaluation

This research was evaluated under a sample of 102 consumers for each test. The number of panelists was selected according to the guidelines by Singh-Ackbarali and Maharaj (2014). The sampling used to select the consumer panel was stratified sampling (Kemp et al. 2011). To assess acceptability, the consumer had to at least: a) be of legal age, b) have no medical impairment, c) have consumed hot beverages at least once in their life, and d) their herbal tea consumption preference was hot tea without sugar. The methodology was employed from

the hedonic rating scale (5 points) under the subjective parameters using the acceptability test type (Kemp et al. 2011; Saint-Denis 2018). The aspects being evaluated were flavor and aroma under the taster's appreciation (1: Dislike a lot, 2: I don't like, 3: Neither like nor dislike, 4: I like, and 5: I like a lot). These sensory attributes were chosen because they are decisive factors for buyers when choosing a product, first because of the positive impression (appearance, branding/packaging, color, flavor, texture, others) required to generate the product towards consumers and, second, for the mechanisms (psychological and physiological) it needs to influence the purchase decision (Chen and Lin 2018). This test aimed to improve or optimize the tea as a commercial product, based on knowing and perfecting the desirable aspects (liking scores) for the consumer.

Statistical analysis

All the results measured in the study were taken in triplicate and shown as mean ± standard deviation (SD) for quantitative data and as counts and percentages for qualitative data. The experimental results were subjected to Shapiro-Wilk and Levene tests to verify the normality and homoscedasticity of the data (population distribution). In this way, an analysis of variance (ADEVA) was carried out at 95% reliability, using a design with factorial interactions AxB. Only for sensory analysis, the Friedman test was used with a significance level of 5%. Differences of means were told significantly when $P<0.05$ for the Tukey test. The degree of fit for the model was determined using the coefficient of determination (R^2) and the root mean square error (RMSE). The statistical software R v.4.3.0 was used for the analysis.

RESULTS AND DISCUSSION

Drying kinetics

Figure 1 shows the variation of moisture content of lemon balm leaves during the drying process at different temperatures and DAV. Initially, the process started with a moisture content of 80.25%, but during the first hour, the free water was rapidly evaporated by the action of heat (Figure 1). Approximately 2.5 h were required to reach equilibrium moisture content. In addition, the DT decreased when the air velocity increased from 1.5 to 3 m s^{-1}, which means that at higher air velocities, the water evaporation rate from the food increased, reducing DT. Some studies

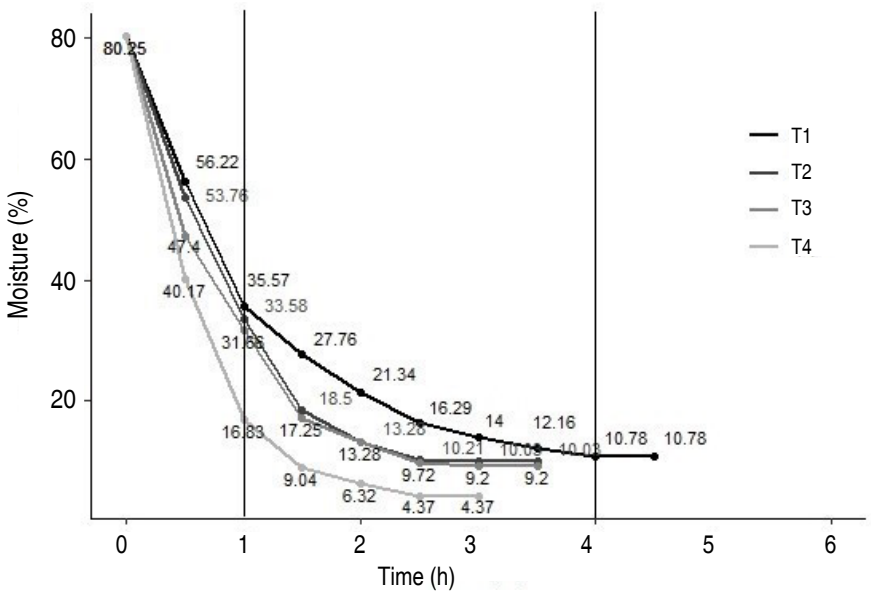

Figure 1. Effect of different temperatures and DAV on lemon balm drying.

have described that increasing temperature can decrease DT (Pasławska et al. 2020; Rudy et al. 2020); however, this study shows that the effectiveness of DAV in reducing DT has a significantly greater impact than increasing temperature alone. This is probably because, during the drying process, the surface water evaporates first since it is more affected by the DAV (Silverira et al. 2019). In such a way, with the elimination of surface water, heat

stimulates the structure of the raw material by convection until it reaches the deepest parts of it (capillarity), which causes the drying rate to increase due to the diffusion of the liquid (which is more influenced by the temperature of the DAV), thus inducing the accelerated release of bound water in the product matrix (Chong et al. 2021). However, this reinforces the existence of a proportional relationship between temperature and DAV, although this behavior may also depend on other factors (dryer type, tray material, perforation diameter, raw material-tray interfacial contact, others) related to the drying process (Argyropoulos and Müllera 2011; Shamekhi-Amiri et al. 2018).

At this point, it is also worth mentioning that the increase in temperature and DAV shortened the convection drying time by approximately 44.44%. This result was comparable to those obtained by Łyczko et al. (2019) in Lavender leaves and Rudy et al. (2020) in *Dracocephalum moldavica* leaves, with a DT reduction of 43.75 and 45%, respectively, but higher than those reported for the lemon thyme (Pasławska et al. 2020). Nevertheless, it was observed that as the drying process progressed, the DAV decreased about the decrease in humidity but was compensated by the temperature gradient. This phenomenon could be explained by the Darcy-type pressure diffusion mechanism according to Fick's law (Łyczko et al. 2019).

On the other hand, the results obtained from modeling using the Page model for the different drying conditions are described in Figure 2 and Table 3. In general, for all drying conditions, the RMSE values were relatively low (<0.04), while for R^2 the values were high (>0.98). Treatment four showed the best results for the statistical model. The high values of the coefficients (R^2 and RMSE) imply good predictive variability between the experimental data and the model. Therefore, the Page model has a good adaptation to predict the drying kinetics of lemon balm leaves. Several studies that address the modeling of the drying process of lemon balm leaves and other varieties, using various techniques, have reported similar results (Chua et al. 2019; Levate et al. 2020), demonstrating the reliability of the model.

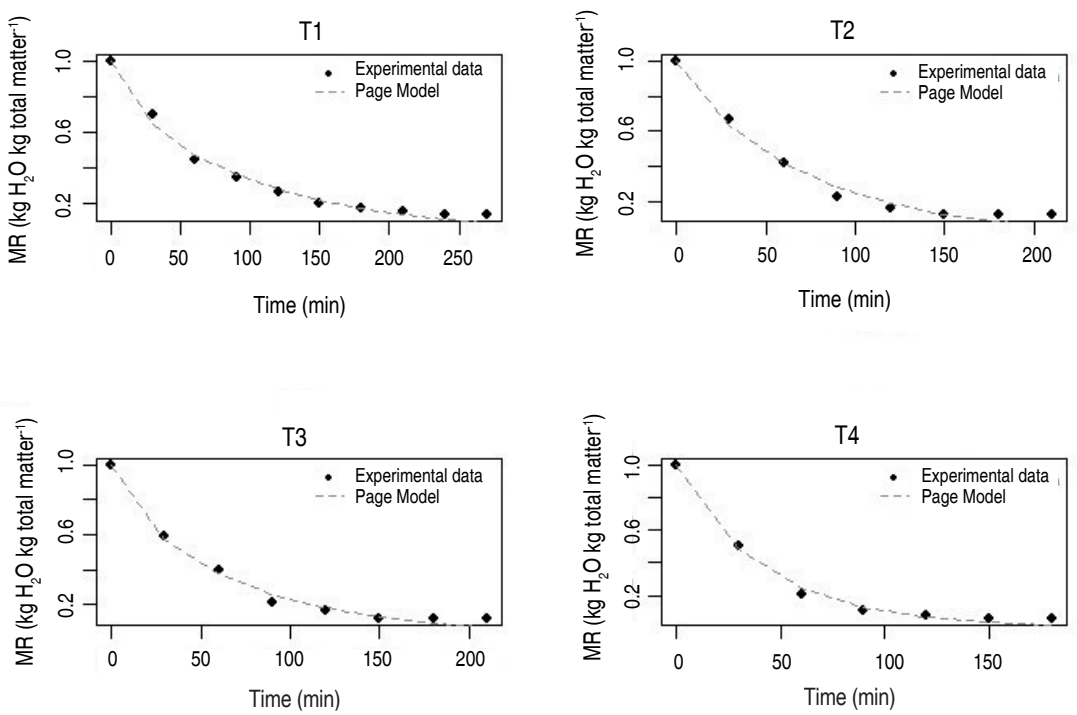

Figure 2. Drying kinetics of *Melissa* leaves at different temperatures and DAV conditions.

Table 3. Results of the coefficients and constants of the Page model for lemon balm leaves' drying process (kinetics).

Drying Conditions	Drying coefficients		Statistical factors	
	κ	η	R^2	SRME
T1	0.0313	0.7730	0.9907	0.0263
T2	0.0195	0.9267	0.9865	0.0351
T3	0.0350	0.8111	0.9923	0.0260
T4	0.0298	0.9432	0.9952	0.0226

As well as the calculation of R and RMSE in this study, it was observed that the parameters κ and η of the Page model increased as the temperature and DAV varied. In particular, the increase in η was related to the rise in DAV, while κ was related to the temperature gradient, as explained by other studies (dos Santos et al. 2017; Levate et al. 2020). However, this could also be explained by the fact that the parameter κ affects the external drying conditions and the parameter η on the internal drying conditions. In fact, from what has been reviewed, this would be the first study that demonstrates that DAV is related to the internal conditions of the drying process. However, more studies would need to be conducted to confirm this hypothesis.

Determination of physicochemical and microbiological composition

Before proceeding to the dehydration of fresh lemon balm, physicochemical and microbiological characterization of the samples of the treatments was carried out. By operational standardization, i.e., based on diameter, all samples were classified with the following characteristics: leaf width averaged 2.8±0.12 cm, leaf length 3.91±0.6 cm, petiole diameter 0.13±0.2 cm, and petiole length 2.58±0.98 cm. Leaves were heart-shaped with toothed margins, green foliage, and a characteristic lime, lemon, or grapefruit aroma. Such attributes were essential to developing the dehydration process, and their results were like those reported in other research (Buitrago-Zapata et al. 2018).

In contrast, the results obtained for the physicochemical characteristics of fresh and dried lemon balm are presented in Tables 4 and 5. Fresh lemon balm leaves presented the highest values in humidity, ash, and a_w (Table 4), while the highest values in EO in dried *Melissa* leaves. On average, after the drying process, the moisture had a value of 11.05±0.53% (wb), the ash content was determined at 0.38±0.09%, the a_w was established at 0.49±0.57, and the EO yield was 1.18±0.07%. The values above were within the range requested by NTE INEN 2392:2017 and other studies (Argyropoulos and Müllera 2011, 2014). Significant differences (P<0.05) existed for both factors and interactions in moisture, % EO, and ash, but no differences were found for a_w and factor B of ash (Table 5).

Table 4. Content of moisture, ash, a_w, and EO of dehydrated *Melissa*.

Treatments	Moisture (%)	Ash content (%)	a_w	Content EO (%)
Fresh plant	80.25±0.29	1.93±0.03	0.96±0.02	0.303±0.036
T1	11.87±0.13^a	0.360±0.02^b	0.50±0.1^a	1.151±0.019^b
T2	10.96±0.03^b	0.490±0.03^a	0.45±0.03^a	1.141±0.002^b
T3	10.82±0.15^b	0.410±0.01^b	0.52±0.02^a	1.136±0.008^b
T4	10.54±0.03^c	0.270±0.03^c	0.44±0.02^a	1.294±0.010^a

All values in the table are expressed as average ± SD. Lowercase letters show statistically significant differences (P<0.05) for the Tukey test.

For fresh leaves, moisture fluctuation was attributed to sample handling, post-harvest resting time, sample collection, and storage methodology (Padilla et al. 2018). The variation in ash is due to the degree of soil contamination by limestone, dicalcium phosphate particles, and pesticides, which could directly impact this quality parameter. Although, the variations could also be related to specific crop processes, i.e., postharvest and storage,

to name a few (Gordanić et al. 2021; Németh-Zámboriné et al. 2019).

In the case of the factors, the a0 and b0 levels proved to be the most appropriate parameters to achieve lower moisture, a_w, and ash but better EO yields (Table 5). Regarding moisture and a_w, the increase in temperature resulting from higher drying velocity reduces the moisture content, a_w, enzyme activity, and DT (Chasiotis et al. 2021). However, the variation in ash content after tray drying is due to the reduction of contaminants (soil, dust, stones, others) resulting from the disinfection process, although it may also be related to the presence of trace contaminants of earthy and environmental material, which are difficult to remove (Mukherjee 2019; Garba and Oviosa 2019). On the other hand, the differences in EO content are believed to be during vapor extraction, where the evaporated water and other volatilized substances are expelled from the drying chamber (Trujillo-Echeverria et al. 2020). Nevertheless, there are other factors, such as the solute/solvent ratio, particle size, solvent polarity, and interfacial contact between the plant matrix and the mechanism performed by the solvent to break the cell walls to extract these compounds, which may be related to the increased yield and higher concentration of lipophilic secondary metabolites (Dulo et al. 2023; Sridhar et al. 2021; Trujillo-Echeverria et al. 2020). Despite this, variations in all compounds of dehydrated lemon balm would be subject to operational variations [including variability of time, DAV, temperature, drying method, and industrial design, among others] (Argyropoulos and Müller 2014), morphological and physiological variations of the crop [e.g., the origin of samples, sowing and harvesting time, fertigation, irrigation, and crop rotation] (Gordanić et al. 2021; Németh-Zámboriné et al. 2019).

Table 5. Analysis of variance for physicochemical and operational parameters of dried *Melissa*.

Moisture: 11.05±0.53% (mean ± SD)					
Source	**Df**	**SS**	**MS**	**F**	***P***
Factor A	1	1.621	1.621	155.21	$1.61\times10^{-06***}$
Factor B	1	1.061	1.062	101.72	$7.97\times10^{-06***}$
A: B	1	0.291	0.291	27.91	$7.44\times10^{-04***}$
Residual	8	0.084	0.010	-	-
Ash content: 0.38±0.09% (mean ± SD)					
Source	**Df**	**SS**	**MS**	**F**	***P***
Factor A	1	0.024	0.024	55.02	$7.49\times10^{-05***}$
Factor B	1	3.0×10^{-05}	3.0×10^{-04}	0.08	0.79^{ns}
A: B	1	0.058	0.058	133.13	$2.89\times10^{-06***}$
Residual	8	0.004	4.0×10^{-04}	-	-
a_w: 0.49±0.57 (mean ± SD)					
Source	**Df**	**SS**	**MS**	**F**	***P***
Factor A	1	3.0×10^{-05}	3.0×10^{-05}	0.01	0.92^{ns}
Factor B	1	0.013	0.013	4.79	0.06^{ns}
A: B	1	5.3×10^{-04}	5.3×10^{-04}	0.19	0.67^{ns}
Residual	8	0.022	0.002	-	-
Content EO: 1.18±0.07% (mean ± SD)					
Source	**Df**	**SS**	**MS**	**F**	***P***
Factor A	1	0.014	0.014	105.3	$7.0\times10^{-06***}$
Factor B	1	0.016	0.016	121.1	$4.14\times10^{-06***}$
A: B	1	0.021	0.021	154.8	$1.63\times10^{-06***}$
Residual	8	0.001	1.0×10^{-04}	-	-

Df: degree freedom, SS: sum of square, MS: mean sum of square, ***: $P<0.001$, ns: not significant.

