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EVALUADORES

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

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EDITORIAL

Food: reflections on some challenges ahead

La alimentación: reflexiones sobre algunos retos a futuro

Aprovechando la invitación para escribir esta nota editorial, me gustaría dedicar las siguientes líneas a revisar algunos de los retos que tenemos como sociedad en cuanto a la cuestión de la alimentación, retos sobre los que las Ciencias Agrarias tienen mucho que decir y aportar. Cabe apuntar que las gafas con las que veo e interpreto el mundo son las de economista, especializado en el campo del desarrollo sostenible desde una perspectiva de Economía Ecológica1 (Álvarez-Cantalapiedra S y Carpintero O 2009).

Antes de abordar los retos sobre la alimentación, me parece que es necesaria una breve contextualización. Desde la visión de diferentes pensadores/as, la humanidad y la mayoría de los sistemas que hemos creado están inmersos en una crisis global y sistémica (Fuentes-González 2023), la cual se manifiesta en problemáticas en diversas dimensiones: social (ética), económica, política (incluyendo los desafíos de las democracias occidentales), institucional, ambiental/ecológica, entre otras. Bajo estas premisas, el hambre, la desnutrición y la alimentación en general, si bien tienen un componente relacionado más directamente con la dimensión ecológica, son cuestiones que están atravesadas por todas esas dimensiones. Ello hace que la búsqueda de soluciones a los problemas de la alimentación no sea una tarea sencilla, todo lo contrario, es compleja.

Por otro lado, a partir del impulso de la Agenda 2030 y los Objetivos de Desarrollo Sostenible (ODS) por Naciones Unidas (2015-2030) (Naciones Unidas 2015), la erradicación del hambre ocupa un lugar central, como ODS 2, en la agenda pública internacional y de los diferentes países. Sin embargo, esto no es nada novedoso, este problema ya estaba en la agenda de desarrollo internacional de los Objetivos de Desarrollo del Milenio (ODM, 2000-2015); es decir, llevamos realizando esfuerzos por eliminar el hambre en el mundo unos cuantos años y no lo hemos logrado ¿Qué se está haciendo bien? ¿qué se está haciendo mal?

En esta sucinta contextualización cabe mencionar al menos dos sucesos globales y uno más nacional que han puesto de relieve la relevancia de la alimentación y los sistemas agroalimentarios. El primer fenómeno internacional fue la pandemia por Covid-19 (2020-2021), la cual sacó a la luz que una parte importante de la población no tenía asegurada una alimentación básica en un contexto de aislamiento y restricciones a la movilidad para controlar el virus. El segundo evento de carácter global es la guerra entre Rusia y Ucrania (iniciada en febrero de 2022 y aún activa), dos países con un peso importante en el comercio internacional de diversos alimentos (especialmente cereales) e insumos agropecuarios; el desabastecimiento generado por esta guerra ha afectado a diferentes países que dependían del comercio de dichos productos. Finalmente, el suceso nacional denominado como “estallido social” que tuvo como escenario diferentes ciudades de Colombia, especialmente la ciudad de Santiago de Cali (Gómez y Herrera 2023), puso de manifiesto que la alimentación es una necesidad básica y fundamental a raíz de los cierres de las ciudades y además se evidenció que hay que fortalecer la de por sí frágil relación entre campo y ciudad (entre zona rural y zona urbana).

Bajo estas consideraciones, los retos que enfrentamos como humanidad y como sociedades más concretas (a nivel territorial, regional o de país) en cuanto a la alimentación son diversos. La población humana ha sobrepasado ya los 8.000 millones de habitantes en el planeta. Cubrir las necesidades de alimentación de esta población no parece ser

un problema, ya que se estima que con la producción de alimentos actual se podría alimentar a 10.000 millones de personas. Entonces, ¿dónde está el problema? Este se ubicaría en la distribución de los alimentos y su desperdicio en toda la cadena alimentaria, cuyo abordaje parece más técnico y económico. Muy relacionado con lo anterior, estaría el problema del hambre y la malnutrición, que padecen 735 millones de personas, es decir 122 millones más que en 2019 antes del inicio de la pandemia del Covid-19, según cifras del último informe de Naciones Unidas al respecto (FAO, IFAD, UNICEF, WFP y WHO 2023). Aquí el problema parece ser más de carácter ético y político, ya que en un mundo en el que existen las capacidades productivas para producir alimentos y haya una parte importante de la población pasando hambre o mal nutrida, choca con cualquier postulado ético y no digamos de voluntad política: por ejemplo, cuando la hay, los recursos y el dinero para la guerra surgen sin ningún inconveniente, cosa que no ocurre para combatir problemas como las hambrunas.

En la producción de alimentos para cubrir las necesidades de la población humana conviven sistemas agropecuarios tradicionales con sistemas agroindustriales con un uso intensivo de diferentes tecnologías e insumos agroquímicos, que siguen las leyes de la oferta y demanda de los mercados internacionales. Uno de los retos centrales es encontrar formas de producción más sostenible, es decir, preservando la capacidad, resiliencia y biodiversidad de los sistemas naturales para poder seguir produciendo alimentos, no solo para las personas sino para el resto de los seres vivientes que dependen de dichos sistemas. En este punto, los estudios indican que el paso de sistemas tradicionales a sistemas industrializados y tecnificados ha llevado a la extracción de mayores flujos materiales y energéticos especialmente a través de los sistemas de producción agroindustriales (lo que se denomina metabolismo agrario (Toledo y González de Molina 2007)). Desde una perspectiva de la Economía Ecológica, la sostenibilidad de estos sistemas debe ser evaluada sobre la base de los límites biofísicos tanto de extracción de recursos como de excreción de desechos en los propios sistemas naturales usados para la producción de alimentos.

Finalmente, y volviendo a los datos del informe de Naciones Unidas ya mencionado, el 29,6% de la población mundial, es decir 2.400 millones de seres humanos, no tienen acceso estable a los alimentos; además en 2021, 3.100 millones de personas, esto es el 42% de la población, no podían permitirse una dieta saludable. Esto nos alerta del problema de la inseguridad alimentaria que padece un tercio de la población mundial, por no mencionar el problema de la poca soberanía alimentaria de muchas comunidades alrededor del mundo a las que se les impone formas de producción y consumo de alimentos a través de las leyes del mercado; el reto es, por tanto, buscar los mecanismos más adecuados para fortalecer la seguridad y, si es posible, la soberanía alimentaria desde enfoques agroecológicos. Así mismo, los datos nos alertan de la relación entre alimentación y salud, para lo cual es necesaria el consumo de una dieta saludable y, tal como hemos comentamos anteriormente, también debería ser sostenible. Aquí entramos en el plano social de las tradiciones, costumbres y hábitos alimentarios y los desafíos estarían en ver si hay algún tipo de dieta que, respetando las costumbres y tradiciones, sea saludable y sostenible, o si son posibles los cambios de hábitos desde dietas altamente calóricas a dietas más saludables. No me gustaría dejar de mencionar el reto que entraña la consideración de la alimentación como un derecho, lo que le daría un estatus político e institucional más allá de una simple necesidad básica.

Las anteriores reflexiones no pretendían ser exhaustivas sobre los problemas o retos relacionados con la alimentación, simplemente buscaban dar unas pinceladas sobre cuestiones que, desde mi punto de vista, se deben abordar a futuro. Para concluir estas líneas me gustaría abogar por el trabajo transdisciplinar (Max-Neef 2004), para abordar los retos enunciados y otros tantos en la cuestión de la alimentación, tanto en el plano académico e investigativo, como en el plano aplicado y de la política pública. La contribución de las Ciencias Agrarias es fundamental en esta tarea, pero también lo es la contribución de las Ciencias Sociales, de las Ciencias Económicas, etc., desde un trabajo transdisciplinar.

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Controlling foodborne pathogens in irrigation water: the effectiveness of zeolite modified with cetrimonium bromide

Control de patógenos transmitidos por los alimentos en agua de riego: la eficacia de la zeolita modificada con bromuro de cetrimonio

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Keywords:
Escherichia coli
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Hexadecyltrimethylammonium bromide
Irrigation
Listeria monocytogenes
Salmonella sp

ABSTRACT

Recurring foodborne outbreaks, attributed to *Escherichia coli* O157:H7, *Salmonella* sp., and *Listeria monocytogenes*, have identified irrigation water as a potential source of contamination, and creating the necessity for safe irrigation water in produce cultivation, as emphasized by the Food Safety Modernization Act (FSMA). In response to this imperative, this study explored the efficacy of surfactant-modified zeolite (SMZ) enhanced with Cetrimonium bromide (CTAB) as a sustainable water purification solution for surface water. The SMZ was assessed to have the capacity to filter contaminated water with high loads of foodborne pathogens. A laboratory study was conducted using a 100 g SMZ column. A liter of phosphate-buffered saline (PBS) was inoculated for each pathogen at 6 log CFU mL^{-1} concentrations. The study found that SMZ modified with CTAB at a concentration exceeding 20% by weight, indicating the ratio of CTAB to the total mass of the modifying solution, could eliminate >6 log CFU mL^{-1} of *Escherichia coli* O157:H7 and *Listeria monocytogenes* and >2 log of *Salmonella* sp. Subsequent field testing in strawberry farms demonstrated the system's effectiveness, displaying significant bacterial reduction when contrasted with unfiltered pond water and sand filtration. The SMZ was able to filter more than 4 log CFU mL^{-1}, from surface irrigation water spiked with a nonpathogenic *Escherichia coli* strain. The results indicate that the SMZ filtration approach holds promise as a remediation tool to control the risks of foodborne disease outbreaks associated with agricultural water.

Palabras clave:
Escherichia coli
Fragaria ananassa
Hexadecyltrimethylammonium bromide
Irrigación
Listeria monocytogenes
Salmonella sp

RESUMEN

Brotes alimentarios han sido atribuidos a *Escherichia coli* O157:H7, *Salmonella* sp., y *Listeria monocytogenes*, y se ha identificado el agua de riego como una posible fuente de contaminación. Esto realza la necesidad de agua de riego segura en productos hortofrutícolas, como lo enfatiza la Ley de Modernización de la Seguridad Alimentaria (FSMA). En respuesta a este imperativo, este estudio exploró la eficacia de la zeolita modificada con surfactante (SMZ) modificada con bromuro de cetrimonio (CTAB) como una solución para la purificación de agua superficial. Se determinó que el SMZ tiene la capacidad de filtrar agua contaminada con altas cargas de patógenos transmitidos por alimentos. Se realizó un estudio de laboratorio utilizando una columna de SMZ de 100 g. Para cada patógeno, se inoculó un litro de solución salina tamponada con fosfato (PBS) a concentraciones de 6 log UFC mL^{-1}. Los resultados revelaron que SMZ, con una concentración de CTAB al 20% por peso total de la solución modificadora, podría eliminar >6 log UFC mL^{-1} de *Escherichia coli* O157:H7 y *Listeria monocytogenes* y >2 log UFC mL^{-1} de *Salmonella* sp. Las pruebas de campo en granjas de fresas demostraron la efectividad del sistema, mostrando una reducción bacteriana en comparación con el agua de estanque sin filtrar o filtrada con arena. El SMZ pudo filtrar más de 4 log UFC mL^{-1} del agua de riego superficial inoculada con una cepa no patogénica de *Escherichia coli.* Los resultados sugieren que la filtración SMZ podría controlar riesgos de brotes por agua agrícola.

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Agriculture, the cornerstone of global food production, relies heavily on water sourced from ground and surface reservoirs. Maintaining the quality of this water is paramount not just for crop yield but also for ensuring the safety of the produce. The microbial content of surface water, due to its dynamic nature, often fluctuates, and at times, environmental runoffs can severely compromise its quality, posing threats to both agriculture and human health (Steele and Odumeru 2004).

In recent decades, a heightened emphasis has been placed on the microbial standards of water utilized in irrigating crops, especially fruits and vegetables. This increased focus stems from recurrent foodborne illnesses related to irrigation water and making a point of concern for public health authorities. Numerous outbreaks, notably those linked with *Escherichia coli* O157:H7 in spinach (CDC 2006), lettuce (Söderström et al. 2008), and *Salmonella* Saintpaul in jalapeno peppers (Barton Behravesh et al. 2011), have underscored the critical role that contaminated irrigation water can play in this alarming scenario.

Responding to the growing concerns over waterborne contaminants and the consequent health risks, the U.S. government enacted the Food Safety Modernization Act (FSMA). Beyond the standard regulations, the FSMA emphasizes agricultural water's safety and sustained sanitary quality. This heightened focus presented implementation challenges, especially for small-scale farmers, a fundamental part of local food supply chains. Their dependence on surface water further complicated their compliance efforts, as this water source can be unpredictable in its quality.

Parallel to these challenges, solutions have been sought in science and technology. Zeolites have emerged prominently in this quest, offering hope to those grappling with water purification concerns. These mineral-laden micro-porous rocks, procured naturally and through laboratory synthesis, boast a unique filtering capability. Historically, their prowess in water treatment has centered around removing heavy metals, a testament to their cost-effectiveness, unparalleled ion-exchange properties, and significant absorption capacity (Perić et al. 2004).

The zeolite's ability to seamlessly integrate cationic surfactants within its negatively charged matrix stands out. Cetrimonium Bromide (CTAB), a quaternary ammonium compound, has gained attention in this context due to its surfactant and antimicrobial properties. It disrupts microbial cell membranes and exhibits biocidal activity, making it useful in disinfectants and sanitation in food processing (Tezel and Pavlostathis 2015). Its modified forms enhance contaminant and pathogen removal from water, crucial for safe irrigation practices. However, it is essential to optimize its use to balance efficacy and safety due to its toxicity. When exposed to elevated concentrations of cationic bonding surfactants, zeolites can transform, forming hydrophobic bonds. Research suggests that by introducing cetrimonium bromide (CTAB) to zeolite, it is possible to shift its polarity, transforming it into a robust ally against pathogenic bacteria (Schulze-Makuch et al. 2003). Furthermore, the uniqueness of their ion exchange capabilities can be tailored to target distinct bacterial adversaries. Fresh avenues of research are probing into the prospect of infusing zeolites with specific antimicrobial agents or compounds, thereby amplifying their effectiveness against pathogens like *Escherichia coli* O157:H7 and *Salmonella* sp. (Wakweya and Jifar 2023). Combining physical filtration with enhanced antimicrobial activity makes zeolites an effective solution. Such an approach could help farmers comply with the FSMA waters rule and provide an effective solution for purifying surface water, which might be susceptible to intermittent contamination. However, there is limited research exploring the use of surfactant-modified zeolite in managing foodborne pathogens in irrigation water. Therefore, this study aims to demonstrate the effectiveness of surfactant-modified zeolite (SMZ) as a filtration substrate, aiding farmers in securing uncontaminated irrigation water.

MATERIALS AND METHODS

Modification of zeolite using cetrimonium bromide (CTAB)

A natural zeolite, branded as Zeobrite Xtreme®, was supplied by Zeobrite® Corporation. The sample contained more than 71% clinoptilolite. The modification of the zeolite was conducted using cetrimonium bromide (CTAB), a cationic surfactant known for its potential to alter zeolite surface polarity, thereby enhancing its efficiency in water treatment (Margeta et al. 2013). The procedure began by preparing solutions, measured as the mass of CTAB relative to the total mass of the solution, resulting in ratios of 0, 5, 10, 15, 20, 25, and 30% CTAB by mass. Following this, the zeolite

was incorporated into each CTAB solution, adhering to a precise mixing ratio of 0.6 mL of CTAB solution per gram of zeolite. This mixture was agitated at a rate of 5.23 rads s^{-1} for 24 h, ensuring thorough interaction between the zeolite and the surfactant. Subsequently, the mixture was dried in a conventional oven (Thermo Scientific™) set at 150 °C for 30 min to remove excess moisture and achieve the desired modification.

After drying, the surfactant modified zeolite with cetrimonium bromide (SMZ-CTAB) underwent a washing step. It was rinsed with tap water until no frothing was observed, indicating the removal of residual surfactant from the zeolite surface. The washed SMZ-CTAB was then dried in environmental conditions, specifically at 25 °C, within a desiccator chamber containing t.h.e® desiccant (MilliporeSigma, Burlington, MA) to maintain a moisture-free environment.

Finally, the dried SMZ-CTAB was sieved using ASTM-graded sieves to obtain a particle size distribution between 355 and 710 µm. This size range was selected based on preliminary assessments (data not shown), demonstrating optimal water treatment application performance. The classification of zeolite particle sizes is illustrated in Figure 1.

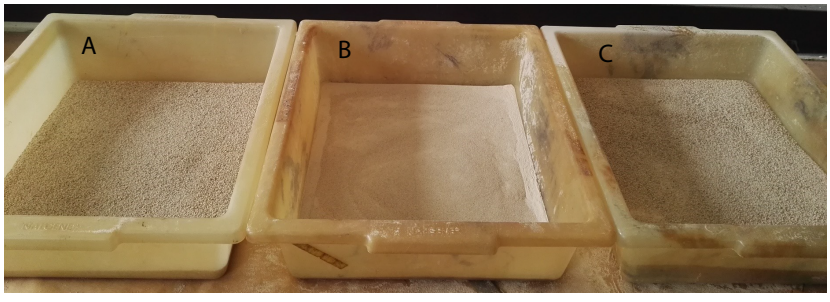

Figure 1. Categorization of the zeolite particles: A. larger than 710 µm, B. smaller than 355 µm, C. optimal range of 355-710 µm.

Microbial culture preparation

To assess the effectiveness of removing bacteria of SMZ-CTAB, three pathogenic strains were chosen: *Escherichia coli* O157:H7 (ATCC 43895), *Listeria monocytogenes* ½ a (Lm F4263, CDC, Atlanta), and a cocktail of nine serotypes of *Salmonella* sp. These *Salmonella* serotypes encompassed: Albert (AR Bank #0401), Cubana (AR Bank #0402), Stanley (AR Bank #0403), Heidelberg (AR Bank #0404), Senftenberg (AR Bank #0405), Corvallis (AR Bank #0406), Concord (AR Bank #0407), Typhimurium (AR Bank #0409), and Infantis (AR Bank #0410).

All strains were preserved at -80 °C using Tryptic Soy Broth (TSB) from Neogen Corporation, Lansing, Michigan, enriched with 20% glycerol by weight. For reactivation, strains underwent successive transfers in Brain-Heart Infusion broth (BHI) from the same supplier. This was incubated for 24 h at 37 °C until reaching a bacterial concentration of 5-6 log CFU mL^{-1}. To ensure culture purity, specific selective media were utilized. *Escherichia coli* O157:H7 was grown on Sorbitol MacConkey Agar with Cefixime-Tellurite supplementation (CT-SMAC, Neogen Corporation, Lansing, Michigan). *Salmonella* sp. was cultivated on Xylose Lysine Deoxycholate Agar (XLD, Neogen Corporation, Lansing, Michigan). *Listeria monocytogenes* was grown on Modified Oxford Agar (MOX, Neogen Corporation, Lansing, Michigan).

SMZ bacterial removal testing

Laboratory-based studies utilizing scaled zeolite filtration setups aimed to identify the optimal concentration of SMZ for eliminating *Escherichia coli* O157:H7*, Listeria monocytogenes* ½ a, and various *Salmonella* sp. serotypes from Phosphate Buffered Saline (PBS). For this purpose, filtration columns were assembled using a 60 mL büchner funnel containing 100 g of SMZ modified with CTAB concentrations ranging from 0, 5, 10, 15, 20, 25, and 30%. To prepare the filtration columns, 500 mL of sterile distilled water was passed through to compact the SMZ, followed by a thorough rinse with an additional 500 mL of water before each filtration. The filtration process involved running 1 L of PBS, pre-inoculated with the target bacteria, through the SMZ-packed columns. To mimic real field irrigation intervals and monitor potential issues, such as

bacterial proliferation within the filter, a 48 h resting period was instituted between consecutive filtrations using the same batch of SMZ. During these intervals, the SMZ was securely wrapped in aluminum foil and stored at a constant temperature of 25 °C.

Bacterial cultures were developed by incubating each strain in 50 mL of BHI for 24 h at 37 °C. Post incubation, they were centrifuged at 4,500 rpm for 10 min. The resulting bacterial pellets were then re-suspended in 1 L of PBS. The initial bacterial concentration before filtration was determined. This inoculated solution was then passed through the SMZ columns of varying CTAB concentrations. Effluent samples were subsequently retrieved to assess bacterial numbers (Figure 2).

For precision, collected samples underwent serial dilution in PBS, spanning 10^0 to 10^{-6} concentrations. A volume of 0.1 mL from these dilutions was plated by duplicating their respective selective media. Utilizing the spread plate methodology, these plates underwent a 48 h incubation at 37 °C. Only colonies numbering between 25 and 250 were considered in the final count. The experimental procedure was replicated three times for each bacterial strain. The filtration effectiveness of SMZ-CTAB was evaluated at intervals of 0, 48, and 96 h.

Scanning electron microscopy (SEM) analysis

The morphological evaluation of the samples was conducted using a Quanta™ 3D Dual Beam™ FEG FIB-SEM (FEI Company, Eindhoven, the Netherlands).

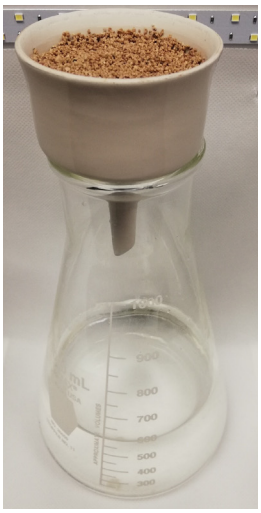

Figure 2. Schematic of the filtration unit featuring a Büchner funnel setup for post-filtration SMZ retention.

This microscopy technique integrates the capabilities of a Focused Ion Beam (FIB) with a high-resolution Field Emission Gun Scanning Electron Microscope (FEG-SEM), enabling detailed structural imaging and material characterization at the nanoscale. For the SEM analysis of the SMZ, samples from the 0, 10, 20, and 30% CTAB were used. These samples were mounted on specimen stubs using conductive silver paint to ensure proper grounding. Subsequently, they were sputter-coated with a thin layer of gold to enhance electrical conductivity and prevent electron charging during microscopy.

After sample preparation, they were loaded into the microscope's chamber, which was then evacuated to a vacuum to facilitate electron travel. The samples were then scanned using a focused electron beam, and the emitted secondary or backscattered electrons were detected to generate high-resolution images, revealing the treated sample's intricate surface topography and features. By analyzing these high-definition SEM images, a better understanding of the morphological changes and interactions induced by the varying SMZ-CTAB concentrations on the sample surfaces.

Field testing of SMZ filtration

To determine the effectiveness of SMZ-CTAB under diverse environmental conditions, field tests were performed to factor in extrinsic elements, including native bacterial

populations, minerals, organic matter, and natural debris typically present in pond water, all of which can influence pathogen filtration.

Location. The field trial was set up at the LSU AgCenter Botanic Gardens, Baton Rouge, LA (coordinates: Lat. 30°24'32.1012"N, Long. 91°6'21.0132" W).

Crop selection. Strawberries (variety: Strawberry Festival) were selected for the study, given their economic significance and vulnerability in Louisiana. These berries are typically harvested directly from the field to their final packaging, emphasizing the importance of pre-harvest pathogen control (Bruchhaus and Hinson 2005).

Planting and maintenance. Strawberry plants were cultivated following guidelines proposed by the LSU AgCenter (Fontenot et al. 2014). Bareroot plants were arranged in double drill rows, spaced 0.4 m apart, and transplanted during the early fall to anticipate the first mid-October frost. Before transplanting, the field was pre-fertilized with 13-13-13 fertilizer (dosed at 4.5 kg per 30 m^{-1} row). Post the initial frost, a weekly addition of 12-9-6 fertilizer (at 0.5 L per 37 m^2) was introduced via drip irrigation. In the absence of rainfall, supplementary irrigation was administered at 25.4 mm per week.

Weed and pest management. A black plastic mulch of 25.4 µm thickness (sourced from Irrigation Mart, Ruston, LA) was utilized for weed control. Regarding pest management, protective measures were tailored based on specific threats, although two applications of Captan® (dosed at 2 L per 4,047 m^2) were routinely applied to manage fungal infections.

Field layout. The entire field spanned an area of 30 x 22 m, subdivided into nine plots, each measuring 1.5 x 3 m (Figure 3). A randomized split-plot design was employed, where six plots were randomly earmarked for each treatment. A buffer zone of 3 m separated each plot to prevent potential cross-contamination.

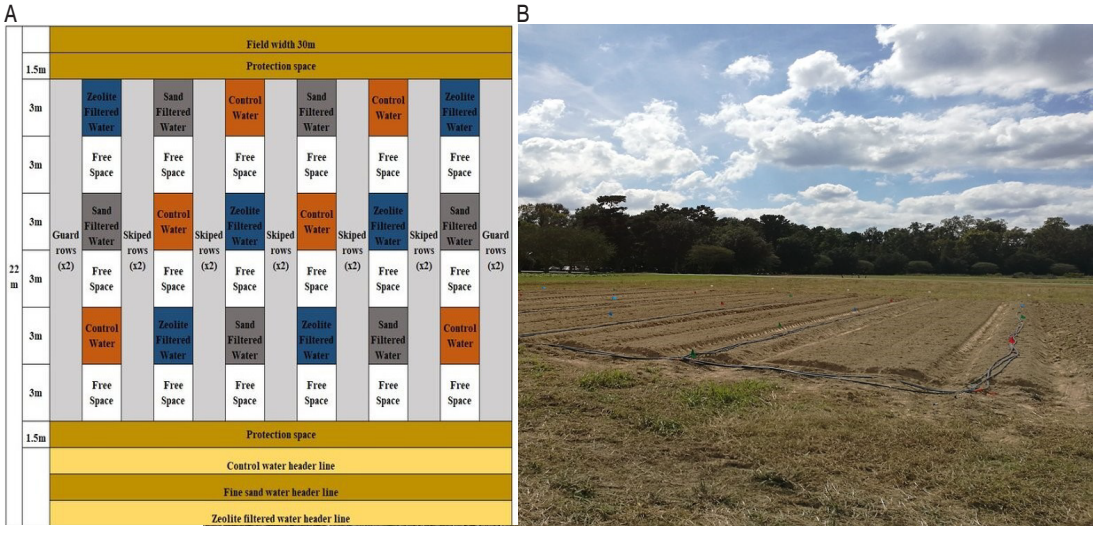

Figure 3. LSU AgCenter Strawberry Field. A. Plot layout. B. Field view, October 2017.

Frost protection. Given the occasional drops in temperature to below -5 °C during the winter season, the strawberry plots were intermittently shielded using floating row covers from AgFabric® (Vista, California).

Filtration system design and execution

A filtration system was designed using the SandPro™ Model 50D pool filters (GAMe, Scottsdale, AZ). Three different treatments of irrigation water were explored. Firstly, there was the control, which used pond water without filtration. Secondly, the Sand-Filtered Water method was applied, where water was directed through a filter filled with 22.68 kg of 20 g silica sand. This specific silica sand, with its particle size range of 0.45-0.55 mm, was chosen for its optimal filtration capabilities. Lastly, the SMZ-CTAB Double Filtration method was introduced. This involved a two-stage process: initially, water was filtered through the silica sand, and subsequently, it passed through a secondary filter

loaded with 11.34 kg of SMZ-CTAB. The results of the previous experiment supported the choice to use a 20% by-weight concentration of CTAB for this field experiment. The waterhead had a diameter of 21.4 mm. After filtration, the water pressure was regulated to 68.95 kPa for the field while the pressure in the tank reached 241.32 kPa. This setup resulted in a flow rate of 2.35 L s^{-1} throughout the system. To maintain the integrity of the treatments and reduce the risk of unintended interferences, distinct header lines were designated for each irrigation system. This strategy also facilitated an enhanced randomization approach in the field. The water source was from a pond, notable for its natural contamination from goose droppings. To simulate pathogenic conditions while ensuring safety, the pond water was spiked with a non-pathogenic strain, *Escherichia coli* ATCC 25922. The inoculation process involved growing a generic *Escherichia coli* culture in BHI broth at 37 °C for 48 h. The culture was centrifuged to form a pellet and re-suspended in 50 mL PBS buffer. This concentrated bacterial solution was mixed with 950 L of pond water, aiming for a resultant concentration of around 5-6 log CFU mL^{-1} of generic *Escherichia coli*. The sampling of the irrigation water was performed after 500 L passed through the system (Figure 4).

Figure 4. Inoculated pond water tank and the filtration system, integrating sand and zeolite.

Microbial sampling and analysis

Strawberries from each plot were harvested monthly after applying the three irrigation treatments. This ensured the immediate capture of microbial responses to each irrigation method. Simultaneously, post-irrigation water samples from each header line were collected into 100 mL sampling cups. Both water and strawberry samples were promptly refrigerated at 4 °C to preserve the microbial state and prevent any potential growth or injury. Once transported to the laboratory, strawberry samples from each plot were pooled to create a composite sample. Two sub-samples weighing 25 g were extracted from this pooled sample and homogenized in 225 mL of buffered peptone water. This step was crucial, as the buffered peptone water counters the potential effects of fruit acidity on bacterial viability and activity.

The homogenized strawberry and water samples underwent microbial analysis to detect Coliforms and *Escherichia coli*. The 3M *Escherichia coli* / coliform Petrifilm™ was employed for this examination, a medium renowned for its precision in quantifying these bacteria. The procedure and interpretation of results derived from the Petrifilm™ strictly adhered to the AOAC™ Official Method 991.14 (AOAC International 1998), which stipulates an incubation period of 24±2 h at 35±1 °C for coliforms, with an additional incubation of 24±2 h for *Escherichia coli*, ensuring the reliability and reproducibility of the bacterial counts obtained. The experimental setup was repeated annually over two years, with each repetition of the experiment spanning the three-month harvest season, during which samplings were carried out monthly.

Water chemical quality analysis

Comprehensive chemical analyses were conducted at the Soil Testing and Plant Analysis Laboratory, Louisiana State University, to understand potential water composition changes from using SMZ. The assessments were focused on detecting variations in specific elemental concentrations.

Parameters examined encompassed Calcium (Ca), Chloride (Cl^-), conductivity, Iron (Fe), Magnesium (Mg), Manganese (Mn), Nitrate (NO_3^-), Potassium (K), Sodium Adsorption Ratio (SAR), Sulfur (S), and Bromide (Br^-). Additional attributes, such as water hardness (based on Ca and Mg levels), alkalinity, and pH, were also assessed to provide a comprehensive profile of the water's chemical quality. Determining alkalinity involved titrating the water sample until it reached a pH=4.5, using 0.02 N of HCl as the titrant. Advanced instrumental analysis was harnessed to ensure accurate determinations of elemental concentrations. Elements like Ca, Cl^-, Fe, Mg, Mn, K, S, and SAR were quantified through Inductively Coupled Plasma- Atomic Emission Spectroscopy (ICP-AES), adhering to the guidelines of the Environmental Protection Agency's ICP-AES Method 200.7, Revision 4.4. The concentration of NO_3^- was ascertained using a direct reading on the Hach DR900 Colorimeter (Hach, Loveland, CO). Bromide (Br^-) levels were determined directly via the HI96716 Photometer (Hanna Instruments, Carrollton, TX).

Statistical Analysis

The experimental design employed was completely randomized. Experiments were replicated three times for the laboratory experiment and the field test twice, and the combined data were analyzed using an Analysis of Variance (ANOVA) through the SAS software package (Version 9.4, SAS Institute Inc., N.C., U.S.A.). Post-hoc tests were performed using the Tukey method to identify differences among treatment groups. Differences were deemed statistically significant at a threshold of $P<0.05$.

RESULTS AND DISCUSSION

This study was to ascertain the capability of SMZ-CTAB, when integrated into a filtration system, to remove foodborne pathogens from irrigation water. A laboratory approach was employed to determine the most effective concentration of SMZ-CTAB in removing pathogens like *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* sp. from sterile Phosphate-buffered saline (PBS).

Filtration of *Escherichia coli* O157:H7

The SMZ-CTAB concentrations ranging from 15 to 30% could remove more than 4.0 log CFU mL^{-1} of *Escherichia coli* O157:H7 from PBS (Table 1). These results align with previous research on zeolite modifications. For instance, the antimicrobial properties of zeolites, especially those modified with metal ions, have been recognized and validated in several studies (Zampino et al. 2011). A similar study using a Copper-activated Zeolite biofilter achieved a >1.5 log CFU mL^{-1} reduction in *Escherichia coli* (Li et al. 2016). They further support the findings that utilizing a zero-valent iron bios and filter coupled with zeolite achieved a significant >6 log removal of *Escherichia coli* O157 (Ingram et al. 2012). After three filtration cycles (96 h), a resurgence in *Escherichia coli* counts was observed. The 100 g of SMZ-CTAB was nearing its saturation point post-filtering 3 L of water. Nevertheless, concentrations above 20% of SMZ-CTAB maintained a removal of >4.0 log CFU mL^{-1} for *Escherichia coli* O157:H7.

Another observation was the pronounced increase in bacterial counts after 96 h of filtration. This trend hints at the

Table 1. *Escherichia coli* O157:H7 counts† in PBS after filtration through SMZ-CTAB.

Treatment SMZ-CTAB (%)	Filtration Time (h)		
	0	48	96
Initial concentration	4.91 ± 0.36^{Aa}	4.60 ± 0.52^{ABb}	4.63 ± 0.39^{Bb}
0	4.80 ± 0.34^{ABa}	4.79 ± 0.60^{Aa}	4.79 ± 0.45^{Aa}
5	4.69 ± 0.25^{Ba}	4.68 ± 0.67^{Ba}	4.55 ± 0.26^{ABa}
10	4.07 ± 0.99^{Cb}	4.02 ± 0.33^{Cb}	4.17 ± 0.02^{Ca}
15	1.62 ± 1.14^{Db}	2.71 ± 0.60^{Da}	2.63 ± 0.93^{Da}
20	1.34 ± 0.95^{Ea}	0.29 ± 0.42^{Eb}	1.22 ± 0.58^{Ea}
25	ND^{Fb}	ND^{Fb}	0.90 ± 0.73^{Fa}
30	ND^{Fb}	ND^{Fb}	0.39 ± 0.28^{Fa}

†Values represent Mean ± Standard Deviation Log CFU mL^{-1}. ND: Nondetectable. Different capital letters in each column indicate significant population differences at $P<0.05$. Different lowercase letters within rows indicate significant differences across the time intervals at $P<0.05$. Limit Of Detection: <10 CFU mL^{-1}.

filtration system nearing its operational limit, emphasizing the need for system maintenance or periodic regeneration, echoed by several filtration studies (Margeta et al. 2013). Table 1 outlines *Escherichia coli* O157:H7 counts in PBS following filtration using various SMZ-CTAB concentrations across different time intervals.

Filtration of *Salmonella* sp.

A preliminary exploration was executed to gauge the effectiveness of a 30% SMZ-CTAB filtration system in eliminating distinct serotypes of *Salmonella* from PBS, as detailed in Table 2. Remarkably, there was no statistically significant variance between the serotypes of *Salmonella* sp. when filtered through 30% SMZ-CTAB. Regardless of the specific *Salmonella* sp. serotype, bacterial counts plummeted to non-detectable levels when subjected to the higher 30% SMZ-CTAB concentration.

Salmonella sp. is renowned for its ability to survive in various environmental conditions, and its prevalence has been a concern in water sources (Eng et al. 2015). It is essential to highlight that the unique membrane compositions of *Salmonella* sp. can lead to variances in susceptibility to surfactants such as CTAB. Bacterial strains, due to differences in their outer membrane structures, may show varying levels of resistance or susceptibility to surfactants and disinfectants (Tezel and Pavlostathis 2015). Quaternary compounds, including CTAB, can potentially compromise bacterial cell membranes, resulting in cell lysis (Gerba 2015). Nonetheless, their effectiveness can be strain-dependent, varying based on the bacterial outer membrane composition.

Table 2. *Salmonella* serotypes counts† in PBS after filtration through SMZ-CTAB.

Salmonella Serotype	Filtration (Log CFU mL^{-1}) at 0% CTAB	Filtration (Log CFU mL^{-1}) at 30% CTAB
Albert	5.845±0.05^{A}	ND^{B}
Stanley	5.75±0.04^{A}	ND^{B}
Senftenberg	5.83±0.19^{A}	ND^{B}
Corvallis	5.82±0.09^{A}	ND^{B}
Cubana	6.14±0.05^{A}	ND^{B}
Concord	5.79±0.10^{A}	ND^{B}
Infantis	6.14±0.04^{A}	ND^{B}
Heidelberg	6.02±0.05^{A}	ND^{B}
Typhimurium	5.86±0.06^{A}	ND^{B}

†Values represent Mean ± Standard Deviation Log CFU mL^{-1}. ND: Not detectable. Different capital letters in each column indicate significant population differences at $P<0.05$. Limit Of Detection: <10 CFU mL^{-1}.

The data (Table 3) revealed patterns, particularly during the initial filtration phase, which indicated a consistent decline in bacterial counts as SMZ-CTAB concentrations increased. Still, the evident differences in microbial counts at reduced concentrations were noteworthy when exposed to a mixture of *Salmonella* sp. serotypes, as displayed in Table 3. Over time, however, the filtration efficiency decreased, with pronounced reductions at elevated concentrations. Owing to their porous nature and high cation-exchange capacity, zeolites have been explored for their role in water treatment and pathogen elimination (Wang and Peng 2010). This potential is amplified when surfactants modify them. Surfactant-modified zeolites (SMZ) show greater effectiveness in pathogen removal from contaminated water than their untreated counterparts, with the underlying mechanism often ascribed to electrostatic attractions and hydrophobic interactions (Tran et al. 2018). Supporting this premise, a study incorporating fractal silver nanoparticles on zeolites yielded significant reductions in both *Escherichia coli* and *Salmonella* sp. populations in sterile water (Guerra et al. 2012). Their outcomes closely mirrored the observations with SMZ modified with CTAB, bolstering the belief that net charges and hydrophobicity remain pivotal mechanisms in bacterial removal. However, while higher concentrations of CTAB show enhanced bacterial removal, too much of it can lead to reduced effectiveness.

High surfactant concentrations might create a condition where the surfactant molecules could start associating with each other rather than the bacteria, thereby reducing their antibacterial effect (Li et al. 2016).

Table 3. *Salmonella enterica* serotype cocktail counts† in PBS after filtration through SMZ-CTAB.

Treatment SMZ-CTAB (%)	Filtration Time (h)		
	0	48	96
Initial Concentration	4.79±0.18^{Aa}	4.76±0.18^{Aa}	4.28±0.07^{Ab}
0	4.63±0.05^{ABb}	4.93±0.03^{Aa}	4.22±0.14^{ABc}
5	4.64±0.05^{ABb}	4.91±0.09^{Aa}	4.33±0.26^{Ac}
10	4.44±0.39^{Bb}	4.83±0.03^{Aa}	4.15±0.17^{ABc}
15	3.34±1.44^{Cc}	4.74±0.01^{Aa}	4.01±0.04^{ABb}
20	3.34±0.71^{Cb}	3.90±0.12^{Ba}	3.93±0.02^{Ba}
25	1.34±1.34^{Dc}	2.99±1.00^{Cb}	4.15±0.03^{ABa}
30	0.33±0.33^{Ec}	2.58±0.48^{Db}	3.02±1.08^{Ca}

†Values represent Mean ± Standard Deviation Log CFU mL^{-1}. Different capital letters in each column indicate significant population differences at $P<0.05$. Different lowercase letters within rows indicate significant differences across the time intervals at $P<0.05$. Limit Of Detection: <10 CFU mL^{-1}.

Moreover, considering *Salmonella* sp. inherent hydrophobicity and its negative surface charge (Ukuku and Fett 2002), it becomes evident why higher concentrations, which augment both net charge and hydrophobicity due to increased CTAB bilayer, render them more efficient in pathogen removal. However, it is pivotal to consider other potential factors, such as *Salmonella* sp. resistance to quaternary ammonia like CTAB, which might also influence removal effectiveness (Tandukar et al. 2013). While the SMZ-CTAB filtration system shows promise in removing various *Salmonella* serotypes, understanding the interplay of bacterial properties, surfactant concentration, and filter characteristics is crucial for optimizing the system's effectiveness.

Filtration of *Listeria monocytogenes*

Listeria monocytogenes susceptibility to surfactants, especially quaternary ammonia compounds like CTAB, has been a focal point of interest in recent studies. When exposed to SMZ-CTAB, even at concentrations as low as 10%, *Listeria monocytogenes* was reduced to non-detectable levels in PBS (Table 4). This high susceptibility can be attributed to *Listeria monocytogene's* inherent sensitivity to the antimicrobial activity of quaternary ammonia compounds (Aarnisalo et al. 2007). This vulnerability could be related to the absence of resistance genes in *Listeria monocytogenes* that protect against surfactants like CTAB (Mereghetti et al. 2000).

Table 4. *Listeria monocytogenes* counts† in PBS after filtration through SMZ-CTAB.

Treatment SMZ-CTAB (%)	Filtration Time (h)		
	0	48	96
0	5.71±0.25^{Ab}	6.02±0.03^{Aa}	5.67±0.19^{Ab}
5	2.59±1.50^{Ba}	1.92±1.61^{Cb}	0.44±0.44^{Bc}
10	ND^{Cb}	0.03±0.03^{Db}	0.23±0.23^{Ca}
15	ND^{Ca}	ND^{Da}	ND^{Da}
20	ND^{Ca}	ND^{Da}	ND^{Da}
25	ND^{Ca}	ND^{Da}	ND^{Da}
30	ND^{Ca}	ND^{Da}	ND^{Da}

†Values represent Mean ± Standard Deviation Log CFU mL^{-1}. ND: Nondetectable. Different capital letters in each column indicate significant population differences at $P<0.05$. Different lowercase letters within rows indicate significant differences across the time intervals at $P<0.05$. Limit of Detection: <10 CFU mL^{-1}.