As a result, the effect of drying parameters is inversely proportional to the physicochemical content of horticultural products. The drying process has a positive effect on increasing the shelf life of lemon balm (Bonazzi and Dumoulin 2011). The glass transitions produced by the drying process affect the quality of dehydrated foods (Badmus et al. 2019). Suitable conditions of temperature and DAV allow for obtaining desorption isotherms in equilibrium within the drying process (Chakraverty and Singh 2014). This means that a given variation of the temperature- DAV-drying time gradients can cause proportional changes in the physicochemical characteristics of the dehydrated food (determined as a hygroscopic solid), which would directly affect the quality and specifics of the final product (e.g., nutritional content, rehydration, and enzyme activity to name a few).

According to Tukey's test at a 95% confidence level, dried Lemon balm physicochemical compounds content was partially significantly different between treatments, except for the a_w, which is directly related to the isothermal process of the drying process. Consequently, treatment T4 offers the best characteristics for the final product, demonstrating that, as with the factors, these characteristics are related since their behavior lasts until the end of the process.

Microbiological analysis

Table 6 shows the total bacteria count in fresh and dried lemon balm leaves. The results show that the bacteriological count value was within the range requested by NTE INEN 2392:2017 (Table 6). No significant differences were found. In the raw material, the presence of pathogens was attributed to the use of organic fertilizers such as cow (or another animal) manure or the use of contaminated water for cultivation (Gómez-Ramirez et al. 2021). However, the lack of controls in the harvesting, post-harvest, storage, and transportation process could also be related to these microorganisms or, even worse, to foodborne diseases (Gallo et al. 2020; Gil et al. 2015).

Table 6. Microbiological analysis.

Treatments	*Escherichia coli* (UFC g^{-1})	*Salmonella* (UFC g^{-1})	*Bacillus Cereus* (UFC g^{-1})	*Clostridium Perfringens* (UFC g^{-1})
Fresh plant	5.33×10^3	3.17×10^3	1×10^3	7×10^3
T1	Lack	Lack	$6x10^1$	Lack
T2	Lack	Lack	$6x10^1$	Lack
T3	Lack	Lack	$6x10^1$	Lack
T4	Lack	Lack	$6x10^1$	Lack
Limit according to NTE INEN 2392:2017	$1x10^2$	Lack	$1x10^4$	$1x10^3$

On the other hand, the absence of microorganisms is attributable to the drying process, which reduces the percentage of moisture and free water necessary for the proliferation and survival of microorganisms (Tinebra et al. 2022). However, the disinfection and sanitization process before drying may have also contributed to the reduction of the microbiological load. Now, although the product is within the parameters by the standard, precautions should be taken with *Bacillus cereus* because it is a highly resistant pathogen; it is present in dust, as well as in the soil, being able to survive in pH media of 4.9–9.3 and even saline solutions of 7%. In addition, it can develop without problems under temperatures from 4 to 48 °C, so it must be kept under quality control in storage to avoid contamination or exposure to bacterial diseases in the consumer (Gil et al. 2015). This should also be considered with other microorganisms since they are characteristic pathogens of these foods.

Color Characteristics

Table 7 shows the results of the average color of the dried leaves of lemon balm. The results were like those obtained by Argyropoulos and Müller (2014). The color analysis was done as additional information to the test of acceptability since it is an essential parameter in consumer decision-making during purchase (Chen and Lin 2018). In addition, the influence of the drying process on the physical characteristics (color of the final product) of the

dehydrated lemon balm was studied to evaluate the impact on the technological quality of the final product.

In this study, the lightness value decreased (P<5%) with increasing drying temperature. a* and b* of the surface of dried lemon balm leaves decreased significantly (P<0.05), which was attributed to non-enzymatic browning (Oliveira et al. 2016). Greater color degradation was observed at temperatures above 45 °C and dry air velocities below 1.5 m s^{-1}, indicating undesirable color changes. The color parameters are improved by increasing the rate of dry airflow due to the increased interfacial contact of the solid with the temperature and the accelerated evacuation of the condensate in the drying chamber.

Table 7. Colorimetry analysis.

Color parameters	Treatments					Sig.
	Fresh plant	T1	T2	T3	T4	
L*	45.40±0.34	46.83±0.20^{a}	44.68±0.22^{b}	42.51±0.44^{d}	43.75±0.26^{c}	
a*	-17.83±0.13	-5.93±0.03^{d}	-5.08±0.08^{c}	-1.39±0.04^{b}	0.57±0.02^{a}	<0.001
b*	24.29±0.40	15.99±0.16^{a}	14.82±0.20^{b}	11.02±0.10^{d}	13.13±0.08^{c}	

*: L value (lightness): 100=white, 0=black, a value (redness): -60 ~ +60, - =green, + =redness, b value (yellowness): -60 ~ +60, - =blue, + =yellow. All values in the table are expressed as average ± SD. Samples with superscript letters indicate significant differences (P<0.05) for Tukey's test.

In general, changes in the color properties of a dried food depend on several physicochemical alterations during the drying process. For example, during food processing with heat treatments, color change is assumed to occur by different processes, such as pigment degradation, ascorbic acid oxidation, and Maillard reaction (Sturm and Hensel 2017). In this study, the color alteration was affected by carotenoid degradation and chlorophyll transformation in lemon balm leaves.

Sensory analysis

A sensory analysis of the dehydrated lemon balm tea was conducted by a panel of 102 consumers. Most of the participants were men (63%). The mean age of the participants was 31.64±14.78 years (range: 19-60). Table 8 shows the scores obtained for each attribute evaluated. Throughout the experiment, the descriptors that consistently scored the highest in terms of aroma and flavor were “neither like nor dislike” and “I don’t like,” while the phrases “I like a lot” and “I like” received the lowest scores. It was found that various consumers had varying degrees of acceptability for lemon balm teas, with a statistically significant difference (P<0.05) observed. The tea samples that consumers liked the most were those of the T4 treatment. The obtained results were like those obtained by da Silveira et al. (2022). When compared to other types of tea, the sensory analysis of lemon balm tea is a subject that has been studied truly little. No additional studies that evaluate the sensory aspects of lemon balm were discovered.

Table 8. Consumer acceptability scores of lemon balm tea in its organoleptic aroma and flavor characteristics.

Attribute	T1	T2	T3	T4
Aroma	2.41±0.64^{b}	2.61±0.69^{b}	2.08±0.82^{c}	3.04±0.84^{a}
Flavor	2.37±0.69^{c}	2.60±0.76^{b}	2.37±0.69^{c}	3.10±0.92^{a}

Lowercase letters show statistically significant differences (P<0.05) for Tukey's test.

Despite these, it is believed that the differences found would be related to various compounds (polyphenolics, antioxidants, flavonoids, and others) and the formation of aromatic substances (Wang et al. 2022), which combine synergistically to produce good aroma and flavor. In this regard, Tang et al. (2020) reported that the attributes of sweetness, bitterness, and astringency were related to the content of polyphenols, flavonoids, amino acids, and triterpenes. The results obtained would support these findings, as the EO content was high, suggesting a direct

relationship between these factors. In addition, in the tasting sessions, consumers mentioned descriptives such as lemon aroma, citrus aroma, herbal aroma, fruity aroma, sweet flavor, bitter flavor, and sour flavor, among others, which would agree with such findings. Nevertheless, other factors responsible for modifying the sensory attributes to consider are crop characteristics, dehydration processes, and preparation of tea (Cárdenas-Mazón et al. 2018).

Lastly, it would be important to conduct more studies to support this hypothesis and increase the consumption of lemon balm tea, especially since lemon balm has been reported to have many beneficial activities for the human body (Petrisor et al. 2022).

CONCLUSION

The findings obtained in this study show that drying parameters have a significant impact on the overall quality of lemon balm and the drying process. Samples dried at 45 °C and 3.0 m s^{-1} achieved lower moisture, a_w, and ash but higher EO yields. The high values obtained in EO were attributed to the particle size (0.03 mm) and the solute-solvent ratio (1:15 w v^{-1}) used in the hydrodistillation process. No like this, crop-specific processes (crop management, postharvest, storage, and food processing, among others) can alter the physicochemical, microbiological, and sensory characteristics of Lemon balm. From the sensory point of view, when the temperature exceeds 45 °C and the dry air velocity is below 1.5 m s^{-1}, there is a decrease in lightness, color degradation occurs, and enzymatic reactions alter the a^* and b^* parameters of the instrumental color. The T4 treatment has resulted in the most preferred tea among consumers. In the drying process, it was observed that temperature has a greater effect in the initial stages of the process, which allows the surface water of the feed matrix to evaporate at a faster rate, while DAV stimulates the internal structure of the raw material by capillarity and diffusion, thus increasing the drying rate and significantly decreasing the drying time. In fact, when the DAV is increased from 1.5 to 3.0 m s^{-1}, the DT decreases by approximately 44%, something that does not happen with increasing temperature alone. Page's model demonstrated an excellent ability to predict drying kinetics under various drying conditions (RMSE <0.04 and R2 >0.98). The findings of this study provide a theoretical basis for optimization of the drying process and improving the quality of the final product. The future perspective is to continuously improve the drying process of herbs, fruits, and vegetables through the optimization of the drying parameters and non-invasive technological systems or chemometric models (predictive algorithms) that allow monitoring or controlling the physicochemical, microbiological, and sensory quality of the final products. More research is needed on the interaction of bioactive compounds and sensory attributes to obtain products with high-quality pharmacological effects oriented to the final consumer.

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Stability of an isotonic beverage based on sweet whey permeate added with cape gooseberry (*Physalis peruviana* L.)

Estabilidad de una bebida isotónica a base de permeado de lactosuero dulce adicionada con uchuva (*Physalis peruviana* L.)

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Keywords:
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ABSTRACT

Carbohydrate and mineral content in whey permeate is similar to that of commercially available sports drinks, most of which are formulated without functional ingredients. The aim of this study was to evaluate the physicochemical stability of two formulations of isotonic beverage from whey permeate obtained by membrane separation (ultrafiltration), added with cape gooseberry fruit (*Physalis peruviana* L.). Physicochemical and microbiological stability, total polyphenol and carotenoid content, antioxidant activity (ABTS and DPPH) and sensory profile were evaluated during two-months of refrigerated storage at 4 °C. The results showed high physicochemical stability (pH, acidity, and total soluble solids) for the functional beverage. Important differences were observed in osmolality, which increased from 304.83 to 324.13 mOsm kg^{-1} for the non-hydrolyzed drink (BIUN) and from 330.1 to 350.53 mOsm kg^{-1} for the hydrolyzed drink (BIUH). The average content of total phenols was 9.64 and 9.72 mg-AG.100 g^{-1} for the BIUN and BIUH beverages, respectively. There was a reduction in the antioxidant activity of the drinks by both DPPH and ABTS analysis during storage time. Total carotenoid content decreased from 0.095 to 0.076 mg β-carotene.100 g^{-1} and from 0.115 to 0.076 mg β-carotene.100 g^{-1} for the BIUN and BIUH beverages, respectively. The sensory profile showed that both drinks had high overall quality.

Palabras clave:
Capacidad antioxidante
Hidrólisis
Osmolaridad
Bebida Isotónica
Almacenamiento
Ultrafiltración

RESUMEN

El contenido de carbohidratos y minerales en el permeado de lactosuero es similar al de bebidas deportivas o isotónicas disponibles a nivel comercial y que, en su mayoría, están formuladas con saborizantes y colorantes artificiales. El objetivo de este trabajo fue evaluar la estabilidad fisicoquímica de dos formulaciones de bebidas isotónicas a partir de permeado de lactosuero obtenido por separación por membranas (ultrafiltración) con adición de uchuva (*Physalis peruviana* L.). Se evaluó la estabilidad fisicoquímica y microbiológica, el contenido de polifenoles y carotenoides totales, la actividad antioxidante (ABTS y DPPH) y el perfil sensorial durante dos meses de almacenamiento bajo refrigeración a 4 °C. Los resultados indicaron una alta estabilidad fisicoquímica (pH, acidez, sólidos solubles totales) para las dos bebidas. Se observaron diferencias en la osmolalidad, la cual incrementó de 304,83 a 324,13 mOsm kg^{-1} para la bebida no hidrolizada (BIUN) y de 330,1 a 350,53 mOsm kg^{-1} para la bebida hidrolizada (BIUH). El contenido de fenoles totales fue 9,64 y 9,72 mg-AG 100 g^{-1} para las bebidas BIUN y BIUH, respectivamente. Se presentó una reducción de la capacidad antioxidante de las bebidas tanto por radicales DPPH como ABTS durante el tiempo de almacenamiento. El contenido de carotenoides totales disminuyó de 0,095 a 0,076 mg β-caroteno 100 g^{-1} y de 0,115 a 0,076 mg β-caroteno 100 g^{-1} para las bebidas BIUN y BIUH, respectivamente. A nivel sensorial, ambas bebidas presentaron una percepción de un producto de alta calidad.

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Sports drinks are non-carbonated water-based beverages, they are generally composed of varying types of monosaccharides, disaccharides, and sometimes maltodextrins, and contain small amounts of minerals (electrolytes) such as sodium, potassium, chloride (Maughan and Murray 2001). Functional sports drinks play an important role in hydrating, improving athletic performance, and preventing or helping specific health conditions (Orrù et al. 2018). The carbohydrate (sugar) concentration and the type of carbohydrate used, the electrolyte content, the osmolality, and the flavoring components can be manipulated to alter the functional characteristics of these beverages (Maughan 2009).

Whey is the liquid obtained after the coagulation of milk proteins during the production of cheese or caseinates (Smithers 2008). It is composed of lactose (5%), water (93%), protein (0.85%), minerals (0.53%), and fat (0.36%) (Pescuma et al. 2010). The composition of whey permeate is similar to that of electrolyte beverages commercialized as sports drinks (Geilman et al. 1992; Silva et al. 2023). Opposite to commercial hydrating drinks that only provide minerals such as sodium, potassium, and chlorine; drinks formulated with whey or whey permeate contain additional electrolytes such as calcium, magnesium, phosphorus, and zinc (El-khair 2009).

There is a limited availability of sports drinks without artificial flavors and colors on the market (Galaz 2013). Citrus flavors have been used in whey-based beverages and have been shown to be very effective in masking the undesirable odor of cooked milk and the salty-sour taste of fresh whey (Chavan et al. 2015). Cape gooseberry (*Physalis peruviana* L.) is considered an exotic fruit with an acidity comparable to that of passion fruit (*Passiflora edulis*), which can be processed for the development of refreshing beverages and other food products (Mendoza and Rodriguez 2012). In addition, this fruit can provide functional compounds due to its high content of total phenols, carotenoids, and considerable antioxidant activity (Rockenbach et al. 2008; Rinaldi et al. 2022).

Membrane technologies are separation methods that have been used in the dairy industry where ultrafiltration is one of the technologies most used for the recovery of whey proteins (Duke and Vasiljevic 2015). Multiple studies have been conducted to increase the value of the permeate produced by whey ultrafiltration; combined with nanofiltration, they have been used to concentrate lactose and demineralize whey (Cuartas-Uribe et al. 2009; Hofmann and Hamel 2023). The use of whey permeate as a source of oligosaccharides has also been studied (Barile et al. 2009). Different proportions of water-whey and water-permeate have been used in the formulation of sports drinks (Beucler et al. 2006; Petrus et al. 2005; O'Donoghue and Murphy 2023). Valadão et al. (2016) developed a sports drink based on hydrolyzed whey from ricotta cheese with an osmolality of 306 mOsm kg^{-1}, including approximately 37% whey, approximately. Ferreira et al. (2020) obtained a beverage with an osmolality of 316 mOsm kg^{-1} using non-hydrolyzed whey permeate. These results indicate the potential of whey permeate as an alternative in the production of isotonic beverages.

Considering the high nutraceutical and functional potential of cape gooseberry fruit (pulp and skin) and the need to develop products that take advantage of whey permeate as a basis for food formulation, this study proposes the formulation of an isotonic beverage using whey permeate (hydrolyzed and non-hydrolyzed) obtained by ultrafiltration and adding cape gooseberry fruit as a nutraceutical source. The stability of the beverages was assessed for two months under refrigeration conditions at 4 °C.

MATERIALS AND METHODS

Raw materials

Sweet whey was provided by AURALAC S.A. (Antioquia, Colombia). Products such as sucrose, stabilizer, and commercial flavoring were supplied by Tecnas S.A. (Medellín, Colombia). Cape gooseberry fruits (*Physalis peruviana* L.) were purchased from a local supplier and selected in compliance with resolution 3929 of 2013 of the Ministerio de Salud y Protección Social de Colombia. The fruit selected had a state of maturity based on color of 4, 5, and 6 (NTC 4580).

The whey presented the following physicochemical characteristics: Acidity: 0.09±0.006% lactic acid (AOAC 947.05), pH=6.53±0.067 (NTC 4592), soluble solids 6.97±0.239 °Bx (AOAC 932.12), lactose 47.9±0.273 g L^{-1} protein 0.85±0.066% (AOAC 990.03), ashes 0.54±0.038% w/w, calcium 366.64±46.817 mg kg^{-1} (NTC 5151), magnesium 72.62±10.153 mg kg^{-1} (NTC 5151). The lactose was quantified by high-performance liquid

chromatography (HPLC) using a chromatograph AGILENT TECHNOLOGIES 1200 series with an AMINEX HPX-87H ion exchange column (300x7.8 mm), and as a mobile phase solution of H_2SO_4 0,008 N at a constant flow of 0.6 mL min^{-1} (Pérez-Escobar et al. 2020). The cape gooseberry whole pulp presented the following physicochemical characteristics: total soluble solids of 15.17±0.10 °Bx (NTC 4580), acidity of 1.636±0.095% citric acid (NTC 4580), pH of 3.46±0.01 (NTC 3651), phenolic acids of 84.14±4.83 AGE 100 g^{-1}, total carotenoids of 33.59±1.278 mg kg^{-1} antioxidant capacity with ABTS 53.6±5.11 mg trolox 100 g^{-1} and DPPH 38.73±5.05 mg Trolox 100 g^{-1}. In addition, products such as sucrose, stabilizer (CMC FGHH, provided by Tecnas S.A.), and commercial flavoring (Passion Fruit MN-Y, provided by Tecnas S.A.) were provided by local companies in the city from Medellin.

Formulation of isotonic beverage

Ultrafiltration permeate (PUF)

Sweet whey was conditioned at 43 °C, skimmed by centrifugation at 8,000 rpm (GEA Westfalia Separator AG, Type: MTA5-00-104), and pasteurized at 63 °C for 30 min. Subsequently, the product was subjected to an ultrafiltration process (Pilot filtration equipment, PERINOX, Series 0114/0115, Model E0FT) at 48 °C, with a concentration factor of 18, pressure and outlet pressure of 1 and 3 bar, respectively. The ultrafiltration pilot plant was equipped with a polyethersulfone spiral semipermeable membrane with a cut size of 10 kDa. The whey permeate by ultrafiltration had the following physicochemical characteristics: Acidity of 0.081±0.004% lactic acid (AOAC 947.05), pH of 66.47±0.15 (NTC 4592), soluble solids of 5.50±0.05 °Bx (AOAC 932.12), lactose of 50.052±0.134 g L^{-1} (Pérez-Escobar et al. 2020), ash of 0.481±0.032% w/w (NTC 5151), protein <2.5% (AOAC 990.03), calcium of 283.063±27.472 mg kg^{-1} (NTC 5151), magnesium of 61.813±5.256 mg kg^{-1} (NTC 5151).

Hydrolyzed PUF

Hydrolyzed permeate was obtained by cold hydrolysis using 0.075 mL L^{-1} of lactase for 20 h, achieving a degree of hydrolysis of approximately 30%. The percentage of hydrolysis was measured using the cryoscopy point method described by Llerena et al. (2019).

Preparation of cape gooseberry (skin and pulp)

The whole fruit was cleaned, disinfected (sodium hypochlorite at 100 mg L^{-1}), and homogenized using an industrial blender (CI TALSA LI30). The resulting product was passed through a USA Standard Mesh (18 mesh=1 mm sieve size) to remove the seeds. The processed fruit was stored frozen at -18 °C for later use.

Preparation of beverages

For the preparation of beverages, preliminary experiments were conducted varying the percentage of PUF and seeking to obtain an osmotic concentration following the regulations for isotonic beverage in normative 2229 of 1994 of the Ministerio de Salud de Colombia. Two formulations were developed: non-hydrolyzed isotonic beverage with cape gooseberry fruit (BIUN) and hydrolyzed isotonic beverage with cape gooseberry fruit (BIUH), formulated with non-hydrolyzed and hydrolyzed PUF, respectively (Table 1). Beverages were prepared by mixing the ingredients and homogenizing at 10.34 MPa (1,500 psi) (Homogenizer, St. Regis CP Division, Series: 3DD13-2941), then pasteurized (62 °C for 30 min), bottled in 250 mL PET flasks, and stored at 4 °C.

Table 1. Formulation of isotonic beverages.

Ingredients	BIUN/BIUH (% w/w)
PUF/hydrolyzed PUF	79.84
Water	14.97
Cape gooseberry (skin and pulp)	2.99
Sucrose	2.00
Stabilizer	0.10
Flavoring	0.10

Physicochemical analysis

Osmolality was determined by cryoscopy procedure and following the NTC 3837 given by ICONTEC (2009), as shown in equation 1:

$$\text{Osmolality}\left(\frac{\text{mOsm}}{\text{kg}}\right)=\left(\frac{\Delta T}{1.858}\right)\times 1{,}000\frac{\text{mOsm}}{\text{kg}} \quad (1)$$

where ΔT is the difference (°C) between the freezing temperature of water and the freezing point of the sample, ΔT was measured with a FUNKE GERBER CryoStar I single sample automatic cryoscope (DIN/ISO/IDF 5764). Determination of pH was measured by potentiometric method, after calibration with buffer solutions of pH=4 and 7 at 20 °C with an OHAUS STARTER 3100 potentiometer (AOAC Method 981.12/90). Acidity was measured by titration (AOAC Method 947.05/90). Total soluble solids (TSS) were expressed as Brix degrees (°Bx) and quantified using a digital refractometer (HI 96801) and following the NTC 4624 given by ICONTEC (1999). Mineral content (calcium, magnesium, potassium, and sodium) was obtained by atomic absorption spectrometry (AOAC 985.35-1988), and total sugars by spectrophotometry UV-VIS (Nielsen 2010). Antioxidant capacity was determined using a modification of the DPPH and ABTS radical cation trapping methods proposed by Bravo et al. (2015). Extraction: 10 g of each sample was taken in a falcon tube and mixed up to 20 mL with solvent (methanol/water, 70:30). The sample was vortexed at 30,000 rpm for 2 min and put in an ultrasonic bath for 10 min, then centrifuged at 8,000 rpm for 15 min. The supernatant was filtered into 25 mL volumetric flasks, completing the volume with solvent. DPPH was determined with 50 µL of the extract were taken in an Eppendorf tube, 950 µL of work solution (0.05 mM DPPH solution) were added, it was stirred at 2,000 rpm in a vortex for 30 s and left to react in darkness for 30 min. The absorbance of the extract was measured at a wavelength of 517 nm using a spectrophotometer (THERMO Scientific, Evolution 60). ABTS was determined with 50 µL of the extract were taken in an Eppendorf tube, 950 µL of work solution (ABTS solution) were added, it was stirred at 2,000 rpm in a vortex and left to react in darkness for 8 min. The absorbance of the extract was measured at a wavelength of 734 nm using a spectrophotometer (THERMO Scientific, Evolution 60). Total phenol content was determined using a modification of the Folin-Ciocalteu reagent method described by Bravo et al. (2015). Finally, 100 µL of the extract were taken in an Eppendorf tube, 400 µL of 0.07 N sodium carbonate solution were added, it was stirred at 15,000 rpm in a vortex, and allowed to stand for 5 min. Then, 500 µL of Folin solution were added and stirred at 15,000 rpm in a vortex. The tube was capped and stored in darkness at room temperature for 2 h. The absorbance of the extract was measured at a wavelength of 760 nm using a spectrophotometer (THERMO Scientific, Evolution 60). Total Carotenoid content was determined using a modification of the method described by Ferreira et al. (2009). A 15 g sample was taken in a falcon tube and stirred with 15 mL of solvent (n-hexane/acetone, 6:4) for 10 min at room temperature and filtered through Whatman No.4 filter paper. The absorbance of the filtrate was measured at a wavelength of 450 nm using a spectrophotometer (THERMO Scientific, Evolution 60).

Microbiological analysis

Counts of total coliforms (AOAC 966.23C 2005), fecal coliforms (AOAC 966.24 2005), aerobic mesophiles (AOAC 988.18 2005), molds and yeasts (AOAC 995.21 2005), and Clostridium sulfite reducer spores (AOAC 972.41 2005) were conducted following the NTC 3837. Analyses were made for two months on beverage samples (BIUH and BIUN) for storage times of 4, 19, 39, and 54 days.

Sensory analysis

The sensory profiles of the beverages were evaluated by the multidimensional approach method, following the Colombian Technical Standards NTC 3501 (ICONTEC 2012), NTC 3932 (ICONTEC 1996), and the Colombian Technical Guides GTC 165 (ICONTEC 2014) and GTC 226 (ICONTEC 2012). A set of relevant descriptors were identified and selected; the intensities were assessed by five trained judges, with an age range between 25 and 60 years. The samples were evaluated under the temperature of 23 °C and relative humidity of 61% (room conditions), and the rating scale from 0 to 5 for all the descriptors was established. Overall quality was rated on a scale from 1 to 3, where 3 is high and 1 is low. Student´s t-test for independent samples was used for statistical analysis.

Statistical Analysis

The study was conducted using a completely randomized design with three replicas. The physicochemical properties of each treatment were followed for two months (days 0,

7, 14, 21, 28, 35, 42, 49, and 56). Bioactive compounds were measured at weeks 0, 4, and 8. Results were analyzed using one-way analysis of variance (ANOVA) with $P<0.05$. Means were compared by the least significant difference method (LSD, $P<0.05$), using Minitab® 19.1.1 (Minitab, LLC., 2021).

RESULTS AND DISCUSSION

Mineral and sugar content

Table 2 shows the results of the proximate analyses. There were statistically significant differences ($P<0.05$) in the content of calcium and magnesium between the two beverages —BIUN and BIUH. This difference may be due to the interaction of calcium and magnesium ions with the enzyme used for the hydrolysis of the BIUH beverage (Zolnere et al. 2017), as well as the effect of additional heat treatment applied to the beverage to inactivate the enzyme (De La Fuente et al. 1999; Rojas Silva 2016).

Ferreira et al. (2020) prepared a beverage based on whey permeate and jaboticaba peel, reporting similar values of total sugars (4.8 g), calcium (34.1 mg), and magnesium (6.2 mg). Hattem et al. (2010) prepared a sports drink from whey permeate added to mango and obtained similar contents of total sugars (6.48 g), calcium (25.1 mg), and magnesium (5.2 mg). In this way, it can be observed that minerals such as magnesium, calcium, potassium, and sodium provided by the sweet whey permeate satisfy the requirements for an isotonic beverage according to Colombian regulations NTC 3837.

Table 2. Content of sugars and minerals in BIUN and BIUH (100 g of beverage).

Component	BIUN	BIUH
Total sugars (g)	5.59±0.87^{a}	5.34±0.37^{a}
Calcium (mg)	28.35±0.64^{a}	32.75±1.06^{b}
Magnesium (mg)	7.10±0.14^{a}	6.50±0.14^{b}
Potassium (g)	0.24±0.16^{a}	0.22±0.12^{a}
Sodium (g)	0.31±0.27^{a}	0.31±0.27^{a}

Average values with different letters present a statistically significant difference ($P<0.05$).

Physicochemical stability

According to Figure 1, the osmolality of the hydrolyzed beverage (BIUH) was higher ($P<0.05$), this is due to the lactose hydrolysis process increasing the concentration of solutes in the beverage. An increase in the osmolality of both beverages was observed over time ($P<0.05$): from 304.83 to 324.13 mOsm kg^{-1} for the BIUN beverage and from 330.1 to 350.53 mOsm kg^{-1} for the BIUH beverage. This increase is due to the usual hydrolysis of sucrose and other more complex carbohydrates present in the samples during storage (Sollanek et al. 2019). Ferreira et al. (2021) obtained similar values to BIUN in osmolarity for an isotonic beverage made from whey permeate and pequi powder.

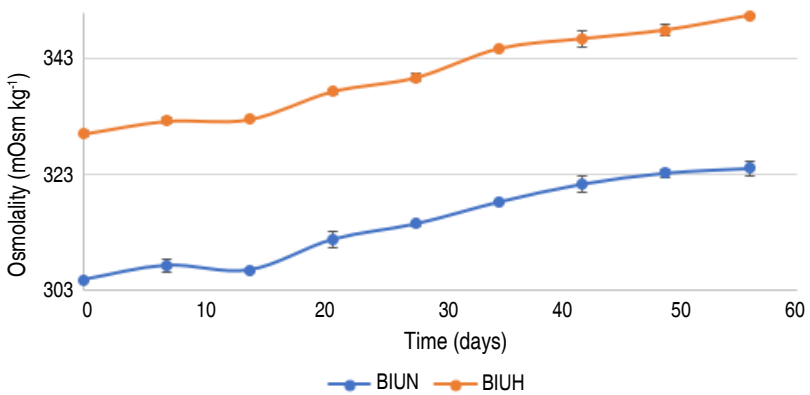

Figure 1. Osmolality stability during storage at 4 °C (confidence intervals).

There were no statistically significant differences (P>0.05) in the beverages during the storage time to total soluble solids (TTS). Figure 2 shows that during the first week, there was a slight increase in TSS in the drinks (between 3 and 4%). This can be attributed to the partial enzymatic hydrolysis of complex carbohydrates present in the samples (Naik et al. 2009). In the second week, the values remained stable between 6.9 and 7.1 °Bx for both drinks.

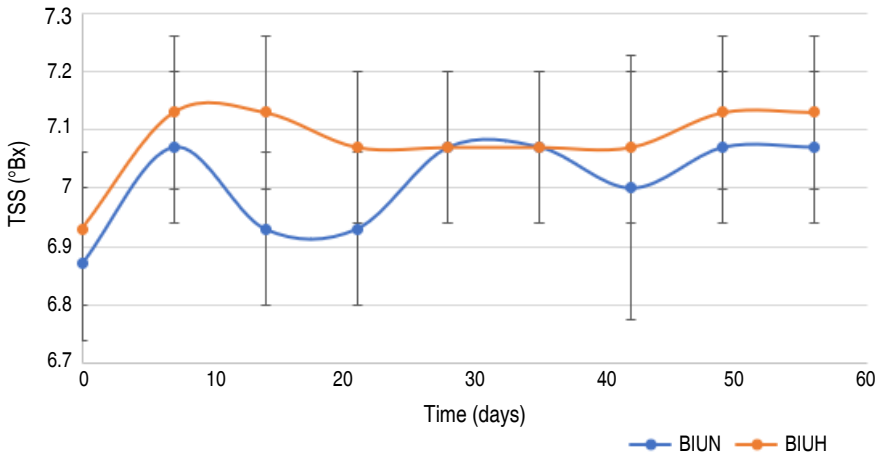

Figure 2. Total soluble solids (TTS) stability during storage at 4 °C (confidence intervals).

According to Figure 3, there were no statistical significance (P>0.05) in the lactic acid content in both beverages nor in storage time. It can be seen that the acidity remained around 0.11% v/v lactic acid during the storage time. Naik et al. (2009) reported comparable results in a whey drink added with watermelon pulp, the slight increase in acidity after 30 days of storage was attributed to the conversion of lactose into lactic acid.