Salmonella sp. and *Escherichia coli* O157:H7 revealed a diminishing trend in filtration efficiency for *Listeria monocytogenes* across subsequent filtration trials. However, it is intriguing that *Listeria monocytogenes* exhibited a more pronounced reduction in the last filtration than other bacteria. At concentrations surpassing 10 and 15%, CTAB successfully eliminated 4.0 log of *Listeria monocytogenes* and *Escherichia coli* O157:H7, respectively.

Table 4 showcases the efficiency of SMZ-CTAB against *Listeria monocytogenes* over time, with almost consistent elimination at higher surfactant concentrations. Given the importance of waterborne transmission in spreading pathogens, these findings underscore the potential of SMZ-CTAB as an effective barrier against bacterial contamination, mainly when optimal concentrations are utilized (Park et al. 2009).

Morphological Analysis of Bacterial Response to CTAB Concentrations

The SEM FIB images (Figure 5) elucidated the extent of the effect of different concentrations of CTAB on bacterial cells. Most bacterial cells displayed intact cell membranes at 0 and 10% CTAB. However, with 20% SMZ-CTAB, bacteria had suffered extensive cell membrane damage due to the bactericidal action of the quaternary ammonium compound. No bacteria were visible at the higher concentration of 30%. This suggests that a filtration device composed of SMZ at 20% CTAB would effectively retain bacteria until they are inactivated. In contrast, a concentration of 30% might release viable but non-culturable bacteria into the system. The bactericidal action of quaternary ammonium compounds, such as CTAB, primarily targets the bacterial cell membrane, causing disruptions and eventual cell death (Wessels and Ingmer 2013).

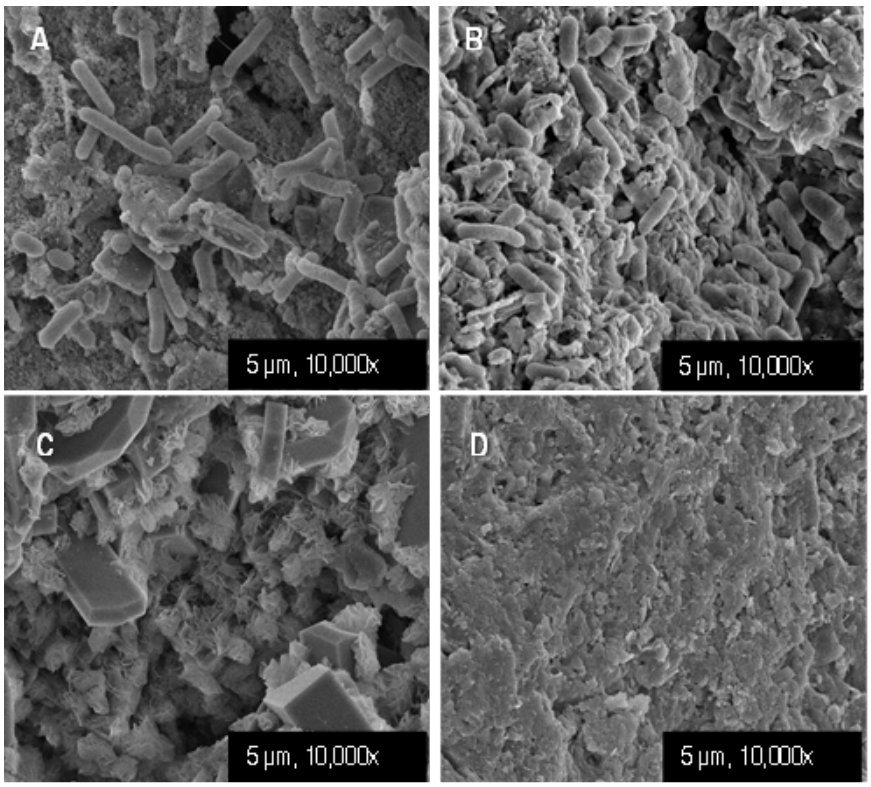

Figure 5. Scanning Electron Microscopy - Focused Ion Beam (SEM-FIB) images of A. SMZ-CTAB at 0%, B. SMZ-CTAB at 10%, C. SMZ-CTAB at 20%, and D. SMZ-CTAB at 30%.

Strawberry field testing

Based on the results of laboratory-scale experiments, 20% CTAB demonstrated high effectiveness in bacterial removal at 96 h and was therefore selected for field trials. The results revealed that the SMZ-CTAB filtration device removed over 4.0 log CFU mL^{-1} of coliform and *Escherichia*

coli (Table 5) over two production cycles, maintaining its efficiency. Notably, merely using sand in the filtration system exhibited no discernible bacterial removal. Only the modified zeolite filtration device ensured that pond water met optimal irrigation levels.

The contamination of irrigation water with pathogens can have severe implications, especially for crops consumed raw, like strawberries (Suslow et al. 2003). The safety of irrigation water is essential for food safety and agricultural systems' overall productivity and sustainability (Havelaar et al. 2015). The results indicated that the levels of *Escherichia coli* were effectively reduced by the SMZ-CTAB treatment to below the recommended threshold of <126 CFU 100 mL^{-1} (Weller et al. 2017).

Strawberry samples from the initial 2017 production cycle were subjected to analyses for *Escherichia coli* and coliform

Table 5. Coliform and *Escherichia coli* removal in pond water filtered through sand and 20% SMZ-CTAB: Petrifilm method.

Coliforms	Control	Sand	Zeolite
February, 2017	4.95 ± 0.02^{ABa}	3.39 ± 0.04^{Cb}	ND^{Cc}
March, 2017	5.41 ± 0.52^{Aa}	5.26 ± 0.60^{Aa}	0.86 ± 0.67^{Bb}
April, 2017	5.66 ± 0.39^{Aa}	5.36 ± 0.45^{Aa}	ND^{Cb}
February, 2018	4.31 ± 0.07^{Ba}	4.10 ± 0.07^{Ba}	1.97 ± 0.10^{Ab}
March, 2018	4.02 ± 0.52^{Ca}	3.86 ± 0.60^{Ba}	ND^{Cb}
April, 2018	4.87 ± 0.39^{Ba}	4.51 ± 0.45^{Aa}	1.15 ± 0.26^{Bb}
Escherichia coli	**Control**	**Sand**	**Zeolite**
February, 2017	4.62 ± 0.12^{Ba}	3.35 ± 0.03^{Cb}	ND^{Cc}
March, 2017	5.41 ± 0.01^{Aa}	5.26 ± 0.11^{Aa}	0.86 ± 0.49^{Bb}
April, 2017	5.66 ± 0.12^{Aa}	5.36 ± 0.03^{Aa}	ND^{Cb}
February, 2018	4.31 ± 0.04^{Ca}	4.10 ± 0.22^{Ba}	1.31 ± 0.02^{Ab}
March, 2018	4.02 ± 0.03^{Ca}	3.86 ± 0.10^{Ca}	ND^{Cb}
April, 2018	4.80 ± 0.04^{Ba}	4.51 ± 0.14^{Ba}	1.15 ± 0.00^{Ab}

†Values represent Mean ± Standard Deviation Log CFU mL^{-1}. ND: Nondetectable. Different capital letters in each column indicate significant population differences at $P<0.05$. Different lowercase letters within rows indicate significant differences across the time intervals at $P<0.05$. Limit Of Detection: <10 CFU mL^{-1}.

counts (Table 6). Notably, the results revealed no statistically significant disparities among the treatments. This outcome may be attributed to the prevalent practice in Louisiana of utilizing plastic covers during cultivation. Such measures exclude direct contact between irrigation water and the fruit. Nevertheless, the region's susceptibility to flooding accentuates the imperative of employing uncontaminated water sources for irrigation (Park et al. 2009). The potential

Table 6. Coliform and *Escherichia coli* in strawberry samples across different harvesting seasons.

Coliforms	Control	Sand	Zeolite
February, 2017	1.73±0.49	1.66±0.58	2.09±0.69
March, 2017	2.28±0.61	2.04±0.70	2.60±0.39
April, 2017	1.90±0.59	1.59±0.57	1.63±0.49
Escherichia coli	**Control**	**Sand**	**Zeolite**
February, 2017	1.66±0.28	1.30±0.00	ND
March, 2017	1.45±0.49	1.48±0.50	2.02±0.67
April, 2017	ND	ND	ND

†Values represent Mean ± Standard Deviation Log CFU mL^{-1}. ND: Nondetectable. Note: No statistical difference was observed between treatments ($P<0.05$). Limit Of Detection: <100 CFU mL^{-1}.

for pathogenic contamination due to flooding can lead to outbreaks, affecting public health and the agricultural economy.

Water Chemical Quality Analysis

A comprehensive view of the chemical profile of irrigation water post-filtration for 2017 and 2018 in Table 7 displays how distinct parameters like alkalinity exhibit variations when processed through the zeolite system, potentially highlighting the ion-exchange properties of SMZ-CTAB. Notably, bromide levels remained low, suggesting no noticeable leaching of bromide post-filtration. However, the analysis of variance shows that parameters between the two years were not statistically significant for any treatments (P<0.05). While zeolite filtration demonstrates the potential to modify the irrigation water's chemical attributes, these changes remain consistent across the years studied. Such consistency suggests that other external or design-related factors influence the slight variations observed. Further research would be beneficial to determine the long-term impacts of zeolite filtration on water quality and its subsequent influence on agriculture.

Table 7. Overall chemical results of irrigation water after filtration through different filtration treatments.

Parameter, unit (mg L^{-1})	Control		Sand		Zeolite	
	2017	2018	2017	2018	2017	2018
Alkalinity	30.91±2.82	31.72±3.45	60.19±26.88	41.48±10.35	78.08±17.08	41.48±3.45
Calcium	9.56±0.36	7.28±0.33	16.08±2.01	6.98±0.14	9.67±3.46	7.02±0.23
Chloride,	9.97±0.85	14.06±0.86	11.35±1.18	14.25±0.08	19.13±0.23	13.78±4.18
Conductivity, (Siemens)	200.97±21.81	130.65±2.12	206.03±31.19	129.65±0.49	263.20±44.80	130.5±0.07
Hardness (Ca, Mg)	29.10±1.04	24.13±1.26	45.49±5.45	23.47±0.29	28.67±10.55	23.91±1.03
Iron	0.07±0.03	0.46±0.02	0.07±0.05	0.31±0.09	0.20±0.16	0.20±0.01
Magnesium	1.27±0.04	1.44±0.10	1.30±0.17	1.47±0.01	1.10±0.47	1.55±0.11
Manganese	0.00±0.00	0.02±0.00	0.00±0.00	0.01±0.00	0.01±0.01	0.01±0.00
Nitrate	1.33±0.00	1.77±0.63	2.07±1.35	1.33±0.00	1.99±0.22	1.33±0.00
pH	8.23±0.98	7.67±0.23	9.31±0.24	7.60±0.07	8.13±0.15	7.64±0.13
Potassium	4.10±1.96	3.99±1.50	3.41±0.88	5.40±0.35	4.34±0.10	7.62±2.41
Salts	128.62±13.96	83.62±1.36	131.86±19.96	82.98±0.32	168.45±28.67	83.52±0.05
SAR	0.77±0.26	0.65±0.00	0.80±0.28	0.66±0.03	4.39±2.51	0.66±0.00
Sodium	9.57±3.14	7.35±0.20	12.50±4.94	7.37±0.24	47.31±20.20	7.37±0.14
Sulfur	0.64±0.26	0.57±0.02	0.76±0.13	0.51±0.02	1.92±0.60	0.48±0.07
Bromide	0.15±0.13	0.16±0.14	0.24±0.17	0.20±0.16	0.05±0.05	0.07±0.05

†Values represent Mean ± Standard Deviation.

CONCLUSIONS

The Food Safety Modernization Act (FSMA) underscores the imperative of maintaining stringent water quality standards in agricultural and food processing settings. In alignment with such directives and the objective of this study, this research evaluated the effectiveness of surfactant-modified zeolite (SMZ) modified with cetrimonium bromide (CTAB) to remove bacteria. These findings indicate that using CTAB (at 20 and 30%) will significantly have bactericidal effects, compromising the integrity of bacterial cell membranes. Such observations are corroborated by SEM FIB imaging, highlighting the impacts of CTAB on bacterial morphology. The filtration media performance across two years reaffirms its reliability. The SMZ-CTAB filtration system emerges as a potential candidate to meet the water quality directives outlined by the FSMA. As the global community grapples with mounting challenges in ensuring food safety, the findings of this experiment provide a pathway that warrants further exploration in the context of scalable water treatment solutions for food production.

ACKNOWLEDGMENTS

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Physiological evaluation of *Sonchus oleraceus* L. seeds with different pre-germinative treatments under high tropical latitudinal conditions

UNIVERSIDAD NACIONAL DE COLOMBIA

Evaluación fisiológica de semillas de *Sonchus oleraceus* L. mediante diferentes tratamientos pre-germinativos en condiciones latitudinales de trópico alto

https://doi.org/10.15446/rfnam.v77n1.107027

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ABSTRACT

Keywords:
Dormancy
Germination physiology
Gibberellins
Scarification
Sowing depth

Sonchus oleraceus L. is an invasive species that negatively affects the quality and yield of different crops. This research aimed to evaluate the physiological behavior of seeds from the weed *S. oleraceus* L., which were subjected to different treatments in three experiments. In experiment 1, different gibberellic acid (GA) concentrations were applied (0, 200, 400, and 600 mg L^{-1}). In the second experiment, apical, basal, and apical-basal cut treatments were carried out on the seeds. In a third experiment, seeds were placed at different sowing depths (0.5, 1, 2, 5, and 10 cm) in peat as the substrate. The results indicated that GA at concentrations higher than 200 mg L^{-1} inhibited seed germination and, consequently, seedling growth. Germination was similar between the 200 mg L^{-1} GA treatment and the control, but germination occurred faster in the control. The apical-basal cut in the seeds generated the highest percentage of germination, the highest average speed of germination, and a significantly lower time of germination than the control; however, the longest shoot was observed in the seeds without a cut, and the longest root was detected in the seeds with the basal cut. Seedling emergence was affected by sowing depth, where it was significantly greater at 0.5, lesser at 2 cm, and, at greater depths, seedlings did not emerge.

RESUMEN

Palabras clave:
Latencia
Fisiología de germinación
Giberelinas
Escarificación
Profundidad de siembra

Sonchus oleraceus L., es una especie invasiva que afecta negativamente la calidad y el rendimiento de diferentes cultivos. El objetivo de esta evaluación fue evaluar el comportamiento fisiológico de las semillas de la maleza *S. oleraceus* L., para lo cual, las semillas fueron sometidas a diferentes tratamientos en tres experimentos. En el experimento 1 se aplicaron diferentes concentraciones de ácido giberélico (0, 200, 400 y 600 mg L^{-1}). En el segundo experimento se realizaron tratamientos de despunte apical, basal, y apical-basal, más un control. En un tercer experimento las semillas se sometieron a diferentes profundidades de siembra (0,5, 1, 2, 5 y 10 cm) en turba como sustrato. Los resultados indicaron que el ácido giberélico en concentraciones superiores a 200 mg L^{-1} presentó un efecto inhibidor de germinación y, por tanto, del crecimiento de las plántulas. La germinación con 200 mg L^{-1} de GA y el control fue similar, pero en el control la germinación ocurrió más rápido. Por su parte, el despunte apical-basal generó el mayor porcentaje de germinación, la mayor velocidad media de germinación, y un tiempo significativamente inferior al control; sin embargo, la mayor longitud de parte aérea fue presentada en las semillas sin despunte, y la mayor longitud de raíz en el despunte basal. La emergencia de las plántulas disminuyó en función de la profundidad de siembra, ya que fue significativamente mayor en 0,5, menor a 2 cm y en profundidades mayores no se logró emergencia de las plántulas.

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Sonchus oleraceus L. (Asteraceae) is an herbaceous species with medicinal properties (Alothman et al. 2018). In folk medicine, the leaf infusions of *S. oleraceus* are employed to treat headaches, hepatitis, bacterial infections, and inflammations, since this species possesses strong anti-inflammatory effects (Vilela et al. 2009); its latex is known for antibacterial properties (Ghaffaripour et al. 2021). It gained attention in agriculture because it is a noxious invasive weed as a result of its genetic diversity and morphological features, together with herbicide resistance (Khalsa et al. 2021; Peerzada et al. 2021; Chauhan and Jha 2020). It is highly competitive, difficult to control in the field, and tends to adapt well to varied crop regions because of its germination and growth in broad temperature regimes and soil conditions (Rojas-Sandoval et al. 2015; Manalil et al. 2018; Ali et al. 2020). It is widely distributed worldwide (Morrison et al. 2021; Peerzada et al. 2021) and, in Colombia, is found in the highlands in various crops (Gámez et al. 2018; Moreno-Preciado and Balaguera-López 2021; Martínez et al. 2022).

S. oleraceus L. is an annual plant that reproduces only by seeds (Khalsa et al. 2021). Its seeds need light to germinate and can germinate at any time of the year (Rojas-Sandoval et al. 2015) and have the potential to emerge all year round from the top 2 cm layer of soil, but emergence is favored when seeds are sown 1 cm deep (Widderick et al. 2010). Khalsa et al. (2021) compiled germination studies on *S. oleraceus* for temperate and subtropical climate conditions, which evidenced different environmental requirements for seeds to germinate. Under tropical conditions, there are few reports, but the germination percentage can be between 41 and 72.5% (Martínez et al. 2022). According to Savaedi et al. (2019), seed germination is highly sensitive to environmental factors. Under high tropical conditions, the details of the germination physiology of this species are unknown. At the same time, this information could be beneficial in comprehensive weed management (Niño-Hernández et al. 2020) optimizing germination when *S. oleraceus* is cultivated as a medicinal species.

The light requirement for germination and germination dependence on chemical signals from the environment (nitrate, gibberellins, ethylene, karrikins, and others) are some of the factors that could trigger germination in small seeds with few storage reserves (Baskin and Baskin 2014), such as those of *S. oleraceus*. If they germinate too deep in the soil, their nutrient reserves are insufficient to sustain the seedling growth to successfully emerge at the soil surface (Fenner 2012). Typically, weed seeds are dormant to ensure their survival by avoiding unfavorable conditions (Bera et al. 2020). They can present physiological dormancy, where applying gibberellins can stimulate germination (Lutts et al. 2016; Savaedi et al. 2019). Gibberellins are hormones that control seed germination through the activation of hydrolytic enzymes and, in photoblastic species, reactions mediated by PhyA and PhyB phytochromes (Barros-Galvão et al. 2019; Savaedi et al. 2019; Castro-Camba et al. 2022). Nevertheless, some seeds require scarification processes to guarantee the entry of water and oxygen to the embryo, such as when they present physical dormancy (Baskin and Baskin 2014).

Therefore, this research aimed to evaluate the physiological behavior of *S. oleraceus* seeds with different pre-germinative treatments under high tropical latitudinal conditions.

MATERIALS AND METHODS

Location and plant material

The research was carried out in the Plant Physiology laboratory of the Faculty of Agricultural Sciences of the Pedagogical and the Technological University of Colombia, Tunja Headquarters, located at 5°33'20.1" N, 73°21'18.0" W, and 2,782 meters above sea level (masl).

For the collection of *S. oleraceus* seeds, visits were made in July, 2018 to crops in the municipalities of Duitama, Nobsa, Paipa, and Tibasosa in traditional agricultural fields of broccoli (*Brassica oleracea* var. italica), cauliflower (*B. oleracea* var. botrytis), lettuce (*Lactuca sativa* L.), cabbage (*B. oleracea* var. capitata), and spinach (*Spinacia oleracea* L.), in addition to harvested fields. Once the species was identified by its leaves, growth habit, and flower, floral structures in optimal health and maturity were harvested; that is, all flowers that were about to release the seeds were collected, identifying this stage by exposure of the pappus since this structure disperses seeds through the air. To facilitate the seed counting and selection methodology, the pappus was removed from the seeds, which facilitated the release of the seeds in a group and allowed the collection of more than 80 seeds per inflorescence. The weight of 1,000 fresh seeds without pappus was 0.2782 g, and the length of each seed

was approximately 3 mm (Figure 1). These seeds were deposited in a resealable bag, thus, making a mixture of seeds between the different crops and municipalities of origin, to guarantee a representative sample of land with an agricultural tradition in the department and, simultaneously, facilitate subsequent handling.

The harvested seeds were left to dry at room temperature (18±1.9 °C) and stored under dark conditions in paper bags for 30 days. The seed sample was used to carry out three experiments, each under a completely randomized design, as described below:

Experiment 1

The seeds were imbibed in solutions with different concentrations of gibberellins (0, 200, 400, and 600 mg L^{-1} of GA_3), which were based on the reports of Niño-Hernández et al. (2020). Four replicates per treatment were used, and each of the 16 experiment units consisted of a Petri dish with 100 seeds. Each gibberellin solution was prepared in distilled water and with the product ProGibb® 10 SP (Bayer S.A, Colombia), whose active ingredient is gibberellic acid (100 g kg^{-1}). The treatments were applied daily to the Petri dishes to moisten the absorbent paper.

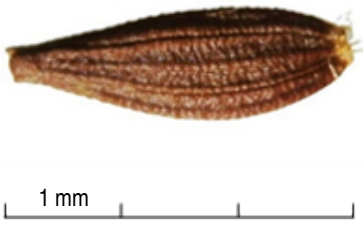

Figure 1. Adult plants and mature sexual propagules of *S. oleraceus* grown in high tropical latitudinal conditions.

Experiment 2

For this experiment, a cut was made to the seeds in the apical part (insertion zone with the pappus), basal part (insertion zone with the floral receptacle), and apical-basal part, and a group of seeds was left intact, without any cut (control), for a total of four treatments with four replicates. Each experiment unit was made up of 100 seeds. Once this procedure was carried out, the seeds were placed in Petri dishes with distilled water and absorbent paper.

Experiment 3

Five treatments corresponding to the sowing of *S. oleraceus* seeds at different depths (0.5, 1, 2, 5, and 10 cm) were evaluated, each treatment presented four replicates. Each replicate consisted of 100 seeds sown in 0.5 L pots with PRO-MIX blonde peat as the substrate, whose physicochemical properties are reported in Moreno et al. (2009). The sowing of each seed was carried out through a 5 mm diameter tube that was inserted into the substrate to the depth required in each treatment. After sowing, the seeds were covered with the same substrate. The substrate was kept moist at field capacity with daily irrigation with distilled water, following the gravimetric method, adapted from Segura et al. (2011).

All the experiments had natural light (211 µmol m^{-2} s^{-1}) conditions, with a photoperiod of 12 h and an average temperature of 18±1.9 °C, which is considered a favorable

temperature for seed germination of this species (Gresta et al. 2010).

Physiological variables

The germination readings and the time in which this occurred were recorded every two days from the germination of the first seed until constant germination (no further germination obtained). This was at 18, 16, and 34 days for experiments 1, 2, and 3, respectively. A seed was considered germinated when the already visible radicle measured at least 2 mm in length. In the sowing depth experiment, the percentage of seedling emergence was measured, understood as the emergence of the hypocotyl on the surface of the substrate. From these data and with the formulas used by Porras et al. (2020), the following variables were calculated: the germination percentage, mean germination speed (germinated seeds d^{-1}), and mean germination time (d).

In experiments 1 and 2, the length (mm) of the aerial part and roots were measured on ten seedlings in each experiment unit. The shoot and root length were measured with the help of a Mitutoyo digital caliper with an approximation of 0.05 mm.

Statistical analysis

The accumulated germination was adjusted to the logistic model according to the methodology used by Cepeda et al. (2021) and Maestre et al. (2023); the first derivative of the model calculated the germination rate as a function of time. This procedure was carried out with R Studio.

The data were subjected to tests of normality of errors (Shapiro-Wilk) and homogeneity of variances (Levene). With these assumptions, an analysis of variance was carried out. In the case of significant differences ($P<0.05$), a mean comparison test was performed using the Tukey test ($P<0.05$) or regression analysis. These analyses were performed with the statistical program SPSS V.19.

RESULTS AND DISCUSSION

Experiment 1

Effect of gibberellic acid

For germination behavior, a logistic model was adjusted at all concentrations (Figure 2 and Table 1). The seeds of the control treatment were characterized by rapid germination during the first seven days, at which time 73.88% of the seeds germinated. The highest speed (44.37 germinated seeds d^{-1}) was obtained four days after sowing. Afterward, germination remained more or less stable until 18 days. With 200 mg L^{-1} of GA_3, the germination began two days later, but the final germination was similar, with a high germination speed for several days. With 600 mg L^{-1}, the percentage and speed of germination were significantly lower throughout the study (Figure 2).

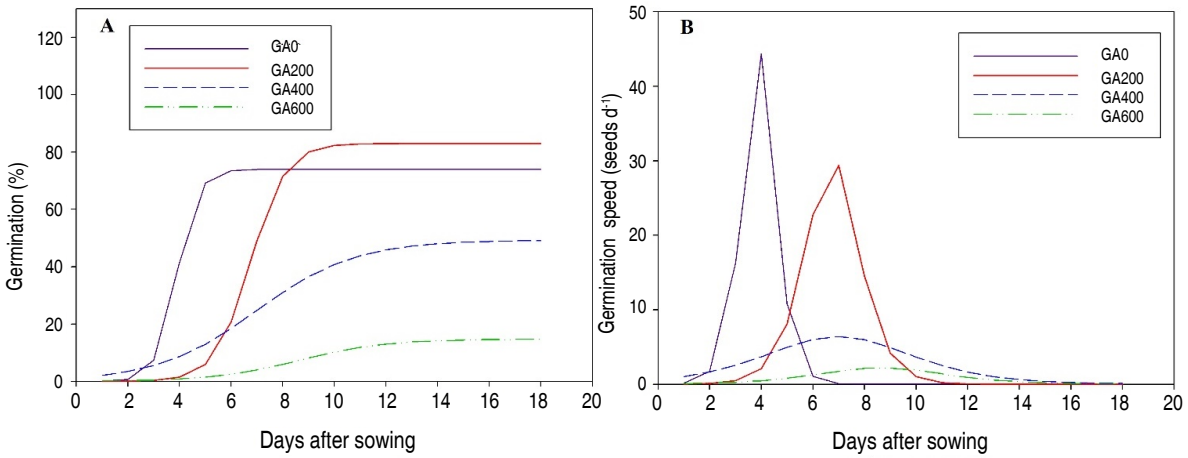

Figure 2. Effect of different doses of gibberellic acid (0, 200, 400, and 600 mg L^{-1}) on accumulated germination (A) and the germination speed (B) of *S. oleraceus* seeds.

Table 1. Fitting equations to the logistic model for the percentage germination of *Sonchus oleraceus*.

Experiment and treatments			
Doses of GA_3 (mg L^{-1})	Equations	RMSE	R^2
0	$Y= 73.92701/(1 + e^{-2.43708*(d-3.89981)})$	5.734**	0.99
200	$Y= 83.01280/(1 + e^{-1.46250*(d-6.75061)})$	0.931**	0.99
400	$Y= 49.22702/(1 + e^{-0.52103*(d-6.97901)})$	1.909**	0.99
600	$Y= 14.70987/(1 + e^{-0.60196*(d-8.66652)})$	0.770**	0.99
Type of scarification			
Control	$Y= 42.09100/(1 + e^{-0.97774*(d-9.13908)})$	2.089**	0.99
Apical	$Y= 68.81148/(1 + e^{-1.78114*(d-7.88151)})$	1.862**	0.99
Basal	$Y= 68.77001/(1 + e^{-0.39613*(d-4.68048)})$	5.965**	0.97
Apical-Basal	$Y= 82.54229/(1 + e^{-0.79438*(d-5.01683)})$	4.193**	0.99
Sowing depth (cm)			
0.5	$Y= 44.82591/(1 + e^{-0.34541*(d-7.78351)})$	4.030**	0.96
1	$Y= 30.06247/(1 + e^{-0.20734*(d-13.86410)})$	1.549**	0.99
2	$Y= 3.72262/(1 + e^{-0.54904*(d-10.95104)})$	0.261**	0.99

** Significant models (P<0.01). RMSE: root means square error.

The final germination percentage presented statistical differences (P<0.05) between the concentrations of GA_3. It was higher with 0 (78.5±3.1%) and 200 mg L^{-1} of GA_3 (83.5±5.61%) and lower with 600 mg L^{-1} (14.75±3.5%); this behavior was adjusted to a quadratic equation (Figure 3A). The mean germination speed (MGS) was also adjusted to the same type of regression. The highest speed was observed with 0 and 200 mg L^{-1} of GA_3 (Figure 3B). The mean germination time (MGT) presented significant differences, and a linear behavior was observed that depended on the increase in GA_3, which was higher with 600 mg L^{-1} (Figure 3C).

The length of the main root was significantly greater in the control seedlings and decreased quadratically as the concentration of GA_3 decreased (Figure 3D). The length of the aerial part was greater with 0 and 200 mg L^{-1} of GA_3, but with 400 and 600 mg L^{-1} of GA_3, this parameter was significantly reduced, described by a second-degree polynomial (Figure 3E).

In the control seeds, endogenous gibberellins can be responsible for inducing germination; these plant hormones, particularly GA_1 and GA_4 induce germination through the activation of hydrolytic enzymes that degrade reserve polysaccharides in the endosperm, such as amylases, and structural polysaccharides of seed coats (Castro-Camba et al. 2022; Gong et al. 2022). Additionally, seeds that germinate faster have the possibility of starting root growth and shooting first, for this reason, in the control treatment, seedlings were also larger in size (Figure 3D, E). However, the applications exceeding 200 mg L^{-1} GA_3 reduced the seed germination percentage in *S. olearaceus* and seedling growth. The adverse effects of GA_3 on seed germination are not frequent but have been reported previously for various species. *S. oleraceus* could react negatively to GA_3 because the 13-hydroxylation pathway negatively affects its biological activity (Magome et al. 2013). To respect, Lee et al. (2022) found that GA_3 did not promote germination while combining GA_1 and GA_4 effectively broke *Amsonia elliptica* seed dormancy. In coffee seeds, GA applications resulted in the release of one or more compounds from the endosperm that further induced cell death in the embryo (Da Silva et al. 2005). These authors argued that the GA-caused inhibition occurs very late during germination and immediately before radicle protrusion. In particular, the mechanism of the GA-induced inhibition of coffee seed germination may involve the release of mannose as the result of mannan degradation by GA, where mannose accumulation could inhibit ATP synthesis and hexose metabolism in seeds (Da Silva et al. 2005).

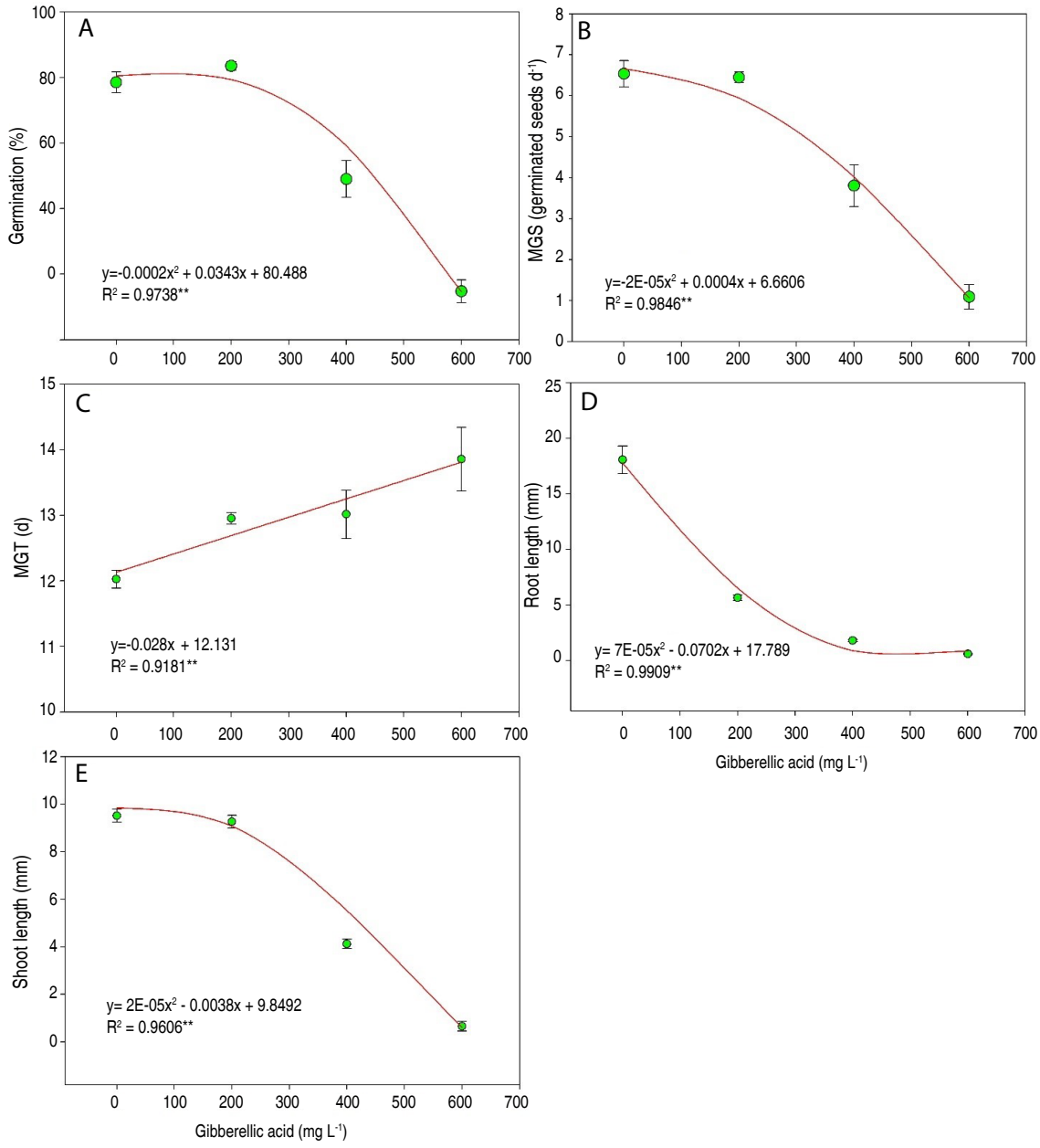

Figure 3. Effect of different concentrations of gibberellic acid (0, 200, 400, and 600 mg L^{-1}) on germination percentage (A), mean germination speed (B), mean germination time (C), length of root (D), and length of shoot (E) of *S. oleraceus*. Vertical bars for each mean indicate the standard error (n=4). **significant regression (P<0.01).

The GA_3 treatments of 125, 250, and, especially, 500 mg L^{-1} of seeds of *Ferocactus histrix* and *F. latispinus* (Cactaceae) had adverse effects on their germination percentages and germination speed, which might be attributed to the use of the supra-optimal concentrations of GA_3 (Amador-Alférez et al. 2013). GA_3 had no positive effects on germination percentage and germination dynamics in *Euptelea pleiospermum* seeds, while light and KNO_3 positively affected germination (Wei et al. 2010). In *Hepatica asiatica* seeds, applications of gibberellic acid (GA_3) promoted embryo growth, reducing morphological dormancy, but had no positive effect on radicle protrusion (Chon et al.

2015). Additionally, the interaction with other hormones might modify the effects of GA on seed germination. Thus, GA_{4+7} and cytoquinin mixture delayed seed germination and reduced seedling formation in eggplant (Neto et al. 2017).

The duration and the temperature of seed storage may also diminish the effects of GA treatment on seed germination (Rivera et al. 2011). In sweet corn, GA applications at a rate of 10-20 mg L^{-1} lost their positive effect on seed germination when the storage time exceeded 60 days, especially when the seeds were stored at 25 °C (Rivera et al. 2011). Some of these processes may have occurred in *S. oleraceus* seeds; however, other studies at the molecular level are needed to understand in more detail the GA inhibition of germination observed in these seeds.

Experiment 2

Type of scarification

For germination behavior in all treatments, initial germination was slow, which then had a marked increase, and, at the end of the experiment, it was slower (Figure 4A and Table 1). Greater germination was observed with the basal-apical cut, with a representative speed in the first 11 days. With the apical cut, germination started late but most of the seeds germinated quickly. For this reason, the peak germination speed was the highest (30.3 germinated seeds d^{-1} on day 8) but extended for a short time. The seeds with a basal cut constantly germinated throughout the experiment, which is why the speed was similar in most of the experiments. However, it was the lowest, even below the control, which might indicate that the embryo received mechanical damage during the basal cut. Nevertheless, this last treatment germinated slower at a lower percentage and only had a representative speed at nine days (Figure 4B).

The final percentage of germination was significantly different between treatments, being higher in the seeds

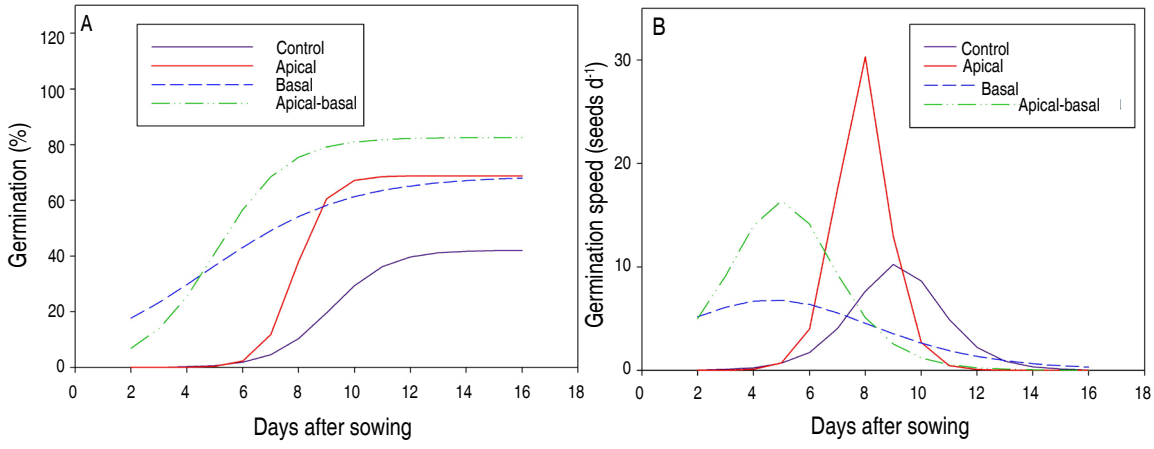

Figure 4. Effect of scarification type on accumulated germination (A) and the germination speed (B) of *S. oleraceus* seeds.

with the two cuts (82.5±3.4%) and lower in the control seeds (Figure 5A). MGS was significantly higher with the basal and apical-basal cut, and the control presented the lowest value (Figure 5B). The most favorable response for MGT was obtained with the basal cut as it presented less time (10.95±0.09 d), followed by the seeds with the apical-basal cut. The opposite response was obtained with the control seeds (Figure 5C).

Root length was significantly affected by the cut type. It was more significant with the basal cut (35.8±1.4 mm) and less with the apical cut (Figure 5D). The control seedlings had the highest growth for shoot length with significant differences in the treatments with an apical cut and an apical-basal, which presented lower values (Figure 5E).

The germination percentages were different in the experiments (Figures 3, 7, and 5) but were close to previous reports for the tropics, which indicated that germination in this species can be between 41 and 72.5% (Martínez et al. 2022). However, in Australia, 65 to 100% of *S. oleraceus* seeds germinated (Widderick et al. 2004).

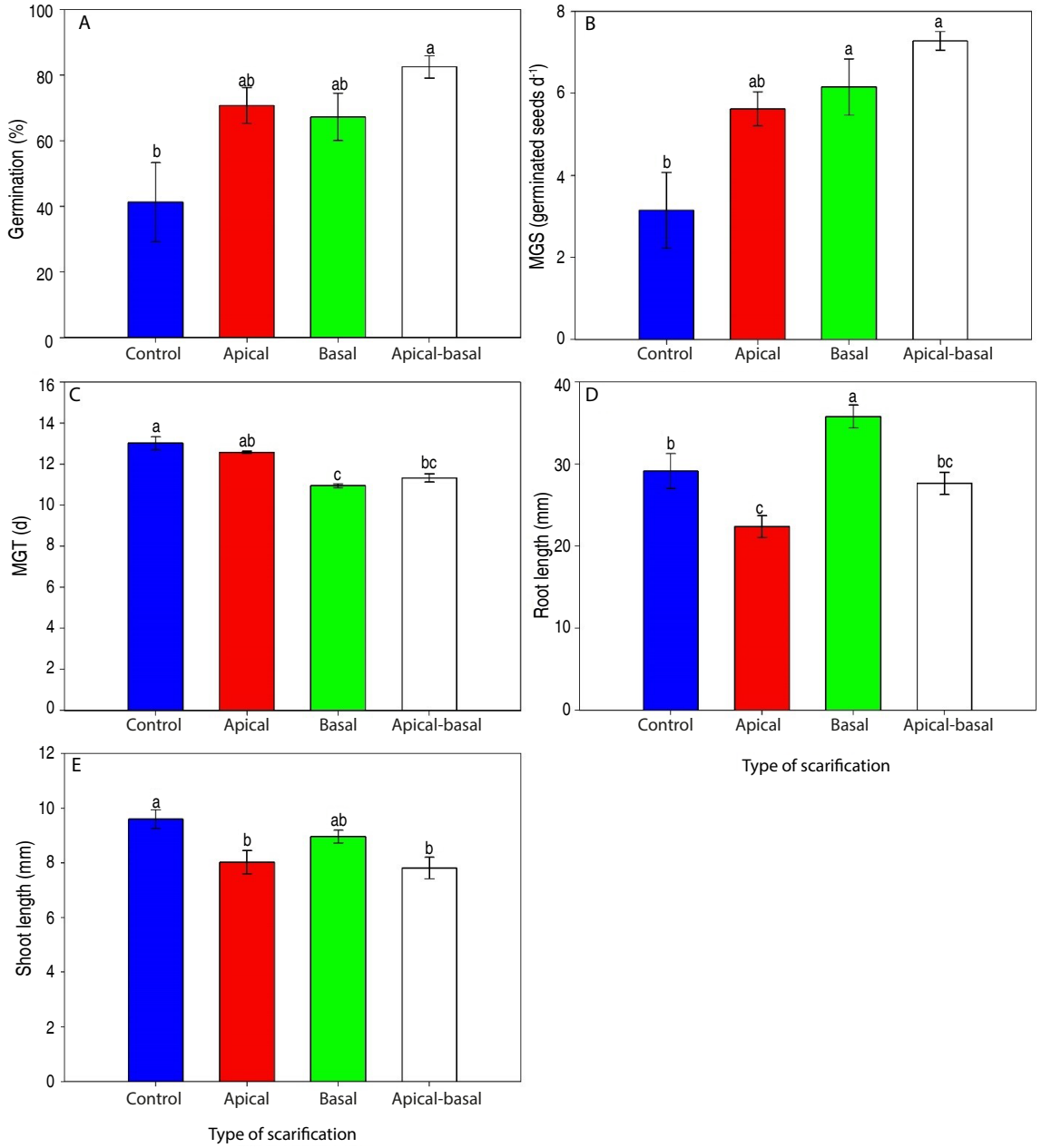

Figure 5. Effect of scarification type on germination percentage (A), mean germination speed (B), mean germination time (C), length of root (D), and length of shoot (E) of *S. oleraceus*. Means followed by different letters indicate significant differences according to the Tukey test (P<0.05). Vertical bars for each mean indicate the standard error (n=4).

The results suggest the presence of exogenous, probably physical dormancy in *S. oleraceous* seeds, since the basal, apical, and, especially, joint apical-basal cuts accelerated seed germination because a cut in the seed coat allows the uptake of water and oxygen into the seed in less time (Baskin and Baskin 2014). Physical dormancy is one of the most common types of dormancy in plant species, where the seed or fruit coats are water-impermeable and unable to imbibe water, limiting the germination process (Baskin and Baskin 2014). For this reason, scarification is one of

the main methods to break this dormancy (Arceo-Gómez et al. 2022; Guo et al. 2022). In nature and field crops, *S. oleraceus* can overcome physical dormancy through day/night temperature changes, high rainfall, microbial activity, soil acidity, or even passage through the digestive tract of some animals, among others, as reported for other species (Baskin and Baskin 2014). If this does not occur, the germination percentages that the species normally present may be sufficient, compensated for by the high number of seeds the plant produces, but also by its easy dispersion (Andrade et al. 2022). It has been reported that the average number of seeds per capitulum in this species is around 140 and the mean number of capitula per plant is 4.4 (Cici and Van Acker 2009).

The basal and apical cuts allow more uptake of water and oxygen to the seeds, which probably explains the higher and faster germination found in *S. oleraceus*. However, it remains unexplained how seedlings obtained with this treatment had a short length since it would be expected that they would be larger as they germinate faster. Partially similar results were found in *Passiflora edulis* Sims and *Passiflora ligularis* seeds, where a basal cut increased germination over the control (Gutiérrez et al. 2011).

Experiment 3
Sowing depth

For germination behavior, the seeds sowed at shallow depths had high percentages of emergence in the first 16 days, which then tended to stabilize until the end of the experiment. This also coincided with a higher speed of emergence, with a maximum peak at eight days. As the depth increased, the percentage and speed of emergence decreased considerably, mainly at a depth of 2 cm (Figure 6). At the three depths, the logistic model described the emergence behavior (Figure 6A and Table 1).

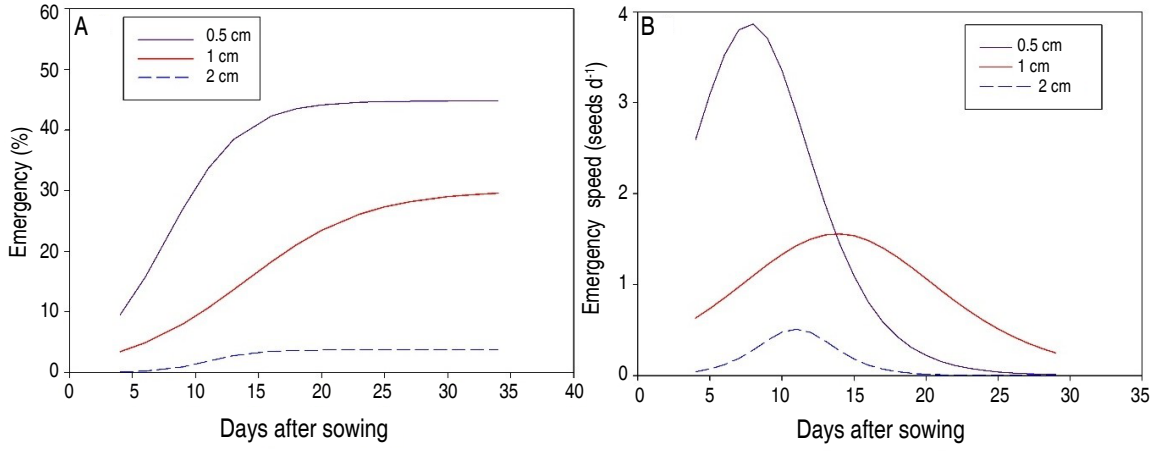

Figure 6. Effect of sowing depth on accumulated emergence (A) and the emergence speed (B) of *S. oleraceus* seedlings.

The final emergence percentage presented statistical differences (P<0.01), and there was a tendency for less emergence with a greater depth. Seedling emergence was no longer evident, even at depths greater than 2 cm (Figure 7A). For this reason, depths 5 and 10 cm in MES and MET were not included. The MES was statistically higher at a depth of 0.5 cm (2.13±0.44 emerged seedlings d^{-1}). The planting depth of 2 cm generated the lowest speed with 0.17±0.07 emerged seedlings d^{-1}. MET had no statistical differences; the times were between 22 and 23 d (Figure 7C). This reduction in the germination percentage and emergence rate at a greater depth could have been caused by external factors, including changes in light regime or lowering temperature with sowing depth (Baskin and Baskin 2014). However, according to Gresta et al. (2010), *S. oleraceus* seeds from Mediterranean populations were indifferent to light conditions during germination and maintained germination over 90% at temperatures between 10 and 35 °C. In this case, it may have been the factor of seed size (small seed reserves) that was responsible for the reduced seed

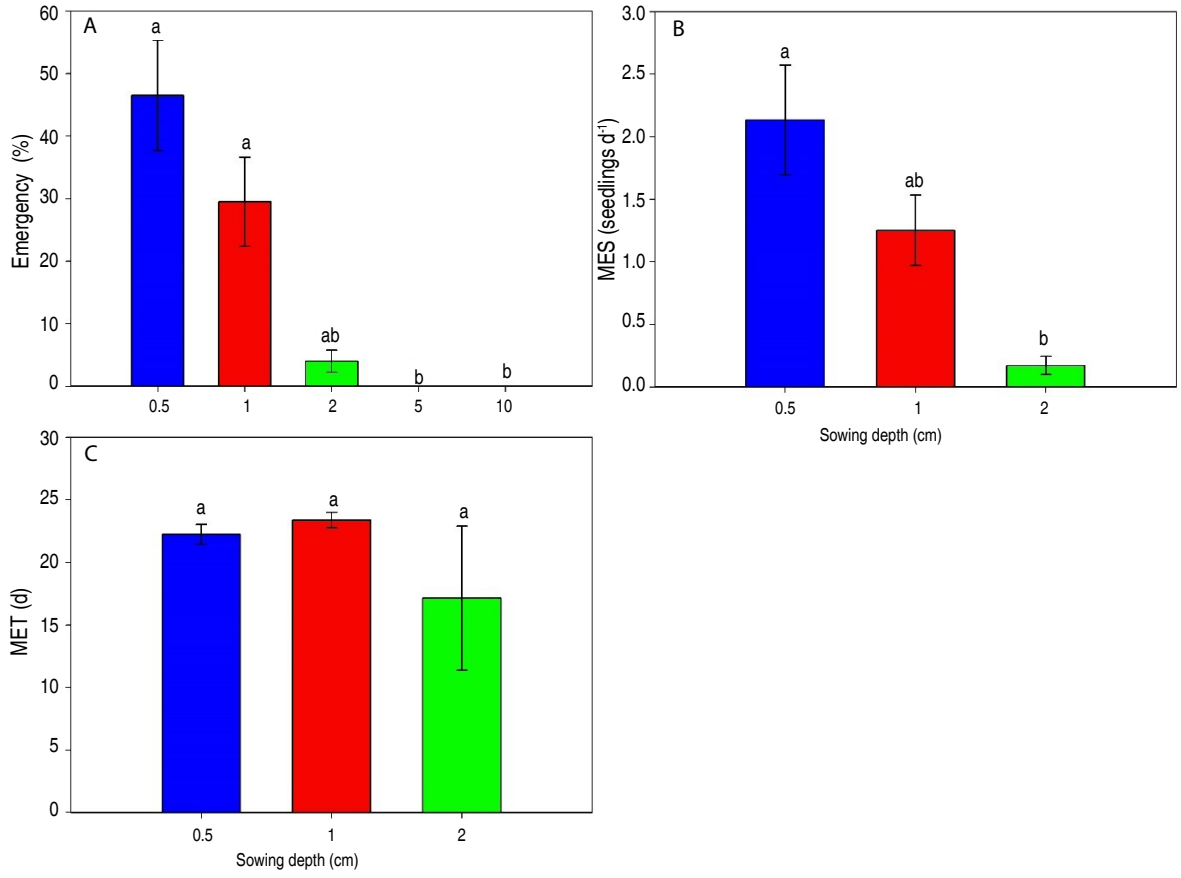

Figure 7. Effect of sowing depth on emergency percentage (A), mean emergency speed (B), and mean emergency time (C) of *S. oleraceus*. Means followed by different letters indicate significant differences according to the Tukey test (P<0.05). Vertical bars for each mean indicate the standard error (n=4).

germination (Fenner 2012). Since *S. oleraceus* achens are small (1 wide and 2-4 cm long), insufficient seed reserves might have limited the seedling emergence from the greatest depth.

The results obtained for *S. oleraceus* agree with Manalil et al. (2018), who indicated that light favored germination, which occurred at the first depths (0.5 to 2 cm). In this respect, Widderick et al. (2010) indicated that *S. oleraceus* can emerge all year round from the top 2 cm layer of soil, where emergence is favored more when seeds are 1 cm deep. *S. oleraceus* seeds found at depths greater than 2 cm, similar to those reported in another species, can remain viable for a variable time and may eventually germinate when the soil layer is turned, a common situation in different agricultural soils. Other seeds can fall to greater depths through the soil pores and finally die if the soil is not disturbed for several years (Baskin and Baskin 2014). Therefore, zero-tillage is an effective method for managing this species since most of the seeds remain in the uppermost 2 cm soil layer, and the seed bank can become smaller with increasing soil depths (Khalsa et al. 2021). On the other hand, Widderick et al. (2010) reported that, in eastern Australia, 2% of seeds were viable on the soil surface after six months, and 12% remained intact at a depth of 10 cm after thirty months. Although germination is increased by light, low germination is possible under darkness, indicating the potential of this species to emerge under conditions of shallow burial or residue cover (Manalil et al. 2018).

Similar to what was found in *S. oleraceus*, Niño-Hernández et al. (2020) reported that *Amaranthus hybridus* tends to

have a greater germination capacity when it is found at a shallower depth in the soil (3 cm). On the other hand, Li et al. (2020) reported that *Periploca sepium* seeds germinated faster at 2 cm, while at depths of 4-5 cm, the speed decreased considerably.

CONCLUSIONS

Gibberellic acid concentrations higher than 200 mg L^{-1} had an inhibitory effect on germination and, consequently, on seedling growth, unusual behavior in nature. Apical-basal cuts on the seeds generated the highest germination percentage (82.5±3.4%), and the process occurred faster than in the control. Seedling emergence was affected by planting depth, where *S. oleraceus* germinated at depths less than 2 cm, at greater depths, seedlings did not emerge. With 0.5 cm the highest percentage was obtained, corresponding to 46.5±8.8%. The results indicated that *S. oleraceus* presents physical dormancy and requires light to germinate. In all experiments, the logistic model described the behavior of germination percentage as a function of time. These results contribute to the knowledge of the physiology and ecology of this species and may be useful for integral management strategies for this weed.

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The influence of soil properties on grass and Kob abundance in Kainji Lake National Park, Nigeria

La influencia de las propiedades del suelo en la abundancia del césped y de Kob en el Parque Nacional del Lago Kainji, Nigeria

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ABSTRACT

The dependence of livestock on grasses as food for sustainability has been established. However, there is limited information on the variation in soil properties on grass and animal abundance at the Kainji Lake National Park (KLNP). Therefore, the impact of soil properties on grassland and Kob were assessed at the Kainji Lake National Park, Nigeria, using an established transect [Gilbert Child (GC), Shehu Shagari (SS), Mamudu Lapai (ML), Hussain Mashi (HM) and Mara Staude (MS)]. The results indicated that variations in soil physical properties were not significantly different. However, Mamodu Lapai (ML) soil had 16.06% higher clay content compared to Hussain Mashi (HM) soils and Mara Staude (MS), and 9.61% compared to Gilbert Child (GC) and Shehu Shagari (SS) soils. Gilbert Child soil had significantly higher soil pH than ML and MS soils. Total organic carbon and total N were lowest in GC, but higher in Na contents. Grass coverage was significantly higher in GC than in MS, while SS, ML, HM, and MS had 9.67, 25.92, 12.96, and 41.97% lower grass coverage, respectively. The cluster size of Kob and the number of Kob sited were significantly higher in GC than in MS, ML, and HM. Grass abundance and Kob activity were higher under sandy soil with high soil pH and Na content but low in TOC and TN. Maintaining grass cover and animal stock at the KLNP requires a proper grazing management strategy that ensures continual maintenance of soil quality for sustainability.

Keywords:
Animals cluster
Grass coverage
Kob
Organic carbon
Sand
Soil pH

RESUMEN

Se ha establecido la dependencia del ganado de los pastos como alimento para la sostenibilidad. Sin embargo, existe información limitada sobre la variación en las propiedades del suelo, el pasto y la abundancia de animales en el Parque Nacional del Lago Kainji (KLNP). Por lo tanto, se evaluó el impacto de las propiedades del suelo en los pastizales y Kob en el Parque Nacional del Lago Kainji, Nigeria, utilizando un transecto establecido [Gilbert Child (GC), Shehu Shagari (SS), Mamudu Lapai (ML), Hussain Mashi (HM) y Mara Staude (MS)]. Los resultados indicaron que las variaciones en las propiedades físicas del suelo no fueron significativamente diferentes. Sin embargo, el suelo Mamodu Lapai (ML) obtuvo un 16,06% más de contenido de arcilla en comparación con los suelos Hussain Mashi (HM) y Mara Staude (MS), y un 9,61% en comparación con los suelos Gilbert Child (GC) y Shehu Shagari (SS). El suelo de Gilbert Child tuvo un pH significativamente más alto que los suelos ML y MS. El carbono orgánico total y el N total fueron más bajos en GC, pero mayores en los contenidos de Na. La cobertura de pasto fue significativamente mayor en GC que en MS, mientras que SS, ML, HM y MS tuvieron 9,67, 25,92, 12,96 y 41,97% menos de cobertura de pasto, respectivamente. El tamaño del grupo de Kob y el número de Kob ubicados fueron significativamente mayores en GC que en MS, ML y HM. La abundancia de pasto y la actividad de Kob fueron mayores en suelos arenosos con alto pH y contenido de Na, pero bajos en TOC y TN. Mantener la cobertura de pasto y el ganado animal en el KLNP requiere una estrategia adecuada de manejo del pastoreo que garantice el mantenimiento continuo de la calidad del suelo para la sostenibilidad.

Palabras clave:
Grupo de animales
Cobertura de pasto
Kob
Carbono orgánico
Arena
pH del suelo

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Grasses form the source of fodder for domesticated and undomesticated animals. Generally, livestock dependence on grasses as feed forms faces deficits of 11.2% green fodder and 23.4% dry crop residues (Roy et al. 2019). The three sources of fodder supply for livestock feeds are crop residues, cultivated fodder, and fodder from common property resources like forests, permanent pastures, and grazing lands (Roy et al. 2019). In Nigeria, grasslands form 37% (about 341,800.82 km^2) of the total land area (Jimoh et al. 2020). The distribution of the grasses varied along different agroecological zones in the country. These variations are based on environmental factors, including the soil fertility status. The fertility of the soil is determined by many soil properties (including pedogenetic factors) their use, and management, which are interrelated (Ramírez-Iglesias et al. 2022). Hence, soil fertility describes the present state of the soil, which means that soil fertility is a combination of soil quality (mineral composition, soil texture, soil structure, soil organic matter (SOM) content, and phosphorus concentration) (Stewart et al. 2019). Also, the soil mineral composition affects the mineral levels found in pasture, and when not in the right proportion, causes an imbalance that will compromise animal productivity (Ramírez-Iglesias et al. 2022). Consequently, these factors and human activities (including fertilization) affect the abundance and quality of grasses (Ramírez-Iglesias et al. 2022). The availability of grasses in any location, without supplementation from other sources dictates or limits the sustainability of the available resources for which livestock are dependent (Lamidi and Ologbose 2014). Eventually, this may result in overgrazing when in short supply. Studies have shown that with adequate rainfall, grass production may substantially reduce when a considerable part of the leaf surface is frequently removed by close grazing without adequate time to regrow before it is harvested again (Kirkman et al. 2023). The extent of grass availability as fodder for livestock was reported to affect the abundance of livestock that depends on grasses (Kirkman et al. 2023). Hence, location and soil fertility are essential in the sustainability of wildlife that depends on grass for sustenance. This is particularly true for antelope (*Kobus kob*) commonly found in parks across the West, Central, and Eastern parts of Africa.

The Kob are herbivorous. They eat grass and reeds and are hunted for sport and food. However, a survey has shown that they are the third most preferred species of bush meat in Central Africa (Folorunso et al. 2019). The abundance of livestock (including Kob) and the maintenance of their population are generally affected by food availability. However, there is a need to evaluate the soil properties that favor the growth and quality of grasses, thereby increasing or decreasing the abundance of Kob. Previous studies on the Kob population and density at the Kainji Lake National Park (KLNP) have not considered soil properties as an essential contributor to the dynamic of Kobs (Olajesu et al. 2019). Hence, the knowledge of the relationship between soil properties, grassland, and grazing activities at the KLNP is lacking. This understanding will enunciate knowledge-based decisions on relevant grassland management strategies required in the different locations at the KLNP. Consequently, the risks that lead to degradation conditions of the park ecosystem are minimized, thus sustaining, or improving the animal population, thereby maintaining the park functions. Therefore, the aim of this study was to evaluate soil properties as they affect the availability of grass and the abundance of Kob in KLNP.

MATERIALS AND METHODS

Description of the study area

The study site was at the Kainji Lake National Park, predominantly explored for park activities. The park is broadly classified into 10 transects based on their vegetation (Ikusemoran and Olokor 2014). The coordinates of the location span between 09°40'N - 10°30'N and 03°30'E - 5°50'E with approximately 5,340.82 sq. km land area. Five of the existing jeep tracks/roads transects and trails transects based on the KLNP master plan (that is, vegetation types and water availability) were randomly selected: Gilbert Child (GC), Shehu Shagari (SS), Mamudu Lapai (ML), Hussain Mashi (HM) and Mara Tsaude (MT) for this study. The transects of 5 km in length were established per site and censured for Kob species, morning (07.00 - 10.00 h) and evening (15.00 - 18.00 h).

Soil sampling

Soil samples were collected at 0-30 cm depth from each transect. Using a grid-line survey technique with 20 m × 20 m, 35 samples were collected for the laboratory analysis of soil physical and chemical parameters. For the terraced landscapes (foot slope, back slope, and shoulder slopes), samples were taken in the middle of the terraces, while for the non-terraced crest and toe slope landscapes, samples

were collected at the maximum and minimum altitudes, respectively, for each transect.

Soil analysis

The soil samples were air dried, ground using a pestle and mortar, and sieved to pass through a 2 mm sieve. The soil chemical properties were determined at the Service Laboratory, Department of Soil Resources Management, University of Ibadan. Soil pH was determined following the FAO (2020) procedure. The organic carbon content was analyzed using the Walkley-Black method described by Poudel (2020). Soil organic matter was calculated by multiplying soil organic carbon by the van Bemmelen conversion factor of 1.724 (Heaton et al. 2016). The total nitrogen content was determined using the Kjeldahl method (Hicks et al. 2022). The available phosphorus was determined following the Olsen procedure (FAO 2021). The cation exchange capacity (CEC) and exchangeable bases were determined after extraction of the samples with 1 N ammonium acetate at pH=7 and the Ca^{2+} and Mg^{2+} were measured by atomic absorption. A flame spectrometer was used for Na and K from the aliquot following the procedures described by IITA (1982). The percent base saturation was calculated by dividing the sum of base cations by the cation exchange capacity and multiplying by 100 (Culman et al. 2019). The particle size distribution of the soil was determined by the Hydrometer method (Beretta et al. 2014).

Estimation of grass cover

The spatial distribution of grass cover (in %) during the study period was determined using a standard procedure through remote sensing by using aerial photographs and satellite images for each of the transects (Varga et al. 2014).

Cluster and number of Kob species

The cluster and number of Kob species were determined three times a week during the dry and rainy seasons by direct observation from a high hide (observatory tower), anthill, and tree formation. The observation of the Kob species was achieved using the King Census method of enumeration for the count with the aid of Zeiss Dialyt 10 by 40 binoculars, camera, and Global Positioning System (GPS) as described by Olajesu et al. (2019). The dry season observation spanned between the months of December - March, and the rain between June - September. The other months were the transition periods between seasons.

Statistical data analysis

Analysis of variance (ANOVA) was carried out to determine significant differences among the treatment means using SAS 9.0 software. Significant means were separated using Duncan's Multiple Range Test (DMRT) at $P<0.05$. Correlations coefficients were carried out for the relationship among the various parameters observed.

RESULTS AND DISCUSSION

Soil properties

The variations in soil physical and chemical properties of the locations under investigation are presented in Table 1. No significant difference was observed in the clay content among the soils from the different locations. However, soil from Mamodu Lapai (ML) had 16.06% higher clay content compared to Hussain Mashi (HM) and Mara Tsaude (MT), and 9.61% more clay content than Gilbert Child (GC) and Shehu Shagari (SS). The Silt content in the soils varied from 100.6 g kg^{-1} in SS and MT to 154.0 in soil from HM, with no significance observed among the locations. The sand content was highest (805.3 g kg^{-1}) in soil from SS, and lowest (638.6 g kg^{-1}) in MT, with no significant difference observed among locations. Soil texture influences the rate at which water is available to the plant (Huntley 2023). This, according to Huntley (2023), determines the pore space in the soil and consequently the movement of water and air through its porosity and permeability. The soil textural classification did not differ among the locations, except for SS with the highest in sand content. The soils from the regions signified similarity in textural classes. The similarities in the soil's textural classifications should not impose any appreciable dissimilarities on grass coverage since they belong to the same classification. However, the distinction in the proportions of sand, silt, and clay contents of the soil may create a slight variation in grass performance, which may likely affect the abundance of animals in the different locations. According to Weil and Brady (2017), soil with relatively higher clay content (such as observed in ML soil) is likely to have better water and nutrient holding capacity and resistance to pH change, but poorer in aeration. The soil is likely to be compacted, which is not adequately suitable for grass development. However, SS soil that was relatively higher in sand content is expected to show relatively opposite qualities to ML. Furthermore, soil with a relatively high percentage of silt and clay particles has a greater erodibility than sandy soil under the same conditions thus leading to poorer grass cover (Johannes et al. 2017).

Table 1. Soil physical and chemical properties across the sites in Kainji Lake National Park.

Parameters	Gilbert Child	Shehu Shagari	Mamudu Lapai	Hussain Mashi	Mara Tsaude	*P* value (<0.05)
Clay (g kg^{-1})	94.0±11.5	94.0±0.0	104.0±10.0	87.3±6.6	87.3±6.6	0.594ns
Silt (g kg^{-1})	120.5±17.6	100.6±13.3	134.0±25.8	154.0±11.5	100.6±13.3	0.306ns
Sand (g kg^{-1})	792.0±20.0	805.3±13.3	762.0±34.1	778.6±17.6	638.6±17.3	0.582ns
Textural classification	Sandy loam	Loamy Sand	Sandy Loam	Sandy Loam	Sandy Loam	-
pH	6.9±0.1^{c}	6.4±0.0^{ab}	6.3±0.0^{a}	6.7±0.1^{bc}	6.3±0.1^{a}	0.006*
Total organic carbon (%)	10.2±2.5	18.0±5.6	12.7±3.0	21.8±1.4	16.5±2.6	0.208ns
Total N (%)	0.9±0.2	1.6±0.5	1.1±0.3	2.0±0.1	1.5±0.2	0.209ns
Available P (mg kg^{-1})	19.7±1.5	19.7±1.1	18.6±0.7	21.7±0.8	21.8±1.0	0.199ns
Exchangeable acidity	0.1±0.0	0.2±0.0	0.1±0.0	0.22±0.0	0.2±0.0	0.231ns
Ca (cmol kg^{-1})	2.2±0.5	2.4±0.4	1.6±0.4	3.4±0.3	3.3±0.6	0.111ns
K (cmol kg^{-1})	0.2±0.0^{a}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{b}	0.2±0.0^{a}	0.007*
Mg (cmol kg^{-1})	0.4±0.0	0.6±0.0	0.4±0.0	0.5±0.0	0.5±0.0	0.463ns
Na (cmol kg^{-1})	0.8±0.0	0.8±0.0	0.7±0.0	0.7±0.0	0.7±0.0	0.217ns
Base saturation	95.9±0.3	94.2±1.4	94.2±1.6	95.8±1.3	94.5±1.2	0.811ns
CEC	4.0±0.6	4.2±0.4	3.3±0.2	5.0±0.2	5.1±0.6	0.068ns

Means in a row followed by the same letters are not significantly different (P<0.05) according to Duncan's Multiple Range Test; ns = Not significant, * = Significant at P<0.05.

The soil's chemical properties varied among the locations (Table 1). The soil pH differed significantly among the study sites under investigation. It differs from 6.3 in ML and MT to 6.9 in GC. Soils from ML and MT had significantly lower soil pH compared to GC and HM. Also, SS soil had significantly higher soil pH compared to GC. Although, these pH values were within the range considered adequate for good grass growth, and nutrient availability (Yue et al. 2017). In their report, soil pH affects the availability and root uptake of nutrients to the crop. However, the ability of grass to thrive under different soil pH differs within crop variety (Yue et al. 2017). The total organic carbon (TOC) did not differ significantly. However, the highest TOC was observed in soils from HM with 53.21, 17.43, 41.74, and 24.31% more compared to GC, SS, ML, and MT, respectively. The TOC serves as a major component of understanding land degradation resulting from diminished soil organic carbon (SOC) stock, hence, loss of soil condition and function (Tessema et al. 2021). This implies that HM had the highest carbon stock followed by GC, while the least was observed in MT. An increase in TOC also implied higher microbial activities and improved soil structure, consequently enhancing soil health (Silva et al. 2014). However, the depletion of TOC in grassland has also been reported to be associated with intensive grazing activity (Ajorlo et al. 2011). Total N in the soils from the locations was not significantly different and had a similar trend as observed for the TOC. The low total N observed in GC could have been a result of overgrazing as reported by Ajorlo et al. (2011) that overgrazing results in the degradation of total soil N. Also, the soil available *P* values were similar in the different locations with no significant difference observed. However, HM and MT had higher available *P* compared to the other locations with the lowest observed in ML soil. The exchangeable acidity ranged from 0.1 (in GC and ML) to 0.22 (in HM soil), with no significant difference among the values. The Ca content was higher in the HM and MT soils and lowest in the ML soil. However, the values were not significantly different. Potassium content varied significantly among the soils from different locations. Soils from GC and MT had significantly higher K compared to the soils from other locations. Soil from SS contained the highest Mg content, while the lowest was observed in soils from GC and ML but were not significantly different. The nutrient compositions in soils from the various locations were similar in trend to the TOC. A similar finding was reported by Gerke (2022), that soil organic matter increases and improves

soil nutrients. This was attributed to the significant role of organic matter in affecting nutrient availability. The soils from the locations did not differ significantly in Na content; however, GC and SS had higher Na than the other locations. High Na in soil was reported to result in a rapid loss of the native SOC because of increased solubility, decomposability, and accessibility, hence, increasing its accessibility and degradability for the microbial population (Singh 2016). However, grasses vary in adaptation to saline conditions (Yue et al. 2017). The high Na content in GC soil can also be responsible for the low TOC, while the Na in SS soil could indicate a possible susceptibility to rapid depletion of the soil TOC. Similarly, the base saturation ranges from 95.8 in HS to 94.2 in SS and ML but was not significantly different. The CEC of the soils did not vary significantly among the locations, but MT had 21.56, 17.64, 35.29, and 1.06% higher CEC than GC, SS, ML, and HM, respectively.

Grass cover and Kob abundance

Grasslands serve as energy capture, soil conservation, hiding cover, bedding cover, nesting cover, and improve habitat quality (Vandever and Allen 2015). They also provide the feed base for grazing and thus numerous high-quality foods. Percent grass coverage across the locations indicated that GC had significantly higher grass coverage compared to MT (Table 2). However, compared to GC, SS, ML, HM, and MT had 9.67, 25.92, 12.96, and 41.97% lower grass coverage, respectively. This trend was similar to what was observed for Na, with GC having higher Na and relatively lower TOC. Hence, the grasses in these locations were enhanced by alkaline conditions (Yue et al. 2017). Furthermore, the higher grass coverage observed could have resulted in overgrazing (by encouraging animal influx) which led to serious grassland degradation (Mligo 2015). They reported that overgrazing results in the depletion of TOC. This explained the differences found in TOC and Na in GC. Consequently, the possibility of a higher level of soil degradation is imperative as reflected in Table 1, whereby the location had lower nutrient status. Soil quality as an indicator of improved crop performance depends on the value of other soil properties considered as the most suitable conditions for a particular crop (Johannes et al. 2017). Also, the cluster size of Kob was significantly higher at GC compared to the cluster size of Kob observed at the other locations.

Table 2. Influence of location differences on grass cover, cluster size of Kob, and number of Kob sited in Kainji Lake National Park.

Parameters	Grass coverage (%)	Cluster size of Kob	No. of Kob sited
Gilbert Child	51.70^{a}	9.19^{a}	61.00^{b}
Shehu Shagari	46.70^{ab}	5.76^{b}	76.00^{a}
Mamudu Lapai	38.30^{bc}	3.08^{b}	15.00^{c}
Hussain Mashi	45.00^{ab}	4.54^{b}	28.00^{c}
Mara Tsaude	30.00^{c}	3.59^{b}	21.00^{c}
SE	3.11	0.85	4.12

Means in a column followed by the same letters are not significantly different (P<0.05) according to Duncan's Multiple Range Test.

Grassland coverage indicates the carrying capacity of the location in supporting a larger number of animals. The result indicated that the GC with significantly higher grass coverage than the other locations, except HM also had a significantly higher cluster size of Kob. The finding was supported by Qi et al. (2017) report, which reported that sustainable livestock production must be accompanied by sufficient grassland size. The high cluster size in GC consolidated that there were higher activities of Kob in this location than in the others. This finding reaffirmed Olajesu et al. (2019) report that GC had a higher cluster size of Kob than the other locations under study. The basis for this assertion is that an increase in cluster size is likely to encourage high reproductive activity, concomitantly resulting in to increase in the Kob population (FAO 2018), due to the realization of an adequate food supply. However, SS had a significantly higher number of Kob sited compared to the sited Kob at GC, which also had significantly higher than the sited Kob at the other locations. This finding was substantiated by the high salt content observed in GC soil. According to Taboada et al. (2011), the level of salt accumulation at the surface of

grazing land is an indication of the extent of animal activity. In their report, salt does not accumulate at the surface of the soil with limited grazing. Surface soil salt accumulation was attributed to the fact that evaporative losses were reduced with a thick layer of litter, thus decreasing the upward flux of water and salts. Hence, only minor salinity fluctuations were detected in the surface soil of pasture with limited grazing. Nevertheless, the fact that higher TOC was observed in SS soil despite similar Na content, indicated limited grazing, which could be a result of better grass quality or more grass coverage at GC.

Correlation coefficient soil physical properties, grass cover, and Kob abundance

The Pearson correlation coefficient among the observed parameters indicated a non-significant correlation between clay content in the soil with silt, and sand but had a negative correlation coefficient with a cluster size of Kob and number of Kob sited (Table 3). Sand content in the sampled soil correlated significantly with the percentage of grass coverage. This finding supported Weil and Brady (2017) and Johannes et al. (2017) report that grasses are favored in well-drained soils with little silt and clay content. A high correlation coefficient also exists between grass coverage, cluster size of Kob, and number of Kob sited.

The soil pH had a significant correlation with percentage (%) base saturation (Table 4). A similar result was reported by Kabala and Łabaz (2018) that %BS increases with increasing soil pH. Also, a very high correlation coefficient

Table 3. Pearson correlation coefficient between the soil physical properties and grass cover, cluster, and number of Kob.

	Clay	Silt	Sand	Grass coverage	Cluster size of Kob
Silt	0.08	-	-	-	-
Sand	0.38	0.36	-	-	-
Grass coverage	0.11	0.25	0.90*	-	-
Cluster size of Kob	-0.09	-0.14	0.51	0.81	-
No. of Kob sited	-0.06	-0.44	0.60	0.73	0.75

* = Correlation is significant at *P*<0.05 level.

existed between soil pH with grass cover score and cluster size of Kob. The relationship between TOC and total N was highly significant. Similarly, total soil OC had a high correlation coefficient with Ca, Mg, CEC, and available P. Total N correlated significantly with available P, Ca, Mg, and CEC, and had significant correlation exchange acidity. A highly significant correlation existed between available P with Ca and CEC observed. Also, Ca had a very high significant correlation with CEC. The soil Na had a highly significant correlation with the number of Kob sited, while the correlation with a cluster size of Kob and grass cover was high, but not significant. Corwin and Lesch (2005) reported that water uptake by the plants under saline conditions was limited, thus increasing the soil's osmotic potential, and causing nutritional imbalances, consequently reducing plants' growth and development. However, the high correlation observed between Na and grass cover, cluster size of Kob and number of Kob sited revealed that the grasses preferred saline conditions and could lead to the deterioration of the location vegetation. Similarly, sodium was reported to play an essential nutrient in animal feed (Johansson 2008). Salt helps in maintaining the right level of mineral balance of the animals and keeps the livestock healthy. There are; however, limits to its requirement to prevent food poisoning (EFSA 2019). Furthermore, studies have indicated that high salinity beyond the threshold level hinders root growth and causes drought symptoms (Vandever and Allen 2015). These salts could compete with the plant roots for available water. Consequently, under drought conditions, soil salts pull water out of plant roots through osmotic pressure, causing them to dehydrate or desiccate. High salt concentrations in the soil could burn young tender roots and prevent normal development. Similarly, intensive grazing causes an increase in topsoil temperatures, thus leading to higher soil water evaporation rates (Taboada et al. 2011). The various correlation was in support of Ajorlo et al. (2011), indicating the effects of varying level of overgrazing activities of Kob across the location on the soil properties of the different locations under investigation.

Table 4. Pearson correlation coefficient between the soil chemical properties, grass cover, cluster, and number of Kob.

`	pH	Total OC	Total N	Ava. P	Exch. acidity	Ca	K	Mg	Na	Base saturation (%)	CEC
Total OC	-0.16	-	-	-	-	-	-	-	-	-	-
Total N	-0.13	0.99**	-	-	-	-	-	-	-	-	-
Ava. P	0.09	0.66	0.69	-	-	-	-	-	-	-	-
Exch. acidity	-0.20	0.93*	0.94*	0.78	-	-	-	-	-	-	-
Ca	0.10	0.74	0.77	0.99**	0.85	-	-	-	-	-	-
K	0.27	-0.50	-0.46	0.29	-0.22	0.20	-	-	-	-	-
Mg	-0.31	0.72	0.70	0.36	0.83	0.46	-0.33	-	-	-	-
Na	0.44	-0.35	-0.36	-0.39	-0.22	-0.34	0.17	0.22	-	-	-
Base saturation (%)	0.94*	0.01	0.05	0.35	-0.04	0.34	0.30	-0.34	0.14	-	-
CEC	0.08	0.69	0.72	0.99**	0.84	0.99**	0.28	0.49	-0.27	0.30	-
Grass coverage	0.79	-0.15	-0.15	-0.34	-0.24	-0.28	-0.16	-0.03	0.75	0.57	-0.29
Cluster size of Kob	0.82	-0.44	-0.43	-0.18	-0.34	-0.17	0.43	-0.16	0.84	0.61	-0.12
No. of Kob sited	0.39	-0.10	-0.11	-0.25	0.03	-0.17	0.03	0.45	0.97**	0.11	-0.11

OC = Organic carbon; Ava. P = Available P; Exch. acidity = exchange acidity; CEC = Cation exchange capacity; *, ** = Correlation is significant at $P<0.05$, t, $P<0.01$ levels, respectively (2-tailed).

CONCLUSIONS

The impact of different soil properties on grass coverage and animal abundance at the KLNP transect under investigation is confirmed by this study. Sand, silt, and clay contents in the locations were similar, but GC and SS soils were sandier than the other locations. Soil from GC with lower TOC and TN, but higher soil pH and Na contents had higher grass coverage and the cluster of Kob sizes. The order of grass coverage and cluster sizes of Kob were GC>SS>HM>ML>MT and GC>SS>HM>MT>ML, respectively. Sand and grass coverage were highly correlated, while soil Na content and the number of Kob sited had a high correlation. Based on the findings of this study, grass coverage, and Kob activity increased under sandy soil, high in soil pH and Na content but low in TOC and TN. However, to maintain good quality grass cover and animal stock at the KLNP, a proper grazing management strategy is critical to ensure continual maintenance of soil quality for sustainability.

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Modeling the grain yield loss and quality assessment of some durum wheat (*Triticum durum* Desf.) genotypes under semi-arid conditions

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Modelización de la pérdida de rendimiento de grano y evaluación de la calidad de algunos genotipos de trigo duro (*Triticum durum* Desf.) que crecen en condiciones semiáridas

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Keywords:
Durum wheat
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Mathematical model
Technological quality

ABSTRACT

This study was conducted at the Experimental Station for Field Crops ITGC in Setif -Algeria during the growing season (2020-2021), to evaluate the parameters of the technological quality efficiency of three durum wheat genotypes {Boussellam (BOS), Oued El Bared (OB), and GTA dur (GTA)} and the efficiency of using mathematical models based on the relation between grain moisture in the field and grain yield to estimate grain yield loss caused by the delayed harvest according to the randomized blocks design with three replications. Results demonstrated that the genotype effects were significant for all technological parameters, the protein content varied from 13.70 to 15.4%; the highest content of protein registered by (OB) was 15.4%. In addition, the values of test weight varied between 79.47 and 81.97 kg hL^{-1}, with a general mean of 80.96 kg hL^{-1}. The study of correlations test showed that there was a significant and positive correlation between the decreased grain moisture in the field and the loss in final grain yield, which suggests that the final grain yield decreases as the grain moisture decreases. This loss can be predicted using a mathematical regression model. The statistical analysis revealed the best agreement between measured and simulated grain yield, with low average absolute error and root mean square error. The grain yield was also well simulated with the observed yield giving a coefficient of efficiency (E) of 0.76, i.e., with a simulation capacity of 76%. Overall, and after the physiological maturity of the grains the mathematical model proved that with the 1% loss of grain moisture, there is a loss of about 0.290 t ha^{-1} of grain yield.