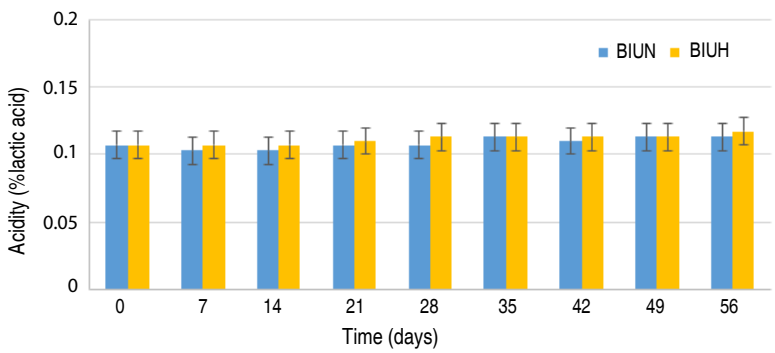

Figure 3. Acidity stability during storage at 4 °C (confidence intervals).

There were statistically significant differences in the pH values during the storage time (P<0.05) but not between the two treatments (Figure 4). The variation in pH can be attributed to the formation of lactic acid during storage; it was observed that the pH of the beverages decreased over time (1.1%). These results differ slightly from those reported by Hattem et al. (2010), when studying sports drinks based on whey permeate and mango and strawberry pulp, pH decreased by around 5% in 15 days of storage at 4 °C. Elsabie and Aziz (2011) found a pH decrease of 10% in a beverage based on permeate and sweet potato after three weeks of storage at 7 °C. These differences may be due to better hygiene conditions during the preparation of the beverages and to lower storage temperature.

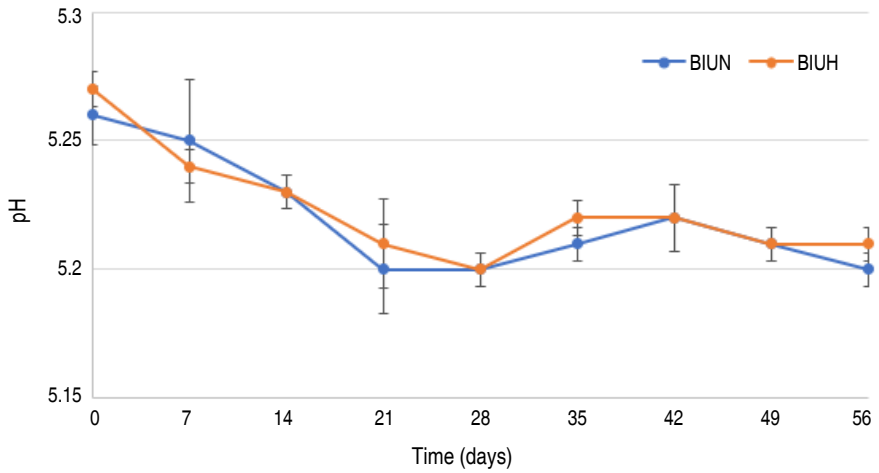

Figure 4. pH stability during storage at 4 °C (confidence intervals).

Bioactive compounds

There were no significant differences (P>0.05) in the content of total phenols between the two treatments (Figure 5). The BIUN beverage presented an average content of 9.64 mg-GAE 100 g^{-1}, while the average content in the BIUH beverage was 9.72 mg-GAE 100 g^{-1}. Porfírio et al. (2020) developed a soft drink with jaboticaba (*Plinia cauliflora*) extract obtaining a 5-fold greater amount of total phenols (48.56 mg-GAE 100 g^{-1}), while Atallah (2015) reported a beverage with mango pulp with 50% less total phenols (4.32 mg-GAE 100 g^{-1}) than the developed beverages.

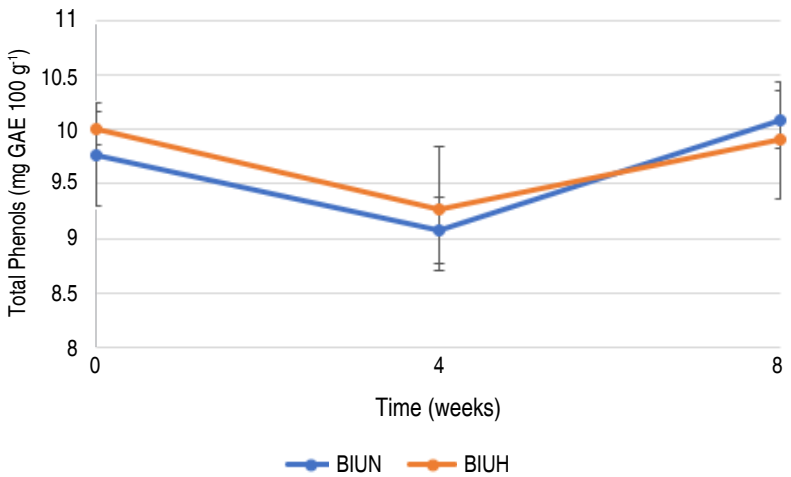

Figure 5. Total phenols stability during storage at 4 °C (confidence intervals).

Statistically significant differences (P<0.05) were found in the total carotenoid content of the BIUH beverage throughout the storage time (Figure 6). However, there was no effect due to the hydrolysis treatment of the beverages (P>0.05). A decrease in the content of total carotenoids is observed during the study, this may be due to the fact that these chemical compounds are susceptible to oxidation and isomerization reactions during storage, due to their highly unsaturated structure (Ferreira et al. 2021). The total carotenoid content decrease in the non-hydrolyzed beverage was about 20%, while the hydrolyzed beverage showed a decrease close to 34%.

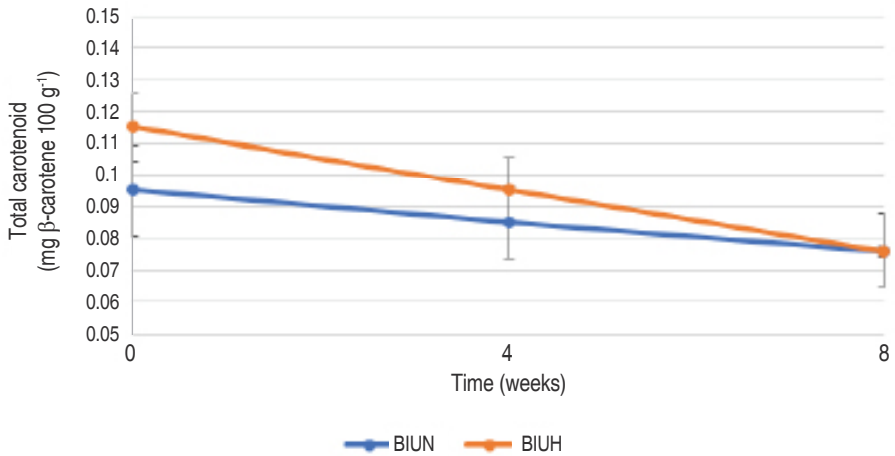

Figure 6. Total carotenoid stability during storage at 4 °C (confidence intervals).

There were no significant differences (P>0.05) in the antioxidant capacity, for both DPPH (Figure 7A) and ABTS radicals (Figure 7B) between the two treatments. During storage under refrigeration, there was a decrease of approximately 30 and 12% for DPPH and ABTS radicals, respectively, after the first 4 weeks; between weeks 4 and 8 there were no differences. The decrease in the antioxidant capacity may be due to the relatively low pasteurization temperature, since it may have not been enough to sufficiently inactivate the enzymes of the food matrix, leading to an alteration of the nutritional properties (Santander et al. 2017).

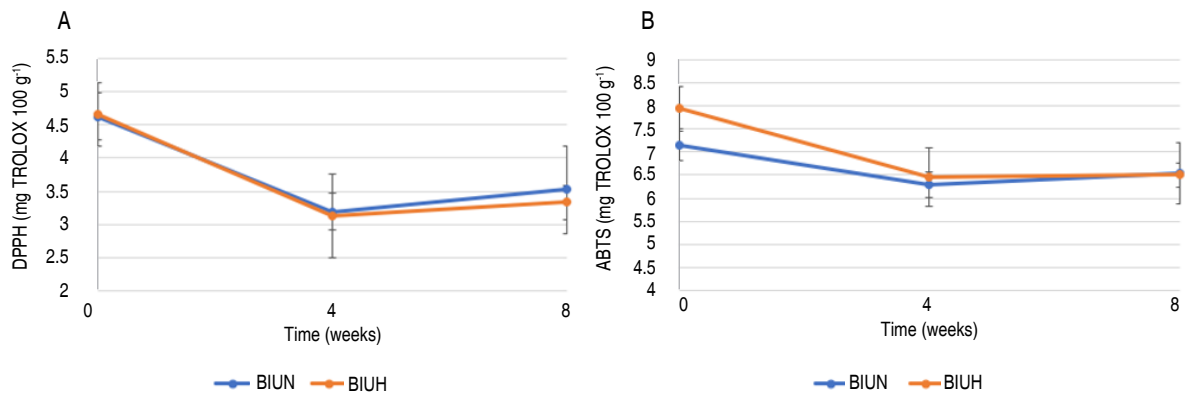

Figure 7. Antioxidant capacity during storage at 4 °C: A. Antioxidant activity by DPPH radical; B. Antioxidant activity by ABTS radical (confidence intervals).

The results found are similar to those reported by Santander et al. (2017), who found that the antioxidant capacity of a whey-based beverage added with tamarillo *(Solanum betaceum)* decreased gradually during storage at 4 °C as storage time increased, and after one month the loss of antioxidant capacity was close to 30%. Other studies such as that of Ferreira et al. (2021) have found that the addition of fruits enriched these types of drinks with nutraceutical components such as antioxidants.

Microbiological stability

According to Table 3, the beverages met the microbiological requirements established in the Colombian technical standard NTC 3837/2009 and normative 2229 (1994) of

Table 3. Microbiological results for isotonic beverages (BIUH and BIUN).

Day	Beverage	Total Coliform count g^{-1}	Escherichia coli count g^{-1}	Aerobic mesophilic count g^{-1}	Molds and Yeasts count g^{-1}	Sulphite-reducing clostridia spore count g^{-1}
4	BIUH	<10	<10	<10	<10	<10
	BIUN	<10	<10	<10	<10	<10
19	BIUH	<10	<10	<10	<10	<10
	BIUN	<10	<10	<10	<10	<10
39	BIUH	<10	<10	<10	<10	<10
	BIUN	<10	<10	<10	<10	<10
54	BIUH	<10	<10	<10	<10	<10
	BIUN	<10	<10	<10	<10	<10

Ministerio de Salud de Colombia. This shows that the use of LTLT (low temperature/long time) pasteurization as heat treatment was sufficient to ensure the safety of both beverages.

Sensory analysis

A set of relevant descriptors that give the maximum information on the sensory attributes of beverages were identified and selected. Aiming to establish a sensory profile, the intensities were assessed by trained judges on a rating scale from 0 to 5 for all the descriptors. Overall quality was rated on a scale from 1 to 3, where 3 is high and 1 is low. Figure 8 shows the sensory profile of the samples.

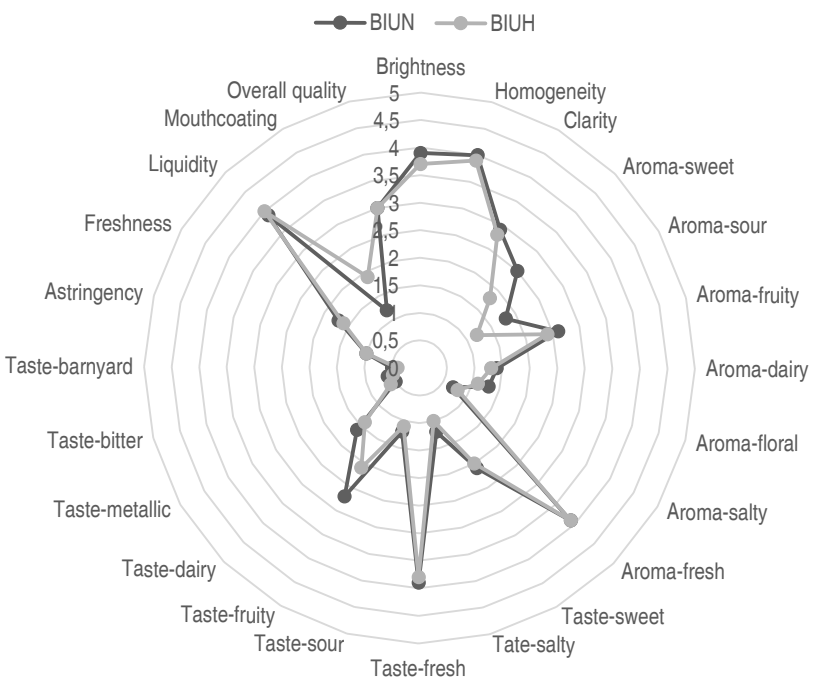

Figure 8. Sensory profile by multidimensional approximation for BIUN vs. BIUH samples.

The BIUN beverage sample presented a fruity flavor of cape gooseberry, yellow fruits, sweet granadilla, and passion fruit, and a balanced flavor. In the first olfactory phase, fruity notes of passion fruit, and dairy, a fruity smell similar to soursop were perceived. The fruity smell was more intense than the taste. The beverage was of high overall quality and had a good balance of notes. The BIUH beverage sample presented fruity notes of cape gooseberry, granadilla, passion fruit, and a slight barnyard flavor that persists over time. The olfactory perception of the fruity attribute was higher than the taste; metallic taste, residual milky notes, and fewer astringent sensations were perceived. Statistically significant differences ($P<0.05$) were found in the attributes of sweet and sour aromas, fruity flavor, and mouth coating, thus obtaining a higher score for the attributes described above in the non-hydrolyzed isotonic beverage sample. Beucler et al. (2006) and Nemati et al. (2020) compared sensory attributes of hydrolyzed and non-hydrolyzed whey permeate and found significant differences in acid, sweet, and astringent taste.

CONCLUSION

The degree of hydrolysis of the whey permeates significantly affected the osmolality of the beverages, this being higher for the hydrolyzed beverage during the entire storage time. Differences in calcium and magnesium content were also found due to the possible interactions of lactase with these minerals. On the sensory profile, the non-hydrolyzed beverage obtained a higher rating in attributes such as sweet and acid smell, fruity flavor, and mouth coating. During storage, the behavior of the beverages was not affected by the degree of hydrolysis, and both beverages presented good physicochemical and microbiological stability but with a tendency to lower the antioxidant capacity properties.

Given the popularity of sports drinks among teenagers and adults, increasing urbanization rates and the proliferation of fitness centers, future studies can be directed to explore new hydrolytic process in whey together the addition of other fruits and vegetables juices or aromatic herbs.

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Squash pulp as a source of carotenoids and dietary fiber in dried handmade spaghetti

Pulpa de calabaza como fuente de carotenoides y fibra dietética en espagueti artesanal seco

https://doi.org/10.15446/rfnam.v77n2.107321

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ABSTRACT

The aim of this study was to evaluate the effect of different levels of dehydrated squash pulp (DSP) (0, 5, 10, 15, and 25 g·100 g^{-1} of flour) and drying temperatures (50 and 60 °C) on the quality characteristics of spaghetti, i.e., dietary fiber, total carotenoid content, color, cooking quality, texture parameters, and sensory acceptance. The results showed that DSP has a total carotenoid content of 44.42 to 277.90 µg·g^{-1} and fiber content of 2.45 to 12.40 g·100 g^{-1}. Additionally, the DSP level decreased the cooking time, increased the loss of solids, and generated a decrease in the fracture, hardness, and elasticity of the spaghetti. Furthermore, the increase from 50 to 60 °C in the drying temperature increased the content of carotenoids up to 6.4%, improving the texture properties while not significantly affecting the cooking quality. The spaghetti formulations containing 5 and 10 g·100 g^{-1} of DSP resulted in improved sensory acceptance. It was possible to develop a new spaghetti type by adding dehydrated squash pulp with better nutritional characteristics such as high dietary fiber content and antioxidants.

Keywords:
Antioxidants
Cooking quality
Drying
Spaghetti
Squash pulp
Texture

RESUMEN

El objetivo de este estudio fue evaluar el efecto de diferentes niveles de pulpa de calabaza deshidratada (DSP) (0, 5, 10, 15 y 25 g·100 g^{-1} de harina) y temperaturas de secado (50 y 60 °C) sobre las características de calidad de los espaguetis, fibra dietética, contenido total de carotenoides, color, calidad de cocción, parámetros de textura y aceptación sensorial. Los resultados mostraron que la DSP tiene un contenido de carotenoides totales de 44,42 a 277,90 µg g^{-1} y contenido de fibra de 2,45 a 12,40 g·100 g^{-1}. El nivel de DSP disminuyó el tiempo de cocción, aumentó la pérdida de sólidos y generó una disminución en la fractura, dureza y elasticidad de los espaguetis. Además, el aumento de 50 a 60 °C en la temperatura de secado incrementó el contenido de carotenoides hasta 6,40%. Mejoró las propiedades de textura sin afectar significativamente la calidad de cocción. Las formulaciones de espagueti que contenían 5 y 10 g de DSP 100 g^{-1} resultaron en mejor aceptación sensorial. Se logró desarrollar una nueva formulación de espaguetis adicionando pulpa de calabaza deshidratada con mejores características nutricionales como alto contenido de fibra dietética y antioxidantes.

Palabras clave:
Antioxidantes
Calidad de cocción
Secado
Espagueti
Pulpa de calabaza
Textura

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Pasta is a product obtained from a dough composed of semolina and water, which is subjected to mixing, extrusion, and dehydration processes (in some cases). The quality of spaghetti depends not only on the quality of the raw materials but also on the processing conditions (Giannetti et al. 2014). Good quality pasta is resistant to cooking, firm, and has low adherence. Pasta is also known for its low nutritional value and dietary fiber content. Durum wheat semolina used in pasta can be enriched with legume flour to produce more nutritious but still high-quality pasta (Ahmad et al. 2018). Because of that, research into spaghetti has focused on three main aspects: extrusion conditions, total or partial replacement of semolina with non-conventional flours, and drying conditions.

The first aspect is important because it influences the final quality. It gives the product greater stability during storage and affects physical characteristics, such as color, texture, cooking quality, and sensory quality. The second aspect can increase nutritional quality since pasta is considered an unbalanced food because of its low contents of fat, dietary fiber, and protein with biological value (lysine and threonine deficiency). Several studies have been carried out on the formulation of spaghetti with unconventional flours (Ahmad et al. 2018; Fiorda et al. 2013; Gull et al. 2015; López-Mejía and Morales-Posada 2020). Various studies have reported the use of dehydrated and ground squash pulp as a partial substitute for wheat semolina due to its contents of protein (4.91–9.63 g 100 g^{-1}), dietary fiber (3.72–35.32 g 100 g^{-1}), minerals (6.01–7.39 g 100 g^{-1}) (Aziah Noor and Komathi 2009; Aydin and Duygu 2015; Mirhosseini et al. 2015), carotenoids (2.82–9.20 mg 100 g^{-1}), and phenolic compounds, as well as its high antioxidant activity (Que et al. 2008) and pleasant orange color.

Different studies have shown that various benefits are obtained by replacing conventional flour with pumpkin pulp flour in pasta formulation. One of the most outstanding is the significant nutritional contribution this plant material provides to the product. Pumpkin pulp presents high alpha and beta-carotene (pro-vitamin) content and high antioxidant capacity (López-Mejía et al. 2020; Buzigi et al. 2021), reducing the risk of degenerative diseases, cardiovascular diseases, cataracts, and certain carcinomas (Buzigi et al. 2021). Moreover, it has been mentioned that it increases the mineral and fiber content, especially the soluble type. Furthermore, pumpkin pulp can help improve important sensory attributes, such as color as it increases redness (a*) and yellowness (b*) due to carotenoid pigments. An increase in flavor acceptance has also been reported. The texture remains acceptable and similar to the samples where wheat was not substituted as long as the DSP (dehydrated squash pulp) addition level in the formulation does not exceed 25–30% (Mirhosseini et al. 2015; Novita Indrianti et al. 2021). However, the partial or total replacement of wheat flour with this vegetable flour remains challenging because some adverse effects on the quality of the pasta are observed. Among them is the decrease in the amount of protein and starch, thus lowering the gluten concentration, which is also interrupted by the presence of fibers. This leads to the absence of a stable network that allows the trapping of starch granules, which leads to increased cooking losses, decreased cohesion and elasticity, and increased adhesiveness. Thus, formulated pasta dough with 50% or more non-wheat flour does not have an appropriate texture for the laminating process (Mirhosseini et al. 2015; Novita Indrianti et al. 2021).

The last aspect, regarding drying, is important because the dehydration process induces the polymerization of the protein network, which, by forming strongly and continuously, prevents the release of the starch exudate and reduces the stickiness of the pasta. Additionally, the drying temperature induces changes in the starch structure, which presents greater damage in dry pasta dried at low temperatures, influencing the adhesiveness and hardness. For this reason, it is considered that pasta dried at high temperatures has a higher cooking quality than pasta dried at low temperatures (Masato et al. 2021), which is one of the most important characteristics for consumers. However, in fortified pasta with alternative flours as an important source of β-carotene, low temperatures (30-60 °C) should be considered to increase the retention of the bioactive compound (Larrosa et al. 2016). Despite the importance of this unit operation in the final quality of spaghetti, few studies that analyze the effect of drying temperature in pasta made from wheat semolina fortified with vegetable flours of high nutritional value have been carried out. Therefore, the aim of this research was to evaluate the effect of low-temperature drying on the properties that determine the quality of spaghetti enriched with dehydrated squash pulp at different levels (5, 10, 15, and 25 g DSP100 g^{-1} of flour).

MATERIALS AND METHODS

Materials

Fresh squashes (*Cucurbita moschata* var. Bolo verde) were purchased in a local market (Cavasa, Candelaria, Palmira, Valle del Cauca, Colombia) and processed to obtain dehydrated pulp. They were conditioned and dried following the methodology reported by López-Mejía and Morales-Posada (2020). The milled durum wheat semolina used was of Italian origin (La Molisana) with a protein ratio of 14 g 100 g^{-1}. All reagents mentioned in this study were analytical grade (95 and 99% v v^{-1}).

Spaghetti preparation

The base spaghetti formulation consists of two components: flour (wheat flour and/or dehydrated squash pulp) (65 g 100 g^{-1} mix) and distilled water (35 g 100 g^{-1} mix). The incorporation of DSP was added in the following amounts: 5, 10, 15, and 25 g 100 g^{-1} of flour. Each component was weighed on a precision balance (Mettler Toledo, PB1502, Switzerland) and mixed manually to achieve a uniform appearance. Subsequently, distilled water was added, and the mixture was kneaded for 15 min until an elastic mass was obtained, which was left to rest for 1 h. Next, the dough was cut with a manual machine (Imperia, Italy, 2015) and length=150.50±0.55 mm.

Spaghetti drying

The drying operation was carried out in a climatic chamber (Memmert, ICH 260L, Germany) under two conditions [Temperature (°C)/Relative Humidity (RH)]: Condition 1) 50 °C/50% RH (30 min) (stage I), 50 °C/70% RH (6 h) (stage II) and 40 °C/50% RH (12 h) (stage III); and Condition 2) 50 °C/50% RH (30 min) (stage I), 60 °C/70% RH (6 h) (stage II) and 40 °C/50% RH (12 h) (stage III). Each drying condition was carried out in triplicate, with an initial load of 300±1 g (moisture content of 35±0.70 g 100 g^{-1}). For each condition, drying ramps were used, carried out in three stages. Stage I was called pre-drying, where the moisture content of the spaghetti was reduced by up to about 20%; this was carried out at a low temperature to avoid cracking the surface and for a short time to prevent the development of fungi. Stage II was called drying and was carried out at a higher temperature than the pre-drying and lasted approximately 5 to 6 h until a moisture content of 12±1 g 100 g^{-1} (w.b.) was achieved. Stage III was called stabilization and consisted of keeping the spaghetti at low temperature and relative humidity conditions to stabilize it for later packaging and storage.

Spaghetti quality

Proximal analysis

Moisture, protein, dietary fiber, fat, and ash contents were determined using AOAC (2005) methods. They were measured in triplicate and expressed on a dry basis.

Total carotenoid content and color

The quantification of total carotenoids (TC) (µg g^{-1}) (dry-based) was performed in fresh, dry, and cooked spaghetti in triplicate. Absorbance at 450 nm (Abs450) was measured on a spectrophotometer (Jenway, 6320D, United Kingdom). The TC concentration concerning the β-carotene concentration was calculated using the equation of Lambert-Beer, with a coefficient of β-carotene in hexane ($E^{1\%}_{1cm}$) of 2,592 (Rodríguez-Amaya 2001). The percentage of total carotenoid retention (TCR) was calculated by the difference from the initial content (fresh spaghetti) (equation 1).

$$TCR = \frac{TC_0 - TC_i}{TC_0} \times 100 \quad (1)$$

Where TC_0 is the total carotenoid content before spaghetti processing (drying and cooking) and TC_i is the total carotenoid content after spaghetti processing (drying and cooking).

The color parameters CIELAB, L* (luminosity: 0–100); b* (color coordinate which indicates yellow + and blue -); a* (color coordinate which indicates red + and green -) were measured using a colorimeter (Konica Minolta, CR-400, Japan) previously calibrated (Y=89.5, X=0.3166, and Y=0.3347). The Chroma (equation 2), tone angle (h°) (equation 3), and color difference regarding dry spaghetti ΔE (equation 4) were calculated. L^*_0, a^*_0 and b^*_0 were measured in fresh spaghetti; and L*, a*, and b* were measured in dry spaghetti.

$$Chroma = \sqrt{a^{*2} + b^{*2}} \quad (2)$$

$$^{\circ}h = artg\frac{b^*}{a^*_0} \quad (3)$$

$$\Delta E = \sqrt{(a^* - a^*_0)^2 + (b^* - b^*_0)^2 + (L^* - L^*_0)^2} \quad (4)$$

Cooking quality

The spaghetti cooking tests were carried out in triplicate for each formulation, according to AACC International Methods (AACC 66-50.01 2010) with some modifications. The tests consisted of immersing 25 g of dried pasta in 300 mL of distilled boiling water (98 °C). The cooking time was the time it took for the spaghetti to be cooked *al dente* (representing pasta with some presence of ungelatinized starch in the inner core). The weight gain (water absorbed by the structure) was determined by the weight difference before and after cooking and was reported as a gram of water 100 g^{-1} of sample. Finally, the solids lost by cooking were evaluated. After cooking, the water level was brought to the initial volume. The dry matter was determined from a sample of 25 mL of cooking water subjected to 105 °C to a constant weight in a furnace (Raypa, EV-50, Spain). The result was expressed as g of solids 100 g^{-1} of sample.

Texture analysis

Fracture tests were carried out on dry spaghetti, and double-cycle compression tests were performed on cooked spaghetti using a texturometer (Shimadzu, EZ-S Test, Japan). The fracture test was performed by incorporating a segment of dry spaghetti on a bending bridge (lower broken core jig 346-51818-01) with an opening of 4 cm, and a compression force was exerted in the center with a probe (toothed pushrod B 1 pc 346-51814-02) at a speed of 30 mm min^{-1} until fracture. The samples consisted of cylinders (1.91±0.15 mm x 70±5 mm) of dry spaghetti (MC=9.10±1.07 g 100 g^{-1}). The breaking force was measured as the maximum peak and the distance at which the sample fractured. The breaking force (σfracture) was calculated using equation 5. The diameter of the sample (1.91±0.15 mm) was measured with an electronic digital calibrator (Mitutoyo, Japan). For the double compression cycle test, the same probe was used. For each test, four strands of spaghetti *al dente* with a diameter of 2.50±1.05 mm were used. The samples were placed perpendicularly to the probe in a base cutting test jig (346-51817-01). The speed of analysis was 30 mm min^{-1}, reaching 100% compression. Through the analysis, six textural parameters were obtained: fracture (N mm^{-2}), hardness (N), cohesiveness (dimensionless), elasticity (dimensionless), gumminess (N), and chewiness (N). The graphs were processed with Origin 8.0 (USA).

$$\sigma\text{fracture}\,(\text{N mm}^{-2}) = \frac{\text{FxL}}{\pi \text{xR}^3} \qquad (5)$$

Where L is the distance between the two supports of the bending bridge (mm); R is the radius of the spaghetti sample (mm); and F is the maximum force at the fracture (N).

Sensory analysis

The spaghetti samples cooked *al dente* were subjected to sensory evaluation by a semi-trained panel of 20 people (9 women and 11 men), aged between 23 and 50. For this analysis, evaluators were selected based on their health status, availability, and fondness for the product (ICONTEC: GTC 165, 2007). Once pre-selected, they underwent screening tests for basic tastes, odors, and colors, and were subjected to paired, duo-trio, and triangular tests with problem substances. The outcomes of these tests were scrutinized using sequential analysis according to ICONTEC: NTC 5278 (2004). Upon selecting the panelists, trials were conducted with the type of sample and test to be applied, ensuring that the results were consistent. Attributes such as color, brightness, odor, elasticity, adhesiveness, firmness, and taste were evaluated in 8 g samples served at room temperature (28 °C) on white polystyrene plates in an illuminated and odor-free space. The rating scale was from 1 to 5, where 5 meant "I like it a lot," 4 "I somewhat like it," 3 "I neither like nor dislike it," 2 "I somewhat dislike it," and 1 "I dislike it a lot." To qualify elasticity, the evaluator was asked to take a piece of spaghetti with both hands and exert contrary forces until it split. For adhesiveness, two pieces of spaghetti were joined and then separated. Finally, firmness was evaluated as the necessary force exerted by the teeth to divide the sample. In this analysis, the attribute was acceptable when the rating was greater than 3. The intent to purchase was estimated by asking the panelists if they would buy the product, to which they responded with "yes," "no," or "maybe," and a comment section was included. Each spaghetti formulation was evaluated on a different day.

Experiment design and statistical analysis

A completely randomized factorial design was carried out to analyze the effect of the drying temperature and the addition of DSP on the spaghetti quality characteristics. A two-way analysis of variance and Tukey's test (P<0.05)

were developed using SPSS. For this purpose, the data were subjected to tests of normality and homogeneity in order to ensure statistical inference. Furthermore, a Pearson correlation test was carried out to analyze the correlation between the percentage of DSP on humidity content and the correlation between sensory attributes evaluated in the product.

RESULTS AND DISCUSSION

Proximal analysis

The DSP flour was characterized by obtaining values of moisture content (13.08±0.07 g 100 g^{-1}), dietary fiber (30.02 g 100 g^{-1}), protein (13.87±0.05 g 100 g^{-1}), ash (8.89±0.10 g 100 g^{-1}), total carotenoids (452.04 µg g^{-1}), phenolic compounds (415±9.19 mg GAE 100 g^{-1}), and CIE L* a* b* coordinates of L*=71.50±0.03, a*=13.60±0.30, and b*=34.72±0.16. According to the analysis of variance, temperature only had a significant effect on the moisture content (P<0.05), while DSP had a highly significant effect on all parameters measured in the proximal analysis (P<0.0001). The interaction between the factors did not significantly affect these variables. According to the homogeneous subset test carried out with the DSP factor, when analyzing the moisture content, there were no significant differences between the control and DSP-5, DSP-10, and DSP-15. At the same time, DSP-25 was significantly higher than the control. This result was attributed to a higher proportion of hydrophilic compounds provided by the DSP (carbohydrates and proteins). This could be corroborated using the Pearson correlation test, in which a value of r=0.457 was obtained (P<0.05). Finally, the moisture content values obtained for each formulation were found to have a maximum value of 13% (d.b.).