Palabras clave:
Trigo duro
Humedad de grano
Producción de grano
Modelo matemático
Calidad tecnológica

RESUMEN

El objetivo de este estudio fue evaluar la eficiencia del uso de modelos matemáticos para estimar la pérdida de rendimiento de grano en algunos genotipos de trigo duro que crecen en condiciones semiáridas en función de la relación entre la humedad del grano en el campo y el rendimiento de grano final. Los resultados de ANOVA demostraron que los efectos de los genotipos fueron significativos para todos los parámetros tecnológicos, el contenido de proteína varió de 13,70 a 15,4%; el mayor contenido de proteína registrado por Oued El Bared fue 15,4%. Además, los valores de peso hectolítrico oscilaron entre 79,47 y 81,97 kg hL^{-1}, con media general de 80,96 kg hL^{-1}. El estudio de la prueba de correlaciones indicó que hubo una correlación significativa y positiva entre la disminución de la humedad del grano en el campo y la pérdida en el rendimiento final del grano, lo que sugiere que el rendimiento final del grano disminuyó a medida que disminuye la humedad del grano. Esta pérdida puede predecirse utilizando un modelo de regresión matemática. El análisis estadístico reveló la mejor concordancia entre el rendimiento de grano medido y el simulado, con un error absoluto promedio y un error cuadrático medio bajo. El rendimiento de grano también fue simulado con el rendimiento observado dando un coeficiente de eficiencia (E) de 0,76, es decir, con una capacidad de simulación del 76%. En general, y después de la madurez fisiológica de los granos, el modelo matemático demostró que a partir del 1% de pérdida de humedad del grano, se pierde alrededor de 0,290 t ha^{-1} de rendimiento de grano.

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Durum wheat (*Triticum durum* Desf.), is one of the oldest cultivated cereal species in the world and one of the staple foods for a balanced diet because it is very rich in calories (149 Cal 100 g^{-1}), starch, proteins, trace elements, and vitamin B1 (Amallah et al. 2016). So, it is recommended to consume Durum wheat widely because it constitutes a large part of humanity's diet at around 35% and provides 15% of its energy needs (IDRC 2010).

Algeria has recorded an average cereals production of around 5.23 million tons over the past three seasons and remains far from the demand estimated at 13 million tons (MADR 2021), durum wheat is the first economically major crop in Algeria which represents 46% of total cereals production grown on 1.6 million hectares (Harrag and Boulfred 2019). However, the durum wheat produced covers only 24 to 55% of the country's annual consumption based on the climate conditions, i.e., approximately 202 kg per capita per year (ITGC 2022), while the residual supplier is essentially provided by imports, to meet the needs croissants, which makes Algeria the world's leading importer of durum wheat (Bessaoud et al. 2019). This situation can be explained by: the main dependence of the Algerian diet on the consumption of durum wheat in its various forms (bread, pasta, couscous, freekeh, aish, baghrir, tamina, borj, rishta, etc.) and on the relatively low grain yields obtained in the country, which is closely linked to the effect of the climate (insufficient and erratic rainfall, low winter temperatures, spring frosts, late-season drought, and sirocco occurrence) (Haddad et al. 2021), with poor application of modern agricultural techniques (including the process and the harvest period) (Di Mola et al. 2021).

Harvesting durum wheat is considered one of the most important stages in the production system, due to its direct relationship with the grain yield and final quality of the grain. Each year, it is observed that there is a big gap between the yields obtained and those estimated because of the high losses registered before and during the harvest which are estimated at 20% for cereal crops (Gustavsson et al. 2011; Lipinski et al. 2013). Harvesting crops with appropriate grain moisture content often leads to a reduction in yield losses, but in the event of a delay in harvesting after physiological maturity, undesirable changes in the technological quality of durum wheat are likely (Forster et al. 2017). Any delay in harvesting will reduce the quality of the harvested grain and may influence the final storage result (Deliberali et al. 2010). Luhmann (2017) also demonstrated that when harvest was delayed beyond grain maturity, durum wheat test weight (Kg hL^{-1}) began to decrease and there was no need to wait for the straws to completely dry out; this will cause a loss of weight, loss of dry matter. One of the options for improving grain quality is early harvesting. For this, the producer must take into account the need and availability of drying, the risk of spoilage, and the energy spent on drying (Embrapa 2011; Alt 2018). The early harvesting process after physiological ripening can be effective in obtaining a suitable industrial quality product, when there are high levels of starch, protein, and water, preventing the grain from being exposed longer to diseases such as mycotoxin-producing fungi (Paul and Lindsey 2014). It is necessary to evaluate whether the grain moisture content at harvest is compatible or not with their characteristics. This study aimed to evaluate the parameters of the technological quality of three durum wheat genotypes sowing under semi-arid conditions and to estimate the efficiency of using mathematical models based on the relation between the grain yield and grain moisture in the field to estimate the grain yield loss caused by the delayed harvest.

MATERIALS AND METHODS

The experiment was carried out at the Experimental Station for Field Crops ITGC in Setif -Algeria- (36°08'N, 5°20'E; 973 m) during the growing season (2020-2021). Three genotypes of durum wheat (*Triticum durum* Desf.) are: Boussellam (BOS), Oued El Bared (OB), and GTA dur (GTA) were shown on 20-12-2020 in randomized blocks design with three replications. The sowing density was adjusted to 300 seeds m^{-2}. The one plot's dimensions were 2.4 m^2, comprising 6 rows, each 2 m long and spaced 0.2 m apart. The soil has a loamy-clay texture with an average organic matter content of 2.8%. The bulk density is 1.35 g cm^{-3}, the field capacity is 25%, and the wilting point is at 12%. The experimental plots were fertilized with 100 kg ha^{-1} of mono-ammonium phosphate (12% N + 52% P_2O_5) before sowing and 80 kg ha^{-1} Urea (46% N) at the tillering stage. The monthly precipitation recorded during the 2020-2021 crop season, from September 1st to June 30th, reached 320.24 mm (Tutiempo Network 2021).

Agronomic and technological measures

Measurements were taken from the onset of physiological

grain maturation until the day of mechanical harvest in the experiment. Samples were collected five times during 13-day intervals after final maturation to evaluate the effect of grain moisture degradation on yield components and technological parameters of durum wheat grain:

Agronomic parameters

Grain yield (GY, t ha^{-1}) was estimated at harvest, the grain yield samples were harvested manually in an area of 1 m^2 for each repetition and then converted into ton ha^{-1}. Measurements of grain moisture content in the field (GMF, %) were conducted using a portable moisture meter "Riceter f 508" type which directly gives the grain moisture in the sample as a percentage.

Technological parameters

The quality analyses were carried out on the grains after the harvest, grain protein content (P, %), the content of yellow berry (non-vitreous grains) (YB, %), the test weight (TW, Kg hL^{-1}), and the content of grain moisture in the laboratory (MIAB, %), using an "Inframatic 9500 Results Plus" type measuring device, which ensures precise measurement of the samples.

Prediction model for estimating grain yield losses based on grain moisture values

To assess grain yield losses due to grain moisture degradation at harvest, a linear regression model was utilized and to evaluate the performance of the model, the following notations were adopted:

Average absolute error (AAE)

The average absolute error (AAE) measures the weighted average magnitude of the absolute errors and can be calculated using the following equation (1) (Willmott and Matsuura 2005).

$$AAE = \frac{\sum_{i=1}^{n} |Oi - Si|}{N} \quad (1)$$

Root mean square error (RMSE)

Root means square error (RMSE) gives the weighted variations in errors (residual) between the simulated and observed values, if the value of AAE and RMSE is close to 0, then the model is perfect. The RMSE was calculated following equation (2) (Willmott and Matsuura 2005).

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n} (Oi - Si)^2}{N}} \quad (2)$$

Index of agreement (D)

The index of agreement can detect additive and proportional differences in the observed and simulated means, the agreement value of 1 indicates a perfect match, and 0 indicates no agreement at all. It was calculated using the equation (3) by Willmott et al. (1985).

$$D = 1 - \frac{\sum_{i=1}^{n} (Si - Oi)^2}{\sum_{i=1}^{n} (|Si - MO| + |Oi - MO|)^2} \quad (3)$$

Coefficient of efficiency (E)

The coefficient of efficiency (E) expresses how much the overall deviation between observed and simulated values departs from the overall deviation between observed values (Oi) and their mean value (MO). The E is unitless and can take values ranging from $-\infty$ to +1, with better model simulation efficiency when the values are closer to +1. It is calculated using the equation (4) (Yan et al. 2015).

$$E = 1 - \frac{\sum_{i=1}^{n} (Oi - Si)^2}{\sum_{i=1}^{n} (Oi - MO)^2} \quad (4)$$

Correlation coefficient (r)

The correlation coefficient is an indicator of the degree of proximity between the observed values and the estimated values of the model. The observed and simulated values are found to be better correlated when the correlation coefficient approaches one. If the observed and predicted values are completely independent, i.e., they are uncorrelated, then r will equal zero. The correlation coefficient was calculated by the following equation (5).

$$r = \frac{\sum_{i=1}^{n} (Oi - MO)(Si - MS)}{\sqrt{\sum_{i=1}^{n} (Oi - MO)^2 \sum_{i=1}^{n} (Si - MS)^2}} \quad (5)$$

Where:
Si = simulated value
Oi = observed value
n = number of observations
MS = mean of simulated value
MO = mean of observed value

A statistical analysis was applied to determine the significant differences between genotypes "one-way analysis of variance (ANOVA)". Fisher's LSD test was used for comparisons of means with CoStat software version 6.4.

RESULTS AND DISCUSSION

Agronomical parameters

The analysis of variance proved that the effects of genotypes are significant for all parameters. The genotype Boussellam registered a grain yield equal to 2,063 t ha^{-1}, followed by Oued El Bared at 2,006 t ha^{-1} which is equal to the general average (2,006 t ha^{-1}), and GTA dur recorded the minimum value of 1,950 t ha^{-1}. The grain yield of the genotype Boussellam exceeded that of the two genotypes, Oued El Bared and GTA dur with 2.76 and 5.48%, respectively (Table 1).

Table 1. Analysis of the variance of the parameters: Grain moisture, grain yield, and quality parameters.

Genotypes	GMF (%)	GY (t ha^{-1})	MIab (%)	P (%)	TW (kg hL^{-1})	YB (%)
Boussellam (BOS)	9.00^{b}	2.063^{a}	8.50^{b}	13.70^{b}	81.97^{a}	10.50^{a}
Oued El Bared (OB)	9.20^{a}	2.006^{ab}	8.60^{a}	15.40^{a}	79.47^{c}	10.33^{a}
GTA dur (GTA)	9.03^{b}	1.950^{b}	8.47^{b}	14.56^{ab}	81.43^{b}	1.00^{b}
Mean	9.08	2.006	8.52	14.55	80.96	7.28
LSD 0.05	0.08	0.098	0.08	0.87	0.15	2.64

GMF, Grain moisture content in the field; GY, Grain yield; MIAb, content of grain moisture in the laboratory; P, Grain protein content; TW, Test weight; YB, Content of yellow berry (non-vitreous grains).

A very significant inter-varietal difference (P<0.01) for grain moisture content in the field, where the BOS genotype registered the minimum value (9%) and the maximum value was recorded by Oued El Bared (9.2%). Low final grain yield values are accompanied by lower relative values in the grain moisture content in the field (Figure 1). The ideal moisture for harvesting durum wheat is 15 to 18%, then the grain must be dried and stored with 13% moisture (Alt et al. 2019). Grain moisture content affects the time of harvest and the drying process (Maiorano et al. 2014). When farmers have a hot air dryer, the harvesting process can be started at 18% moisture content (Metz 2006). In Algeria, due to the lack of artificial drying, durum wheat harvesting generally begins when the moisture content of the grains is around 13% and continues down to 9%, coinciding with high temperatures in the air and the lack of a combined harvester.

Five samples were collected after the physiological maturity of the grains, expressed in five dates during a time interval of 13 days, commencing with the first sample on "Date 1" and concluding on "Date 5" to quantify the grain yield (t ha^{-1}) and their respective grain moisture content (%). A consistent decline in grain moisture content (GMF) was observed from Date 1 to Date 5, coinciding with a decrease in grain yield (GY). The highest GMF recorded were 13.00, 13.03, and 13.47%, corresponding to maximum grain yields of 3,201, 3,032, and 2,726 t ha^{-1} for the BOS, OB, and GTA genotypes, respectively. In contrast, the lowest GMF were recorded on Date 5, with values of 9.00, 9.20, and 9.03% for the BOS, OB, and GTA genotypes, respectively, and these conditions resulted in the lowest grain yields of 2,063, 2,006, and 1,956 t ha^{-1} for each respective genotype (Figure 1).

According to Parvej et al. (2020), grain yield losses can fluctuate based on multiple factors, such as the geographical region, prevailing weather conditions, the specific type of wheat under cultivation, and the length of the harvest delay. The results of this study indicated after 13 days of physiological ripening, grain moisture losses at Boussellam reached 30.77%, this corresponds to a loss of grain yield of 35.55% equivalent to 1,138 t ha^{-1}, GTA dur lost 32.96% of grain moisture which corresponds to 28.47% (0.776 t ha^{-1}) loss of grain yield and at Oued El Bared the grain moisture losses reach 29.39% and it has lost 33.84% of its grain yield i.e., 1,026 t ha^{-1} for 13 days (Figure 1). Indeed, after physiological maturation, each day of delay leads to a loss of 2.73% (0.088 t ha^{-1}), 2.60% (0.079 t ha^{-1}), and 2.19% (0.060 t ha^{-1}) of the grain yield for Boussellam, Oued El Bared and GTA dur, respectively.

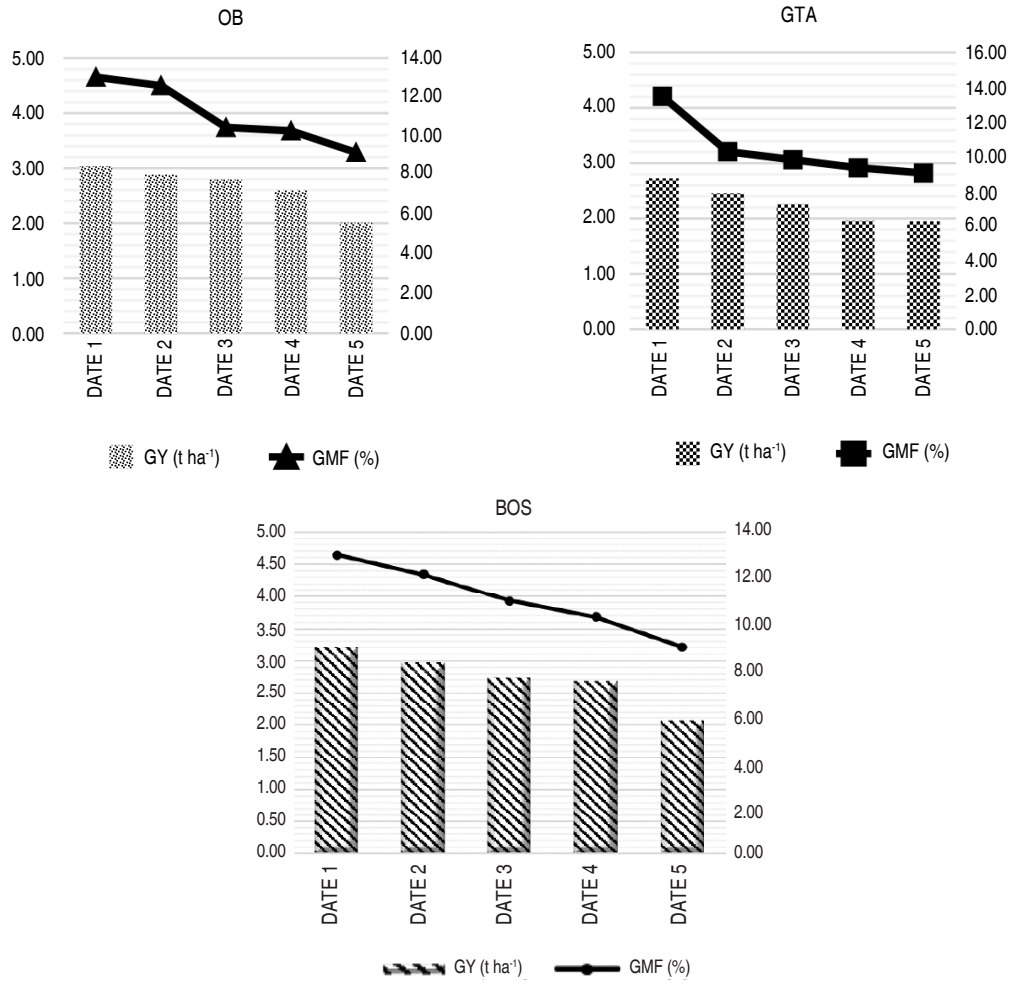

Figure 1. Grain yield loss (GY) as a function of grain moisture degradation in the field (GMF). BOS, OB, and GTA are abbreviations of Boussellam, Oued El Bared, and GTA dur, respectively.

Technological parameters

The grain protein content shows significant differences (P<0.05) between the genotypes tested, it varied between 13.7 and 15.4% with an average of 14.55%. The grain protein content was significantly higher for the Oued El Bared genotype (15.4%) followed by no significant differences in the GTA dur genotype (14.56%), while grain protein content was significantly lower for Boussellam genotype (13.7%) (Table 1).

This inter-varietal variability highlights a genetic divergence between the varieties studied. The genetic diversity of endosperm reserve protein richness has been widely analyzed by several authors, according to Serra et al. (2021) attributed protein synthesis to the expression of several genes so that each genotype has a wide spectrum of variants. Allelic is responsible for its qualitative profile.

As well as the analysis of variance indicates that there is a very highly significant difference (P<00.1) between the genotypes for the test weight (TW), this explains why the test weight is a parameter under genotypic control, but it is also affected by climatic conditions at the time of grain filling. It is noticed that the test weight was significantly the highest for Boussellam genotype (81.97 kg hL^{-1}) while it was significantly the lowest for OB and GTA genotypes (79.47, 81.43 kg hL^{-1}) with significant differences respectively (Table 1). In most countries, durum wheat is marketed based on its physical properties, established by grading systems such as test weight (Forster 2016). In addition,

the dimensions of the grain of wheat, as well as the size of the caryopsis, constitute very important factors that influence the semolina yield. At the regulatory scale, the test weight of the three genotypes meets the first-degree requirements of the American and Canadian grading systems (GIPSA 2020; CGC 2021).

For the content of yellow berry (non-vitreous grains), the difference between the varieties was very highly significant (P<0.001). The mean value of yellow berry was significantly higher for (BOS) and (OB) genotypes without significant differences between them (10.50, 10.33%, respectively) so they presented more non-vitreous grains, while it was significantly lower for (GTA) genotype (1.00%) (Table 1). That means (GTA) genotypes developed a tolerance to yellow berry and characterized by high proportions of vitreous grains and minimal starchy kernels rates (1%). However, it was acceptable for Tilley et al. (2012) who defined the vitreousness of durum wheat as high (above 75%), medium (between 60 and 75%) and low (below at 60%). Annicchiarico et al. (2006), claim that durum wheat is faced with a lack of technological and commercial quality stability, due in particular to variations in important criteria such as non-vitreous kernels (penalizing semolina value) and black point.

Indeed, in durum wheat, the relationship between content of yellow berry and milling performance is complex, but it can be said that in general, starchy grains give less coarse semolina and more flour, which reduces their ability to milling (Heinze et al. 2016).

Correlations between the different parameters studied and estimation of inter-varietal genetic diversity

The results indicated that there are significant and positive correlations between grain moisture content in the field and grain yield in GTA dur r=0.90* and very significant and positive in Boussellam r=0.97** (Table 2); these results suggest that a decrease of grain moisture is generally linked to a decrease in grain yield, and this relationship is particularly strong in the Boussellam genotype.

Table 2. Correlation coefficient between grain moisture, grain yield, and quality parameters for the three genotypes studied.

Genotypes	Parameters	GMF (%)	GY (t ha^{-1})	Mlab (%)	P (%)	TW (kg hL^{-1})	YB (%)
Boussellam	GMF (%)	1	-	-	-	-	-
	GY (t ha^{-1})	0.97**	1	-	-	-	-
	Mlab (%)	0.95*	0.85^{ns}	1	-	-	-
	P (%)	0.79^{ns}	0.92*	0.60^{ns}	1	-	-
	TW (kg hL^{-1})	-0.46^{ns}	-0.37^{ns}	-0.64^{ns}	0.21^{ns}	1	-
	YB (%)	-0.72^{ns}	-0.77^{ns}	-0.57^{ns}	0.15^{ns}	-0.15^{ns}	1
		GMF (%)	**GY (t ha^{-1})**	**Mlab (%)**	**P (%)**	**TW (kg hL^{-1})**	**YB (%)**
Oued El Bared	GMF (%)	1	-	-	-	-	-
	GY (t ha^{-1})	0.87^{ns}	1	-	-	-	-
	Mlab (%)	0.99***	0.86^{ns}	1	-	-	-
	P (%)	0.89*	0.83^{ns}	0.91*	1	-	-
	TW (kg hL^{-1})	-0.51^{ns}	-0.32^{ns}	-0.55^{ns}	0.18^{ns}	1	-
	YB (%)	-0.51^{ns}	-0.67^{ns}	-0.46^{ns}	0.68^{ns}	-0.43^{ns}	1
		GMF (%)	**GY (t ha^{-1})**	**Mlab (%)**	**P (%)**	**TW (kg hL^{-1})**	**YB (%)**
GTA dur	GMF (%)	1	-	-	-	-	-
	GY (t ha^{-1})	0.90*	1	-	-	-	-
	Mlab (%)	0.98**	0.96**	1	-	-	-
	P (%)	0.67^{ns}	0.89*	0.78^{ns}	1	-	-
	TW (kg hL^{-1})	-0.01^{ns}	0.09^{ns}	0.04^{ns}	0.15^{ns}	1	-
	YB (%)	0.28^{ns}	-0.12^{ns}	0.11^{ns}	0.38^{ns}	-0.14^{ns}	1

GMF: Grain moisture content in the field; GY: Grain yield; MlAb: content of grain moisture in the laboratory; P: Grain protein content; TW: Test weight; YB: Content of yellow berry (non-vitreous grains). *P<0.05, **P<0.01, ***P<00.1, ns: not significant.

Parvej et al. (2020) indicated that delaying the harvest of wheat beyond its physiological grain maturity can indeed lead to grain yield losses, and it emphasizes the importance of timely harvesting to maximize both yield and quality. According to Scariot et al. (2018), managing grain moisture content through effective drying and storage practices is also essential in reducing both pre-harvest and post-harvest losses.

At Boussellam there was a positive and significant correlation between grain moisture estimated by the mobile moisture meter and that estimated in the laboratory r=0.95*. GTA dur and Oued El Bared showed very significant and very highly significant correlations with r=0.98** and r=0.99***, respectively. These results proved the effectiveness of the mobile moisture meter in estimating changes in field moisture (Table 2).

Positive and significant correlations are observed between total protein content and grain yield in Boussellam and GTA dur, r=0.92*, and r=0.89*, respectively, while Oued El Bared marked a positive correlation and not significant (r=0.83). This positive relationship between grain protein content and grain yield suggests that plant nutrition and growth conditions were suitable for grain yield and grain protein accumulation. In addition, the grain could act as a sink, promoting the translocation of proteins to the grain. These results help contribute to the development of wheat varieties with improved yield potential and desirable protein content to meet the needs of different end uses, such as bread making or pasta production.

Pandey (2014) showed a strong correlation between the protein and carbohydrate content of various cereals and legumes and the moisture content of the grain. Starch is considered a dominant constituent of cereal grains; protein is the second component that plays a similar role in terms of water absorption and water binding. The Oued El Bared genotype highlighted this correlation, in which the protein content and the grain moisture content in the field and content of grain moisture in the laboratory are positively and significantly correlated r=0.89* and r=0.91*, respectively, according to Deliberali et al. (2010), any delay in harvesting will reduce the quality of harvested grain and may affect the final storage outcome. No correlation was observed for Boussellam and GTA dur genotypes concerning grain moisture content and protein content. Dorrian et al. (2023) found that the variation in protein content based on the harvest date was no greater than 0.20%. They also stated that, from a physiological perspective, moisture content would not be expected to have a significant effect on grain protein because protein accumulation is anticipated to be at its highest and stable at maturity.

Estimating grain yield losses based on grain moisture

The relationship between grain moisture in the field (GMF) and grain yield (GY) is illustrated in Figure 2A. Based on the mean values of the treatments, a model was generated based on grain moisture in the field to estimate and predict the grain yield loss. The results indicated a very highly significant correlation with r=0.87*** (P<0.001). Regression models that empirically fit the linear grain moisture-grain yield relation indicate that 76% of the variation in grain yield is due to the effect of grain moisture change. Linear regression gives an equation (6), that is, after the physiological maturity of the grain, 1% loss of grain moisture, there is a loss of about 0.290 t ha^{-1} of grain yield.

$$GY(t\ ha^{-1}) = 0.2293 \times GMF(\%) + 0.0611 \quad (6)$$

The evaluation of model performance

To check the goodness of yield prediction by the linear model proposed, average absolute error (AAE), root mean square error (RMSE), index of agreement (D), coefficient of efficiency (E), correlation coefficient (r), and the T-test (student test) were calculated to evaluate the performance of prediction models in this study.

Observed and simulated grain yield correlated very well giving an r=0.87 a slope of 0.76 and D of 0.93 (Table 3, Figure 2B) indicating that the model explained 76% of the relationship between observed and modeled durum wheat grain yield with an agreement (D) of 93%. The good agreement between measured and simulated is also reflected in the statistical analysis, with low average absolute error and root mean square error. The grain yield was also well simulated with the observed yield giving a coefficient of efficiency (E) of 0.76, i.e., with a simulation capacity of 76%.

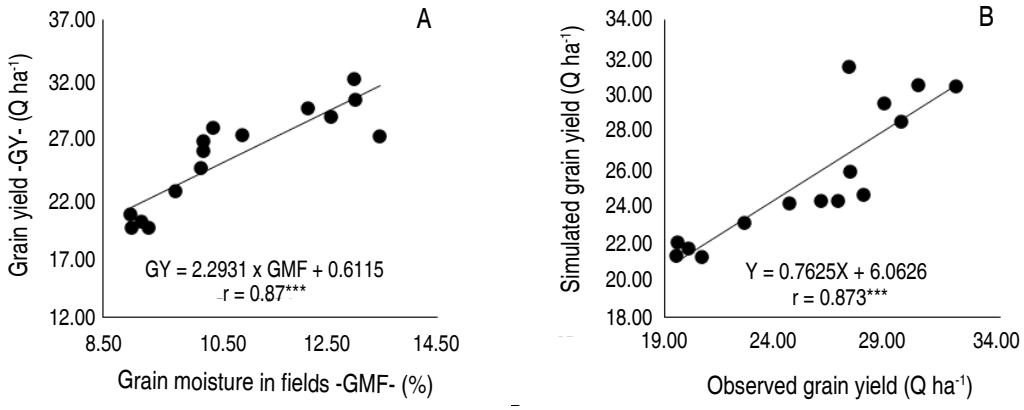

Figure 2. Linear relationship between grain moisture in the field (GMF) and grain yield (GY) in (A), observed and simulated grain yield (B).

Table 3. Derived statistical indices to assess the performance of the regression model in predicting grain yield from grain moisture in the field.

Treatments	Grain Moisture in Field (%)	Grain yield (t ha^{-1})	
		Observed	Simulated
1	13.00	3.201	3.042
2	12.17	2.960	2.851
3	11.00	2.731	2.584
4	10.30	2.677	2.423
5	9.00	2.063	2.125
6	13.03	3.032	3.050
7	12.60	2.884	2.950
8	10.47	2.790	2.462
9	10.30	2.597	2.423
10	9.20	2.006	2.171
11	13.47	2.726	3.149
12	10.27	2.456	2.415
13	9.80	2.259	2.308
14	9.33	1.956	2.201
15	9.03	1.950	2.132

AAE	RMSE	D	E	T-test	r
0.161 t ha^{-1}	0.196 t ha^{-1}	0.93	0.76	0.99	0.87***

AAE, average absolute error; RMSE, root mean square error; D, index of agreement; T-test, student test; r, correlation coefficient.

The Studied T-test showed that the simulated grain yield was not significantly different (P>0.05) from the observed grain yield, with RMSE and AAE of 0.196 and 0.161 t ha^{-1}, respectively (Table 3). Leng and Hall (2020) used a regression model to simulate changes in historical maize yield from observations, with r and RMSE of 0.51* and 7.52, respectively. The RMSE and AAE values when expressed as a percentage of the average observed grain yield were 7.68 and 6.31%, respectively. Overall, the difference between the simulated and observed grain yield was <0.0001%, the performance of the model concerning RMSE was estimated at 92.32%, and the accuracy to AAE was 93.69%, which

suggests that the model has high precision in predicting grain yield under semi-arid conditions, surpassing the results of Mkhabela et al. (2011), who developed a regression model as a predictor and reported that the model explained 47 to 80% of grain yield variability in prairie spring wheat, with RMSE ranging from 6 to 34% of average yield.

CONCLUSIONS

The study provides valuable insights into estimating grain yield losses in durum wheat under semi-arid conditions through a mathematical model based on grain moisture. Genotype variations were significant in key parameters such as protein content, test weight, and content of yellow berry when the BOS genotype exhibited the highest proportions of GY, TW, and YB (2,063 t ha^{-1}, 81.97 kg hL^{-1}, and 10.50%, respectively) with the lowest protein content (13.70%), OB. On the other hand, showed values significantly close to BOS in terms of GY and YB but had higher protein content (15.40%) and lower TW (79.47 kg hL^{-1}), GTA dur demonstrated a tolerance to yellow berry (1.00%), featuring a higher proportion of vitreous grains. This highlighted the importance of genotype selection in crop management. The study establishes a strong positive correlation between decreasing grain moisture in the field and subsequent grain yield loss. This relationship can be predicted accurately using a robust mathematical model. Statistical analysis confirms the model's effectiveness with low average absolute error (0.161 t ha^{-1}) and root mean square error (0.196 t ha^{-1}), resulting in a 76% simulation capacity as indicated by the coefficient of efficiency. Furthermore, the research emphasizes the critical role of timely harvesting after grain physiological maturity to minimize yield loss. In summary, this study equips durum wheat farmers in semi-arid regions with valuable tools and insights for optimizing grain yield and quality through genotype selection and harvest timing. The developed model's high precision and accuracy make it a valuable resource for durum wheat cultivation in challenging semi-arid environments.

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Evaluation of microorganisms response to soil physical conditions under different agriculture use systems

Evaluación de la respuesta de los microorganismos a las condiciones físicas del suelo en diferentes sistemas de uso agrícola

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Keywords:
Soil biology
Soil degradation
Soil microorganisms
Soil quality

ABSTRACT

Soils with appropriate water and air conditions improve microbiological activity, but agriculture frequently deteriorates soil's physical requirements and impairs microbes' survival. The main aim of this study was to evaluate the relationship between physical soil conditions and microbiological activity under different agricultural use and management systems in the warm tropical climate of Colombia. To evaluate physical soil conditions, bulk density (Bd), total porosity (Pt), macroporosity (MPt), water content, temperature, and mechanical penetration resistance (MPR) were measured on surface soil (0 to 10 cm) at three different use and management systems: irrigated rice (IR), pasture (P) and native forest with cacao (FC). The measured biological properties were the carbon of microbial biomass (C-MB) and the presence of bacteria and fungi. Soil organic carbon (SOC) was determined to calculate the microbiological quotient (MQ). The C-MB was significantly higher in FC (132.25) and P (148.11) but lower in IR (41.61). No significant differences were observed between the soil use system in the count of bacteria and fungi. The MQ (0.44) was higher in IR (0.22), revealing a significant effect of the soil use system on microbiological activity. Less anthropic intervention and permanent plant covers, such as FC and P, enhance microorganism's survival, evidenced by higher C-MB content, correlated with soil Bd and MPt. The abundance of bacteria and fungi in the soil is affected by the physical conditions, primarily by Pt and MPt. However, fungi survive better with less water content, existing a differentiated effect of the physical conditions of the soil.

Palabras clave:
Biología de suelo
Degradación de suelo
Microorganismos del suelo
Calidad de suelo

RESUMEN

Los suelos con condiciones apropiadas de agua y aire mejoran la actividad microbiológica, pero la agricultura frecuentemente deteriora las condiciones físicas del suelo, lo que perjudica la supervivencia de los microbios. El objetivo de este estudio fue evaluar la relación entre las condiciones físicas del suelo y la actividad microbiológica, bajo diferentes sistemas de uso y manejo agrícola en clima tropical cálido de Colombia. Para evaluar las condiciones físicas del suelo, se midieron la densidad aparente (Bd), la porosidad total (Pt), la macroporosidad (MPt), el contenido de agua, la temperatura y la resistencia mecánica a la penetración (MPR), en suelo superficial (0 a 10 cm) en tres sistemas de manejo: arroz irrigado (IR), pasto (P) y bosque nativo con cacao (FC). Se midió el carbono de la biomasa microbiana (C-MB), la presencia de bacterias y hongos. Se determinó el carbono orgánico del suelo (COS) para calcular el cociente microbiológico (MQ). El C-MB fue significativamente mayor en FC (132,25) y P (148,11), pero menor en IR (41,61). No se observaron diferencias significativas entre los sistemas de uso del suelo en el conteo de bacterias y el conteo de hongos. El MQ (0,44) fue mayor en IR (0,22), revelando un efecto significativo del sistema de uso del suelo sobre la actividad microbiológica. La menor intervención antrópica y la cobertura vegetal permanente, como FC y P, potencian los microorganismos, evidenciados por un mayor contenido de C-MB, que se correlaciona con el Bd y el MPt del suelo. La abundancia de bacterias y hongos es afectada por las condiciones físicas, especialmente por Pt y MPt. Sin embargo, los hongos sobreviven mejor con menor contenido de agua, existiendo un efecto diferenciado de las condiciones físicas del suelo.

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Microorganisms represent less than 5% of the soil organic matter (SOM). Still, they are the most active and essential living component, responsible for the dynamics of organic residue decomposition, mineralization of nutrients, degradation of xenobiotic compounds, soil aggregation, and the development of symbiotic relationships with plants to promote their growth (Dalal 1998; Kaschuk et al. 2010). Furthermore, they are essential for the soil to perform its multiple productive and environmental service functions since they participate in almost all the known metabolic reactions of the soil (Voroney and Heck 2015; Paolini 2018).

Different soil uses and management systems can differentially affect the microorganisms that inhabit it, thus modulating the ecosystem services they provide. It is also known that this biological component of the soil responds quickly to environmental changes as an indicator of high sensitivity to edaphic changes produced by management practices (Di-Ciocco et al. 2014).

Many types of agricultural land use have been identified to cause a drastic decrease in total soil microbial biomass and a reduction of total SOC. The latter occurs due to the decomposition of the SOM by microorganisms and is strongly affected by the chemical and physical conditions of the soil (Kaurin et al. 2018).

Dexter (2004) pointed out that the biological status of soil depends very strongly on the prevailing physical conditions, mainly due to poor water infiltration, poor aeration, and poor conditions for root growth. These are also symptoms associated with decreased biological activity in soils. Cui and Holden (2015) found that soil structure strongly correlates negatively with soil respiration and enzyme activity, indicating a decline in microbial activity with a worse soil structural quality.

For this reason, various studies have evaluated the activity of microorganisms in different types of soil use and management. Morais et al. (2016) assessed the effects of crop systems and coverage on soil microbial communities, observing that the number of microorganisms on the soil surface was higher associated with zero tillage. Zagal et al. (2002) evaluated soil microbial activity in different annual crop rotation systems, registering a significant correlation between microbial activity and the rotation system with soil management that enhanced the organic carbon content.

It is known that an essential function of soil is the conduction of water and air, which is necessary for the remarkable growth of roots and microbiological communities. Therefore, microbial processes in soil largely depend on pore structure and soil compaction conditions (Morais et al. 2016). Ishak et al. (2016) found that soils with low compaction contain more soil pores predominated with macropores that enhance soil capacity to retain soil water and provide soil oxygen diffusion, thereby increasing microbial activity. However, in the compacted soils, low microbial activity was observed.

Valenzuela and Visconti (2018) found that the soil bulk density, the content of clays, and the content of silts are physical characteristics that can be used as variables to explain the relationship of physical soil conditions with the stabilization processes of the SOM; therefore, since the decomposition and stabilization of the SOM is a process carried out by soil microorganisms, physical soil conditions are involved (Six et al. 2004).

Recent studies have emphasized that biological characteristics of the soil, such as the total organic carbon, the carbon of the microbial biomass, and the microbial quotient, can gradually increase or decrease depending on the use and management of soil, which is essential for the evaluation of a sustainable agricultural system (Assis et al. 2017; Zornoza et al. 2018). Indeed soil management practices can affect soil microorganism communities (Brevik et al. 2015), and concerning soil physical conditions. Ishak et al. (2016) observed that microbial activity was higher in uncompact soils and decreased as soil compaction increased. In addition, the amount of soil microbes expressed as microbial biomass may be influenced by the amount of soil organic matter, climatic factors, land use, and physical-chemical characteristics of soil (Iglesias 2009). That is the reason why studies must be carried out in those fields.

In this context, this study was conducted to assess the relationship between physical soil conditions and microbiological activity under different agricultural use and management systems in the warm tropical climate of Colombia.

MATERIALS AND METHODS

Study area

The study was conducted in the Zulia River Irrigation District in the rural area of Cucuta, Colombia. The site has a mean annual temperature of 29 °C, the mean annual precipitation is 1,900 mm, and the climate is classified as Tropical Dry Forest, according to Holdridge (IGAC 2006a). On the same farm, located at 8°10' 22.4" N and 72°29' 03.5" W, and at an altitude of 80 meters above sea level (masl), three different soil agricultural use and management fields were selected, delimited, and characterized according to their uses: irrigated rice (IR) cultivation, native forest with cocoa (FC), and pasture (P). According to IGAC (2006a), the selected fields are in a soil classified as Typic Udifluvents.

The management system of the plot with irrigated rice IR involved cultivation with mechanized tillage in flooded soil (muddy soil) and flood irrigation with periodic water replacement. The sowing was manual and broadcasted with a pre-germinated seed of the Fedearroz 2000 variety; chemical fertilization was broadcasted manually and based on urea, diammonium phosphate, potassium chloride, and complete fertilizer, to contribute 150 kg ha^{-1} of N, 80 kg ha^{-1} of P and 120 kg ha^{-1} of K. Selective pre-emergent and post-emergent herbicide applications and chemical pest and disease controls were performed by back sprayers manually. The harvest was mechanized, and the grain was retrieved from the field in packages transported by a tractor. After the harvest, the straw residues were burned.

The native forest with cacao FC involved agroforestry management of cacao trees (*Theobroma cacao*) with a shade of tall tropical trees such as Ceiba (*Ceiba pentandra*), Jobo (*Spondias mombin*), Apamate (*Tabebuia rosea*) and the Higuerón (*Ficus luschnathiana*). The management was reduced to maintenance pruning of the cacao once per year and harvesting of pods twice per year. No fertilizers, herbicides, insecticides, or fungicides were applied.

The plot management with pasture P involved star grass paddocks (*Cynodon nlemfuensis*) destined for grazing cattle, with weed control manually and without chemical fertilization or irrigation.

Soil sampling

The study consisted of a single-factor experiment with the use and management system as variables (three treatments). Three composite soil samples were taken in each treatment with two repetitions. A 1-hectare plot was allocated for a systematic soil sampling for each treatment. This was carried out, marking three equidistant points on a 100 m long diagonal transept. At each sampling point, a cross with 2 m on each side was marked on the soil to extract subsamples from the intersection, and at the ends of the cross, thus, a composite sample of surface soil (0 to 10 cm depth) was conformed. From the composite soil sample, 1 kg was taken and stored in plastic bags for refrigeration (4 °C) at the laboratory. Also, two undisturbed samples at the same soil depth were taken at each sampling point using metal cylinders 5 cm high and 5 cm in diameter.

Evaluation of soil physical, chemical, and biological properties

To record the differences between fields on soil sampling conditions, the surface soil temperature was measured using a mercury thermometer, and the volumetric moisture content was measured with a dielectric sensor. The mechanical resistance to penetration (MRP) was measured at the surface soil (0 to 10 cm depth) at the same time of soil sampling, using a hand penetrometer, with a conic point of 2 cm^2. At the laboratory, the physical properties of the soil were determined, such as particle size analysis by the agitation and sedimentation method of modified Bouyoucos (IGAC 2006b). The Bd and the total porosity were measured by the Uhland-type metal cylinder method, where the core soil sample is saturated with water in immersion trays before weighing at the saturated condition and then dried in the oven at 105 °C for dry soil weight. The MPt was measured as the porous space of diameter >30 µm; using the tension table method, which consists of a porous membrane plate to which suction is applied through a column of water suspended at 100 cm (-10 kPa) (Pla 1983; Pla 2010). The pH and electrical conductivity (EC) were measured in a soil/water suspension (1:1). The cation exchange capacity (CEC) was determined by the standard method of ammonium acetate 1 N and pH=7 (IGAC 2006b).

Within 48 h of soil sampling, the refrigerated composite soil samples were used to determine the carbon of the C-MB by the substrate–induced incubation method modified by Lozano et al. (2005). Also, the oxidable SOC was determined on the composite soil samples by acid digestion

with the potassium dichromate oxidation method (IGAC 2006b).

Evaluation of the abundance of bacteria and fungi in the soil

Within 24 h of soil sampling, the refrigerated composite soil samples were used to determine the total cultivable populations of fungi and bacteria in the studied soils 10^{-1} dilutions were prepared by weighing 10 g of soil and adding sterile saline solution (0.85% NaCl), which were homogenized by vigorous stirring on an orbital shaker for 10 min. A preliminary test with serial dilutions from 10^{-2} to 10^{-6} was conducted to select the final dilution to use in round Petri plates of microbe cultures (Vanegas et al. 2013).