For a fixed drying time of 6 h, an inversely proportional relationship was evident between the drying temperature and moisture content (Table 1), which could be corroborated by the Pearson correlation test performed, which revealed a highly significant value of r (r=-0.556) (P<0.01). The moisture content decreased when the drying temperature increased from 50 to 60 °C. This result is related to the increase in collision energy between molecules due to the increase in temperature, which increases the diffusivity of the water toward the surrounding medium.

Table 1. Physicochemical properties of the spaghetti on a dry basis as a function of % DSP and drying temperature.

T (°C)	DSP (g 100 g^{-1} flour)	Moisture content (g 100 g^{-1})	Protein Content (g 100 g^{-1})	Dietary Fiber (g 100 g^{-1})	Raw Fat (g 100 g^{-1})	Ash Content (g 100 g^{-1})
50	0 (control)	10.29±0.07^{a}	19.10±0.21^{c}	0.52±0.03^{a}	1.06±0.13^{a}	1.15±0.06^{a}
	5	11.14±0.07^{a}	18.43±0.42^{b}	2.50±0.02^{b}	1.22±0.01^{b}	1.48±0.07^{b}
	10	10.17±1.40^{a}	17.96±0.40^{a}	4.96±0.11^{c}	11.42±0.04^{c}	2.10±0.13^{c}
	15	10.34±0.63^{ab}	18.26±0.31^{a}	7.37±0.12^{d}	1.80±0.03^{d}	2.88±0.08^{d}
	25	11.43±0.06^{b}	17.75±0.06^{a}	12.38±0.13^{f}	2.31±0.03^{f}	3.91±0.13^{f}
60	0	8.40±0.08^{a}	19.34±0.25^{c}	0.51±0.02^{a}	1.02±0.02^{a}	1.19±0.15^{a}
	5	8.38±0.01^{a}	18.79±0.07^{b}	2.45±0.03^{b}	1.17±0.01^{b}	1.39±0.06^{b}
	10	8.61±0.11^{a}	17.50±0.04^{a}	4.88±0.09^{c}	1.37±0,06^{c}	1.87±0.05^{c}
	15	9.35±0.50^{ab}	17.60±0.28^{a}	7.35±0.16^{d}	1.79±0.03^{d}	2.79±0.15^{d}
	25	10.95±1.93^{b}	17.59±0.59^{a}	12.40±0.44^{f}	2.35±0.09^{f}	3.81±0.08^{f}

T: Temperature (°C); DSP: Dehydrated squash pulp incorporated in the spaghetti formula (g 100 g^{-1} flour). The data correspond to the average ± standard deviation (SD) (n=3). Different letters in the same column indicate significant differences (P<0.05, Tukey's test) due to the effect of the DSP.

Concerning protein content, it was observed that DSP-10, DSP-15, and DSP-25 were not significantly different, although they were significantly lower than the control. At the same time, DSP-5 was significantly higher than all DSP formulations but lower than the control. In contrast, fat increased significantly from 1.17 to 2.35 g 100 g^{-1}, which indicated the contribution of fatty acids that raw vegetable material provides to the formulation. A similar trend was evidenced for ash content due to the richness in minerals such as calcium, phosphorous, potassium,

and zinc that DSP provides to the formulation (Ponka et al. 2015). This trend was also observed for dietary fiber: up to 25 times higher in DSP-25 than the control.

Color and total carotenoid content

According to the analysis of variance, the factors, both independently and in their interaction, had a highly significant effect on the color CIEL* a* b* coordinates (P<0.001). However, temperature did not have a significant effect on the total carotenoid content (P>0.05). A similar result was reported by Hidalgo et al. (2010), who found no significant loss of this bioactive compound during the drying of pasta. The greatest loss occurs during the extrusion process (a stage that was not considered in this study since it was handmade pasta). In contrast, DSP significantly influenced this variable (P<0.05). DSP had a significant effect on the total carotenoid content of the spaghetti. The added DSP is rich in carotenoid pigments, such as β-carotene, which provide functional properties and nutritionally enrich the product. The spaghetti obtained in this study had a total carotenoid content ranging from 44.42 to 277.90 µg g^{-1}, and the total carotenoid content of DSP-25 was 12 times higher than that of the control.

Analyzing the values of the a* coordinate that expresses the reddish tones for positive values, it was observed that this increases significantly with the addition of DSP, presenting the highest value in DSP-25. On the other hand, the b* coordinate that expresses yellow tones for positive values decreases significantly with increasing DSP, lowering the yellow tone. This behavior is related to the increase in total carotenoid content in the formulation, reaching a value of 269.52 µg g^{-1} for DSP-25. The above is reflected in the values of °h and Chroma, since °h decreases, approaching orange tones, and Chroma also decreases, meaning that the color reduces its purity (Table 2). Moreover, when analyzing the effect of temperature on these coordinates, a decrease in L*, a*, and b* was observed, indicating an increase in orange tones. This is attributed to a greater elimination of moisture, which leads to the carotenoids becoming more concentrated in the food matrix. The ΔE of the fresh samples decreases due to the effect of temperature, while it increases due to the effect of DSP (ΔE=17.77–23.88: 50 °C; 17.20–22.19 60 °C). It was noted that the DSP-25 spaghetti had a dark brown tonality, which could be attributed to the Maillard reactions caused by the thermal treatment and sugar-sugar-amino acid interactions.

Table 2. CIEL* a* b* color parameters and total carotenoid content measured in the dried pasta.

T(°C)	DSP	L*	a*	b*	C	°h	TC
50	0 (control)	57.63±1.81^{C}	0.59±0.02^{A}	44.39±1.40^{C}	44.39±1.40^{D}	89.23±0.00^{E}	22.39±3.63^{A}
	5	49.17±0.01^{B}	3.07±0.00^{B}	37.87±0.01^{B}	37.99±0.01^{BC}	85.68±0.01^{D}	44.42±5.29^{A}
	10	47.81±0.01^{B}	4.46±0.00^{C}	36.82±0.01^{B}	37.09±0.01^{C}	83.12±0.06^{C}	110.56±5.98^{B}
	15	46.15±0.08^{A}	5.84±0.01^{D}	35.54±0.06^{A}	36.02±0.06^{A}	80.66±0.02^{B}	139.49±11.86^{C}
	25	45.15±0.01^{A}	8.15±0.00^{E}	34.77±0.01^{A}	35.71±0.01^{AB}	76.81±0.00^{A}	261.15±26.57^{D}
60	0 (control)	58.12±3.23^{C}	0.60±0.03^{A}	44.76±2.48^{C}	44.76±2.48^{D}	89.23±0.00^{E}	23.33±3.63^{A}
	5	45.33±0.41^{B}	2.34±0.02^{B}	34.91±0.32^{B}	34.99±0.32^{BC}	86.32±0.12^{D}	44.75±4.30^{A}
	10	47.75±0.01^{B}	4.84±0.00^{C}	36.77±0.01^{B}	37.09±0.01^{C}	82.76±0.00^{C}	101.02±4.85^{B}
	15	43.21±0.01^{A}	5.06±0.00^{D}	33.28±0.01^{A}	33.66±0.01^{A}	81.51±0.00^{B}	150.40±28.45^{C}
	25	43.49±0.01^{A}	8.23±0.00^{E}	33.49±0.01^{A}	34.49±0.01^{AB}	76.16±0.00^{A}	277.90±32.42^{D}

T: Temperature (°C); DSP: dehydrated squash pulp (g 100g^{-1} flour); C: Color saturation; °h: Hue Angle; and TC: Total Carotenoids (µg g^{-1}). The data correspond to the average ± standard deviation (n=3). Different letters in the same line indicate significant differences (P<0.05, Tukey's test) because of the effect of the DSP.

Cooking quality

According to the analysis of variance, temperature did not have a significant effect (P<0.05) on total carotenoid retention (TCR) and solids lost in the cooking water (SP). In contrast, it significantly affected cooking time and weight gain (P<0.05). On the other hand, the DSP factor

significantly (P<0.05) affected all response variables and the interaction between both factors (T * DSP).

The effect of DSP on the test of homogeneous subsets showed that TCR increased as the DSP value decreased in the formulation, reaching values of 90.4% for the control sample, while for DSP-25, it obtained values of 22.6%. This can be explained by the increase in the concentration of carotenoids in the matrix, meaning that these are more available to leach into the liquid cooking medium since it is also at boiling temperature. Likewise, the increase in DSP leads to a weakening of the network that forms the protein and starch, facilitating the loss of solids in the cooking water. Therefore, this decrease in TCR values is not necessarily related to degradation by the temperature of the cooking water. Indeed, Oduro-Obeng et al. (2021) mention an increase in the availability of these compounds due to cooking, which also increases their accessibility after consumption. Additionally, although it was not measured in this study, an isomerization of the pigment could have occurred, mainly due to the effect of the applied heat treatment and exposure to light, which also influences its bioavailability (Khoo et al. 2011).

Spaghetti cooking time varied from 3 to 6 min. This time was shorter than that reported by Minarovičová et al. (2018), who obtained values between 5.9 and 7 min. This difference depends on many factors, such as the level of substitution of wheat flour by DSP, the thickness of the spaghetti, the conditions of pasta production (extrusion and drying), the amount of starch, and the proportion of the rest of the components, such as protein and dietary fiber. Regarding the effect of adding DSP, a decrease in cooking time was observed when the level of DSP was increased. This behavior was similar for both drying temperatures (50 and 60 °C). However, the cooking time was greater in spaghetti dried at 50 °C using the lower contents of DSP (5 and 10 g 100 g^{-1} flour). Piwińska et al. (2016) observed no significant variation in pasta samples dried at different temperatures, reporting a cooking time of 6.50 min for each drying condition (50 and 60 °C). This response variable allowed us to detect that the DSP requires less cooking time (3–6 min) than the traditional formula (100% wheat flour) (8–15 min). Similar results were obtained by Padalino et al. (2013), who reported a cooking time of 7 min for spaghetti produced with yellow pepper flour (15% w w^{-1}), and by López-Mejía and Morales-Posada (2020), who reported a decrease in cooking time due to the effect of adding DSP, reaching values of 5.12, 6.90, and 7.25 min. This may be due to the physical disruption of the gluten matrix caused by the fiber, facilitating water absorption and consequently decreasing cooking time.

Concerning weight gain, the values obtained (0.90–1.35 g g^{-1}) were lower than those reported by López-Mejía and Morales-Posada (2020) (1.38–1.78 g g^{-1}). The differences with other authors may be attributed to the particular characteristics of DSP since the dehydrated pulp used by Minarovičová et al. (2018) had a lower protein content (8.20±0.10 g 100 g^{-1}) than the one used (13.87±0.05 g 100 g^{-1}) in this study, which means that the obtained DSP can help to form a greater protein network, thus helping to maintain the integrity of the starch while it swells during cooking. On the other hand, the effect of DSP on weight gain was significant when the level of DSP was increased from 15 to 25 g 100 g^{-1}. However, lower weight gain values were obtained for spaghetti dried at 60 °C. According to Piwińska et al. (2016), this behavior could be because temperature promotes a stronger protein network formation, further limiting starch swelling. In contrast, pasta with 100% wheat flour presents weight gain values of 1.76–2.02 g g^{-1} greater than those obtained in this study due to its high level of starch and gluten (Padalino et al. 2013).

Similarly, the solids lost in the cooking water were 0.01 and 0.045 g g^{-1} (Figure 1C), lower than those reported by Minarovičová et al. (2018) (0.05–0.07 g g^{-1}). These values are desirable for the pasta to be considered high quality (0.07–0.08 g g^{-1}) (Añón 2007). Regarding the effect of DSP, it was noted that the solids lost in the cooking water were not significantly different from the DSP-5, DSP-10, and DSP-15 formulations. However, DSP-25 had a significantly higher value (0.045 g g^{-1}). This result was associated with the solubilization of carbohydrates like starch and soluble fiber (pectin) due to the weakening of the network formed by gluten (Brennan 2008).

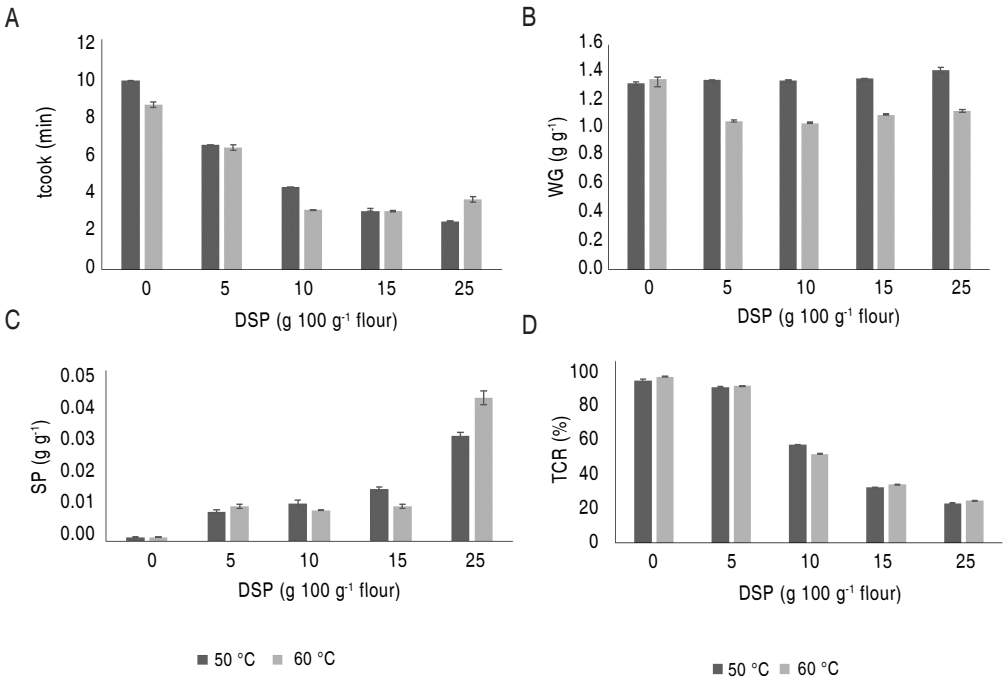

Figure 1. Spaghetti cooking quality as a function of the drying temperature and the percentage of dehydrated squash pulp. A. Cooking Time (t_{cook}), B. Weight Gain (WG), C. Solids Lost by Cooking (SP), and D. Total Carotenoids Retention (TCR). Mean ± SD (n=3).

Texture analysis

Texture is an important part of consumer acceptance and is one of the criteria that defines pasta quality. According to the analysis of variance, both temperature and the DSP had a significant effect (P<0.05) on all the texture variables and the interaction of both factors. Results showed that elasticity and gumminess were higher in the control formulation (DSP-0) (Table 3). The formulations closest to this value were those corresponding to DSP-5 and DSP-15. The other formulations were significantly less elastic and did not show significant differences (Table 3).

Table 3. Dry spaghetti fracture stress and cooked spaghetti texture profile according to drying temperature and DSP addition level.

T (°C)	DSP	Fracture (N·mm^{-2})	Hardness (N)	Cohesiveness	Elasticity	Gumminess (N)	Chewiness (N)
50	0	49.63±0.27^{e}	5.03±0.00^{e}	0.68±0.00^{e}	1.13±0.00^{c}	3.42±0.00^{d}	3.87±0.00^{b}
	5	35.34±0.02^{d}	2.53±0.03^{d}	0.47±0.01^{c}	0.79±0.01^{b}	1.20±0.04^{c}	0.94±0.04^{a}
	10	33.34±0.012^{c}	2.33±0.02^{e}	0.57±0.01^{b}	0.76±0.01^{a}	1.33±0.00^{b}	1.01±0.02^{a}
	15	30.37±0.03^{b}	2.64±0.01^{c}	0.64±0.02^{d}	0.72±0.01^{b}	1.69±0.05^{c}	1.22±0.06^{a}
	25	20.33±0.03^{a}	1.13±0.01^{a}	0.48±0.01^{a}	0.73±0.01^{a}	0.55±0.00^{a}	0.40±0.01^{a}
60	0	55.05±0.06^{e}	8.08±0.13^{e}	0.83±0.03^{e}	1.88±0.10^{c}	6.71±0.30^{d}	12.63±1.06^{b}
	5	40.35±0.01^{d}	2.66±0.01^{e}	0.64±0.01^{c}	0.78±0.01^{b}	1.70±0.02^{c}	1.33±0.00^{a}
	10	38.35±0.01^{c}	2.47±0.11^{b}	0.47±0.01^{b}	0.67±0.03^{a}	1.15±0.02^{b}	0.78±0.05^{a}
	15	33.34±0.02^{b}	2.36±0.01^{c}	0.54±0.01^{d}	0.76±0.02^{ab}	1.27±0.01^{c}	0.96±0.01^{a}
	25	27.34±0.02^{a}	2.41±0.00^{a}	0.47±0.01^{a}	0.66±0.01^{a}	1.13±0.01^{a}	0.75±0.02^{a}

T: Drying temperature (°C); DSP: dehydrated squash pulp (g·100 g^{-1} flour). The data correspond to the average ± standard deviation (n=3). Different letters in the same line indicate significant differences (P<0.05, Tukey's test) because of the effect of the DSP.

Regarding the fracture stress and hardness, all the values obtained for each of the formulations were significantly different, being higher in the control formulation (σfrac=52.34 N·mm^{-2} and hardness=6.56 N). The formulation that was closest to the control was DSP-5 (σfrac=37.84 N·mm^{-2} and hardness=2.59 N). Lastly, the cohesiveness was significantly different in all the formulations, whereby DSP-15 was the closest formulation to the control. In Table 3 shows more clearly the texture parameters evaluated for dried and cooked samples in the function of the percentage of DSP and drying temperature. Independently of the drying temperature, the incorporation of DSP produced a 37% decrease in the breaking force of the dried spaghetti. On the other hand, when the drying temperature was elevated from 50 to 60 °C, a noticeable effect in breaking force was noted, which could be due to an increase in the denaturalization of the proteins, promoting the reticulation of glutenine and gliadine with disulfide bonds. In other words, more disulfide bonds are formed at higher temperatures. This strengthens the network, resulting in the product's characteristic rigidity (Ogawa et al. 2015).

Regarding the elasticity parameter (Table 3), values of 0.67 and 0.79 were obtained, showing a decrease of 15% when the proportion of DSP increased. The remaining texture parameters, such as hardness, cohesiveness, gumminess, and chewiness, also decreased significantly with the increase of DSP. Aukkanit and Sirichokworrakit (2017) also reported that adding DSP increased hardness values and decreased cohesion and elasticity values. The added DSP is rich in fiber and lacks gluten; therefore, the spaghetti structure may be interrupted. Furthermore, electronic microscopies conducted on pasta with DSP showed an increase in the porosity of the transversal section due to the increase of DSP and an increase in the size of the pores, which causes a lack of continuity in the structure (López-Mejía and Morales-Posada 2020).

Additionally, the decrease of cohesivity of the spaghetti in the function of the percentage of DSP increase indicates that the structure's integrity was affected by the inclusion of fibers present in DSP, which yields a less compact structure. This behavior decreased gumminess, indicating the strength needed to break down the food to be ready for swallowing. On the other hand, the drying temperature did show a significant effect on hardness and cohesivity (Table 3). DSP-25 spaghetti dried at 60 °C showed greater hardness and breaking force. This is because the temperature caused the gluten network to increase in strength. However, the effect of drying temperature on hardness cannot be noted in cooking because water absorption diminishes the effect of temperature on the hardness of the spaghetti (Ogawa et al. 2015).

Sensory Acceptance

Sensory evaluation allows the possibility of using alternative raw materials for manufacturing new, enriched, available, and easily obtained food products. Besides having appropriate quantities of proximal composition, a new product must also have good acceptance. If the food is rejected based on its sensory qualities, its consumption will not be considered an option. According to the analysis of intersubject effects, temperature only affected the acceptance of flavor, while DSP affected the acceptance of color, brightness, elasticity, and flavor. The interaction of both factors only had a significant effect on flavor. This was higher in DSP-5 dried at 50 °C.

All formulations evaluated for sensory attributes obtained scores greater than 3, the minimum score for the attributes to be considered acceptable (Table 4). The attributes of color, brightness, elasticity, and flavor presented significant differences between the evaluated spaghetti formulations. The formulations that had greater acceptance in color, brightness, and flavor were DSP-5 and DSP-10. The DSP-5 and DSP-15 formulations had greater acceptance in terms of elasticity. Regarding consumers' intent to purchase, DSP-5 and DSP-10 came closer to category 3 (the consumer would buy), while DSP-15 and DSP-25 came close to category 2 (the consumer might buy). It is important to remember that 10 out of 20 evaluators considered that the samples should be evaluated at the temperature at which the spaghetti should be consumed. This was a technical limitation during the development of the sensory analysis, considering the small size of the sample (8 g) and the speed at which it cooled down.

According to the Pearson correlation test carried out, the products with good color acceptance also presented good scores for brightness (r=0.649; P<0.01) and <elasticity (r=0.406; P<0.01). It was also observed that the products with good adhesiveness presented good firmness (r=0.397; P<0.01). If the correlations are only analyzed regarding intent to purchase, the attributes that influenced the most were color (r=0.545; P<0.01), brightness (r=0.563; P<0.01), and elasticity (r=0.561; P<0.01).

Table 4. Results of the sensory evaluation.

T	DSP	Color	Smell	Brightness	Elasticity	Adhesiveness	Firmness	Flavor	Intent to purchase
50	0	3.13±0.55^{c}	3.40±0.17^{a}	3.53±0.21^{ab}	4.83±0.29^{a}	4.67±0.58^{a}	4.67±0.58^{a}	3.63±0.40^{a}	2.63±0.55^{a}
	5	4.17±0.15^{ab}	4.23±0.71^{a}	4.03±0.15^{a}	4.11±0.35^{ab}	3.13±0.45^{a}	4.03±0.15^{a}	4.27±0.43^{a}	2.74±0.51^{a}
	10	4.38±0.16^{a}	4.05±1.00^{a}	3.63±0.55^{ab}	3.72±0.71^{ab}	3.37±1.27^{a}	3.83±0.57^{a}	3.95±0.52^{a}	2.31±0.65^{a}
	15	3.20±0.88^{abc}	3.30±0.95^{a}	3.02±0.81^{ab}	3.26±0.70^{ab}	3.54±0.68^{a}	3.86±0.56^{a}	3.38±1.20^{a}	1.97±0.74^{a}
	25	3.43±0.62^{bc}	3.07±1.18^{a}	2.53±0.77^{b}	2.84±1.17^{b}	3.47±1.03^{a}	3.76±0.88^{a}	3.07±0.92^{a}	1.86±0.74^{a}
60	0	2.80±0.72^{c}	3.12±0.33^{a}	3.52±0.39^{ab}	4.46±0.45^{a}	3.95±0.42^{a}	4.65±0.58^{a}	3.56±0.52^{a}	2.60±0.42^{a}
	5	4.60±0.06^{ab}	4.00±0.97^{a}	4.15±0.67^{a}	4.35±0.67^{ab}	3.5±1.39^{a}	4.25±0.79^{a}	4.05±0.76^{a}	2.80±0.42^{a}
	10	4.63±0.36^{a}	4.15±1.04^{a}	3.90±0.72^{ab}	3.55±1.10^{ab}	3.57±1.18^{a}	4.30±0.8^{a}	4.20±0.77^{a}	2.60±0.60^{a}
	15	3.55±1.10^{abc}	3.70±1.03^{a}	3.20±0.83^{ab}	3.70±0.80^{ab}	4.10±0.72^{a}	4.15±0.59^{a}	3.55±1.23^{a}	2.15±0.81^{a}
	25	3.20±1.05^{bc}	3.30±1.13^{a}	2.85±0.74^{a}	3.20±1.10^{b}	3.60±0.99^{a}	3.80±0.83^{a}	3.30±0.88^{a}	2.05±0.69^{a}

T: Drying temperature (°C); DSP: dehydrated squash pulp (g·100 g^{-1} flour). The data correspond to the average ± standard deviation (n=20). Different letters in the same line indicate significant differences (P<0.05, Tukey's test) due to the effect of the DSP.

CONCLUSIONS

Increasing the substitution of dehydrated squash pulp (DSP) from 5 to 25 g·100 g^{-1} primarily led to notable improvements in the product's dietary fiber and total carotenoid content. The incorporation of DSP not only enhanced the product's nutritional aspects but also could play a significant role in promoting human health due to its contribution of antioxidant pigments, such as beta-carotene. This precursor to vitamin A is essential for various bodily functions, including maintaining healthy vision, immune system support, and skin health, while also protecting against oxidative stress and reducing the risk of chronic diseases. This demonstrates the potential of DSP-enriched spaghetti as a valuable addition to a balanced diet. Moreover, increasing the proportion of DSP led to a reduction in cooking time for the spaghetti. It is worth noting that spaghetti formulations containing 5 and 10 g·100 g^{-1} of DSP exhibited superior sensory acceptance. Interestingly, consumers preferred pasta dried at 50 °C, highlighting that these DSP levels and drying temperatures are well-suited for creating spaghetti with enhanced nutritional attributes while maintaining taste and ensuring overall consumer satisfaction. Based on the results presented in this work, it is suggested that future studies concentrate on examining whether different varieties of squash or processing methods for Dehydrated Squash Powder yield varying nutritional profiles and sensory qualities. This exploration could offer consumers a wider array of options. Additionally, investigating consumer behavior and preferences in relation to pasta enriched with DSP may provide valuable insights. By focusing on these research domains, the full potential of DSP-enriched products to promote human health and sustainable food systems can be further revealed.

Finally, regarding the study's limitations, it would be crucial for future research to examine the impact of thermal treatments, including drying and cooking, not only on carotenoid retention but also on the formation of isomers that could influence their bioavailability. In addition, it is important to consider various strategies to improve the texture of the pasta, such as incorporating hydrocolloids to counteract the effects of adding dehydrated pumpkin pulp on the ultrastructure. This approach would make it possible to further increase the fiber and carotenoid content, while ensuring optimal sensory acceptance and superior cooking quality.

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Short article

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Bioactive compounds and physicochemical attributes of loquat fruits in Mexico

Compuestos bioactivos y atributos fisicoquímicos de frutos de níspero en México

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Total sugar content

ABSTRACT

The loquat is a fruit found in some regional markets of Mexico, and information on its quality is limited. The physicochemical properties and antioxidant potential of loquat fruit pulp produced in the states of Mexico, Oaxaca, and Veracruz were evaluated. The fresh weight (fw) of the fruits was about 15 g. The acidity of the pulp showed variations between 0.60 and 0.93%. The Total soluble solids values were between 14.5 and 17.3 °Bx. The concentration of total phenols was 3.5 mg g_{dw}^{-1}, and the flavonoids represented 62% of the bioactive compounds. The content of carotenes was higher in fruits from the State of Mexico (75.4 μg EβC g_{dw}^{-1}) that achieved the largest fruit size. In the ABTS and FRAP assays, Veracruz fruits had the highest antioxidant capacity but the smaller fruit size. The total sugar mean content was 6.8% fw. In general, the loquat fruit was of commercial small size, and in the tropical conditions of Veracruz attained high phenolic contents.

Palabras clave:
Capacidad antioxidante
Carotenoides
Eriobotrya japónica
Calidad del fruto
Plántulas de níspero
Contenido de azúcares totales

RESUMEN

El níspero es un fruto que se encuentra en los mercados regionales de México, y la información sobre su calidad es limitada. Se evaluaron las propiedades fisicoquímicas y el potencial antioxidante de la pulpa de frutos de níspero producidos en los Estados de México, Oaxaca y Veracruz. El peso fresco (pf) de los frutos fue de alrededor de 15 g. La acidez de la pulpa presentó variaciones entre 0,60 y 0,93%. Los valores de sólidos solubles totales estuvieron entre 14,5 y 17,3 °Bx. La concentración de fenoles totales fue de 3.5 mg g_{dw}^{-1}, y los flavonoides representaron el 62% del total de compuestos bioactivos. El contenido de carotenoides fue mayor en frutos del Estado de México (75.4 μg EβC g_{dw}^{-1}). En los ensayos ABTS y FRAP, los frutos de Veracruz presentaron la mayor capacidad antioxidante. El contenido medio de azúcar total fue de 6,8% pf. En general, el tamaño de la fruta en términos comerciales fue pequeño, y los frutos de Veracruz alcanzaron el mayor contenido de compuestos fenólicos.

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The loquat (*Eriobotrya japonica* Lindl.), also known as the Japanese plum (Costa et al. 2022), is an evergreen tree belonging to the Rosaceae family, originally native to China (Shah et al. 2023). Its cultivation has extended to various regions including Brazil, India, Japan, and Turkey (Xu and Chen 2011; Costa et al. 2022). In Colombia, loquat trees can be found growing in public urban forests in Bogota (Escobedo et al. 2015) and Medellin (Vergara-Navarro et al. 2007). Across Europe, the fruit is commonly sold in regions markets, while loquat trees adorn home gardens in Valencia, Spain, and Perugia, Italy. In Portugal, it grows in the Algarve region. However, in New Zealand, the loquat has occasionally been considered an invasive species (Tennyson et al. 1997).

This fruit is typically enjoyed fresh and is classified as non-climacteric (Alos et al. 2017). It is also used for the preparation of home remedies, and the chemical composition of its pulp shows anticancer, antiinflammatory, hypoglycemic (Li et al. 2009), antiviral, and hypolipidemic activities (Sagar et al. 2020).

In Mexico, there are few commercial orchards and mainly it grows in home gardens in mild temperate zones and tropical zones. It is produced commercially in the states of Mexico and Oaxaca, but it is widely distributed in the state of Veracruz. Most of the loquat trees in Mexico were propagated by seeds, and there are no cultivars originated and registered in Mexico. The fruit harvest period is from the end of October to January.

Most of the loquats at maturity are yellow and not many trees produce fruit of orange color in México. The volatiles of fruit aroma, total soluble solids (TSS), and titratable acidity (TA), have been used to estimate the optimum maturity of loquat (Shah et al. 2023) but the external color of the fruit has been established as the main determining factor of the harvest season (Chávez-Reyes et al. 2013), and it is attractive at the time of marketing.

The loquat tree has been little studied in Mexico despite that it grows in various climates and soils. In Guatemala, loquat seedling trees have been selected to produce fruits with adequate fruit size and good flavor for supermarkets (Cruz-Castillo et al. 2006), while in Mexico the production is for local markets and self-consumption.

The loquat fruit contains sugars, organic acids, polyphenols, and carotenoids among other compounds (Ding et al. 2001). In the ripe fruit, most of the sugars are fructose, sucrose, glucose, and sorbitol (Xu et al. 2010). The malic acid represents the majority of the loquat organic acids (Famiani et al. 2015).

In Mexico, there are few studies on the phenolic compounds of loquat fruits (Chávez-Reyes et al. 2013). The phenolic compounds are considered the most important antioxidant components in the loquat fresh fruit, and together with the fruit size, color, firmness, sugar content, and organic acids determine its quality parameters (Xu et al. 2014). The phenolic profile and the fruit quality of loquat are influenced by genetic and environmental aspects (Zhou et al. 2011). In Mexico, the evaluation of physicochemical, morphological, and biochemical properties of loquat covering several regions has not been studied.

This study aimed to investigate the morphology, physico-chemical properties, and nutraceutical potential of loquat fruits cultivated in three distinct states of Mexico.

MATERIALS AND METHODS

Selection of trees

The loquat fruits were collected from seedling trees of about 20 years old, randomly selected in three states of the Mexican Republic in the communities of San Agustín Etla, Oaxaca (mild temperate zone); Acultzingo, and Ixhuatlan del Café in Veracruz (tropical zone); and Temascaltepec de González and Coatepec Harinas, State of Mexico (mild temperate zone). The harvest time of mature fruits between October and November 2020 was determined by the yellowish color of the peel (Figure 1).