Cultures on the Petri plates were made from 0.1 mL of each selected dilution and placed on the surface of the specific growth culture medium. Nutrient agar (NA) was the bacteria-specific medium (dilutions 10^{-3}, 10^{-4}, and 10^{-5}) and Rose Bengal agar for fungi whose selected dilutions were 10^{-2}, 10^{-3}, and 10^{-4}. For each soil sample, this was done in triplicate. The culture plates with nutrient agar for bacterial growth were incubated for 24 h at 37 °C, and those of Rose Bengal for fungal growth were incubated between 5 to 7 days at 28 °C. Quantification was performed using the plate count technique and was expressed as the decimal logarithm of the colony forming units (CFU) per gram of soil (Calvo et al. 2008). Finally, the microbiological quotient was calculated as the ratio of C-MB to the SOC (Paolini 2018; Kaschuk et al. 2010).

Data analysis

Data were analyzed using Statgraphics Centurion statistical version XVI software using an analysis of variance (ANOVA) after checking the normality and variance homogeneity assumptions. In case of violation of these assumptions, the data were analyzed using the Kruskal Wallis non-parametric test. In the comparison of means, the least significant difference test was applied at a confidence level of 0.05%. Additionally, correlation graphs and Pearson's correlation coefficient were used to establish the degree and type of association between variable pairs. A multivariate analysis was made to interpret each variable's variance and influence.

RESULTS AND DISCUSSION

Physical and chemical properties of surface soil

The particle size analysis results in the studied fields (Table 1) show that soils are of fine texture; the plot with FC has a silty clay loam texture, while the soils with IR and P have clay texture.

Table 1. Particle size analysis and textural class of soil in each plot.

Particle type	Irrigated rice (g kg^{-1}) (n=3)	Native Forest with Cacao	Pasture
Sand (0.05 to 2.0 mm)	174.0	87.0	117.0
Silt (0.002 to 0.05 mm)	336.0	385.0	549.0
Clay (< to 0.002 mm)	489.8	528.0	334.0
Textural class	Clay	Clay	Silty clay loam

Results obtained from the physical properties of soils in each use and management system (Table 2) show limitations due to compaction; given that soils in IR and P had high soil Bd values according to their texture (Dexter 2004). The lowest Bd was observed in the FC, with a value that can be by the texture. Regarding the Pt, it is observed that the soil of the three use and management systems shows a porous space reduction (Table 2). The IR and P presented MPt values below 0.10 (10%), which indicates physical degradation by poor aeration (Dexter 2004; Pla 2010). A different situation was observed in the soil under the FC system, where the MPt was 0.12 (12%), which indicates a favorable physical condition.

The volumetric water content registered in the surface soil was near saturation in the IR, while the FC had a 21.3% lower soil moisture in comparison with IR, while the driest soil was P with 32.9% less water content. The surface soil temperature was the same for IR and P with a value of 39.3 °C and was slightly lower in the FC at 38.7 °C.

Table 2. Soil physical properties in each production system.

Soil use and management	Bd* (Mg m^{-3})	Total Porosity (V V^{-1})	Macropores (V V^{-1})	Volumetric Moisture content (%)	Temperature (°C)	MRP* (MPa)
			n=6			
Irrigated rice	1.47±0.13^{b}	0.39±0.02^{b}	0.08±0.02^{a}	34.89±2.54^{b}	39.33±0.58^{a}	1.58±1.04^{a}
Native forest with ocoa	1.30±0.14^{a}	0.36±0.02^{a}	0.12±0.03^{b}	27.47±1.47^{ab}	38.67±0.58^{a}	3.11±0.39^{b}
Pasture	1.33±0.18^{a}	0.39±0.05^{b}	0.08±0.02^{a}	23.41±0.91^{a}	39.33±0.58^{a}	5.00±0^{c}

*Bd: Bulk Density, *MPR: Mechanical resistance to penetration. Different letters represent means with significant differences (P<0.05).

Using the Bd, MPt, and MRP as indicators to evaluate soil compaction in each plot, the soil of P revealed physical degradation because of high Bd, low MPt, and high MRP. Also, the soil of IR can be considered compacted since Bd and MPt are out of normal ranges as considered by Pla (2010). Although the soil of IR presented the lowest MRP, it is understood that the high moisture content and high clay content influenced this physical behavior.

However, the evaluation of the soil's physical properties evidenced a degradation in the soil used with IR because of management practices such as tillage with flooding, which generate compaction of the soil, resulting in high Bd and low MPt (Table 2) as other studies have reported (Ruiz et al. 2005; Valenzuela et al. 2015). The soil in P uses evidenced compaction generated by the trampling of animals during the grazing process (Medina 2016).

The analysis of the chemical properties of the soil (Table 3) revealed that the pH was higher in the FC (5.77), which indicates a moderately acidic soil condition. The pH is classified as strongly acid for the IR and P, with values of 5.47 and 4.76, respectively. The electrical conductivity results in the soil of the three use systems showed values between 34.53 and 166.73 µS cm^{-1} (Table 3), indicating no salinity problems.

Table 3. Chemical properties of the soil according to each use system.

Soil use and management	pH (1:1)	EC* (1:1) (µS cm^{-1})
Irrigated Rice (IR)	5.47±0.22	34.53±4.41
Native forest with cocoa (FC)	5.77±0.10	48.57±29.14
Pasture (P)	4.76±0.00	166.73±255.73

*EC: electric conductivity (n=6).

Impact of soil use system on the activity of microorganisms

The results of the variables measured to know the impact of each soil use system on the activity of the microorganisms are presented in Table 4.

The statistical analysis of the results for SOC expressed a statistically significant difference between the means, resulting in the IR soil having the lowest content of SOC and the P and FC soils constituting a homogeneous group with higher SOC values.

The SOC content was statistically higher in the P and the FC (Table 3). In the P system, it may be due to adding organic matter from animal manure and residues from pasture. In the FC case, it may be due to the contribution of litter from shade trees and cacao trees. Furthermore, in these two systems, there is no soil disturbance by tillage and no residue burning that could negatively affect the SOC (Vimal et al. 2017; Valenzuela and Visconti 2018). According to the C-MB analysis, a statistically significant difference between the means was found, resulting in the soil in IR having the lowest content of C-MB and the soils in P and FC constituting a homogeneous group of higher content in C-MB. The C-MB quantifies the global quantity of microorganisms present in the soil (Sánchez et al. 2005), and the results show that the system of use in P and FC presented

significantly higher values of C-BM compared to the IR (Table 3). This can be associated with the higher SOC content since it contributes to increasing the activity of microorganisms; by constituting a source of energy and nutrients for microorganisms, which contributes to their development and higher microbiological activity (Sánchez et al. 2005; Anderson and Domsch 1989). It also confirms what Paolini (2018) affirms, that microbial biomass is associated with greater incorporation of organic matter and nutrients into the soil.

Table 4. Selected soil biological characteristics in the 0-10 cm soil layer in each system.

Soil use and management	SOC* (g kg^{-1})	C-MB (mg C kg^{-1})*	CFU* Bacterias (Log CFU g^{-1})	CFU Fungi (Log CFU g^{-1})	MQ*
			n = 9		
Irrigated Rice	19.43±1.68[a]	41.61±7.85[a]	5.50±0.43[a]	4.98±0.04[a]	0.22±0.05[a]
			n=9		
Native forest with cocoa	30.90±4.84[b]	132.25±28.27[b]	5.68±0.50[a]	5.04±0.48[a]	0.44±0.13[b]
			n = 9		
Pasture	34.05±1.21[b]	148.11±1[b]	5.83±0.07[a]	5.43±0.25[a]	0.44±0.06[b]

( ): standard deviation, ***SOC**: soil organic carbon, ***C-MB**: carbon of microbial biomass, ***CFU**: colony forming units. ***MQ**: microbiological quotient, Different letters represent means with significant differences (*P*<0.05).

Additionally, the results coincide with Núñez et al. (2012), who reported high C-BM values for grassland with intensive grazing (297 mg C kg^{-1}) and grassland with light grazing (291 mg C kg^{-1}), indicating that the grazing systems improve the biological activity of soils. The ANOVA did not show a significant difference between the soil use system on the count of bacteria (Log CFU bacteria) or fungi (Log CFU fungi). However, the use of IR reported the lowest value in the bacteria and fungus count. In comparison, using P reported the highest value in the count of bacteria and the count of fungi (Table 3).

On the other hand, this coincides with the study by Hernández et al. (2013), who found a decrease in the bacterial populations present in the soil in different cultivation systems with the application of agrochemicals.

Hernández and Lizarazo (2015), when studying populations of soil bacteria in conserved areas of Paramo and cultivated soils, reported an average of cultivable bacteria of 8.90 log CFU g^{-1} of soil, and 3.60 log CFU g^{-1}, respectively. Thus, the importance of soil use and management over the quantity and diversity of microbial populations was demonstrated.

The results of the fungus count allowed observation that the soil in use with IR had the least amount of cultivable fungi (Table 3). This behavior could be associated with the compaction of the soil and the lower SOC content, which affects the survival of the microorganisms (Calvo et al. 2008). When observing that the use of P showed a more significant amount of fungi, it is related to the fact that the largest abundance of fungi is found in the superficial layers of soil, where the microclimate, environment, and availability of nutrients are favorable for their development and growth (Lavelle and Spain 2001).

In the case of the MQ, the analysis of variance allowed us to find a significant effect of the soil use system on this parameter, resulting in the use of IR showing the lowest microbial quotient. In contrast, the uses of FC and P showed a higher quotient, confirming that the IR use and management system has degraded soil microbiological activity.

Relationship between soil Physical conditions and soil microbiological activity

The evaluation of the relationship between the physical conditions of soil and the parameters of microbiological activity, using the correlation graph between Bd and C-MB (Figure 1), reveals that the C-MB decreased while the Bd increased, which means that these two parameters are inversely proportional; because an increase in Bd implies that the soil is more compact, and therefore the oxygen supply to microorganisms is decreased, (Ishak et al. 2016; Visconti and Valenzuela 2023).

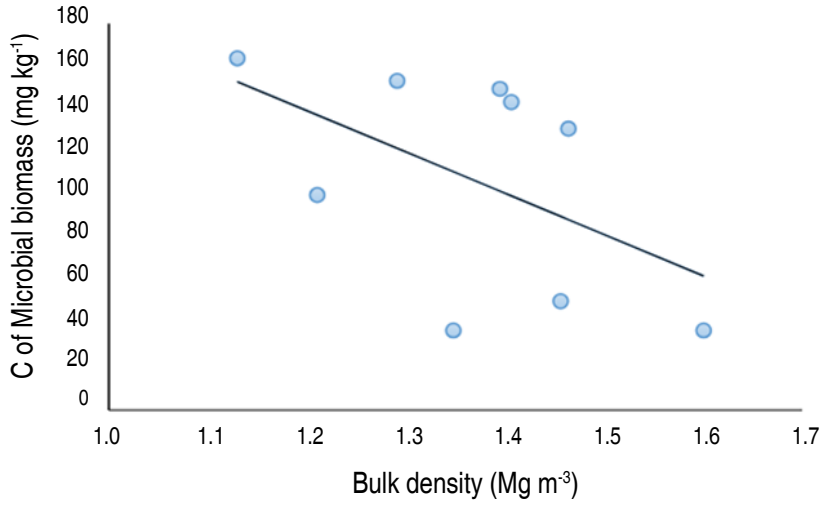

Figure 1. Correlation graph between the bulk density (Bd) and the carbon of microbial biomass (C-MB) (Pearson correlation=-0.53).

In the relationship between the macropores and the CFU of bacteria (Figure 2), it is observed that increases in soil macropores corresponded to slight increases in the number of colonies forming units of bacteria, indicating that they are directly proportional since soil macropores allow an increase in soil aeration, thus increasing the availability of O_2 for bacteria (Cui and Holden 2015).

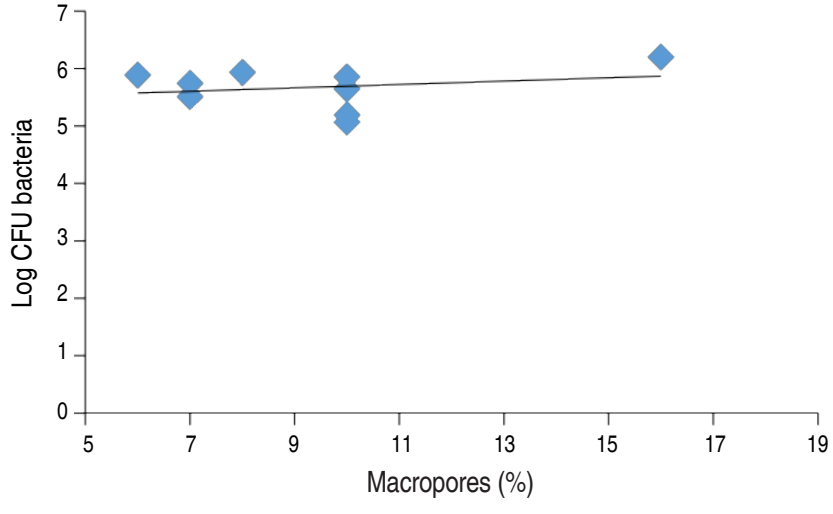

Figure 2. Correlation graph between Macropores and Log of CFU of Bacteria (Pearson correlation= 0.55).

Morugán-Coronado et al. (2019) pointed out that changes in soil moisture affect soil microbes' survival. In this case, the relation between soil moisture and the CFU of Fungi (Figure 3) shows that increasing the soil moisture content slightly decreases the number of CFU of Fungi.

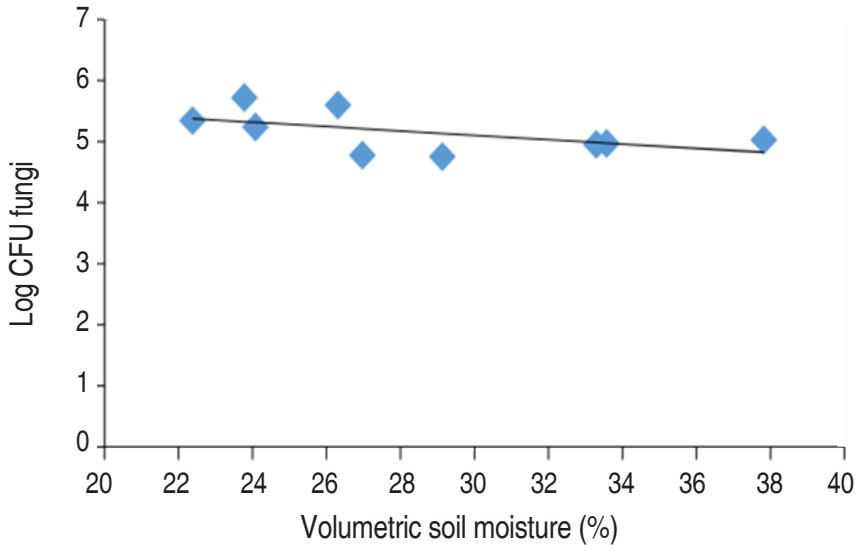

Figure 3. Correlation graph between volumetric soil moisture and Log of CFU Fungi (Pearson correlation=-0.54).

In Figure 4, it was evident that increases in soil moisture content are related to marked decreases of C-MB, which can be explained considering that the excess of water in soil displaces the air and, with this, the availability of O_2 for the microorganisms, reducing their survival, (Siebielec et al. 2020; Visconti and Valenzuela 2023).

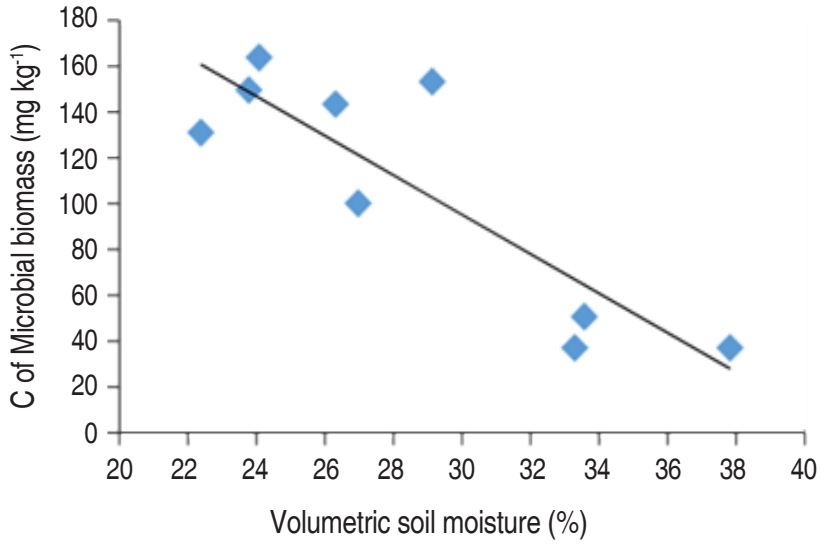

Figure 4. Correlation graph between soil moisture and C-MB (Pearson correlation=-0.86).

In addition, the correlation analysis between the soil's physical variables and the soil's microbiological activity confirmed that the decrease in quantity and size of pores due to soil compaction affects soil microorganisms, which implies a danger to soil biodiversity (FAO 2015).

On the other hand, it is considered that the response of soil microorganisms to the increase of macropores is because of the improvement of soil aeration and drainage (Lipiec et al. 2012; Santamaría et al. 2018). Furthermore, the destruction of soil macropores leads to a higher

proportion of micropores, which hinders the movement of water, creating conditions of water excess and oxygen deficit that affects the activity of microorganisms (Figure 2) (Ishak et al. 2016).

The correlation between soil moisture and fungi count (Figure 3), showed that increasing soil moisture decreases the number of fungal colony-forming units. Calvo et al. (2008) expressed that excess moisture in soil saturates the pores and creates conditions of lack of O_2. Furthermore, Santamaría et al. (2018) explained that water is necessary for all living organisms. Still, fungi survive better in semi-arid regions and suffer more when soil moisture is high enough to provoke oxygen shortage in the soil. In this same sense, Vanegas et al. (2013) expressed that the excess of water inhibits the survival of the microorganisms; as found in this study (Figure 4), since under flooding, the air in the soil pores is replaced by water and oxygen is rapidly consumed by root respiration and microbial activity (Morugán-Coronado et al. 2019).

The analysis of principal components for the variables measured in this study has allowed us to interpret that the thirteen variables are grouped into three main parts capable of explaining 77.07% of the variability in the data (Table 5).

Table 5. Principal component analysis for the variables studied.

Component number	Eigenvalue	Percentage of variance	Accumulated percentage
1	5.29	40.75	40.75
2	2.55	19.58	60.33
3	2.18	16.73	77.07

Interpreting the weight with which the variables are grouped in each component allows us to analyze the influence of the measured physical characteristics on the presence of soil microorganisms.

The principal component analysis interprets that the variables C-MB, SOC, Fungi, and bacteria count are grouped with greater weight in component one. This allows it to point out the critical influence that the content of organic matter has on the activity of soil microorganisms. Therefore, the SOC directly influences biological activity and largely determines the variability of the parameters related to soil microorganisms' activity, as Zhao et al. (2019) mentioned.

In the case of component two, the variables porosity, macropores, and fungi count are grouped with greater weight. This indicates the critical influence of the physical soil conditions involved with the soil aeration and drainage relationships and the presence and survival of fungi in the soil, confirming that these microbes grow better in dryer conditions.

Finally, in component three, it was found that the variables with the greatest weight were: soil temperature, soil moisture, pH, and electrical conductivity, which indicates that climatic conditions had a direct influence on the environment that the soil offers to microorganisms, and this affects their survival, as mentioned in other studies conducted by Liu et al. (2018).

CONCLUSIONS

According to the results of this study, soils with less anthropic intervention and in which a permanent plant cover exists, such as the use of forest with cocoa and pasture, presented the highest soil organic carbon content, which favored the presence of microorganisms in the soil, evidenced by a higher carbon content of microbial biomass. This also coincides with better soil physical conditions, expressed by bulk density and macroporosity.

The results also indicated that, among the fungi and bacteria, the first can survive better with less soil water content, so physical soil conditions have a differentiated effect on each microorganism. Especially the total amount of pores and their size distribution, due to their role in aeration and water retention. As a result, this research emerges as promising material for evaluation of the microorganisms under different conditions environmental in agriculture use systems.

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By-products of the cocoa agribusiness: high value-added materials based on their bromatological and chemical characterization

Subproductos de la agroindustria del cacao: materiales de alto valor agregado en función de su caracterización bromatológica y química

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ABSTRACT

Worldwide, cocoa agribusiness generates about 48 million tons of cocoa pod husk residues annually, and 700 thousand tons of cocoa bean shell, by-products of the pulping and roasting processes. These residues, if not used, can represent an environmental problem. The aim of this research was to identify the potential and use of these by-products through their bromatological composition, lignocellulosic content, aromatic compounds, fatty acid profile, polyphenol and methylxanthine content, and antioxidant capacity. Extraction was made from cocoa husk and shell with a mixture of acetone-water (70:30), applying sonication (40 kHz 15 min^{-1}) and maceration (4 h). Total polyphenols (Folin-Ciocalteau method), antioxidant capacity (DPPH and ABTS), catechin, epicatechin, caffeine, and theobromine (HPLC) were quantified. Aromatic compounds and fatty acid quantifications were evaluated through GC/MS-QP. Cocoa husk and shell stood out for their content of fiber, protein, lignocellulosic material, and fatty acids (oleic, linoleic, elaidic, and stearic). Volatile compounds such as pyrazines, esters, alcohols, and aldehydes were identified in the residues. The results showed that the husk had a higher content of total polyphenols than the shell (26.64 mg GAE g^{-1} vs. 19.18 mg GAE g^{-1}). The shell exhibited higher values of epicatechin (21.64 mg g^{-1}), theobromine (15.41 mg g^{-1}), and caffeine (4.96 mg g^{-1}) compared to the husk (6.07, 0.53, and 0.52 mg g^{-1}, respectively). Due to their composition, these by-products can be used by different industries, and contribute to obtaining a higher added value and to the solution of environmental problems due to their use.

Keywords:
Agro-industrial wastes
Husk
Methylxanthines
Polyphenols
Proximal composition
Shell

RESUMEN

La agroindustria de cacao a nivel mundial genera cerca de 48 millones de toneladas al año de residuos de cáscara de la mazorca y 700 mil toneladas de cascarilla, subproductos de los procesos de despulpado y tostión. Estos residuos, si no son aprovechados, pueden representar una problemática ambiental. El objetivo de esta investigación fue identificar el potencial y aprovechamiento de estos subproductos por medio de su composición bromatológica, contenido lignocelulósico, compuestos aromáticos, perfil de ácidos grasos, contenido de polifenoles y metilxantinas y capacidad antioxidante. La extracción se realizó sobre la cáscara y cascarilla de cacao con una mezcla de acetona y agua (70:30), aplicando sonicación (40 kHz 15 min^{-1}) y maceración (4 h). Se cuantificaron polifenoles totales (método Folin-Ciocalteau), capacidad antioxidante (DPPH y ABTS), catequina, epicatequina, cafeína y teobromina (HPLC). Los compuestos aromáticos y cuantificación de ácidos grasos se evaluaron mediante GC/MS-QP. La cáscara y cascarilla de cacao se destacaron por su contenido de fibra, proteína, material lignocelulósico y ácidos grasos (oleico, linoleico, elaídico y esteárico). Se identificaron compuestos volátiles en los residuos como pirazinas, ésteres, alcoholes y aldehídos. Los resultados mostraron que la cáscara tiene un mayor contenido de polifenoles totales que la cascarilla (26,64 mg GAE g^{-1} vs. 19,18 mg GAE g^{-1}). La cascarilla exhibió mayores valores de epicatequina (21,64 mg g^{-1}), teobromina (15,41 mg g^{-1}) y cafeína (4,96 mg g^{-1}) en comparación con la cáscara (6,07, 0,53 y 0,52 mg g^{-1}, respectivamente). Estos subproductos, por su composición, pueden ser aprovechados por diferentes industrias, contribuyendo a obtener un mayor valor agregado y a la solución de problemas ambientales por su utilización.

Palabras clave:
Residuos agroindustriales
Cascarilla
Metilxantinas
Polifenoles
Composición proximal
Cáscara

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The cacao tree belongs to the family Malvaceae, genus *Theobroma* L., and is native to the upper Amazon basin, specifically Colombia, Brazil, Peru, and Ecuador. Cocoa beans are used to produce chocolate, cocoa beverages, and as an additive in confectionery, and ice cream, among others. Likewise, they have cosmetic and pharmaceutical applications due to their anti-inflammatory, antidepressant, and antioxidant properties (Vásquez et al. 2019).

Around five million farmers in the world grow cocoa, and it is the main source of income for more than 40-50 million people. According to the International Cocoa Organization (ICCO), world cocoa production in the 2021-2022 season was 4.8 million tons; Ivory Coast, Ghana, Brazil, Ecuador, and Indonesia are some of the main producers. In 2019, ICCO ratified Colombia as an exporter of fine and aromatic cocoa. This maintains the competitiveness of the national beans and the country's qualification as a producer and exporter of fine cocoa by 95%. According to the National Cocoa Growers Federation, in the years 2021-2022, the country recorded a production of 62,741 tons of cocoa beans and kept the upward trend of the last 10 years. The main producing departments were Santander (36.8%), Arauca (16.9%), Antioquia (8.3%), Tolima (5.8%), Huila (5.7%), and Nariño (5.4%). The Agricultural Risk Management Unit published that approximately 65,000 families are cocoa producers.

The generation of large amounts of organic waste —also called residual biomass— is the main drawback that agribusiness, as a whole, faces, which is why strategies are proposed to add value to the by-products according to their characteristics and their possible applications. The world cocoa production indicates that approximately 50 million tons of residual biomass are produced, in most cases they are left on plantations, thus causing environmental and phytosanitary problems (Sarmiento-Vásquez et al. 2021).

The processing and transformation of cocoa consists of four fundamental stages. In the shelling stage, the pods are manually opened to extract the beans, which are covered by mucilage. Subsequently, in the fermentation stage, the mucilage is degraded by microbial action, and the generation of precursors of the aroma and flavor characteristic of chocolate is promoted. In the drying stage, the moisture of the beans is reduced by up to 7% approximately. Finally, in the roasting stage, some chemical changes continue as a result of what happened in the fermentation and drying. Aromatic compounds are generated by the action of temperature due to amino acid degradation reactions and interaction with reducing sugars in the bean. These processes generate by-products such as shell, husk, and exudates from the fermentation process, which represent approximately 74-86% of the total weight of the fruit (Figure 1) (Aprotosoaie et al. 2015; Vásquez et al. 2019).

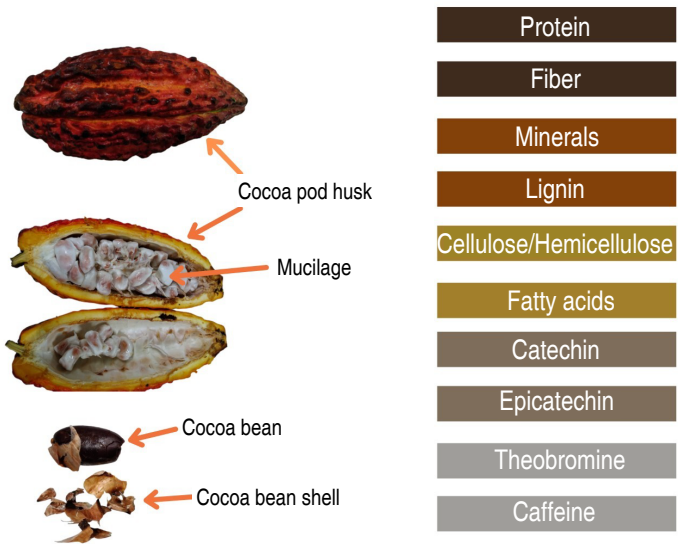

Figure 1. Cocoa by-products and compounds of interest for use.

The husk is the outermost part of the cocoa and is obtained when the cocoa beans are separated from the pod, this constitutes between 52-70% of the total weight of the fruit. Ten tons of husk are generated for each ton of dried cocoa beans (Campos-Vega et al. 2018). The shell is the part that covers the cocoa bean, that is separated from it before or after the roasting process (Rojo-Poveda et al. 2020) and represents about 12-20% of the weight of the dried bean. Considering world production, approximately 700 thousand tons of shell would be produced per year (Okiyama et al. 2017). The mucilage (white pulp) envelops the beans, it constitutes between 30 and 45% of the weight of the pod and is separated from the bean in the fermentation stage, generating about 100-150 L of exudates per ton of cocoa beans (Vásquez et al. 2019).

Currently, different sectors have focused on these by-products to use them and strengthen the development of high-value-added products; for the extraction and application of structural components (cellulose, hemicellulose, lignin, and remaining minerals), biomolecules (theobromine, antioxidants, phenolic compounds, and complex carbohydrates), ant to explore their heat capacity for bioenergy generation (Vandenberghe et al. 2022). These residues can also generate beneficial effects on human health because they contain bioactive substances such as polyphenols and methylxanthines. Their composition could be included in different matrices to develop new products in the market (Grillo et al. 2019). In this context, this research seeks to analyze the potential of cocoa by-products —shell and husk— by bromatological analysis, aromatic compounds, fatty acid profile, polyphenol content, methylxanthines, and antioxidant capacity to identify their application in biorefinery from a bioactive substance's perspective.

MATERIALS AND METHODS

Raw material

Agro-industrial residues from *Theobroma cacao* L. cultivar CCN 51 were used, specifically from the pulping stage (husk), and transformation stage (shell) —i.e., roasting— harvested in 2020 by local regional farmers in Cundinamarca, Colombia. The by-products were subjected to a drying process in a MEMMERT UFE600 forced convection oven at 40 °C for 16 h up to a humidity of about 7%; subsequently, the particle size was reduced in a V-MOLM hammer mill and sieved using a 60 mesh (250 microns) (Nguyen and Nguyen 2017; Pico Hernández et al. 2019).

Bromatological and lignocellulose composition

The moisture and ash content were determined according to AOAC 931.04 and 972.15 methods, respectively. Calcium, potassium, magnesium, manganese, and zinc contents were determined by atomic absorption spectrometry. The phosphorus content was determined by UV-VIS spectrophotometry. The crude protein (Kjeldahl method), fat, and crude fiber content were determined according to the MRE-001 standard. The content of Klason lignin (insoluble acid) was determined based on the TAPPI T 222 om-02 standard, and the content of cellulose and hemicellulose was evaluated according to the ASTM D1695-07 standard.

Aromatic compounds

They were evaluated following the methodology described by Rojas M et al. (2020) with some modifications, using gas chromatography-mass spectrometry equipment (Shimadzu GC/MS-QP 2010 Ultra). Volatiles were extracted using the solid phase microextraction technique (HS-SPME) with a StableFlex Divinylbenzene/Carboxen/Polydimethylsiloxane fiber (DVB/CAR/PDMS). The fiber was preactivated at 250 °C for 30 min. The samples were subjected to initial heating of 10 min at 60 °C, the fiber was exposed to the headspace for 30 min at about 66 °C, and the desorption time was 5 min at 250 °C. The analysis was performed using a Restek Rtx-5MS column (30 m x 0.25 mm ID x 0.1 µm) with helium as carrier gas and a constant flow of 1 mL min^{-1}. The temperature started at 50 °C for 3 min following a gradient of 230 to 10 °C per minute and was maintained at 230 °C for 40 min in the splitless injection mode. The mass spectra of the sample compounds were compared to the National Institute of Standards and Technology database (NIST Library, Gaithersburg, MD, US).

Lipid content analysis

Total lipid extraction was performed. For that, 13 mL of dichloromethane/methanol (2:1% v/v) were added to the sample, the mixture was stirred (Vortex LAB SCIENCE Mixer V8) and centrifuged (HERMLE Z366K) at 10,000 rpm for 10 min and subsequently filtered. 2 mL of 0.73% sodium chloride was added to the obtained extracts, stirred, and centrifuged at 3,500 rpm for 10 min; two phases were formed, and the lower phase was taken

to the forced convection oven (MEMMERT UFE 600) at 45 °C until the dried lipid fraction was obtained.

The fatty acid methyl esters were obtained following the methodology described by Villarreal-Peña et al. (2012). For that, 5 mL of sulfuric acid/methanol (1:2% v/v) were added to the glass tubes with the dried total lipids and stirred for 60 min at 60 °C to form the methylation reaction. Subsequently, 2 mL of hexane was added. Fatty acid methyl esters were evaluated using a mass-coupled gas chromatograph (Shimadzu GCMS-QP 2010 Ultra) and a DB-WAX column (30 m x 0.250 mm x 0.25 µm, Agilent J&W), the carrier gas was helium. An injection volume of 1 µL, dichloromethane solvent, injector temperature 225 °C, column flow 1.21 mL min^{-1}, and Split 3:1 were set. The temperature ramp was 100 °C, 4 min, climbed to 3 °C min^{-1} to 193 °C and finally increased to 1.5 °C min^{-1} to 240 °C, 10 min with a total run time of 76 min. The equilibration time of the system was 3 min, and the data scanning started at 4 min. Fatty acids were identified by comparing retention times of characteristic peaks generated by 37 methyl ester standards (FAME Mix Supelco 37). Quantification was performed by calibration curves for each fatty acid and the data were expressed in terms of percentage of the total area.

Sonication-assisted extraction

An extraction by sonication and maceration was performed to evaluate the content of total polyphenols, and antioxidant capacity (DPPH and ABTS), and to identify the bioactive compounds by HPLC of the cocoa husk and shell. In a test tube, 8 mL of acetone-water (70:30, v/v) were added to 400 mg of sample, then the mixture was subjected to a Branson 3800 CPXH ultrasonic bath for 15 min at 40 kHz ultrasonic radiation. After sonication, maceration was performed, and the samples were subjected to constant stirring for 4 h at room temperature. The extracts were centrifuged (HERMLE Z366K) at 10,000 rpm for 15 min, the supernatants were removed and stored at -30 °C until use.

Total polyphenol content

The total polyphenol content was determined using the Folin-Ciocalteau reagent. 50 µL of the extract, 425 µL of distilled water, and 125 µL of the reagent were added to each test tube and stirred. Subsequently, 400 µL of 7.1% sodium carbonate (Na_2CO_3) solution was added. The tubes were left in the absence of light for 1 h. Absorbance was recorded at 760 nm on a THERMO SCIENTIFIC G10S UV-VIS spectrophotometer. The calibration curve was performed using gallic acid solutions at concentrations between 0-500 mg L^{-1} (R^2=0.997). The results were calculated from equation 1 and expressed in mg gallic acid equivalent per gram of dry sample (mg GAE g^{-1}).

$$\frac{\text{mg GAE}}{\text{g}} = \frac{C \times V_{total} \times DF}{DW} \quad (1)$$

Where:
C = Gallic acid concentration (mg L^{-1})
V_{total} = Total volume used in extraction (µL)
DF = Dilution factor
DW = Dry weight of the sample used in the extraction (g)

Antioxidant capacity

The ability of the samples to trap the DPPH (2.2-diphenyl-1-picrylhydrazyl) radical was evaluated by decreasing the absorbance to 517 nm. 10 µL of the extract and 990 µL of a DPPH solution—which was prepared in methanol (0.05 mM)— were added to a test tube. The tubes were shaken and stored in the dark for 30 min. Absorbance was measured at 517 nm. The calibration curve was constructed with Trolox solutions at concentrations between 200 and 1800 µmol L^{-1} diluted in methanol (R^2=0.994).

The cationic radical $ABTS^{\bullet+}$ was generated by the oxidation reaction of ABTS (2.2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) with potassium persulfate for 16 h in the dark. In a test tube, 990 µL of ABTS reagent was reacted with 10 µL of the extract. The tubes were shaken and allowed to react in the dark for 7 min. Absorbance was measured at 734 nm. The calibration curve was constructed using Trolox solutions at concentrations between 200 and 2200 µmol L^{-1} diluted in ethanol (R^2=0.993).

The results for DPPH and ABTS were expressed as percent inhibition and µmol Trolox equivalent per gram of dry sample (µmol Trolox g^{-1}), according to the following equations (2) and (3):

$$\%\text{inhibiton} = \frac{A_1 - A_2}{A_1} * 100 \quad (2)$$

Where:
A_1 control absorbance and A_2 sample absorbance.

$$\frac{\mu mol\ Trolox}{g} = \frac{C \times V_{total} \times DF}{DW} \quad (3)$$

Where:
C = Trolox equivalent concentration (µmol L^{-1})
V_{total} = Total volume used in extraction (µL)
DF = Dilution factor
DW = Dry weight of the sample used in the extraction (g)

Identification of bioactive compounds by HPLC
It was conducted using cocoa husk and shell extracts following the methodology described by Carrillo et al. (2014) with some modifications. High-performance liquid chromatography (HPLC) equipment (Shimadzu Prominence LC-20AT) with a diode detector (PDA, SPD 20AT) was used. The column employed was the C18 Zorbax Eclipse Plus Agilent (150 mm x 4.6 mm, 5 µm) and a Zorbax NH2 4-Pack pre-column (12.5 mm x 4.6 mm, 5 µm).

The analysis was performed in gradient mode using 1% acidified water with acetic acid (A) and 1% acidified acetonitrile (B) as mobile phases. The program started with 0% B, 5.3 min; 0-90% B, 8.7 min; 90-70% B, 3 min; 70-30% B, 2 min; 30-0% B, 2 min; and 0% B, 4 min at a flow of 1.3 mL min^{-1} and a total run time of 25 min. The injection volume was 10 µL, and the oven temperature was 40 °C. The reading was performed at 280 nm. To identify catechin and epicatechin, a calibration curve between 5 and 200 ppm of the standards (catechin R^2=0.997 and epicatechin R^2=0.996) was conducted.

Similarly, a calibration curve was conducted between 5 and 75 ppm of the theobromine (R^2=0.997) and caffeine (R^2=0.998) standards. Mobile phases were 1% acidified water with acetic acid (A) and 1% acidified acetonitrile (B) following an initial gradient of 10% B, 0-0.02 min; 20% B, 0.02-5.3 min; 25% B, 5.3-8 min; 27% B, 8-10 min; 30% B, 10-12 min; 40% B, 12-15 min; 60% B, 15-17 min; 30% B, 17-19 min; and 0% B, 19-25 min. The injection volume was 15 µL and the temperature was 30 °C. The reading was performed at 280 nm.

Statistical Analysis
The statistical analysis was carried out by the STATGRAPHICS Centurion XVI software version 16.1.18, applying the mean comparison —Tukey test with a significance level of P<0.05— to the analysis results of lignocellulosic content, polyphenols, antioxidant capacity, and methylxanthines. All analyses were performed in triplicate.

RESULTS AND DISCUSSION

Bromatological composition of cocoa shell and husk
Table 1 presents the bromatological composition of the cocoa husk and shell. The values of fat, crude protein, zinc, and manganese contents were higher in the shell than in the husk, while the content of crude fiber was higher in the husk. These results are consistent with those presented by Yapo et al. (2013), these authors found a higher protein value in husks from hybrid cocoa in Abidjan, Ivory Coast, as well as a higher fat content. However, Villamizar-Jaimes and López-Giraldo (2017) found similar fat content values in husks from clone CCN-51 collected in San Vicente de Chucurí-Santander, Colombia.

The value of crude protein in cocoa shells (Table 1) is consistent with that found by Nsor-Atindana et al. (2012) in shells obtained from a manufacturing company in Wuxi, China, and by Fakhlaei et al. (2020) in shells from Jengka Pahang, Malaysia. Agus et al. (2018) reported a higher protein and ash content in Jengka Termerloh, Malaysia. The cocoa shell can be used for animal feed thanks to its protein content along with other constituents such as fiber and minerals.

Grassia et al. (2019) and Yapo et al. (2013) studied the proximal composition of cocoa beans and reported a higher fat content (33-56.54 g 100 g^{-1}) relative to cocoa husk and shell, while the protein content (9.9-12.96 g 100 g^{-1}) was lower compared to shell. The ash content in the husk and shell was higher than in the bean (0.9-3.71 g 100 g^{-1}). Furthermore, the fiber contents in the husk and shell were higher than the fiber values reported by Vázquez-Ovando et al. (2016) in cocoa beans from Mexico (3.13 g 100 g^{-1}) and Venezuela (4.23 g 100 g^{-1}). The fiber content gives nutritional value to the husk; thus, it can be used to obtain value-added products (Villamizar-Jaimes and López-Giraldo 2017). The fiber content (Table 1) was similar to that reported by Vriesmann et al. (2011) in husks from Itabuna, Brazil, and by Pico Hernández et al. (2019) in husks from clone CCN-51 collected in Colombia.

Regarding the shell, the fiber content agrees with that reported by Fakhlaei et al. (2020), while Agus et al.

(2018) report a lower fiber content; however, they found a similar fat content. Due to its high fiber and low-fat content, the shell has been of great interest in the food industry. Its potential to be used as a functional ingredient in confectionery, bakery, and low-calorie products to achieve a high-fiber diet by decreasing and controlling cholesterol and glucose levels in the blood has been evaluated (Okiyama et al. 2017).

Table 1. Bromatological composition of cocoa husk and shell.

Parameter (g 100 g^{-1})	Cocoa pod husk			Cocoa bean shell		
	This study	[1]	[2]	This study	[3]	[2]
Fat	0.52±0.2	1.5±0.13	2.27±0.25	3.88±0.2	5.48±0.51	6.28±0.47
Crude protein	4.3±1.3	8.6±0.09	8.91±0.29	17.6±1.3	17.17±0.25	15.35±0.46
Crude fiber	36.8±0.1	36.6±0.01	-	27±0.1	28.81±0.63	-
Ash	8.81±0.34	6.7±0.06	7.92±0.39	9.20±0.09	10.78±0.19	8.03±0.76
Calcium	0.39±0.3	0.254±11.31	0.61±0.08	0.32±0.3	-	0.42±0.01
Phosphorus	0.21±0.12	-	0.71±0.09	0.56±0.12	-	0.59±0.02
Magnesium	0.17±0.02	0.1109±0.01	0.49±0.06	0.57±0.02	-	0.25±0.01
Potassium	3.29±0.02	2.768±51.97	5.82±0.37	2.86±0.02	-	2.17±0.07
Manganese (mg kg^{-1})	<5	35.72±0.03	-	56±10.0	-	-
Zinc (mg kg^{-1})	54±6.0	39.74±0.06	-	215±6.0	-	-

[1]Vriesmann et al. (2011), [2]Yapo et al. (2013), and [3]Fakhlaei et al. (2020). [-] unreported data.

The differences between some parameters in the bromatological composition can be attributed to the origin and variety of the samples, the climatic conditions, the agronomic management of the crop, the fermentation and roasting process, and the processing conditions to which they were subjected (Zapata et al. 2015; Grillo et al. 2019).

Minerals such as phosphorus, calcium, magnesium, and potassium are required for cells to maintain their vital functions; their presence in the cocoa husk makes it suitable to be used as a source of these minerals (Campos-Vega et al. 2018). The contents are similar to those reported by Vriesmann et al. (2011) and Yapo et al. (2013). Moreover, the shell has been identified as a good source of minerals including potassium, magnesium, calcium, phosphorus, copper, and zinc (Cinar et al. 2021). The calcium, phosphorus, and potassium content found in the shell were similar to those reported by Yapo et al. (2013) in samples from a hybrid cocoa plantation derived from Forastero in Abidjan, Ivory Coast, while the magnesium content was lower.

Cinquanta et al. (2016) evaluated the content of zinc (48.8 mg kg^{-1}) and manganese (30.1 mg kg^{-1}) in cocoa beans from Ghana and obtained values lower than those found in the shell. Microelements such as zinc and manganese present in cocoa husk, shell, and beans depend on fertilization in plantations, which is why variations can be attributed to the type of fertilizer and the richness of these microelements in the soil (Lares et al. 2014).

Figure 2 shows the lignocellulosic content of the cocoa husk and shell. The cellulose content showed significant differences —being higher in the husk— while the hemicellulose content was higher in the shell. Similarly, Vásquez et al. (2019) reported a higher content of cellulose in the husk compared to the shell, while the lignin content was higher in the shell.

Budaraga and Putra (2020) presented similar values of cellulose, hemicellulose, and lignin in husks from Indonesia. Concerning shell, Wijaya and Wiharto (2020) reported higher lignin and cellulose values and a lower value of hemicellulose content in samples from Enrekang,

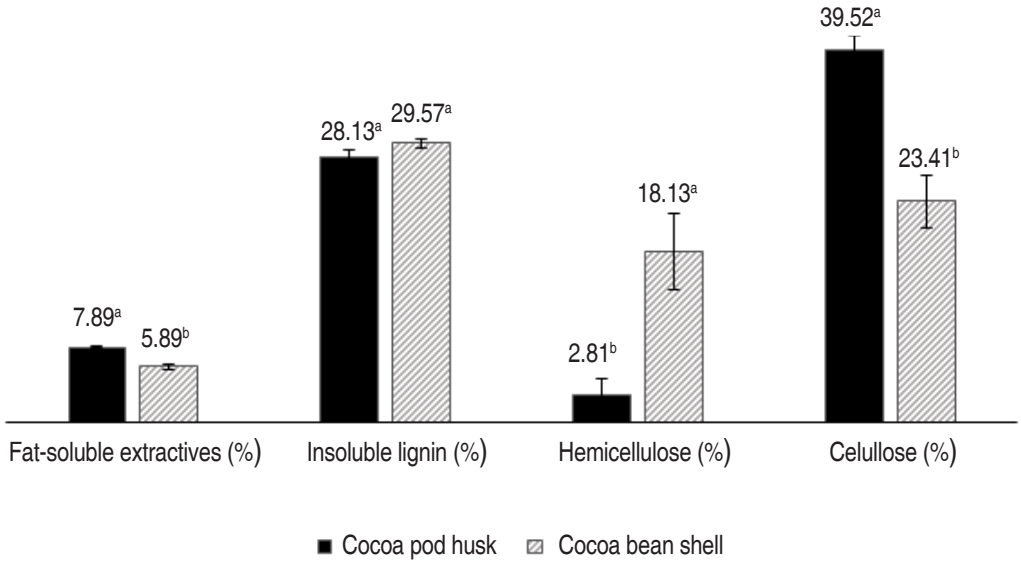

Figure 2. Lignocellulosic content of cocoa husk and shell. Different letters a and b indicate significant differences (P<0.05).

Indonesia. Gómez Hoyos et al. (2020) reported a lower lignin content in shells from Colombia.

These residues containing lignocellulose are a source of biomass and therefore can be used to produce biofuels, energy, and products of interest for various economic sectors, by applying pretreatments to degrade the structure and obtain substrates rich in fermentable compounds in addition to cellulose to develop biodegradable materials. According to Sakagami et al. (2008), phenolic compounds retained in the lignin fraction of the husk exhibit high antimicrobial and antiviral activity, and some of the components have been used against the HIV-1 virus obtaining promising results.

Fatty acid profile

In the cocoa husk, palmitic, linoleic, and oleic fatty acids were the most prominent; palmitic acid was the highest with 36.92%, as detailed in Table 2. Conversely, in cocoa shell, oleic, elaidic, and stearic acids were predominant, elaidic acid stood out with the highest proportion, 28.68%. The husk showed a higher percentage of saturated and polyunsaturated fatty acids, while the shell had a higher content of monounsaturated fatty acids.

Carta et al. (2020) reported the lipid profile of cocoa husk collected from an Italian food industry. The palmitic acid content was lower, while the percentages of oleic, linoleic, and linolenic acids were higher. The percentage of saturated fatty acids (53.59%) was higher and that of unsaturated fatty acids was lower (46.41%).

Lessa et al. (2018) evaluated the fatty acid profile in cocoa shells from Bahia, Brazil, and reported a higher content of palmitic acid and stearic acid, obtaining a content of oleic acid similar to the one reported in this research. The percentage of saturated fatty acids (64%) was higher, and that of monounsaturated (28.7%) and polyunsaturated (6.03%) fatty acids was lower. Similarly, Botella-Martínez et al. (2021) mentioned that the main fatty acids obtained from the shell of the forastero variety cultivated in Ghana were palmitic, stearic, and oleic acids.

Grassia et al. (2019) identified the fatty acids of cocoa beans in the criollo variety from Ecuador and the hybrid variety from Ghana, reporting the content of three major acids in cocoa beans: palmitic acid, with values lower than that found in cocoa husk; stearic acid and oleic acid, with values higher than that found in the cocoa shell; and lower contents in the main fatty acids i.e., linolenic acid and linoleic acid. The percentage of saturated fatty acids (60.60-63.92%) was higher. The percentage of monounsaturated (32.56-36.62%) was higher than that of the husk and lower than that of the shell. The percentage of polyunsaturated acids (2.08-3.59%) was lower.

Table 2. Profile of fatty acids in the cocoa husk and shell by GC-MS analysis.

Fatty acids	Cocoa pod husk (%)	Cocoa bean shell (%)
Lauric acid C12:0	0.40	0.14
Myristic acid C14:0	1.02	0.87
Pentadecanoic acid C15:0	0.38	0.13
Oleic acid C18:1 Cis-9	10.09	27.27
Palmitic acid C16:0	36.92	1.21
Palmitoleic acid C16:1	0.35	-
Margaric acid C17:0	0.44	0.34
Cis-10-heptadecanoic acid C17:1	0.12	-
Stearic acid C18:0	4.66	26.01
Elaidic acid C18:1 Trans-9	0.28	28.68
Linoleic acid C18:2 Cis-9,12	36.35	9.14
Linolelaidic acid C18:2 Trans-9,12	-	0.25
Linolenic acid C18:3 Cis-9,12,15	3.75	1.12
Arachidic acid C20:0	1.01	1.76
Heneicosanoic acid C21:0	0.23	-
Behenic acid C22:0	1.73	1.58
Tricosanoic acid C23:0	0.82	0.33
Lignoceric acid C24:0	1.47	1.16
Total saturated fatty acids	49.08	33.53
Total monounsaturated fatty acids	10.84	55.95
Total polyunsaturated fatty acids	40.10	10.51

According to Rachmawaty et al. (2018), stearic and oleic acids have antimicrobial activity; the latter is also attributed to an antioxidant and anticancer potential. That is why cocoa by-products that contain these acids can be used in the food industry as fat substitutes for certain foods or to obtain products with antioxidant and antimicrobial properties. For example, stearic acid is suitable for producing emulsifiers, emollients, lubricants, cosmetics, and personal care products (Tulashie et al. 2022). In another study, resveratrol and fatty acids, such as linoleic, present in cocoa husk possess skin-lightening properties and show UVB sunscreen potential (Campos-Vega et al. 2018).

Identification of aromatic compounds

Pyrazines, aldehydes, ketones, alcohols, esters, furans, acids, pyrroles, and terpenes are some of the volatile organic compounds that characterize the aroma of cocoa (Barbosa-Pereira et al. 2019). The amount and type of volatile compounds in cocoa beans are important to define their quality, as well as their commercial value based on the unique and complex flavors and aromas of chocolate (Utrilla-Vázquez et al. 2020). Table 3 presents the aromatic compounds identified in the cocoa husk and shell with their respective description of the aroma reported in the literature. Identifying them can contribute to the search for potential alternatives to obtain new products and add value to these residues.

The taste and aroma of different cocoas are the result of several factors, among them, the most important is the genetic origin, followed by the environment where the trees grow. It is influenced by the edaphoclimatic conditions and vegetation, in addition to factors such as primary management (soil preparation, agronomic management) and post-harvest (pulping, fermentation, drying, roasting).

A study conducted by Barros (2017) identified 50 volatile compounds in cocoa husks from Pará, Brazil, out of which 16 were alcohols, 11 hydrocarbons, 8 aldehydes, 7 ketones, 5 esters, 2 amines, and isovaleric acid. Values greater than those identified in the husk.

Table 3. Aromatic compounds identified in the cocoa husk and shell by SPME-GC-MS.

Compounds	Cocoa bean shell		Cocoa pod husk		Odor description	Reference
	RT* (min)	Area	RT* (min)	Area		
Alcohols						
2-Nonanol	3.416	139,779	-	-	Waxy, green, creamy, citrus orange	[1]
2,3-Butanediol	4.043	854,609	-	-	Fruity, creamy, buttery	[2]
2-Heptanol	6.479	195,823	6.479	1,564,031	Citrus, earthy, oily	[1]
1-Hexanol	-	-	5.698	377,339	Fruity, green, herbaceous	[1]
2-Nonanol, acetate	-	-	16.273	473,399	-	
Phenylethyl Alcohol/2-Phenylethanol	12.649	479,709	-	-	Flowery, honey-like, rose, lilac, caramel, sweet	[1]
2-Butanol, 3-methyl-	-	-	2.870	267,656	Flavoring agents	[3]
Aldehydes						
Benzaldehyde	8.110	238,745	-	-	Bitter, almond-like, burnt sugar, grass, earthy	[1]
Benzeneacetaldehyde	10.522	92,146	-	-	Honey, flowery, sweet	[4]
Nonanal	12.381	380,637	12.384	604,671	Tallowy, soapy-fruity, fatty, waxy, pungent	[1]
Hexanal	-	-	4.275	746,698	Green, fermented	[1]
2-Hexenal	-	-	5.367	594,426	-	
Octanal	-	-	9.293	135,366	Orange peel, oily, fatty, soapy	[1]
(E)-2-Octenal	-	-	10.962	77,509	Fatty, waxy	[1]
(E)-2-Nonenal	-	-	14.081	81,327	Green	[1]
Decanal	-	-	15.484	134,363	Sweet, orange, waxy	[1]
Furfural	4.853	467,357	-	-	Sweet, bread, potato-like	[5]
Ketones						
Acetoin	2.990	108,773	-	-	Buttery, sour milk, caramel	[2]
2-Heptanone	6.194	158,800	6.167	471,336	Fruity, banana-like, green	[1]
2-Decanone	15.043	99,528	-	-	Floral	[1]
6-methyl-5-Hepten-2-one	-	-	8.754	126,556	Pungent, green	[1]
2-Nonanone	-	-	11.954	232,340	Hot milk, green, fruity	[6]
Esters						
Butanoic acid, butyl ester/Butyl butyrate	9.066	86,616	-	-	Fruity	[2]
Acetic acid, 2-phenylethyl ester	16.919	223,898	-	-	Floral, fruity, sweet	[7]
Benzeneacetic acid, methyl ester/ Methyl 2-phenylacetate	14.554	261,519	-	-	Honey, sweet, jasmine	[1]
Furans						
2-Furanmethanol	5.347	261,551	-	-	Burned, faint burning-like	[5]
Pyrazines						
2,5-Dimethyl-Pyrazine	6.829	573,608	-	-	Cocoa, roasted nuts, green, rum	[6]
2,3-Dimethyl-Pyrazine	6.945	264,926	-	-	Caramel, cocoa, sweet, baked, hazelnut, roasted, earthy	[1]

Table 3

Compounds	Cocoa bean shell		Cocoa pod husk		Odor description	Reference
	RT* (min)	Area	RT* (min)	Area		
Trimethyl-Pyrazine	9.283	1,779,145	-	-	Cocoa, roasted nuts, peanuts	[6]
3-ethyl-2,5-Dimethyl-Pyrazine	11.553	781,799	-	-	Potato, roast, earthy	[1]
Tetramethyl-Pyrazine	11.791	2,807,599	-	-	Coffee-milk, roasted	[7]
2,3,5-Trimethyl-6-ethylpyrazine	14.000	525,600	-	-	Candy, sweet, chocolate, cocoa, hazelnut, roasted	[1]
2-Isoamyl-6-methylpyrazine	16.819	152,189	-	-	-	[1]
2,3-diethyl-5-methylpyrazine	13.848	105,351	-	-	Earthy, roasted	[8]
3-(3-methyl butyl)-2,5-dimethylpyrazine	18.644	103,346	-	-	Fruity	[9]
Pyrroles						
1-(1H-pyrrol-2-yl)-Ethanone,	11.107	102,790	-	-	Chocolate, hazelnut	[2]
Terpenes/terpenoids						
D-Limonene	10.135	282,400	-	-	Citrus	[2]
cis-linalool oxide	11.407	403,751	-	-	Sweet, floral, creamy	[7]
trans-Linalool oxide (furanoid)	-	-	11.890	398,197	Floral, citrus, fruity	[8]
Hydrocarbons						
(-)-Aristolene	-		18.652	1,183,975	-	
Copaene	-		20.550	104,564	-	
Gamma-Muurolene	-		21.321	77,205	-	

*RT: retention time. [1]Barbosa-Pereira et al. (2019), [2]Utrilla-Vázquez et al. (2020), [3]Valle-Epquín et al. (2020), [4]Rodriguez-Campos et al. (2011), [5]Calva-Estrada et al. (2020), [6]Hinneh et al. (2019), [7]Gil (2018), [8]Aprotosoaie et al. (2015), [9]Scalone et al. (2019).

Barbosa-Pereira et al. (2019) conducted a study to classify and characterize the volatile compounds of cocoa shells obtained from beans from different cultivars and collected in various geographical origins. They identified a total of 101 compounds comprising 15 aldehydes, 9 ketones, 4 sulfur compounds, 8 esters, 2 hydrocarbons, 3 furans, 21 pyrazines, 7 alcohols, 4 pyrroles, 10 terpenes, 10 acids, 3 lactones, and 5 other compounds. Values greater than those obtained in the shell.

Compounds such as acetic acid, 2-phenylethyl ester; benzeneacetaldehyde; 2-heptanol; 2-phenylethanol; and trimethyl-pyrazine enhance the shell as a food ingredient, so it is important to describe and study its volatile composition to define the quality and flavor of the product. For instance, shell has been used to produce chocolate flavor with a real chocolate aroma using enzymatic technology to make products such as cookies and bread. For its part, the husk has demonstrated potential as an inert support for the bioconversion of secondary metabolites by fungal strain, that is, obtaining volatile fragrance components in a solid-state fermentation system (Campos-Vega et al. 2018).

Polyphenols, methylxanthines, and antioxidant capacity

The total polyphenol content and antioxidant capacity of the husk and shell are presented in Table 4. The content of total polyphenols and the antioxidant capacity, DPPH, and ABTS, in the husk showed significant differences with respect to the shell; the values of the husk are higher. The total antioxidant capacity of the soluble fractions of the husk exhibits greater synergistic interactions between various compounds with antioxidant properties, including low molecular weight phenolic compounds, medium molecular weight proanthocyanindines, and oligosaccharides and/or polysaccharides derived from the cell wall matrix peptide (Yapo et al. 2013).

Table 4. Content of total polyphenols, DPPH, and ABTS in cocoa husk and shell.

Analysis	Cocoa pod husk	Cocoa bean shell
Total polyphenols (mg GAE g^{-1})	26.64±3.85[a]	19.18±1.89[b]
DPPH (µmol Trolox g^{-1})	157.85±2.82[a]	116.27±3,92[b]
% inhibition	72.73±1.94[a]	55.30±2.00[b]
ABTS (µmol Trolox g^{-1})	214.67±6.82[a]	113.91±6.75[b]
% inhibition	72.00±4.13[a]	40.58±2.39[b]

Different letters in the same row indicate significant differences (P<0.05).

The total phenol content can vary due to environmental factors such as sample origin, variety, maturity, climate and processing, roasting, tempering, alkalization, and storage (Hernández-Hernández et al. 2018). It depends on extraction methods, solvent polarity, and sample/solvent ratio (Campos-Vega et al. 2018), which in turn affects the antioxidant properties of a given extract (Schinella et al. 2010).

Sotelo et al. (2015) performed an extraction process in husks of the clone TSH 565 from Tierralta, Córdoba (Colombia) by stirring with ethanol-water acidified with 1% HCl and obtained a lower content of polyphenols of 19.26±0.46 mg GAE g^{-1}. Quiroz-Reyes et al. (2013) obtained a similar content of polyphenols (25.34±1.82 mg GAE g^{-1}) using a first extraction with water-methanol and a second phase with acetone-water in husks from Mexico.

With respect to the cocoa shell, Hernández-Hernández et al. (2019) obtained a lower polyphenol content (11 mg GAE g^{-1}) in samples of different genotypes from Mexico using a methanol-water extraction with HCl acidified stirring. Lessa et al. (2018) reached a similar polyphenol content (21.2 mg GAE g^{-1}) in shells from Bahia, Brazil, using an alcoholic solution of hydroethanol as solvent.

In another study, Hernández-Hernández et al. (2018) applied the extraction by maceration technique to cocoa beans from Mexico, using ethanol-water acidified with HCl as a solvent and a second extraction with acetone-water. They reported a polyphenol content of 49.46±2.50 mg GAE g^{-1}, higher than that found in cocoa husk and shell.

The DPPH and ABTS methods were used to calculate the antioxidant capacity in the extracts, which varies according to the composition of the raw material and the system used for the extraction. Both methods are influenced by many factors; therefore, it is necessary to use several methods to include various mechanisms that contribute to the antioxidant action.

Rachmawaty et al. (2019) performed maceration extraction on cocoa husks from West Sulawesi, Indonesia. They used acetone-water and 70% ethanol as solvents and evaluated DPPH antioxidant capacity (percent inhibition). They obtained values of 61.58 and 69.19% for ethanol and acetone-water, respectively. The result obtained using acetone-water as solvent was very similar (Table 4). The acetone extract generates a higher DPPH buffering activity than that of ethanol because the content of phenolic compounds is easily soluble in acetone compared to ethanol. Sotelo et al. (2015) applied the ultrasonic extraction technique with acidified ethanol-water (1% HCl) to husks of the clone TSH 565 to analyze their ABTS antioxidant capacity and obtained a value greater than 229.61 µmol Trolox g^{-1}.

Jokić et al. (2018) applied the subcritical water extraction technique (Temperature 170 °C) to cocoa shells from a chocolate factory in Osijek, Croatia, to obtain bioactive compounds by evaluating their antioxidant capacity by DPPH inhibition percentage. They obtained a similar value of 56.11%, while Lessa et al. (2018) obtained a higher DPPH inhibition percentage, 79.2%, in shells from Bahia, Brazil.

Zapata et al. (2015) used methanol maceration extraction on cocoa beans of the clone CCN 51 from Cauca, Colombia, to determine the trapping activity of the ABTS radical, which yielded a value of 384.36 µmol Trolox g^{-1}. García-Alamilla et al. (2017) found a DPPH inhibition percentage of 84.83% in cocoa beans from Tabasco, Mexico, using an aqueous

extraction by shaking for 2 h. According to the two previous investigations, the cocoa beans had a higher antioxidant capacity than the husk and shell.

Table 4 shows that the higher the content of total polyphenols, the higher the antioxidant capacity. It indicates a possible correlation between these variables. The extracts obtained from the cocoa husk and shell have antioxidant and antimicrobial activity attributed to polyphenols and methylxanthines. Therefore, these by-products are promising and of great interest in various fields of application such as human health, the food and cosmetics industries, and bioremediation.

The concentrations of polyphenols (catechin and epicatechin) and methylxanthines (theobromine and caffeine) obtained by HPLC are presented in Figure 3. Rojo-Poveda et al. (2020) reported an interaction between flavonoids and cocoa methylxanthines, which helps increase the plasma concentration of epicatechin metabolites and improve the vascular effects attributed to flavonoids.

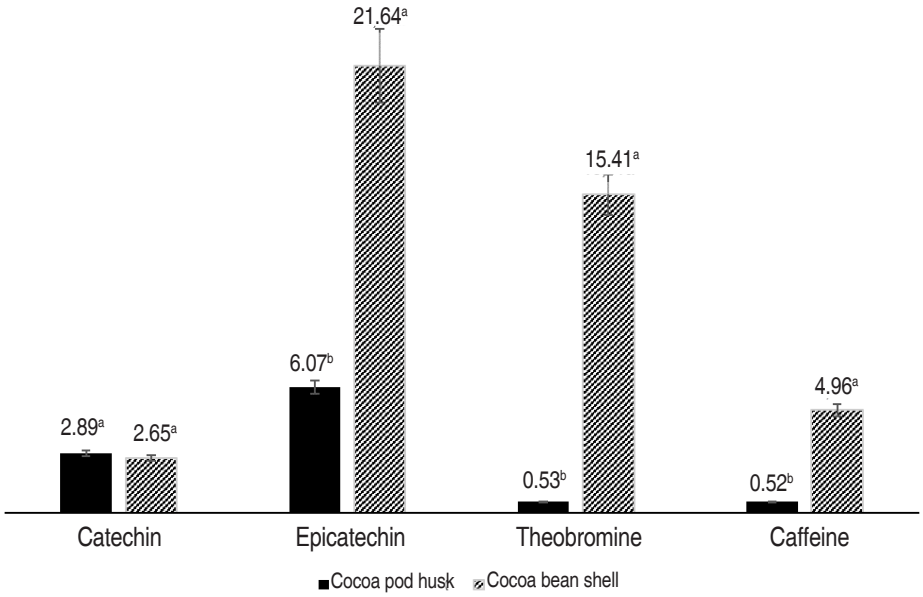

Figure 3. Phenols and methylxanthines in cocoa husk and shell extracts by HPLC. Values were expressed in mg g^{-1} of dry extract. Different letters a and b indicate significant differences (P<0.05).

Cocoa husk presented a significant difference in the concentrations of epicatechin, theobromine, and caffeine compared to shell; however, the large amount of husks discarded by the cocoa industry makes it a cheap, renewable, and sustainable source for the extraction of these compounds.

The extraction process, solvent, and extraction time greatly influence the concentration of phenols and methylxanthines. The genotype, origin, degree of maturity, and processing of cocoa also influence the variation of concentrations. According to Carrillo et al. (2014) when acids are added to the HPLC mobile phase, the separation of the polyphenols improves, since it allows the reduction in ionization of both hydroxyl and carboxyl phenolic groups.

Sotelo et al. (2015) evaluated the concentration of epicatechin, caffeine, and theobromine in the husk extracts obtained by stirring and ultrasound with and without HCl. Stirring and ultrasound extraction yielded the highest values, both with HCl, without significant differences between them. The values of epicatechin (0.342-0.350 mg g^{-1}), caffeine (0.039-0.043 mg g^{-1}), and theobromine (0.032-0.038 mg g^{-1}) were lower.

Rahayu et al. (2019) applied ethanol microwave-assisted extraction to the cocoa husk from Indonesia and evaluated

the concentration of catechin in the extract. They obtained a concentration range between 47.80-51.03 mg L^{-1} of catechin, greater than that found in the husk extract obtained by sonication-assisted acetone (28.88 mg L^{-1}). Quiroz-Reyes et al. (2013) conducted a study in Mexico and determined the concentration of catechin and epicatechin in the bean and cocoa shell by extraction with maceration and ultrasound, using water-methanol and acetone-water as solvents. The values of catechin (maceration 0.28 mg g^{-1} and ultrasound 0.32 mg g^{-1}) and epicatechin (maceration 2.64 mg g^{-1} and ultrasound 2.77 mg g^{-1}) in the shell were lower, while the concentrations of catechin (maceration 4.62 mg g^{-1} and ultrasound 4.26 mg g^{-1}) and epicatechin (maceration 132.88 mg g^{-1} and ultrasound 144 mg g^{-1}) in the cocoa beans were higher.

Hernández-Hernández et al. (2018) carried out a study on cocoa beans and cocoa shells in Mexico evaluating the concentrations of theobromine, catechin, and epicatechin extracts obtained with acidified water and stirring methanol. The concentrations of theobromine (12.00 mg g^{-1}) and epicatechin (17.70 mg g^{-1}) in the shell were similar, while those of the fermented cocoa bean (theobromine 9.79 mg g^{-1} and epicatechin 6.13 mg g^{-1}) were lower. The concentration of catechin was lower in both the bean (1.48 mg g^{-1}) and the shell (1.2 mg g^{-1}).

CONCLUSIONS

Cocoa husk and shell had a similar lignin content. Protein and fat content were higher in the shell, while the husk had a higher cellulose content. In the shell, pyrazines, esters, and oleic, elaidic, and stearic fatty acids predominated, in contrast to the husk, where aldehydes, alcohols, and palmitic, oleic, and linoleic fatty acids prevailed. Cocoa residues are known for the presence of polyphenols, which are correlated with antioxidant capacity. The husk presented a higher content of total polyphenols (26.64 mg GAE g^{-1}), antioxidant capacity DPPH (157.85 µmol Trolox g^{-1}) and ABTS (214.67 µmol Trolox g^{-1}) than the shell (19.18 mg GAE g^{-1}, 116.27 µmol Trolox g^{-1}, and 113.91 µmol Trolox g^{-1}, respectively). The shell had higher values of epicatechin (21.64 mg g^{-1}), theobromine (15.41 mg g^{-1}), and caffeine (4.96 mg g^{-1}) than the husk (6.07, 0.53 and 0.52 mg g^{-1}, respectively).

The bromatological and chemical characterization of cocoa shells, and husk is essential to understand their potential as high-value-added by-products in various industries, such as food, pharmaceuticals, and cosmetics. Thanks to its properties, it has potential application as an ingredient in bakery and pastry products, in the production of biofuels, or in obtaining extracts with antioxidant and antimicrobial properties. The use of these by-products can benefit cocoa farmers, protect crops from contamination, and promote the sustainable development of the entire cocoa industry. For future research, it is proposed to conduct an economic, social, and environmental study of the use of cocoa by-products, investigate the development of new products and innovative applications, evaluate alternative methods to extract bioactive compounds —including the evaluation of the antioxidant capacity by other methods such as FRAP, ORAC, or superoxide anion— and explore the properties of mucilage and its potential for various applications.

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Inoculation and microelements: two important factors for enhanced conidiogenesis of *Trichoderma asperellum* in solid and liquid fermentation

Inoculación y microelementos: dos factores importantes para mejorar la conidiogénesis de *Trichoderma asperellum* en fermentación sólida y líquida

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Trichoderma asperellum

ABSTRACT

The standardization of cultivation processes that allow high levels of conidia growth and formation is required to formulate *Trichoderma* products to combat fungal diseases in agronomically important crops. This study evaluated the effects of inoculation using different inoculum concentrations ($1.0x10^5$, $1.0x10^6$, and $1.0x10^7$ conidia mL^{-1}) and inoculum volumes (10, 30, and 50 mL). Later, it evaluated the effect of adding microelements ($CaCO_3$, KH_2PO_4, $MgSO_4*7H_2O$, and $(NH_4)_2SO_4$) on the conidiogenesis of two strains of *Trichoderma asperellum* (GRB-HA01 and GRB-HA02) in solid-state and liquid fermentation processes. After 12 days of fermentation, the highest conidiogenesis values for *Trichoderma asperellum* GRB-HA01 ($6.9x10^9±5.7x10^2$ conidia g^{-1}) and *Trichoderma asperellum* GRB-HA02 ($1.3x10^9±1.4x10^2$ conidia g^{-1}) were achieved using an inoculum volume of 10 mL at a concentration of $1.0x0^7$ conidia mL^{-1}. Adding $CaCO_3$ (1 g g^{-1}), resulted in the highest conidia concentrations for *Trichoderma asperellum* GRB-HA01 ($3.0x10^{11}±2.5x10^2$ conidia g^{-1}) and *Trichoderma asperellum* GRB-HA02 ($8.6x10^{10}±1.1x10^1$ conidia g^{-1}), reducing fermentation times to 9 days. The conidiogenesis obtained with liquid fermentation was lower and affected *Trichoderma asperellum* GRB-HA01 ($3.1x10^7±1.1x10^2$ conidia g^{-1}) and *Trichoderma asperellum* GRB-HA02 ($3.1x10^9±2.8x10^2$ conidia g^{-1}). This study showed that inoculation and adding microelements were important factors in the conidiogenesis processes of *Trichoderma asperellum* GRB-HA01 and GRB-HA02. Additionally, it was evidenced that solid-state fermentations are more efficient than liquid fermentation processes.

Palabras clave:
Carbonato de calcio
Conidio
Fermentación
Inóculo
Fosfato dihidrogenado de potasio
Trichoderma asperellum

RESUMEN

La estandarización de los procesos de cultivo que permite altos niveles de crecimiento y formación de conidios es necesario para la formulación de productos de *Trichoderma* para combatir enfermedades fúngicas en cultivos de importancia agronómica. Este estudio evaluó los efectos de la inoculación utilizando diferentes concentraciones de inoculo ($1,0x10^5$, $1,0x10^6$ y $1,0x10^7$ conidios mL^{-1}) y volumen de inóculo (10, 30 y 50 mL). Posteriormente, se evaluó el efecto de la adición de microelementos ($CaCO_3$, KH_2PO_4, $MgSO_4*7H_2O$ y $(NH_4)_2SO_4$) sobre la conidiogénesis de dos cepas de *Trichoderma asperellum* (GRB-HA01 y GRB-HA02) en procesos de fermentación en estado sólido y líquido. Después de 12 días de fermentación, los valores más altos de conidiogénesis para *Trichoderma asperellum* GRB-HA01 ($6,9x10^9±5,7x10^2$ conidios g^{-1}) y *Trichoderma asperellum* GRB-HA02 ($1,3x10^9±1,4x10^2$ conidios g^{-1}) se lograron utilizando un volumen de inóculo de 10 mL con una concentración de $1,0x10^7$ conidios mL^{-1}. La adición de $CaCO_3$ (1 g g^{-1}) generó las mayores concentraciones de conidios para *Trichoderma asperellum* GRB-HA01 ($3,0x10^{11}±2,5x10^2$ conidios g^{-1}) y *Trichoderma asperellum* GRB-HA02 ($8,6x10^{10}±1,1x10^1$ conidios g^{-1}), reduciendo los tiempos de fermentación a 9 días. La conidiogénesis obtenida con la fermentación líquida fue menor y afectó a *Trichoderma asperellum* GRB-HA01 ($3,1x10^7±1,1x10^2$ conidios g^{-1}) y *Trichoderma asperellum* GRB-HA02 ($3,1x10^9±2,8x10^2$ conidios g^{-1}). Este estudio demostró que la inoculación y la adición de microelementos fueron factores importantes durante los procesos de conidiogénesis de *Trichoderma asperellum* GRB-HA01 y GRB-HA02. Adicionalmente, se evidenció que las fermentaciones en estado sólido son más eficientes que los procesos de fermentación líquida.

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Bio fungicide production is important for phytosanitary crop management in agronomy and forestry because bio fungicides act as alternatives to chemically synthesized fungicides (Chandrashekara and Manivannan 2012). *Trichoderma* is one of the most widely used antagonist fungi to control different agronomic diseases, representing around 60% of the biological control agents registered worldwide (Coban and Sargin 2019). *Trichoderma* is a natural inhabitant in soil and has been widely cultivated industrially as a biological control product. Several commercial *Trichoderma* spp. products are available for plant disease management, containing various propagules as active components, such as conidia, chlamydospores, and vegetative mycelium (Coban and Sargin 2019). However, conidia are the most used propagules in biocontrol programs due to their ability to withstand extreme conditions, including high and low temperatures (Cumagun 2017). Conidia are asexual reproductive structures and a primary survival and dispersal mechanism for this genus (Cumagun 2017).

Conidial biomass can be produced by implementing either submerged or solid substrate cultivation techniques. *Trichoderma* conidia obtained from solid-state fermentation exhibits a higher tolerance to abiotic stress compared to propagules or biomass derived from liquid fermentation (Sriram et al. 2011). Solid-state fermentation (SSF) has allowed the productive conidia scaling of various species of the *Trichoderma* genus (Rayhane et al. 2020). Additionally, solid-state fermentation improves bioactive production with biofungicidal potential, which generates bioproducts (Torres et al. 2015; Rayhane et al. 2020) including enzymes (lipases, cellulases, and chitinases) (Urbina et al. 2019) metabolites (mycotoxins, lactones such as 6- pentyl- alpha-pyrone(6-PP) (Ramos et al. 2008). SSF reduces production costs, increases process efficiency, is resistant to contamination, and is an easily scalable method (Gonzalez and Vicente 2016).

Studies have shown several physicochemical factors that promote conidiogenesis. They include concentration and sources of macronutrients, like carbon (Alarcon and Utia 2020) and sources of nitrogen (Gezgin et al. 2020). Moreover, adding mineral salts like NaCl, KCl, and $CaCl_2$ has been found to have a positive effect on conidia production (Dastogeer et al. 2018), along with maintaining a pH range of 5.5-6.5 (Raut et al. 2013). Culture conditions, such as light and temperature, have also been noted as important factors (Steyaert et al. 2010). It is also essential to consider the type of containers and culture spaces to allow for proper gas exchange. Some studies have shown that mycelial lesions can affect conidiogenesis processes due to the accumulation of volatile organic compounds (VOCs) fungi produce (Adnan et al. 2019). To improve conidia production in *Trichoderma* through liquid and solid fermentations, it is essential to identify specific inoculation parameters and add microelements. This will enable the provision of practical recommendations that can benefit Colombian agriculture by enhancing conidia production.

This study aimed to assess the impact of the type of inoculation on conidia production through solid-state fermentation evaluating different concentrations of conidia and inoculum volumes, of conidia production. Based on the findings, the best conditions were used to evaluate the impact of adding microelements ($CaCO_3$, KH_2PO_4, $MgSO_4*7H_2O$, and $(NH_4)_2SO_4$) on the conidiogenesis of two *Trichoderma asperellum* strains (GRB-HA01 and GRB-HA01) in solid and liquid state fermentations.

MATERIALS AND METHODS

Microorganisms

This study included two *Trichoderma asperellum* (GRB-HA01 and GRB-HA02) strains Universidad de Medellin Biodiversity, Biotechnology and Bioengineering Research Group (GRINBIO) donated.

General culture conditions

To activate the strains and prepare conidia inoculum, fungi were cultivated on potato dextrose agar (PDA) at a concentration of 39 g L^{-1} and pH=6.0 previously sterilized at 12 °C, 15 psi, 15 min. Conidia inoculum was obtained by surface washing using an aqueous solution containing sterile Tween 80 at 0.01% v/v from activated cultures on PDA agar with 8 days of growth. The concentration was adjusted to test conditions using a hemocytometer.

For solid-state fermentation, Bae et al. (2016) proposed a modified methodology in which 200 g of unsupplemented rice were placed in polyethylene bags and the substrate was hydrated with distilled water at a 2:1 ratio (rice: water). Then, the bags were sealed with cotton and spring to allow gas exchange, and sterilized at 121 °C, 15 psi, 15 min. The cultures were incubated under laboratory conditions for

12 days with alternating photoperiods of 12-hour diffused light and 12-hour darkness at 28±2 °C. The polyethylene bags were hand-shaken every 24 h during cultivation.

Determination of the effect of inoculum concentration and volume on conidiogenesis in solid culture

To evaluate the effect of conidia concentration in the inoculum on the conidiogenesis of *T. asperellum* GRB-HA01 and *T. asperellum* GRB-HA02 strains, the inoculum was prepared at concentrations of $1.0x10^5$, $1.0x10^6$, and $1.0x10^7$ conidia mL^{-1}. Subsequently, to determine the combined effect of the inoculation volume, rice substrates were inoculated at ratios of (10, 30, and 50 mL per 200 g of un-supplemented rice).

Determination of the effect of microelements on conidiogenesis in solid culture

To evaluate the effect of adding microelements on conidia production for *T. asperellum* GRB-HA01 and *T. asperellum* GRB-HA02 strains, a rice substrate was supplemented before sterilizing the medium with one of the following salts (% w/w): 2.5 ammonium sulphate $(NH_4)_2SO_4$; 1 calcium carbonate $CaCO_3$; 2 magnesium sulphate; $MgSO_4*7H_2O$; or 0.5 potassium dehydrogenate phosphate KH_2PO_4. Additionally, the effect of mixing the salts was evaluated, and a culture without supplementation was used as a control. After sterilization, the culture was inoculated using the best result obtained during the evaluation of the concentration and volume of inoculum, 10 mL of conidia suspension, and a concentration of $1.0x10^7$ conidia mL^{-1}.

Determination of the effect of microelements on conidiogenesis in liquid culture

To determine how adding microelements affected conidia production for *T. asperellum* GRB-HA01 and *T. asperellum* GRB-HA02 strains, liquid fermentation was carried out using potato dextrose broth (PDB) as a culture medium at a concentration of 39 g L^{-1} and peptone at 0.01% (w/v). Each treatment was enriched before sterilizing the medium with (% w/v): 2.5 $(NH_4)_2SO_4$; 1 $CaCO_3$; 2 $MgSO_4*7H_2O$; and 0.5 KH_2PO_4, respectively. The pH values of the media were adjusted to 5.5. The combined effect of microelements a salt mixture and a control culture without supplementation was evaluated. Fermentations were conducted in a 250 mL Erlenmeyer flask using 100 mL of medium. Flasks were sealed with a cotton plug and sterilized at 121 °C, 15 psi, for 15 min. Subsequently, the cultures were inoculated with 10 mL of a mycelial suspension from activated cultures in a PDB medium with 10 days of growth. Submerged fermentations were incubated in a rotary shaker at 100 rpm for 12 days at room temperature (28±2 °C), with a photoperiod of 12-h light and 12-h darkness.

Conidia sampling, counting, and determining yield

To determine conidia production based on concentration, inoculum volume, and enrichment with microelements in solid fermentations, a sample of 1 g of colonized substrate was taken on days 3, 6, 9, and 12 for each of the treatments. The samples were dissolved in 10 mL of water sterilized with Tween 80 at 0.01% (v/v), and then, shaken at 100 rpm for 24 h. The conidia concentration for each treatment was determined using a hemocytometer. To evaluate how adding microelements affected conidiogenesis in liquid cultures, a sample of 1.5 mL was taken on days 3, 6, 9, and 12, and conidia were counted using a hemocytometer.

Yields generated in solid (Y_{px-S}), and liquid (Y_{px-L}) fermentation were determined based on the number of inoculated conidia for various inoculation strategies and adding microelements. They were calculated based on the total conidia produced per 200-g culture bag of rice, and the number of conidia produced per flask culture with 100 mL of PDB for each treatment about the control.

Statistical analysis

A completely randomized design (CRD) was used to determine the effect of inoculum concentration and volume on conidiogenesis. The factors were three levels of inoculum concentration ($1.0x10^5$, $1.0x10^6$, and $1.0x10^7$ conidia mL^{-1}), three levels of inoculum volume (10, 30, and 50 mL) and the effect of microelement addition on conidiogenesis in solid and liquid media block design with six levels ($CaCO_3$, KH_2PO_4, $MgSO_4*7H_2O$, $(NH_4)_2SO_4$), a mixture of all microelements, and a negative control without salts was used. Each experiment was carried out using three replicates and two repetitions in time. To conduct analyses, variables were initially described using descriptive statistics and later analyzed using parametric tests. In all cases, variables were expressed as the mean standard deviation. To determine the effects of the treatments on the variables, an analysis of variance (ANOVA) was conducted, followed by a Tukey test. Before any statistical analysis, Levene's tests were performed

to determine homogeneity, and the Shapiro-Wilk test was used to evaluate data normality. The value $P<0.05$ was used as a statistical criterion to reveal significant differences among treatments with 95% confidence. All data were analyzed using the IBS SPSS Statistics Version 25 statistical program.