Morphological characterization of fruits

The polar (PD) and equatorial (ED) diameters were measured with a digital vernier caliper (Mitutoyo model CD-6”CSX). The weight of fresh fruits (FW), skin (FS), pulp (FP), and dry weight of seeds (SW) were recorded using an OHAUS® CS200 digital scale. In addition, the number of seeds per fruit was quantified. Subsequently, the pulp/fruit ratio (FP/FW) was calculated. In total, 960 fruits were evaluated considering 320 fruits in each of the three states of the Mexican Republic.

Figure 1. Loquat fruits were harvested showing a yellow color on their skin.

Physicochemical analysis of the pulp

The total soluble solids of the loquat juice were measured using an Atago thermocompensated refractometer (AOAC 1990). Measurements were made in triplicate and the results were expressed in °Bx. To measure the pH, and acidity, 1 g of the fresh pulp was mixed with distilled water (10 mL) and extracted by vortexing (1 min, 3,000 rpm, in a Vortex synergy, WVR International), sonication was performed by 15 min with an Ultrasonic Cleaner 8890, Cole Parmer®, and incubation in a Prendo® INO-650 M Orbital Incubator by 30 min, at 30 °C. The mixture was centrifuged (15 min, 4,000 rpm, SOLBAT® J-600, Mexico) and the supernatant was transferred to a 10 mL volumetric flask and made up to volume with distilled water. The pH was measured from the extract using a digital potentiometer. The acidity was determined in a 5 mL aliquot of the extract, titrating with 0.01 N NaOH, until reaching a pH=8.0 (AOAC 1990). The acidity results were expressed in percentage (%) of malic acid (Famiani et al. 2015).

Antioxidant properties

The content of total phenols and flavonoids, as well as the antioxidant capacity of the loquat fruit pulp, were determined in extracts. Lyophilized and powdered pulp (0.6 g) were mixed with 80% methanol (25 mL) and extracted by vortexing (3 min, at 3,000 rpm, Vortex synergy, WVR International). Then, after adjusting to pH=3±0.3, the mixture was sonicated (15 min, ultrasonic cleaner 8890, Cole Parmer), incubated (30 min, at 30 °C, Prendo INO-650M Orbital Incubator), and centrifuged (15 min, 4,000 rpm, SOLBAT® J-600 centrifuge, Mexico). The supernatant was calibrated with 80% methanol to obtain a final volume of 25 mL. The extracts were prepared in triplicate and stored in amber bottles under refrigeration. For the analyses, 96-cell microplates were used, the extracts were evaluated in quadruplicate and the absorbances were measured in a multidetector microplate reader with Gen5 software (Biotek Instruments Inc. Winoosky, VT, USA).

The total phenolic content was determined with the Folin-Ciocalteu method (Singleton and Rossi 1965) adapted to microplates. The extract (25 µL) was mixed with distilled water (125 µL), Folin-Ciocalteu reagent (20 µL), and 20% sodium carbonate (30 µL). The reaction mixture was stirred and allowed to stand for 30 min in the absence of light. The calibration curve was prepared with gallic acid (2.5-29.5 µg mL^{-1}). Absorbances were measured at 760 nm.

For the total flavonoid content (mg g_{dw}^{-1}) (Kubola and Siriamornpun 2011), loquat pulp extract (0.5 mL), distilled water (2.5 mL), and 5% $NaNO_2$ (0.15 mL) were mixed and allowed to settle in a falcon tube. $AlCl_3.6H_2O$ (0.3 mL) and 5% NaOH (1 mL) were then added, and vortexed (3,000 rpm, 3 min). The calibration curve was prepared with catechin (5-29.5 µg mL^{-1}). In each cell of a microplate, 200 µL of the reaction mixture were added and the absorbances were measured at 510 nm.

The antioxidant capacity was evaluated by the ABTS and FRAP assays. For the ABTS test (μmol ET g_{dw}^{-1}) (Re at al. 1999) an aliquot of 20 µL of the extract was mixed with 180 µL of the $ABTS^{\bullet+}$ solution, and after 15 min of reaction the absorbance at a wavelength of 734 nm was measured, using $ABTS^{\bullet+}$ (200 µL) as a control. The calibration curve was prepared with trolox (4.99-59.93 µM).

The FRAP assay (Benzie and Strain 1996) was adapted to microplates. An aliquot of 20 µL of standard or sample were mixed with 180 µL of FRAP solution and 60 µL of distilled water. 260 µL of FRAP were used as a blank. The calibration curve was prepared with trolox (3.8-46 µM). Absorbances were measured at 595 nm.

Total sugars

The phenol-sulfuric acid method (Yue et al. 2022) was used. The lyophilized pulp (0.1 g) was diluted in distilled water (50 mL). It was shaken in a vortex (2,500 rpm, 3 min), centrifuged (3,500 rpm, 10 min) and the supernatant was calibrated to 10 mL with distilled water, later dilutions were made in the proportions 1:8, 1:10, 1:15 and 1:20 for the different samples. In glass tubes, the sample extract (300 µL), 5% phenol solution (300 µL) and concentrated sulfuric acid (1.5 mL) were mixed, the mixture was left to stand for 1 h. The calibration curve was prepared with glucose (15.2-75.3 µg mL^{-1}). For the analysis, 96-well microplates with lids were used, in each well 200 µL of the reaction mixture was added and the absorbances were measured at 490 nm (Rao and Pattabiraman 1990) to obtain the total sugars in % fresh weight, considering that the loquats had about 85% of humidity.

Carotenoids

Loquat pulp (0.1 g) was mixed with 10 mL of hexane-acetone 3:2 v/v. The samples were vortexed (3,000 rpm, 1 min) and subsequently incubated (9 min, 30 °C). Finally, the mixture was centrifuged (1277 xg, 15 min) and the supernatant was calibrated to 10 mL with the extraction mixture. The absorbance was measured at 450 nm in a spectrophotometer. The calibration curve was prepared with β-carotene in a concentration range of 0.5-4 µg mL^{-1} (Ordoñez et al. 2009). The carotenes concentration was reported in µmol ET g_{dw}^{-1}.

Statistical analysis

The experiments were analyzed with a completely randomized block design for the three Mexican regions. An ANOVA analysis of variance and comparison of means of treatments was applied in the morphology of fruits, physicochemical and phytochemical properties of the fruit pulp (Tukey $P<0.05$) using the statistical package Infostat version 2015.

RESULTS AND DISCUSSION

Fruit morphology

The PD and ED of the fruits were different for the three regions. The loquat fruit of the State of Mexico, achieved higher length and wide than those of Oaxaca and Veracruz ($P<0.05$). Morton (1987) observed similar fruit lengths to those found in this study, and Aslmoshtaghi and Shahsavar (2013) reported similar values for ED and FW. Higher morphological values in length and wide were found in loquats of Turkey (Okatan et al. 2022). The loquat orchards sampled in the state of Mexico originated from seeds of selected fruit with large fruit size may influenced the large loquat fruit size recorded in the State of Mexico.

In the fruit, the pulp was the main component compared with the skin and the seeds (Table 1). The fruits of Veracruz had lower FW, FP, FS and SW, compared to those of the State of Mexico ($P<0.05$). In contrast, Gentile et al. (2016), and Feng et al. (2007), reported higher values for loquat fruit weights and diameters in fruits from Mediterranean countries and China, respectively. There were significant differences ($P<0.05$) for the FP/FW ratio, and the fruits of Veracruz had the lowest percentages, while those of the State of Mexico, had the highest. Thus, the loquats from Veracruz had less pulp weight, and the seeds and skin attained a higher percentage in the fruits compared with fruits from the other two regions. In general, the FP/FW values are similar to those indicated by Lin et al. (1999). Ercisli et al. (2012), determined that the weight of the fruit pulp is always greater than the seed, representing FP/FW of 80% which is higher than the found in the present study (Table 1).

Loquat fruit size is considered important in the European market. Fruit with large sizes achieve better prices (Costa et al. 2022). According to the quality standards for loquat fruit of the Ministry of Agriculture, Fisheries and Food of Spain (MAPA 1990), the fruits of Oaxaca (14.6 g) and Mexico (19.8 g) were classified as small, and the fruits from Veracruz were inadequate for the

Table 1. Morphological measurements of loquat fruits in three states of the Mexican Republic.

State	PD (mm)	ED (mm)	FW (g)	FS(g)	FP(g)	SW(g)	FP/FW (%)
Oax	32.6±0.3^{bt}	27.4±0.2^{b}	14.6±0.4^{b}	1.9±0.05^{b}	10.1±0.3^{b}	2.5±0.07^{b}	66.9±0.4^{b}
Ver	26.1±0.4^{c}	21.9±0.3^{c}	8.6±0.4^{c}	1.3±0.05^{c}	5.6±0.3^{c}	1.8±0.08^{c}	64.0±0.4^{c}
Mex	35.1±0.3^{a}	30.5±0.2^{a}	19.8±0.3^{a}	2.6±0.04^{a}	14.0±0.3^{a}	3.2±0.06^{a}	70.2±0.3^{a}
HMSD	1.11	0.79	1.26	0.15	1.0	0.23	1.28

[t]Values with the same letter within columns are statistically equal based on Tukey's test (P<0.05) ± standard deviation. Oax: Oaxaca; Ver: Veracruz; Mex: State of Mexico; PD: Polare diameter; ED: equatorial diameter; FW: Fruit weight; FS: Fruit skin; FP: Fruit pulp; SW: seed weight. HMSD: Honest minimum significant difference.

European market. In Turkey (Ozturk and Ozturk 2018), fruit of 16 g is also considered of small size. In Mexico, people are accustomed to consuming small-sized fruits.

The fruits studied were harvested from seedling trees without agronomic management. Practices such as foliar application of B (Ali et al. 2022), fruit thinning (Lin et al. 1999), branch scratching, application of growth regulators (Agustí et al. 2007), introduction of cultivars, and selection of seedling trees with large fruit size (Cruz-Castillo et al. 2006) could support the development of the loquat fruit for markets with better payment. The loquat is not considered a tropical fruit tree. The small fruit size of the loquat fruit from Veracruz may be related to climatic factors, all the trees studied were under tropical conditions from 300 to 1,500 m altitude. The information about loquat fruit growth in the tropics is scarce.

Physicochemical properties

In general, loquat fruit has acidity values between the range of 0.3-0.6% (Dhiman et al. 2021), and the fruits from the Oaxaca State presented 0.6%. The other fruits evaluated had juice acidity between 0.8 and 0.9% (Table 2). The pH values of loquat were similar to those found by Ali et al. (2020). The TSS of the fruits from Veracruz and Oaxaca were similar and higher (P<0.05) than those from the State of Mexico. Similar values of TSS were reported by Hasegawa (2010), Ercisli et al. (2012), Xu et al. (2014), Xu and Chen (2011), and Toker et al. (2013). The total sugar contents

Table 2. Physicochemical measurements of loquat fruits from three different states of the Mexican Republic.

State	TSS (°Bx)	pH	Total sugars (%, FW)	Acidity (%)
Oax	16.6±0.4^{at}	4.1±0.05^{a}	7.48±0.68^{a}	0.6±0.06^{b}
Ver	17.3±0.4^{a}	3.7±0.05^{c}	6.00±1.25^{a}	0.9±0.07^{a}
State of Mex	14.5±0.3^{b}	3.9±0.04^{b}	7.00±0.77^{a}	0.8±0.05^{ab}
HMSD	1.27	0.15	3.37	0.20

[t]Values with the same letter within columns are statistically equal based on Tukey's test (P<0.05) ± standard deviation. HMSD: Honest minimum significant difference.

values obtained for fruit produced from seedling trees in Mexico were slightly less than those found by Hasegawa et al. (2010) in Brazilian cultivars.

Bioactive compounds and antioxidant capacity

The fruits from Veracruz and the State of Mexico presented higher total phenolic content (TP) (P<0.05). The flavonoids (TF) were higher in loquat fruits from Veracruz, and this compound represented more than 50% of the total phenolic content (Table 3). The highest values of the ABTS test corresponded to the fruits of Veracruz. The values determined by FRAP were similar for the fruit in the three states of the Mexican Republic (P<0.05) (Table 3).

In general, values for the antioxidant capacity, total content of phenols, and flavonoids were similar to those obtained in China by Xu et al. (2014), and Xu and Chen (2011), and in fruits of a local market in Mexico City (Chávez-Reyes et al. 2013). The concentration of total phenolic compounds in this study were similar to that reported by Ercisli et al. (2012), and Chávez-Reyes et al. (2013). Rivas et al. (2020), showed that there is a positive correlation between the

total phenolic content, flavonoids and antioxidant capacity, therefore, these compounds contributed greatly to the antioxidant capacity. In the present study, a trend of greater antioxidant capacity was observed as the phenolic content increased, especially in the fruits from Veracruz and the State of Mexico (Table 3).

Table 3. Content of total phenols (TP), total flavonoids (TF) and antioxidant capacity (FRAP and ABTS), total sugars, and total carotenoids in three states of Mexico.

State	TP (mg g_{dw}^{-1})	TF (mg g_{dw}^{-1})	ABTS (μmol ET g_{dw}^{-1})	FRAP (μmol ET g_{dw}^{-1})	Carotenoids (μg g_{dw}^{-1})
Oax	3.2±0.1[bt]	2.1±0.1[b]	24.5±0.9[b]	20.2±1.1[a]	49.5±6.5[ab]
Ver	3.8±0.2[a]	2.6±0.2[a]	28.2±1.4[a]	22.5±1.5[a]	30.3±8.4[b]
Mex	3.8±0.1[a]	2.1±0.1[b]	23.9±0.8[b]	20.6±0.9[a]	75.4±7.3[a]
HMSD	0.4	0.3	3.5	4.1	28.3

[t]Values with the same letter within columns are statistically equal based on Tukey's test (P<0.05) ± standard deviation. HMSD: Honest minimum significant difference.

The variation in the phenolic content can be influenced by environmental factors (Zhou et al. 2011; Friedman et al. 2009; Arámbula et al. 2010). In the present study, fruits from Veracruz under tropical conditions had the smallest size, but higher concentrations of phytochemicals (Table 3). Thus, a breeding program to increase fruit size will allow better characteristics of fruits from Veracruz. Loquat trees are adapted to subtropical or mild temperate climates (Costa et al. 2022), and their growth in tropical conditions may provoke stress and an increase of phenolic compounds in the fruit (Bryant and Julkunen-Tiitto 1995; Gershenzon 1984; Okatan et al. 2022).

Total carotene content (Table 3) was higher in the loquat fruits of the State of Mexico. Ercisli et al. (2012) and Ferreira et al. (2009) found similar values for total carotene content. In the loquat pulp the carotene responsible for the yellow and orange colors are β-carotene and β-cryptoxanthin (Ding 1998). The synthesis of carotene and its accumulation in the fruit is influenced by environmental factors (Costa et al. 2022) and occurs during fruit ripening. When the content of carotenes in the pulp of the loquat fruit increases, the acidity decreases, and the total soluble solids and the content of glucose, sucrose, fructose and sorbitol, abscisic acid, increase (González et al. 2003; Shah et al. 2023).

CONCLUSION

The loquat fruits harvested in the State of Mexico had the larger weight and dimensions but had medium size according to international commercial standards. This fruit also had low TSS. In contrast, the loquat fruits from Veracruz that showed smallest size showed higher TSS and higher concentrations of phenolic compounds and antioxidant capacity. However, the content of carotenes was lower. Loquat fruits from Veracruz and the State of Mexico showed higher acidity. The concentration of flavonoids in the loquat fruits was higher than 50% of the total phenolic content in all the fruits evaluated. The fruit studied was harvested from seedling trees with a lack of agronomic management, then, the fruit quality can be improved in Mexico. This is the first study showing bioactive compounds of loquat fruits in three States of the Mexican Republic. The physicochemical and bioactive compounds of the loquats through these regions could support studies aiming to the breeding of loquat trees to produce adequate fruit size in the State of Mexico and Oaxaca and/or the development of nutraceutical products derived from loquat fruit from Veracruz where higher total phenols and flavonoids contents were determined.

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