RESULTS AND DISCUSSION

Evaluation of the volume and concentration of inoculum to produce conidia of *T. asperellum* strains in solid fermentation

The production kinetics of conidia for *T. asperellum* GRB-HA01and GRB-HA02 (Figure 1A and 1B) showed differences in conidia production responses resulting from the application of different volumes and concentrations of conidia in the inoculum. In this study, the highest conidia production and the best yields were achieved at 12 days when the cultures were inoculated with $1.0x10^7$ conidia mL^{-1} at a volume of 10 mL, both for *T. asperellum* GRB-HA01 ($6.9x10^9 \pm 5.7x10^2$ conidia g^{-1}, Y_{px-S} of 700 conidia per conidia inoculated) and for GRB-HA02 ($1.3x10^9 \pm 1.4x10^2$ conidia g^{-1}, Y_{px-S} of 130 conidia per conidia inoculated). The positive effect of a rise in initial conidia concentration in *Trichoderma* inoculum has been reported by authors like Coban and Sargin (2019). Their study evaluated the effect of conidia inoculum concentration for *Trichoderma harzianum* at concentrations of $1.0x10^4$, $1.0x10^5$, $1.0x10^6$, $1.0x10^7$, and $1.0x10^8$ UFC mL^{-1}, achieving a maximum concentration of $1.0x10^9$ CFU mL^{-1} at a $1.0x10^8$ UFC mL^{-1} inoculum concentration. However, conidia production values are similar compared with those found in this study for *T. asperellum* GRB-HA01 ($6.9x10^9 \pm 5.7x10^2$ conidia g^{-1}) and GRB-HA02 ($1.3x10^9 \pm 1.4x10^2$ conidia g^{-1}).

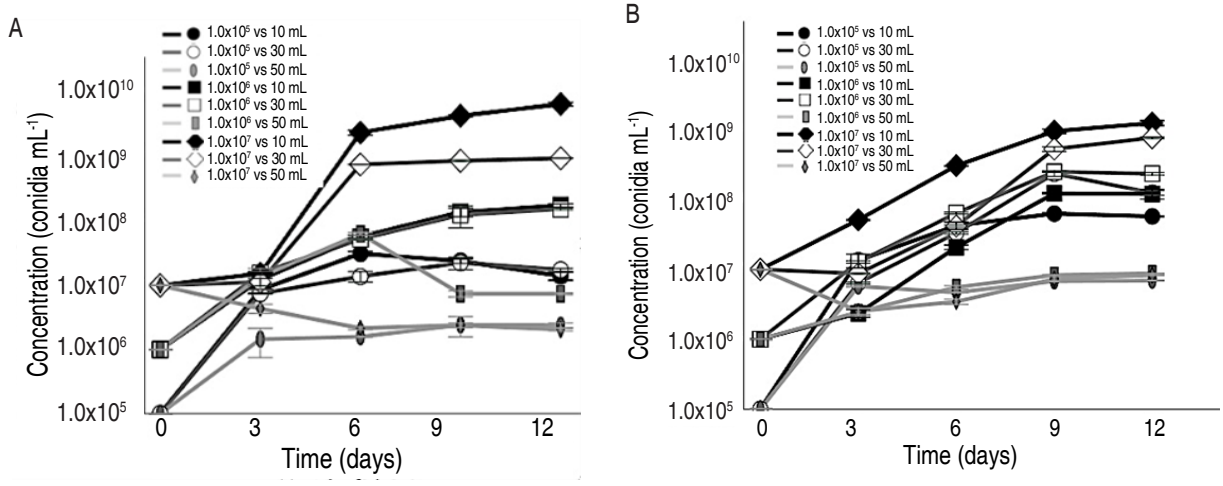

Figure 1. Conidia production kinetics with different inoculum volumes and concentrations of A. *T. asperellum* GRB-HA01, B. *T. asperellum* GRB-HA02.

When comparing the results obtained using concentrated inoculum $1.0x10^7$ conidia mL^{-1} using 10 mL with those obtained at concentrations of $1.0x10^6$ conidia mL^{-1} (GRB-HA01: $1.3x10^8 \pm 1.1x10^2$ conidia g^{-1}; Y_{px-S}: 130 conidia per conidia inoculated; GRB-HA02: $1.2x10^8 \pm 2.1x10^2$ conidia g^{-1}; Y_{px-S}: 120 conidia per conidia inoculated) and $1.0x10^5$ (GRB-HA01: $4.0x10^7 \pm 3.1x10^2$ conidia g^{-1}; Y_{px-S}: 400 conidia per conidia inoculated; GRB-HA02: $7.6x10^7 \pm 2.8x10^2$ conidia g^{-1}; Y_{px-S}: 760 conidia per conidia inoculated), it was observed that conidia production decreased 10 times for GRB-HA01 and GRB-HA02 when the concentration was $1.0x10^6$ conidia mL^{-1} and decreased 100 times for GRB-HA01 and GRB-HA02 for $1.0x10^5$ conidia mL^{-1}. Statistical data analyses of the inoculum concentration and volume for *T. asperellum* GRB-HA01 and GRB-HA02 showed that inoculum concentration significantly and positively affects conidia production ($P<0.05$). However, inoculum volume did not have any significant effect. When comparing the means of the inoculum concentration using a Tukey analysis, $1.0x10^7$ conidia mL^{-1} was the concentration that best promoted conidia

production compared to concentrations of $1.0x10^5$ and $1.0x10^6$ for each of the evaluated volumes. Results were similar to those reported by Alarcon and Utia (2020), who evaluated three doses of Tricho-D based on *Trichoderma harzianum* (0.2, 0.25, and 0.3 kg ha^{-1}) during field tests on potato crops. They observed that the highest dose (0.3 kg ha^{-1}) allowed greater substrate colonization; therefore, a higher presence of conidia, improving crop productivity and reducing the degree of severity generated by *Rizhoctonia solani* by 75% compared to a control that showed a 60% reduction.

When inoculum was increased to 30 mL for the highest conidia concentration ($1.0x10^7$ conidia mL^{-1}), conidia production decreased to $7.0x10^8 \pm 2.3x10^2$ conidia g^{-1} (GRB-HA01) at 6 days, and $8.0x10^8 \pm 2.7x10^2$ conidia g^{-1} (GRB-HA02) at 9 days, decreased the $Y_{px\text{-}S}$ to 10 conidia per conidia inoculated, which is 10 times lower. The highest inoculum volumes (50 mL), regardless of the initial conidia concentration, generated the lowest final conidia production, with values of $8.0x10^6$ ($1.0x10^5$ conidia mL^{-1}), $6.0x10^6$ ($1.0x10^6$ conidia mL^{-1}), and $2.0x10^6$ conidia g^{-1} ($1.0x10^7$ conidia mL^{-1}), and $Y_{px\text{-}S}$ of 80, 6 and 0.2 conidia per conidia inoculated for GRB-HA01 and values of $1.0x10^6$ ($1.0x10^5$ conidia mL^{-1}), $1.0x10^7$ ($1.0x10^6$ conidia mL^{-1}) and $2.0x10^7$ conidia g^{-1} ($1.0x10^7$ conidia mL^{-1}). However, reducing conidia concentration at high volumes improved the conidia production response in GRB-HA01. When comparing the poor results achieved by higher inoculum volumes and those generated at concentrations of $1.0x10^7$ conidia mL^{-1} and 10 mL, conidia production per gram of inoculated substrate was 100 times greater in the latter for *T. asperellum* GRB-HA01 and GRB-HA02. An analysis of the different volumes using a Tukey test demonstrated that the use of concentrated inoculum at volumes of 10 and 30 mL achieved the highest conidia production. In contrast, inoculum volumes of 50 mL least favored the conidiogenesis processes in both strains of *T. asperellum*. Studies such as Domingues et al. (2000) support the results rendered in this study, showing that inoculum size plays an important role in fungi morphology and is related to metabolite growth and production. This effect has also been reported by Chakravarthi et al. (2020) who show that high concentrations of conidia inoculum led to the formation of filamentous mycelium. The same effect was observed in this study when volumes of 50 mL were used showing an increase in the formation of filamentous mycelium by increasing inoculum concentration. Chakravarthi et al. (2020) show that small inoculums produce pellets that help dispersion in the substrate and improve growth, protein production, and conidiogenesis. This behavior is influenced by various endogenous signals and environmental factors, which affect fungi physiology, biochemistry, and development.

Effect of microelements on conidiogenesis of *T. asperellum* GRB-HA01 and GRB-HA02 in solid culture

There were important macroscopic differences in rice-grain color and coverage in *T. asperellum* GRB-HA01 and GRB-HA02 cultures after adding different microelements ($CaCO_3$, KH_2PO_4, $MgSO_4*7H_2O$, $(NH_4)_2SO_4$ and micronutrient mixture). Conidia counts generated in the culture indicated that $CaCO_3$ and KH_2PO_4 increased conidiogenesis in *T. asperellum* GRB-HA01. However, *T. asperellum* GRB-HA02 was not as sensitive to the presence of $MgSO_4*7H_2O$, $(NH_4)_2SO_4$, and all the salt mixtures, for which the results matched the control. In this study, $CaCO_3$ was the microelement that generated the greatest rice-grain coverage and promoted green tone generation for *T. asperellum* GRB-HA01 and *T. asperellum* GRB-HA02, which is a good conidiogenesis process indicator. Conidia production kinetics (Figure 2A and 2B) confirmed that $CaCO_3$ was the molecule that had the most positive impact on conidia production of *T. asperellum* GRB-HA01 ($3.0x10^{11} \pm 2.5x10^2$ conidia g^{-1} and a Y_{px_S}: 30,000 conidia per conidia inoculated), and *T. asperellum* GRB-HA02 ($8.0x10^{10} \pm 1.1x10^1$ conidia g^{-1} and a Y_{px_S}: 8,000 conidia per conidia inoculated). Although KH_2PO_4 also increased conidia production of *T. asperellum* GRB-HA01 ($1.2x10^9 \pm 1.9x10^2$ conidia g^{-1} and a Y_{px_S}: 120 conidia per conidia inoculated) and *T. asperellum* GRB-HA02 ($4.0x10^9 \pm 4.9x10^3$ conidia g^{-1} and a Y_{px_S}: 400 conidia per conidia inoculated), the conidiogenesis was 10 times better for *T. asperellum* GRB-HA01. Adding $CaCO_3$ to the culture media improved conidia production in GRB-HA01 ranging from $1.0x10^6$ to $1.0x10^2$ when it was compared with $(NH_4)_2SO_4$, $MgSO_4*7H_2O$, a microelement mixture, and negative control, for which production of $1.4x10^6$; $1.4x10^8$; $1.2x10^7$ and $1.0x10^8$ conidia g^{-1} and a Y_{px_S}: 0.1, 14, 1.2, and 10 conidia per conidia inoculated was achieved, respectively. In this study, statistical analysis demonstrated that the use of microelements in solid fermentation had a significant effect on conidiogenesis processes ($P<0.05$) for *T. asperellum* GRB-HA01 and GRB-HA02. Comparing the means of data using a Tukey test showed that $CaCO_3$

is a microelement that improves conidia production. It reduces the times in which *T. asperellum* GRB-HA01 and GRB-HA02 achieved complete substrate colonization. According to Martinez (2007), it has been possible to demonstrate that the presence of CO_2 is a determining physical–chemical factor that causes variation in the *Trichoderma* population. In addition, $CaCO_3$ is a cofactor that does not lead fungus metabolism to generate conidia. However, the presence of calcium ions produces an increase in osmotic pressure in fungal cells inducing sporulation (Krystofova et al. 1995). Šimkovič et al. (2008) showed this effect and they evaluated the effect of calcium ion (0-1.0 mol L^{-1}) concentration, observing an increase in conidiogenesis ($3.0x10^8$ conidia mL^{-1}) by adding 0.1 mol L^{-1} Ca^{2+} and represented another path to conidia *Trichoderma viride* formation.

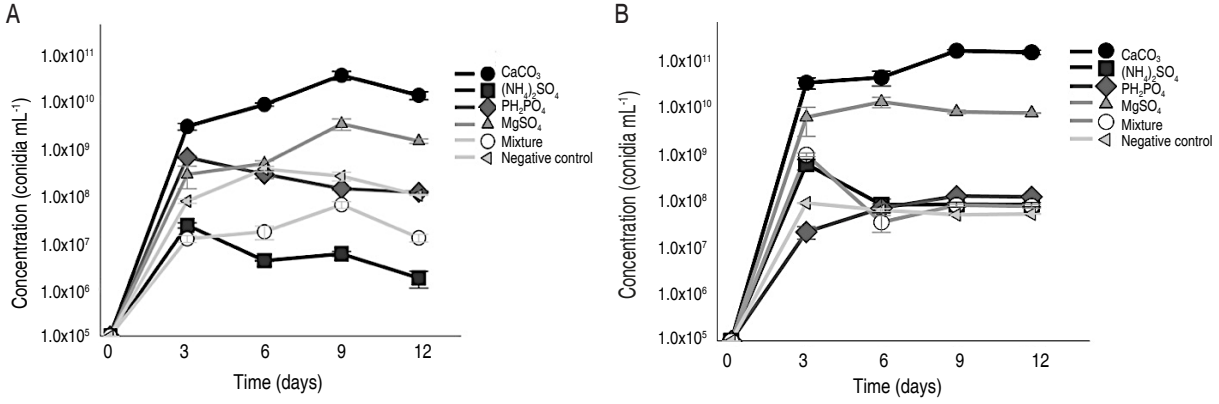

Figure 2. Conidia production kinetics with different microelements. A. *T. asperellum* GRB-HA01, B. *T. asperellum* GRB-HA02 in solid cultures.

Adding different microelements to the substrate during the kinetics of conidia production gave a maximum conidia production after 9 days of culture, with values for *T. asperellum* GRB-HA01 of $3.0x10^{11}$, $1.4x10^6$, $1.4x10^8$, $1.2x10^9$, $1.2x10^7$, and $1.0x10^8$ conidia g substrate^{-1} when $CaCO_3$, $(NH_4)_2SO_4$, $MgSO_4$*7H2O, KH_2PO_4, a mixture of all salts and negative control were added, respectively. Afterwards, values only varied by $3.3x10^{11}$, $3.0x10^6$, $2.4x10^8$, $3.5x10^9$, $1.9x10^7$, and $1.4x10^8$ conidia g substrate^{-1} when the exponent was not changed. A similar behavior was observed for *T. asperellum* GRB-HA02 obtaining values of $8.0x10^{10}$, $5.9x10^7$, $1.3x10^7$, $4.0x10^9$, $2.5x10^7$, and $2.4x10^7$ conidia g substrate^{-1} when $CaCO_3$, $(NH_4)_2SO_4$, $MgSO_4$*$7H_2O$, KH_2PO_4, a mixture of all salts and a negative control were added, respectively, and their values only varied by $8.7x10^{10}$, $6.9x10^7$, $1.2x10^7$, $4.5x10^9$, $2.6x10^7$, and $2.8x10^7$ conidia g substrate^{-1} without changes in the exponent. The cultivation time in which the maximum coverage of the rice grains was reached was 9 days, which is ideally the moment to start the drying process favoring reduced production times. Microelement evaluation for *T. asperellum* GRB-HA01 suggests that adding a salt mixture s and $(NH_4)_2SO_4$ (Figure 2A and 2B) reduced the conidiogenesis process to values of $1.2x10^7$±$1.6x10^2$ and $1.4x10^6$±$1.2x10^2$ conidia g^{-1} with Y_{px_S}: 1.2 and 0.1 conidia per conidia inoculated, respectively. These two treatments rendered the lowest values when compared with other treatments, including a negative control where values of $1.0x10^8$±$1.5x10^2$ conidia g^{-1} Y_{px_S}: 10 conidia per conidia inoculated (substrate without microelements) were achieved. While the lowest values of conidia production using *T. asperellum* GRB-HA02 were observed in treatments with $MgSO_4$*$7H_2O$, $(NH_4)_2SO_4$, and salt mixtures, achieving similar values to those obtained in the control ($2.4x10^7$±$1.0x10^2$ conidia g^{-1}; Y_{px_S}: 2.4 conidia per conidia inoculated). On a macroscopic scale, metabolism was directed towards biomass production. According to Šimkovič et al. (2008), the presence of Mg^{2+} ions inhibit conidiogenesis in fungi because they decrease osmotic pressure in cells. The macroscopic scale shows that adding $(NH_4)_2SO_4$ does not help sporulation processes. It is possible that ammonium (NH_4) is used as a source of nitrogen and redirects cell metabolism toward biomass generation. Finally, the mixture of all microelements did not allow GRB-HA01 and GRB-HA02 to achieve good growth and sporulation, possibly due to the generation of an osmotic imbalance in

cells. Studies such as Adnan et al. (2019) and the results obtained in this study show that conidial responses vary greatly among species because each species has specific nutritional requirements that define metabolism and growth. However, there are metabolic adaptations specific to each species's environment that change conidia production.

Effect of adding microelements on conidiogenesis in liquid culture

Large differences were found in fungi conidia production when different microelements were added to the culture. *T. asperellum* GRB-HA02 was the microorganism that showed the highest conidia production in media containing the salt mixture of ($3.1x10^9$±$2.8x10^3$ conidia mL^{-1}) (Figure 3B), with yields of 310 conidia per conidia inoculated. In contrast, GRB-HA01 treatments that used microelement mixtures presented lower values ($1.2x10^8$±$9.4x10^3$ conidia mL^{-1}), with a Y_{px-L} of 12 conidia per conidia inoculated. $CaCO_3$ ($3.0x10^7$±$1.1x10^2$ conidia mL^{-1}) was the only treatment that was below the negative control ($4.3x10^7$±$6.9x10^7$ conidia mL^{-1}), with a Y_{px-L} of 3.0 conidia per conidia inoculated. The maximum conidia production for *T. asperellum* GRB-HA01 (Figure 3A) was achieved by adding KH_2PO_4 ($1.3x10^9$±$2.8x10^2$ conidia mL^{-1}), giving a Y_{px-L} of 130 conidia per conidia inoculated.

In general, when the salts weren't appropriate or when they were not added to the culture media, conidia production of the *T. asperellum* GRB-HA01 and GRB-HA02 strains decreased by 80-98% achieving Y_{px-L} of 0.7–48 conidia per conidia inoculated. For other treatments, using GRB-HA01, a maximum conidia concentration (conidia mL^{-1}) achieved values of $1.9x10^8$±$8.9x10^3$ ($(NH_4)_2SO_4$), $2.6x10^8$±$8.8x10^4$ ($MgSO_4$), $3.0x10^7$±$1.1x10^2$ ($CaCO_3$), and $1.2x10^8$±$9.4x10^3$ (mixture of all microelements) with Y_{p-L}: 19, 26, 3 and 12 conidia per conidia inoculated, respectively. In *T. asperellum* GRB-HA02 cultures, the effect of reducing conidia production was higher and the maximum concentration had values of (conidia mL^{-1}) $9.6x10^7$±$2.7x10^3$ (KH_2PO_4), $3.3x10^8$±$8.4x10^4$ ($(NH_4)_2SO_4$), $4.8x10^8$±$4.7x10^2$ ($MgSO_4$), $6.6x10^8$±$1.4x10^2$ ($CaCO_3$), and $1.3x10^7$±$8.4x10^4$ (control) with Y_{px-L}: 9.6, 33, 48, 66, and 1 conidia per conidia inoculated, respectively.

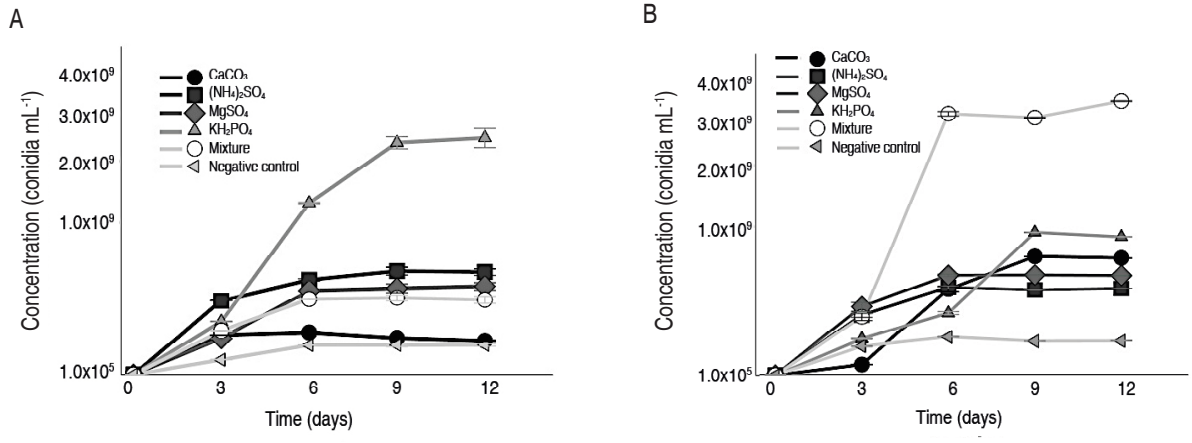

Figure 3. Conidia production kinetics in *T. asperellum.* A. GRB-HA01 and B. GRB-HA02 in liquid fermentation.

In this study, the highest conidia production in *T. asperellum* GRB-HA01 and GRB-HA02 strains was achieved in 6 days, after production was kept constant or reduced. For instance, with GRB-HA01, media supplemented with NH_4SO_4 changed from $4.8x10^8$ to $1.8x10^8$ conidia mL^{-1}, and in cultures enriched with microelement mixture, concentration decreased from $2.6x10^8$ to $1.2x10^8$ conidia mL^{-1}.

Statistical data analyses showed that microelements significantly affected conidia production (P<0.05), achieving maximum production after 6 days of culture. However, Tukey's analysis demonstrated that KH_2PO_4 had a significant positive effect on conidia production for *T. asperellum* GRB-HA01 (P<0.05), achieving values of $1.3x10^9$±$2.8x10^2$ conidia mL^{-1}, while for *T. asperellum* GRB-HA02 the mixture of all treatments favored this conidia production process (P<0.05), with values of $3.1x10^9$±$2.8x10^3$ conidia mL^{-1}. These results were similar to those Gonzalez and Vicente (2016) obtained. They evaluated different nitrogen sources and achieved maximum conidia

production ($1.16x10^9$ conidia mL^{-1}) using 5 g of KH_2PO_4 and 1.3 g of $MgSO_4*7H_2O$, and the lowest production was using $(NH_4)NO_3$, and $(NO_4)*2SO_4$ at concentrations in the order of $1.0x10^7$ and $1.0x10^8$ conidia mL^{-1}, respectively. This is similar to the results achieved in this study for *T. asperellum* GRB-HA01 using $(NH_4)_2SO_4$ ($1.9x10^8 \pm 8.9x10^3$ conidia mL^{-1}), $MgSO_4$ ($2.6x10^8 \pm 8.8x10^4$ conidia mL^{-1}), $CaCO_3$ ($3.0x10^7 \pm 1.1\ x10^2$ conidia mL^{-1}), and the mixture with all microelements ($1.2x10^8 \pm 9.4x10^3$ conidia mL^{-1}), and for *T. asperellum* GRB-HA02 with the addition of KH_2PO_4 ($9.6x10^7 \pm 2.7x10^3$ conidia mL^{-1}), $(NH_4)_2SO_4$ ($1.4x10^8 \pm 5.1x10^2$ conidia mL^{-1}) and $MgSO_4$ ($4.8x10^8 \pm 4.7x10^2$ conidia mL^{-1}).

When comparing the results of fermentation in liquid state and solid media, important differences were observed in conidia production for *T. asperellum* GRB-HA01 and GRB-HA02. The addition of micronutrients improved conidia production, reaching the highest values at 9 days of culture in solid fermentations in media enriched with $CaCO_3$ (GRB-HA01: $3.0x10^{11} \pm 2.5x10^2$ conidia g^{-1}; GRB-HA02: $8.0x10^{10} \pm 1.1x10^1$ conidia g^{-1}). In contrast, conidia production in liquid fermentations generated a reduction in conidia concentration (GRB-HA01: $3.0x10^7 \pm 1.1x10^2$ conidia mL^{-1}; GRB-HA02: $6.6x10^8 \pm 1.4x10^2$ conidia mL^{-1}), with values similar to the control. The difference in conidia concentrations in solid and liquid fermentations allowed an increase of 10,000 times more for GRB-HA01 and 100 times for GRB-HA02 in a solid medium. A different response was evidenced at 6 days of culture during conidia production in liquid fermentations. Hence, the highest conidia concentration in GRB-HA01 was reached with the media supplemented with KH_2PO_4 ($1.3x10^9 \pm 2.8x10^2$ conidia mL^{-1}), while for GRB-HA02 it was achieved in the media containing micronutrient mixture ($3.1x10^9 \pm 2.8x10^2$ conidia mL^{-1}). However, the study showed values were lower than those in solid fermentations. Hölker et al. (2004) have also observed this effect, and they report that conidia obtained in solid fermentations are more resistant to desiccation and more stable in a dry state. In contrast, the germination capacity of spores obtained in submerged cultures decreases rapidly. In addition, in solid-state fermentations, there is less catabolic repression, less demand for water, and greater conidia productivity.

The results of this study indicate that *Trichoderma* sporulation processes are the result of biotic and abiotic factors. It is important to carry out a similar evaluation for all *Trichoderma* species to enhance conidia production. Monga (2001) and Adnan et al. (2019) observed that the production of chlamydospores, hyphae, and conidia or biomass production of *Trichoderma* species are less dependent on exogenous media and that generating conidia from a wide range of *Trichoderma* species like *T. harzianum*, *T. viride*, *T. koningii*, *T. saturnisporum* and *T. polysporum* has rendered that it depends on environmental acidity and nutrient-deficient systems.

CONCLUSIONS

This study highlights the importance of inoculation as a method to evaluate the conidiogenesis processes of *T. asperellum* GRB-HA01 and GRB-HA02. Results demonstrate that higher conidia production in *T. asperellum* can be achieved by inoculating cultures with $1.0x10^7$ conidia mL^{-1} and a volume of 10 mL. Adding $CaCO_3$ to a culture medium in solid fermentations also led to the highest conidia production for both strains, while adding $(NH_4)_2SO_4$ and a salt mixture had a negative effect on conidiogenesis. Under liquid culture conditions, the highest conidia production was observed in *T. asperellum* GRB-HA02 after six days of culture in media containing a mixture of all salts, while *T. asperellum* GRB-HA01 produced the highest conidia production after nine days of culture adding KH_2PO_4. Nonetheless, conidia production in liquid culture was less than that of conidia production obtained in solid fermentations for both strains. Overall, this study highlights the importance of optimizing inoculation and microelement addition in both solid and liquid fermentation to enhance conidiogenesis in *Trichoderma asperellum*, which could have significant implications for the development of more efficient sustainable processes to produce bioactive compounds and biocontrol agents.

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HS-SPME GC/MS Volatile profile of the onion *Allium fistulosum* L. variety Pereirana, cultivated in Colombia

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Perfil volátil HS-SPME GC/MS de la cebolla *Allium fistulosum* L. variedad Pereirana, cultivada en Colombia

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ABSTRACT

The study presents a characterization of the volatile organic compounds found in both raw and the essential oil of the "Pereirana Onion," an endemic Colombian Welsh onion variety for which the composition has not been previously reported. The analysis was conducted using four distinct fibers through the HS-SPME/GC-MS method. The results revealed that chopped Pereirana onions release as many as 29 different compounds, with concentrations up to 20 times higher than those observed in other evaluated onion species (Biónica, Veleña, and Veleña Sonsón). Most of these compounds are sulfur-based, including dipropyl disulfide, (E)-1-(Prop-1-en-1-yl)-2-propyldisulfane, disulfide, methyl 1-(methylthio) propyl, dipropyl trisulphide, and (E)-1-(Prop-1-en-1-yl)-3-propyltrisulfane. Furthermore, steam extraction of essential oils from Pereirana onions led to the identification of up to 70 different compounds. Simple correspondence analysis (SCA) revealed that Veleña and Veleña Sonsón onion species share common compounds but significantly differ from Biónica and Pereirana varieties cultivated in Risaralda, Colombia. These findings suggest potential applications in the pharmaceutical, agricultural, and food industries, paving the way for future research and industrial utilization.

Keywords:
GC-MS
HS-SPME
VOCs
Welsh onion

RESUMEN

El estudio presenta una caracterización de los compuestos orgánicos volátiles encontrados en la "Cebolla Pereirana", una variedad endémica de cebolla de rama en Colombia para la cual no se había informado previamente sobre su composición. El análisis se realizó utilizando cuatro diferentes fibras por la técnica HS-SPME/GC-MS. Los resultados revelaron que las cebollas Pereirana picadas liberan hasta 29 compuestos diferentes, con concentraciones hasta 20 veces más altas que las observadas en otras especies de cebolla evaluadas (Biónica, Veleña y Veleña Sonsón). La mayoría de estos compuestos son de origen organosulfurado, incluyendo el disulfuro de dipropilo, (E)-1-(Prop-1-en-1-il)-2-propildisulfano, disulfuro, metil 1-(metiltio) propilo, trisulfuro de dipropilo y (E)-1-(Prop-1-en-1-il)-3-propiltrisulfano. Además, la extracción de aceites esenciales mediante arrastre por vapor de las cebollas Pereirana permitió identificar hasta 70 compuestos diferentes. El análisis de correspondencia simple (SCA) reveló que las especies de cebolla Veleña y Veleña Sonsón comparten compuestos comunes, pero difieren significativamente de las variedades Biónica y Pereirana cultivadas en Risaralda, Colombia. Estos hallazgos sugieren aplicaciones potenciales en las industrias farmacéutica, agrícola y alimentaria, allanando el camino para futuras investigaciones y utilización industrial.

Palabras clave:
CG- EM
MEFS
COVs
Cebolla de rama

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Global onion production in 2022 was 93.23 million tons (MT), being China (23.91 MT), India (19.42 MT), Egypt (3.12 MT), USA (3.03 MT), and Iran (2.35 MT) as the top five producers (Kumar et al. 2022). In Colombia, Welsh onion (*Allium fistulosum*) has been grown since 1950 and is grown from 1,500 to 3,000 meters above sea level (masl), between 12 and 20 °C. According to the Colombian statistics department, in 2019, in Colombia, 16,922 ha of Welsh onion were harvested, with a production of 414,554 tons (DANE 2019).

The Department of Risaralda is the third largest producer of onions in Colombia, with 50,000 tons per year, cultivated since 1960 (Jaramillo and Vallejo 2014). Given the environmental conditions that did not favor cultivation, the plant adapted to the environment, giving rise to a unique material, widely accepted by consumers, called 'Cebolla Pereirana'. This plant was morpho-agronomically characterized by exhibiting thin, dark, and violet pseudo-stems, dark green leaves, intermediate foliage, high tillering, and abundant serosity in leaves, as well as by being resistant to breaking and a cause of strong irritation of the eyes when the leaves are squeezed (Polanco and Pérez 2018).

Fresh onions are a cornerstone of global cuisine due to their distinctive flavor, which is derived from sulfur compounds found in the volatile fraction, commonly referred to as onion oil. This oil is obtained via steam distillation of crushed onions, yielding a complex mixture predominantly composed of mono, di, tri, and tetrasulfides with various alkyl groups (Hosoda et al. 2003). As a result, onion oil has been traditionally and extensively utilized in the food industry to augment the flavors of processed foods (including soups, meats, and ready-to-eat meals), sauces, salad dressings, and dips. This method effectively bypasses the difficulties associated with managing large volumes of fresh produce (Lawless 2012). Given the widespread use of both fresh onions and onion essential oil, this study incorporates both materials.

The genus *Allium* has a wide variety of sulfur compounds responsible for taste and smell, with marked differences in volatile organic compounds (VOCs) in the various onion species. The compound responsible for generating this variety of compounds is S-alk(en)yl-L-cysteine sulfoxides (ACSOs), thanks to the enzymatic activity of allinase. This pattern of sulfur compounds is common to most *Allium* species. The VOCs profile is concentrated, and the result is a mixture of sulfur-containing compounds including thiosulfinates, mono-, di- and tri-sulfides as well as specific compounds such as the lachrymatory or tear factor, thiopropanal S-oxide (Colina-Coca et al. 2013).

The sulfur compounds are extracted using solvent-free liquid-liquid extraction, hydro distillation, or headspace solid-phase microextraction (HS-SPME). The extraction process can be regarded as a dynamic equilibrium involving the target compounds evaporating into the headspace and their subsequent adsorption onto and desorption from the fiber. The process depends partly on the chemistry and physical dimensions of the fiber coating (exposed area and film thickness), in addition to the aerodynamics inside the headspace (Peng et al. 2020).

The HS-SPME/GC-MS technique used to analyze VOCs is performed with small sample amounts, and avoids the use of solvents, promoting sustainable chemistry and detecting a wide variety of compounds simultaneously (Licen et al. 2021). The SPME is based on the equilibrium of partition of compounds of interest, between the matrix and the extraction phase that covers the surface of the fused silica fiber. This fiber should be selected according to the selectivity of the compounds to be assessed, adapting the time of exposure, equilibrium temperature, and dynamic or static mode, which is decisive in obtaining adequate detection limits.

The efficiency of this technique depends on the polarity of the fiber, and polydimethylsiloxane (PDMS) coated fibers have been satisfactory in most cases. Previously, the VOCs of fresh leaves of *Allium fistulosum* and *Allium tuberosum* were studied with HS-SPME/GC-MS and PDMS fibers, finding dipropyl disulfide (67%), 1-propenylpropyl disulfide (23%), and dipropyl trisulphide (6%) (Hori 2007). The genus *Allium* has also been studied with CAR/PDMS fibers, detecting characteristic sulfides such as propyl mercaptan, dimethyl disulfide, 2,5-dimethylthiophene, and allyl propyl disulfide (Zhang and Li 2007).

The present study contributes to the knowledge of the endemic material 'Cebolla Pereirana', which has its production system and is a product highly appreciated by consumers for its attractive spicy flavor and intense smell

for culinary applications. However, there is no evidence of studies reporting the volatile compounds present in this onion variety. This is the first study assessing VOCs using HS-SPME/GC-MS, comparing four fibers and four onion varieties (Pereirana, Biónica, Veleña, and Veleña Sonsón) grown in Pereira, Department of Risaralda, Colombia.

MATERIALS AND METHODS

Onion samples

The samples were taken in Pereira, Colombia, during the second semester of 2021, at Castilla and Carmela farms, both at coordinates 4°45'21.4"N 75°36'32.0" W (4.7559450, -75.6088910). The sampling was performed randomly in a zig-zag route, taken between four and six shoots (stems, leaves, and roots) per hectare (4 ha) (Silva and Uchida 2000), of each of the four Welsh onion varieties, namely: (a) Pereirana; (b) Veleña; (c) Veleña Sonsón; and (d) Biónica.

Volatile organic compound analysis

Fresh sample preparation

The samples were transported at 4 °C and stored in a refrigerator at the same temperature. The assay was conducted three times. The leaves and stems of each variety weighing 1 g were finely cut with a knife and stored in a hermetically-sealed SPME vial (D'Auria and Racioppi 2017). HS-SPME-GC-MS Analysis of onion (*Allium* cepa L.) and shallot (*Allium ascalonicum* L.). Food Res, 1(5), 161-165. For each sample, the HS-SPME analysis was performed at a temperature of 45 °C with a time of 5 min of homogenization and 15 min of exposure to the fibers divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), polyacrylate, polydimethylsiloxane-divinylbenzene (PDMS/DVB), and polydimethylsiloxane (PDMS).

Extraction of Essential Oil from the Pereirana Variety Onion

The leaves and stems were chopped and taken to the steam-dragging equipment. The assay was conducted three times (Boutekedjiret et al. 2003; Peredo et al. 2009) with a sample ratio of solvent 1:1. The distillate was re-extracted with ethyl acetate and concentrated in a rotary evaporator. The solvent was evaporated in a rotary evaporator at 40 °C at 240 mbar. Subsequently, 3 µL of the essential oil were diluted (1:1,000) with distilled water, stirring at 6,000 rpm, and brought into equilibrium for 20 min at 40 °C (Peredo et al. 2009). Then, the SPME fiber was exposed in the headspace for 30 min at 40 °C (triplicated analysis) (Soto et al. 2015). The qualitative tentative analysis was performed by comparison with the mass spectra of the NIST14 and NIST14s libraries. The quantification method was carried out by integrating the area under the curve, represented as a percentage of the total area (% Area).

Analysis of the fresh sample and essential oil

Instrumentation

The SPME fibers used were polydimethylsiloxane (PDMS, 100 µm), polyacrylate (85 µm), polydimethylsiloxane-divinylbenzene PDMS/DVB 65, divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS 50/30 µm) from Supelco® Bulletin 925F-SPME (Sigma-aldrich 2009). The fibers were conditioned according to the manufacturer's instructions. GC-MS analyses were performed on Shimadzu GC2010 Plus + QP2020 equipment, with an SH-Rxi-5Sil MS column (30 m x 0.25 mm x 0.25 µm).

Compounds were desorbed through the injection port with helium as carrier gas (Grade 5.0), at a linear velocity of 35.0 cm s^{-1}. The run starts with a temperature of 40 °C (2 min), increasing 3 °C min^{-1}, up to 90 °C (3 min), and then increasing 3 °C min^{-1}, up to 150 °C (5 min), and finally, there is a heating rate of 10 °C min^{-1}, up to 280 °C (1 min) for a total run time of 60.67 min. Mass spectra were obtained by electron impact (EI) at 70 eV. The temperatures of the ionization source and the transfer line were 220 and 250 °C, respectively. The qualitative tentative analysis was performed by comparison with the mass spectra of the NIST14 and NIST14s libraries. The quantification method was carried out by integrating the area under the curve, represented as a percentage of the total area (% Area).

Statistical analysis

A descriptive analysis of the variables 'type of onion' and 'type of compound' was performed using the IBM® SPSS® Statistics 25 software, both on a nominal scale. The qualitative data was presented through a contingency table, indicating the attributes for each variable, type of onion (Biónica, Pereirana, Veleña, and Veleña Sonsón), and type of compound (present in the chromatographic analysis).

A simple correspondence analysis was performed to jointly study these two qualitative variables. It was considered appropriate for dealing with contingency tables in which

the existing cases are represented in the categories of each of the qualitative variables under study, i.e., type of onion and type of compound. The association test used was the chi-square test of independence (χ^2), which provided information on the relationship that could exist between the two variables (López 2004).

RESULTS AND ANALYSIS

Prior studies have similarly characterized volatile organic compounds in onions, utilizing multivariate statistical techniques, including cluster analysis, principal component analysis, and discriminant analysis, to identify compound groups associated with specific onion varieties (Taglienti et al. 2021; Cozzolino et al. 2021). Volatile organic compounds in onions are responsible for their distinct sensory attributes and global culinary popularity. However, these compounds vary with onion variety, underscoring the importance of their comprehensive characterization (Bello et al. 2013).

This study aimed to characterize four onion varieties in Pereira, Colombia, by employing chromatographic analysis to detect volatile compounds and conducting statistical analyses to correlate compound types with onion varieties.

Analysis of fibers in fresh onion variety Pereirana

Using DVB/CAR/PDMS, PDMS, PDMS/DVB, and polyacrylate fibers, the Pereirana variety was assessed to determine which fiber was the most optimal for the analysis. In the process, it was possible to observe 23 compounds according to their mass spectra of the NIST14 and NIST14s libraries. The VOCs profile determined by the four fibers can be observed in Table 1.

Table 1. Compounds extracted from the Pereirana variety onion through the four fibers evaluated.

Code	Compounds	Reference ions	$t_{R\ (min)}$
	Monosulphide		
F1	Propyl mercaptan	76, 47	2.19
	Disulfides		
F2	Dipropyl disulfide	43, 150	18.53
F3	Disulfide methyl propyl	60, 122	10.19
F4	(E)-1-(Prop-1-en-1-yl)-2-propyldisulfane	41, 148	18.92
F5	Disulfide methyl 1-(1-propenylthio) propyl	115, 81	42.13
F6	1-Methyl-2-(1-(propylthio) propyl) disulfane	117, 75	41.53
F7	Diisopropyl disulfide	43, 150	19.05
F8	1-Allyl-2-isopropyldisulfane	41, 43	17.82
	Trisulphides		
F9	Trisulfide dipropyl	43, 75	30.76
F10	(E)-1-(Prop-1-en-1-yl)-3-propyltrisulfane	41, 148	18.90
F11	(Z)-1-(Prop-1-en-1-yl)-3-propyltrisulfane	74, 41	31.09
F12	1-Allyl-3-propyltrisulfane	73, 116	31.25
	Tetrasulphide		
F13	6-Ethyl-4,5,7,8-tetrathianonane	73, 149	50.46
	Other sulphurised compounds		
F14	2,4-Dimethyl-5,6-dithia-2,7-nonadienal	69, 129	50.04
F15	Ethanethioic acid, S-propyl ester	43, 74	7.71
	Other		
F16	Cyclopentaneacetic acid, 3-oxo-2-pentyl-, methyl ester	83, 83	45.43
F17	7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin	243, 213	51.62
F18	Brassilato de etileno	55, 98	54.25
F19	Tetracosa-2,6,10,14,18-pentaen-22-ol, 2,6,10,15,19,23-hexamethyl-23-methoxy-, alltran	73, 69	56.30
F20	1,3-Benzodioxole, 5-propyl	135, 77	41.34
F21	Diethyl Phthalate	149, 177	42.35
F22	Ethylene brassylate	83, 82	45.33
F23	1,4-Benzenediol, 2-[(1,4,4a,5,6,7,8,8a-octahydro-2,5,5,8a-tetramethyl-1-naphthalenyl)methyl]-, [1R-(1.alpha.,4a.beta.,8a.alpha	191, 119	45.67

Among the 23 compounds observed, 65.22% had the characteristic of being sulfurous, namely: F1 (1 monosulphide); F2 to F8 (7 disulfides); F9 to F12 (4 trisulphides); F13 (1 tetrasulphide); and F13 to F14 (other sulphurised compounds). The other eight compounds belonged to aromatic groups or esters. Disulphides and trisulphides were the most observed compounds in the four fibers with retention times of less than 30 min.

The observed compounds were grouped in Table 2, where it is determined that the most indicated fiber to perform the analysis of the various varieties of Welsh onion was PDMS, since it had the greatest affinity with most of the compounds present in the onion variety Pereirana, observing 14 mostly sulfurous compounds. On the other hand, with DVB/CAR/PDMS, PDMS/DVB, and polyacrylate fibers, it was possible to observe nine, five, and eight compounds, respectively.

The compounds observed in most fibers were propyl mercaptan, dipropyl disulfide, methyl propyl disulfide, and (E)-1-(Prop-1-en-1-yl)-2-propyldisulfane (Figure 1).

Table 2. Compounds extracted from the Pereirana Variety Welsh Onion using the four evaluated fibers.

Fibers	Compounds																						
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16	F17	F18	F19	F20	F21	F22	F23
DVB/CAR/PDMS	■	■	■	■									■	■		■	■	■					
PDMS	■	■	■	■	■	■	■	■	■	■	■	■		■	■								
PDMS/DVB	■	■	■	■															■				
Polyacrylate		■		■												■			■	■	■	■	■

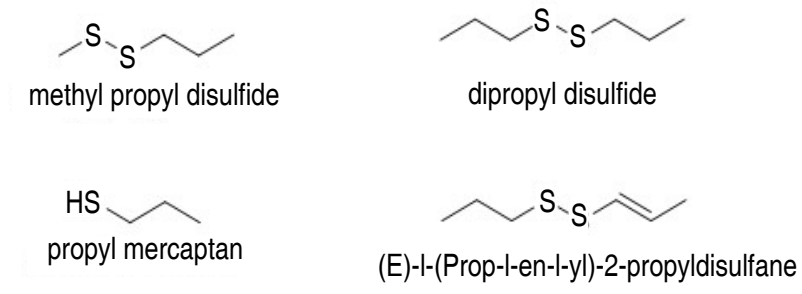

Figure 1. Main compounds observed in the four fibers.

Table 2 shows that compounds F2 (Dipropyl disulfide) and F4 ([E]-1-[Prop-1-en-1-yl]-2-propyldisulfane) were the only ones found in the four assessed fibers. Both are aliphatic sulfur compounds with a very similar molar mass. These compounds, together with F1 (Propyl mercaptan) and F6 (1-Methyl-2-[1-(propylthio)propyl]disulfane) were detected in a study of *Allium ascolanicum* L. as the most abundant (Indrasari et al. 2021).

PDMS fiber is preferred for the extraction of non-polar analytes, whereas, on the other hand, polyacrylate fiber is preferred for more polar compounds. PDMS/DVB or CAR/DVB fibers can be used for the extraction of polar VOCs with low molecular weight and, in addition, for obtaining increased retention capacity. Although the stationary phase is fundamental, factors such as the concentration of the analytes in the sample, temperature, pH, agitation time, and addition of salts should also be considered (Garcia-Esteban et al. 2004).

Analysis of the extraction results with PDMS fiber in the four varieties of Welsh onion

The four varieties of Welsh onion were analyzed by the PDMS fiber. The main components observed, according

to their mass spectra from the NIST14 and NIST14s libraries, are grouped in Table 3, in which 29 compounds were found in the PDMS fiber, mostly sulfurous.

It is possible to observe one monosulphide, nine disulfides, five trisulphides, one tetrasulphide, three sulfur compounds, and 10 different compounds such as ketones or ester derivatives. Disulphides and trisulphides were the most abundant. A descriptive analysis of the bar graph (Figure 2) allowed observation that the onion variety Biónica had the highest number of VOCs, followed by Pereirana and Veleña Sonsón. The variety Veleña had the lowest VOC content.

Table 3. VOCs observed in the PDMS fiber in the different varieties assessed by GCMS HS-SPME.

Code	Compounds	$t_{R\ (min)}$	Compound Area (%)
	Monosulphide		
C18	Propyl mercaptan	2.19	6.61
	Disulfides		
C1	Dipropyl disulfide	19.05	63.56
C2	(E)-1-(Prop-1-en-1-yl)-2-propyldisulfane	19.19	6.07
C3	Disulfide, methyl 1-(methylthio)propyl	24.31	0.56
C7	1-Methyl-2-(1-(propylthio)propyl)disulfane	41.53	0.15
C8	Disulfuro, metil 1-(1-propeniltio)propil	42.13	1.23
C19	Disulfide methyl propyl	10.18	0.90
C21	1-(1-Propenyl)-2-(4-thiohept-5-yl)disulfide	41.52	0.15
C20	Diisopropyl disulfide	19.05	1.95
C25	1-Allyl-2-isopropyldisulfane	17.90	0.34
	Trisulphides		
C4	Trisulfide dipropyl	30.91	12.13
C5	(E)-1-(Prop-1-en-1-yl)-3-propyltrisulfane	31.47	0.61
C26	(Z)-1-(Prop-1-en-1-yl)-3-propyltrisulfane	31.09	0.13
C27	1-Allyl-3-propyltrisulfane	31.25	0.23
C28	1,2,4-Trithiolane, 3,5-diethyl-	31.47	0.61
	Tetrasulphide		
C11	6-Ethyl-4,5,7,8-tetrathianonane	40.46	0.47
	Other sulphurised compounds		
C10	2,4-Dimethyl-5,6-dithia-2,7-nonadienal	50.04	0.91
C17	2,3-Bis(ethylthio)hexane	23.24	0.42
C29	Ethanethioic acid S-propyl ester	7.71	0.34
	Other		
C9	3(2H)-Furanone, 5-methyl-2-octyl-	45.20	0.37
C6	2-Tridecanone	38.67	1.21
C12	Morpholine, 4-octadecyl	50.57	0.12
C13	1-Heptadecanamine, N, N-dimethyl-	51.52	0.09
C14	1,6,10-Dodecatriene-3-carboxylic acid, methyl ester	54.70	0.21
C15	9-Octadecenoic acid, methyl ester, (E)-	54.80	0.18
C16	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	55.14	0.15
C22	3(2H)-Furanone, 2-hexyl-5-methyl-	45.21	0.05
C23	Squalene	56.25	0.17
C24	Heneicosane	58.11	0.08

Compounds such as dipropyl disulfide (E)-1-(Prop-1-en-1-il)-2-propyldisulfane, methyl-1-(1-propenylthio) propyl disulfide, dipropyl trisulphide, and (E)-1-(Prop-1-en-1-il)-3-propyltrisulfan were present in the four varieties, with an area percentage for the Pereirana onion of 65.67, 6.28, 1.08, 12.53, and 0.63%, respectively. This result is in line with those corresponding to a previous study (Bastaki et al. 2021), according to which the active compounds of the genus were

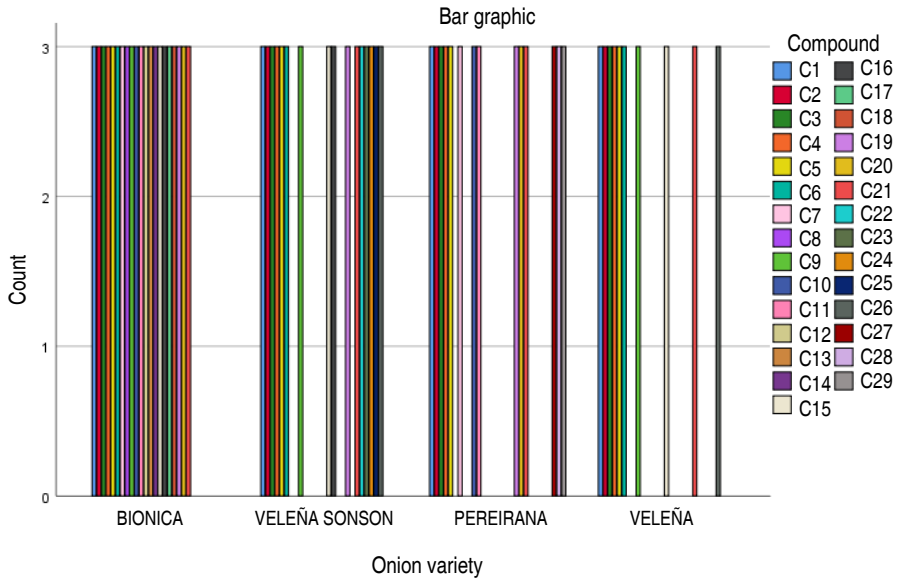

Figure 2. Bar graph of the 29 compounds obtained from the four varieties of Welsh onion.

Allium disulfide of diallyl disulfide, diallyl trisulphide, diallyl sulfide, dipropyl disulfide, dipropyl trisulphide, 1-propenyl propyl disulfide, allyl methyl disulfide, and dimethyl disulfide that could have antioxidant and antimicrobial properties.

The Pereirana onion was differentiated by containing 1-Allyl-3-propyltrisulfane, (Z)-1-(Prop-1-en-1-yl)-3-propyltrisulfane, 1-Allyl-2-isopropyldisulfane, ethanethioic acid S-propyl ester, and organo-sulfur compounds, whereas the other varieties differed in having ketones and ester derivatives. Figure 3 presents a chromatogram for the PDMS fiber, whose most abundant peak with t_R of 19.05 min corresponded to dipropyl disulfide, which in the Pereirana onion had 20 times the relative abundance concerning the other varieties. This way, it was necessary to dilute the extract since it saturated the mass detector. The compound had reference ions of 43 and 150 atomic mass units (mass spectrum illustrated in Figure 3).

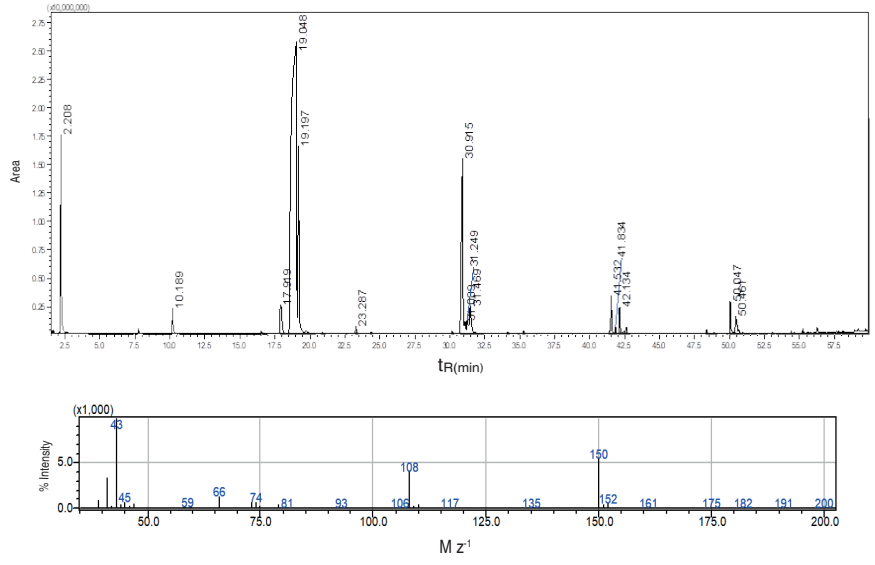

Figure 3. PDMS chromatogram of the Pereirana onion and the respective dipropyl disulfide ions (19.048 t_R).

The compounds identified in this study are comparable to those found in the Welsh onion *A. Fistulosum*, as reported by Choi (2019) using the HS-SPME/GC-MS technique. The predominant compounds identified by the author included (Z)-1-propenyl propyl sulfide, (Z)-propenyl propyl disulfide, (E)-propenyl propyl disulfide, dipropyl trisulfide, and propyl propanthiosulfonate.

Subsequently, a correspondence analysis was performed to describe the relationship or independence between the two nominal variables under study—type of onion and type of compound—and determine the relationships between categories, defining similarities or dissimilarities between the two variables. This procedure allowed a grouping described by the relationship between the categories of the variables that were consistent. To that end, the chi-square test (χ^2) was performed,the results of which are illustrated in Table 4.

Based on the results presented in Table 4, it can be observed that the significance obtained through the chi-square statistic (χ^2) was 0.000, which rejects the null hypothesis of independence and accepts that there was a relationship between the two variables. Below is Figure 4 in which the four varieties of onion are observed and two varieties that are closely related are Veleña and Veleña Sonsón.

Table 4. Data obtained from the chi-square test (χ^2).

	Value	df	Asymp. Sig (2- sided)
Pearson's chi-squared	149.58[a]	84	0.000
Likelihood ratio	166.33	84	0.000
Linear association	0.146	1	0.702
No valid cases	183	-	-

[a]116 cells (100% expected a count less than five. The minimum expected count was 0.49).

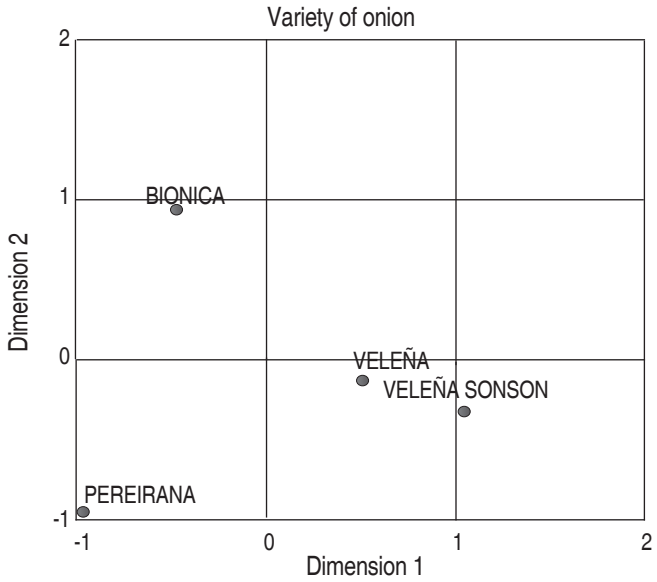

Figure 4. Graph for the variable 'type of onion'.

The associated compounds are illustrated in a column points chart (Figure 5) for a type of compound. It can be observed that the compounds C25 and C26 were di and trisulphides with a molar mass between 148 and 180 g mol^{-1}, from which 1-Allyl-2-isopropyldisulfane can be highlighted. This compound has been found in onion oil together with dipropyl disulfide and dipropyl trisulphide; however, the concentrations decreased as the temperature increased, which made the aroma change significantly (Tian et al. 2021).

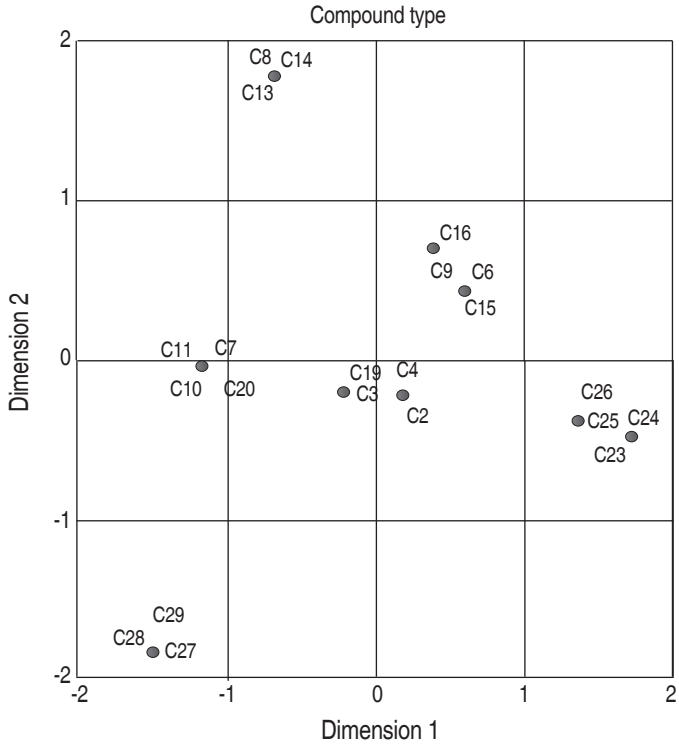

Figure 5. Column points chart for the type of compound.

Associated compounds C6, C9, C15, and C16 were observed, of which the first two had the characteristics of being methyl ketones. Methyl ketones include 2-undecanone and 2-tridecanone, which are important in the flavor and fragrance industry. These compounds have a variety of natural and commercial functions, including pheromones and natural insecticides in plants. Depending on their concentrations, they can generate a protective effect against insect attacks, which may indicate that the varieties Biónica, Veleña, and Veleña Sonsón had some protection against insects (Antonious 2013).

Compounds C1, C3, C4, and C19 gave rise to another group, and compounds C1, C4, and C19 have been reported in previous studies by HS-GC-MS (D'Auria and Racioppi 2017), for example, the Dipropyldisulphide was reported in 51.41% for *Allium cepa* L. and 58.57% for *Allium ascalonicum* L.

The presence of this group of compounds could be used for soil biofumigation and plant growth stimulation since they can control pathogens and increase fruit productivity by 15 to 20%. This is a potential biofumigant that could be used to replace methyl bromide, a substance that is characterized by destroying the ozone layer (Arnault et al. 2013).

In studies on essential oils of onions, *Allium porrum* L. (*Alliaceae*) was characterized by the presence of dipropyl disulfide, dipropyl trisulphide, and dipropyl tetrasulphide. The results obtained suggest that the presence of the allyl group was essential for the antimicrobial activity of these sulfur derivatives when they are present in *Allium* or other species (Casella et al. 2013).

Compounds such as diallyl disulfide, diallyl trisulphide, diallyl sulfide, dipropyl disulfide, dipropyl trisulphide, 1-propenylpropyl disulfide, allyl methyl disulfide, and dimethyl disulfide correspond to the most active compounds of the *Alliaceae* family, which have been proven to have medicinal properties such as antibacterial and antioxidant activities thanks to the organo-sulfur compounds, which are believed to prevent the development of cancer, cardiovascular and neurological diseases, diabetes, liver diseases, as well as allergies and arthritis (Bastaki et al. 2021). These organo-sulfur

compounds have demonstrated their versatility in various applications. Patel et al. (2018) explored their potential in promoting hair growth when incorporated into a shampoo formulation. Additionally, Sharma et al. (2018) highlighted their effectiveness as antibacterial agents, while Hannan et al. (2010) investigated their role as bacterial inhibitors in food products. Furthermore, Khater et al. (2009) suggested their suitability as a potential insect repellent. These findings underscore the multifaceted utility of organo-sulfur compounds in diverse fields.

Through the correspondence analysis, it was possible to show that there is a significant relationship between the variable "onion variety" and the variable "type of compound" and in Figure 6 it can be seen for each variety of onion the compounds that are associated with it, and which therefore characterize it.

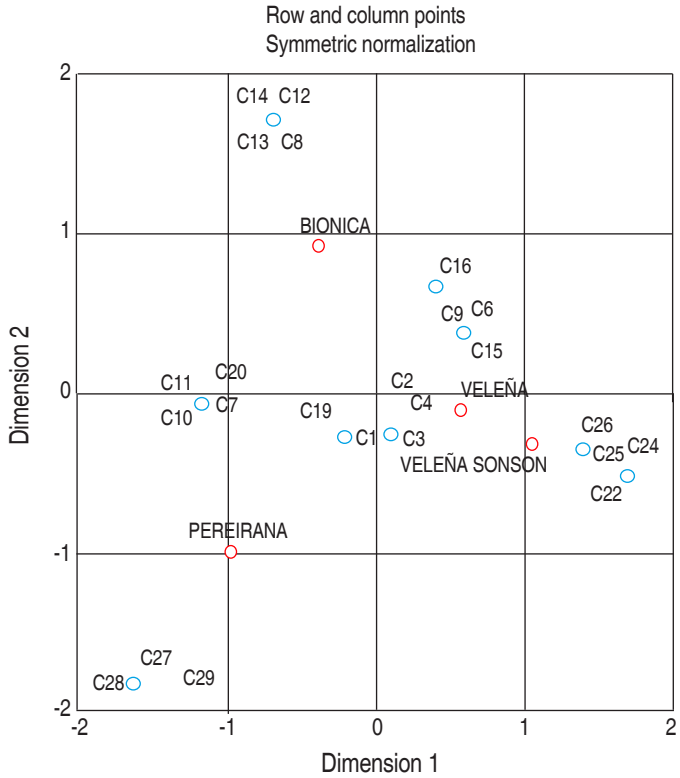

Figure 6. Bispace graph: Relationship between the type of compound and the variety of onion.

Study of the essential oil of Pereirana onion

A fluid liquid oil was obtained with a characteristic odor of onion, a light-yellow color, distinctive properties of essential oils (Rojas et al. 2009), and a yield of 0.028%, which was above that reported by other authors 0.0053% (El-Tohamy et al. 2009), 0.0045% (Yassen and Khalid 2009; Pino et al. 2000) and lower than that reported by Dron et al. (1997), which obtained 0.032% in extraction by supercritical fluids. Table 5 shows the compounds observed with the PDMS and polyacrylate fibers, which were the ones that extracted the greatest number of compounds. The result is presented by comparison with the NIST14 and NIST14s libraries. The compounds with more than 85% coincidence with the spectrum of the library are presented. The relative areas for each compound are also presented concerning the total automatically integrated.

The main compound of the essential oil of Welsh onion was dipropyl disulfide (number 31, with 15.38%). Studies presented by Cantrell et al. (2020) for onion Cepa oil extracted with dichloromethane (DCM) managed to extract up to 27 organosulfur compounds, including dipropyl disulfide in great abundance. According to Gao et al. (2019) who used the SPME fiber with phase 50:30 µm DVB/

CAR on PDMS, sulfocompounds are the main functional compositions of onion and its oils, exhibiting significant bioactivity in human health. According to this experiment, volatile sulfocompounds, such as disulfide, trisulfide, tetrasulfide, thiophene, and dithiane with dimethyl groups, were the main components of onion oils.

Table 5. Chemical Composition of the Essential Oil from the Pereirana Variety Onion.

No	Compounds	CAS	t_R (min)	SI (%)	PDMS Area (%)	Polyacrylate Area (%)
1	Propyl acetate	109-60-4	3.89	95	0.51	0.25
2	1,1-Dietoxietano	105-57-7	4.19	96	0.33	0.13
3	Sec-butyl acetate	105-46-4	4.88	93	0.10	-
4	Isobutyl acetate	110-19-0	5.28	98	1.57	0.82
5	Ethyl butyrate	105-54-4	6.08	93	0.12	-
6	n-Butilo acetato	123-86-4	6.49	96	-	0.25
7	Ethyl 2-methyl butyrato	7452-79-1	7.76	93	0,09	0.04
8	3-Hexen-1-ol, (Z)	928-96-1	7.98	86	-	0.02
9	3-Hexen-1-ol, (E)	928-97-2	8.42	85	-	0.05
10	Amyl acetate	628-63-7	8.82	93	1.24	-
11	Isoamyl acetate	123-92-2	8.83	95	1.07	0.52
12	m-Xylene	108-38-3	9,40	91	-	0.05
13	Cyclopentane, 1-ethenyl-3-methylene-	61142-07-02	9.40	93	0.11	-
14	3,4- dimethyl thyophene	632-15-5	9,86	92	0.39	0.27
15	Isopropyl methyl disulfide	40136-65-0	11.09	93	0.36	0.22
16	α-Pineno	80-56-8	11.23	92	0.14	-
17	(Z)-Methyl-1-propenyl disulfide	23838-18-8	11.41	88	0.3	0.11
18	Dimethyl trisulfide	3658-80-8	12.76	87	-	0.03
19	1-Heptanol	111-70-6	13.03	88	-	0.05
20	β-Myrcene	123-35-3	13.91	90	0.09	0.03
21	Decane	124-18-5	14.47	90	0.05	-
22	Octanal	124-13-0	14.56	94	0.09	0.07
23	Limonene	138-86-3	15.79	92	0.13	0.05
24	4-methyl decane	2847-72-5	17.41	93	0.05	-
25	2-methyl decane	6975-98-0	17.62	92	0.05	-
26	2-propyl-1-Heptanol	10042-59-8	17.91	93	0.10	-
27	1-Octanol	111-87-5	17.92	94	-	0.10
28	(E)-1-Allyl-2-(prop-1-in-1-yl) disulfide	122156-02-9	19.25	89	0.10	0.06
29	Tridecane	629-50-5	19.49	95	-	0.34
30	Undecane	1120-21-4	19.54	95	1.66	-
31	Dipropyl disulfide	629-19-6	19.87	96	15.38	6.08
32	1-Allyl-2-isopropyl disulfate	67421-85-6	20.19	91	2.65	1.24
33	Metil propil trisulfuro	17619-36-2	22.16	91	0.45	0.28
34	(E)-1-Metil-3-(prop-1-en-1-il) trisulfan	23838-25-7	22.69	87	0.07	0,03
35	3-methyl-1-hexanol	13231-81-7	23.68	90	0.25	-
36	Nonyl acetate	143-13-5	23.73	86	-	0.39

Table 5

No	Compounds	CAS	t_R (min)	SI (%)	PDMS Area (%)	Polyacrylate Area (%)
37	Methyl 2-oxo nonanoate	56275-54-8	24.42	91	0.16	0.11
38	Dodecane	112-40-3	25.47	93	0.12	-
39	Decanal	112-31-2	25.72	94	0.32	0.15
40	Cyclohexyl Isothiocyanate	1122-82-3	27.00	95	0.43	0.30
41	3-Ethyl-5-Methyl-1,2,4-Trithiolane	116505-59-0	27,13	86	0.39	0.24
42	3,3,8-trimethyl decane	62338-16-3	29.60	88	0.05	-
43	2-Undecanone	0112-12-9	30.73	94	12.16	5.63
44	2-Undecanol	1653-30-1	31.12	96	0.78	0.56
45	2-Methoxy-4-vinylphenol	7786-61-0	31.31	85	-	0.12
46	2,4-Decadienal	25152-84-5	31.72	87	-	0.04
47	Dipropyl trisulfide	6028-61-1	32.14	96	2.15	1.35
48	(E)-1-(Prop-1-in-1-yl)-3-propyltrisulfan	23838-27-9	32.54	90	4.19	3.45
49	2,4-Octanediona	14090-87-0	34.58	92	-	1.79
50	4-Methyl dodecane	6117-97-1	35.76	92	0.12	-
51	Methyl 1-(methyl thio) propyl disulfide	53897-66-8	36.47	89	0.16	0.09
52	2-nonyl acetate	14936-66-4	36.65	85	0.18	0.26
53	2-hexyl-5-methyl-3(2H)-furanon	33922-66-6	37.37	93	1.42	0.99
54	4,6-dimethyl dodecane	61141-72-8	39.60	93	0.14	-
55	2-Tridecanone	0593-08-08	39.96	94	6.82	3.61
56	3,7- dimethyl decane	17312-54-8	40.14	92	0.16	0.04
57	2-Tridecanol	1653-31-2	40.24	93	-	0.15
58	Dipropyl tetrasulfide	52687-98-6	42.67	86	0.20	0.13
59	1-Methyl-2-(1-(propyltio)propyl)disulfide	126876-21-9	42.82	85	0.37	0.14
60	(Z)-3-hexen-1-yl benzoate	25152-85-6	42.96	94	0.17	0.11
61	trans-3,6-diethyl-1,2,4,5-tetratian	934273-77-5	43.89	90	1.99	1.06
62	cis-4,6-diethyl-1,2,3,5-tetratian	137363-91-8	44.04	88	1.92	1.26
63	Hexadecane	544-76-3	44.55	93	0.20	-
64	5-methyl-2-octyl-3(2H)-furanone	57877-72-2	46.83	95	0.63	0.45
65	Dioctyl ether	629-82-3	47.80	86	0.06	0,04
66	2-Nonadecanone	629-66-3	49.02	86	0.06	0.04
67	Pentadecanal	2765-11-9	49.55	86	0.03	-
68	Benzyl benzoate	120-51-4	50.75	90	0.04	0.05
69	1-(1-propenylthio)propyl propyl disulfide	143193-11-7	51.64	87	0.08	-
70	4,7-Diethyl-1,2,3,5,6-pentatiopane	878997-71-8	52.83	89	0.21	-

Area (%)=relative percentage of the compound in the essential oil; SI (%)=percentage of coincidence with the NIST14 and NIST14s libraries.

Essential oils are concentrated and contain more than twice as many components as fresh samples. They can be used to confer the aroma and flavor of onions without the difficulties of handling large quantities of fresh products. It is plausible to anticipate that the acquisition of new knowledge about native species will yield numerous benefits for humanity, offering new services and products, as well as raw materials for various industries (Lead et al. 2005). In this context, the volatile extracts of the Pereira onion are identified as a raw material of interest

due to their high concentration of sulfur compounds. These compounds have been attributed with properties useful for insecticide applications (Denloye 2010), and in the pharmaceutical industry as promising anti-allergy, antihistamine, anti-inflammatory, antioxidant agents, and as preventatives for brain edema with neuroprotective potential (Kuete 2017).

Onion extracts can also be utilized in the cosmetic industry due to their propensity to induce tissue regeneration and facilitate wound healing (Celano et al. 2021). Other potential applications include use as an oil oxidation stabilizer, an ingredient in bakery products, pasta, and noodles, a quality improver for meat products, a substrate for enzyme production, and as a raw material for tea. However, extensive research is necessary before these potential applications can be commercialized or scaled up at an industrial level (Kumar et al. 2022).

CONCLUSIONS

The results underscore the efficacy of the HS-SPME/GC-MS method for the analysis of volatile compounds in the essential oil of the Pereirana variety Welsh onion. This method enabled the separation and characterization of up to 70 compounds using four solid-phase microextraction fibers. Notably, the polyacrylate fiber demonstrated a significant affinity for sulfur compounds, with dipropyl disulfide being the most abundant, constituting 65.67% of the total.

The study revealed that the concentration of volatile compounds in the Pereirana variety of Welsh onion exceeds that of other varieties by up to 20 times. As a result, this variety emerges as a promising plant material warranting further research. Its potential applications span across various industries, encompassing food, pharmaceutical, agricultural, and cosmetics.

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Genome-wide association study on growth traits in Colombian Hair Sheep

Estudios de asociación en genoma completo para caracteres de crecimiento en Ovinos de Pelo Colombiano

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Keywords:
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ABSTRACT

The Colombian hair sheep have characteristics of great interest, among the following: high capacity for adaptation, good fertility, high prolifically, and low presence of diseases, which have been little studied. Currently, genome-wide association studies (GWAS) have been widely used to detect and locate candidate genes. However, in sheep, there is a low number of investigations carried out in GWAS, because the available information is limited, compared to that of other species. This research aimed to conduct a genome-wide association study on muscle growth traits using the Illumina OvineSNPs50 BeadChip array. A GWAS using 54.241 single nucleotide polymorphisms (SNPs) was conducted in Ethiopian (44 individuals), Sudan (63), and Pelibuey (60) breeds of Creole hair sheep to evaluate eight growth traits. Quality control was performed using a linear regression model in PLINK. Moreover, a functional analysis was done in the KEGG database using the *Ovis aries* (sheep) genome v.3.1. In total, 44.396 SNPs that passed quality control were used for the analysis. The 10 most significant SNPs were identified for each trait. The functional analysis allowed the annotating of four candidate genes, namely CEP135, EMCN, PAM, and PIAS2, as the most relevant genes for the traits assessed. Additionally, 27 genes associated with phenotypic traits were considered promising and could also be influencing growth traits. This is the first GWAS on Colombian hair sheep to report genomic traits associated with muscle growth traits. Four candidate genes (CEP135, EMCN, PAM, and PIAS2) associated with eight growth traits were identified by genome-wide association in colombian hair sheep.

Palabras clave:
Ovino criollo
GWAS
Peso vivo
Microarreglo
SNPs
Ganancia de peso

RESUMEN

Los Ovinos de Pelo Colombiano tienen características de gran interés, entre ellas se resaltan: alta capacidad de adaptación, buena fertilidad, alta prolificidad y baja presencia de enfermedades que han sido poco estudiadas. Actualmente, los estudios de asociación de genoma completo (GWAS) han sido ampliamente usados para detectar y localizar genes candidatos. Sin embargo, en ovinos se cuenta con un bajo número de investigaciones al respecto, debido a que la información disponible se encuentra limitada, en comparación con la de otras especies. El objetivo de esta investigación fue realizar un análisis genómico asociado a caracteres de crecimiento muscular, mediante el uso del microarreglo OvineSNP50 BeadChip de Illumina. Se realizó un GWAS empleando 54,241 polimorfismos de nucleótido simple (SNPs), en ovinos de pelo criollo, en el cual se evaluaron las variedades raciales Etíope (44), Sudán (63) y Pelibuey (60), para ocho caracteres de crecimiento. Se llevó a cabo un control de calidad a través del programa PLINK, por medio de un modelo de regresión lineal. Posteriormente se hizo un análisis funcional, empleando el genoma ovino *Ovis aries* v.3.1, en la base de datos de KEGG. Después de haber realizado el control de calidad, se analizaron 44,396 SNP. Se identificaron los 10 SNPs más significativos para cada carácter. A través del análisis funcional se logró anotar cuatro posibles genes candidatos (CEP135, EMCN, PAM y PIAS2), como los más importantes en los caracteres evaluados. Adicionalmente, se encontraron 27 genes asociados a los caracteres fenotípicos, los cuales pueden ser prometedores y podrían estar influyendo en los caracteres de crecimiento. Este trabajo es el primer GWAS en ovinos de pelo colombiano en el país, que ha asociado caracteres fenotípicos de crecimiento muscular con caracteres genómicos. El GWAS permitió identificar cuatro posibles genes candidatos (CEP135, EMCN, PAM y PIAS2), asociados a ocho caracteres de crecimiento.

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Sheep are an important component of the environment based on economic and conservation perspectives (Knapik et al. 2017). These animals can adapt to landscapes that are difficult to cultivate and play an important role in food security, household economies, and cultural development of Colombian indigenous and farmer communities (Corpoica 2007). Traditionally, in the country, Colombian hair sheep have been used for meat production in extensive systems (Zuleta et al. 2009). These animals are highly diverse since crosses between different breeds date back to the colonization (Vergara et al. 2017). There are two varieties of sheep, which producers refer to as Ethiopian, or cherry red-haired sheep, and Sudan, or yellow-haired sheep; however, polychromy is common among sheep, with animals found in cherry red, yellow, white, black, and combinations of the above, due to the introduction of animals from West Africa, Ethiopia, Aruba, Curacao, and other Caribbean islands (Zuleta et al. 2009). Furthermore, in Colombia, exotic breeds are also used and crossed with Creole breeds to obtain hybrid vigor for meat production (Vergara-Garay et al. 2016).

The increasing popularity of sheep meat production worldwide has prompted the sheep industry, producers, and geneticists to address the importance of growth and meat production in this species (Zuleta et al. 2009). Growth variables, such as body weight at different stages (Al-Mamun et al. 2015; Gholizadeh et al. 2015), have been evaluated as important growth indicators (Kemper et al. 2012). Given the low productive parameters in Colombian hair sheep (Romero et al. 2002), strategies to understand and improve these indicators must be implemented. In this regard, genomics-based approaches represent a valuable tool (Casas and White 2015).

The development of high-throughput SNP (single nucleotide polymorphism) genotyping has enabled the development of GWAS to identify candidate genes for traits of interest (Al-Mamun et al. 2015; Gholizadeh et al. 2015; Kominakis et al. 2017). Despite the discovery of various candidate genes through GWAS in recent years, a small number of these have been found in sheep due to the limited availability of genomic information on sheep (Zhang et al. 2013). Furthermore, most research has been conducted on exotic breeds, while studies on native sheep are scarce (Casas and White 2015). GWAS are expected to contribute to identifying SNPs associated with traits of interest; therefore, this study aimed to analyze associations between growth traits and genomic traits using the Illumina OvineSNPs50 BeadChip array.

MATERIALS AND METHODS

Study animals

The study population comprised 167 individuals of Colombian hair sheep (females and males) belonging to Sudan (OPC_S), Pelibuey (OPC_P), and Ethiopian (OPC_E) breeds. The number of individuals from each breed was 63, 60, and 44, respectively. The animals were in the departments of Cesar, Cordoba, and Valle del Cauca. The technical, scientific, and administrative procedures for research with animals, were adjusted to the regulations of Law 84 of 1989 and with resolution 8430 of 1993 (National Congress of Colombia).

Growth traits

The phenotypic growth traits assessed were birth weight (BW) measured during the first 24, adjusted weaning weight to 90 days (AWW) (equation 1), adjusted final weight adjusted to 1-year-old (AFW) (equation 2), pre-weaning daily weight gain (WGPRE) and post-weaning daily weight gain (WGPOS), fat thickness (FT), loin depth (LD), and loin eye area (LEA). The last three traits were measured at approximately 1 year of age, through a MyLab™One VET ultrasonograph (Esaote, Maastricht, Netherlands).

$$AWW = \left[\frac{(WW - BW}{WA}\right](100) + BW \qquad (1)$$

Where AWW is adjusted weaning weight to 90 days, WW is weaning weight, BW is birth weight, and WA is weaning age (days).

$$AFW = \left[\frac{(FW - WW}{AFWD}\right](365) + WW \qquad (2)$$

Where AFW is adjusted final weight adjusted to 1-year-old, FW is final weight, WW is weaning weight, and AFWD is the age at the final weight (days).

Samples and genotyping

Blood samples (10 mL from each animal) were obtained through jugular venipuncture and collected in vacutainer tubes with EDTA. Genomic DNA was extracted from the

blood samples using Gene JET Genomic DNA purification (Thermo Fisher Scientific). The DNA was quantified through spectrophotometry using NanoDrop® ND-1000 (Thermo Fisher Scientific). DNA genotyping was performed on an Illumina OvineSNPs50 BeadChip array, targeting 54,241 SNPs, according to the Infinium® Assay Super II Illumina® protocol for use on the HiScan®SQ System.

Quality control was performed in PLINK v1.9b5.2 (Purcell et al. 2007) by excluding individuals who were missing more than 5% of genotyping information, as well as SNPs with call rates below 95%, allele frequencies below 5%, and Hardy-Weinberg equilibrium $P<0.001$.

Genome association analysis

The association analysis between the genotype and eight phenotypic traits was based on a linear regression model (equation 3) using 44,396 filtered SNPs. The equation was adjusted according to the genomic location of the markers using Wald's standard statistic to calculate the *P* value for each SNP.

$$Y = X\beta + \in \quad (3)$$

Where Y is the vector of n phenotypic observations (BW, AWW, AFW, WGPRE, WGPOS, FT, LD, and LEA) for 167 individuals, X is a covariable matrix (n x p); where n are individuals and p are SNPs, β is a vector of p effects (SNPs), and ϵ is a vector of n residuals.

Post-GWAS analysis

Using Haploview v4.2 (Barrett et al. 2005), SNPs were filtered for each trait based on a probability value of $P<0.001$ to obtain a matrix of significantly associated SNPs, which was used in further analyses. Next, SNPs were classified according to *P*-value and correlation coefficient (R^2) using GWASTools (Gogarten et al. 2012) in R version 3.1 to identify the 10 most relevant SNPs associated with each phenotype studied.

Additionally, Genome Data Viewer was used to search the *Ovis aries* genome version 4 for growth genes, namely calpain (CAPN1), calpastatin (CAST), growth hormone (GH), growth hormone receptor (GHR), leptin (LEP), myostatin (MSTN), type 1 insulin-like growth factor (IGF-1), fatty acid-binding protein 9 (FABP9), and fatty acid-binding protein 4 (FABP4). SNPs located in these genes were identified for each trait assessed. On the other hand, a linkage disequilibrium value was established with a LD=0.001, under a window of 50,000 bp, using the SNPRelate 0.9.12 package (Zheng et al. 2012) from Bioconductor in the R program, to establish a set of filtered SNPs in equilibrium to avoid the strong influence of the groups of SNPs in the principal component analysis (PCA).

Functional analysis

The most significant genomic associations were searched in the KEGG database using *Ovis aries* genome v.3.1 in ReactomePA Bioconductor package (Yu and He 2016) in R to infer the biological processes of the genes involved. Furthermore, the DAVID database was used to obtain biological functions from additional databases, such as Gene Ontology, UniProt Keywords, InterPro, Kegg Pathway, Smart, and Pir SuperFamily. Finally, the KEGG identifiers retrieved were mapped to metabolic pathways in *Ovis aries*.

RESULTS AND DISCUSSION

In this research, a GWAS was conducted in 167 animals belonging to three breeds of Colombian hair sheep to determine associations between eight phenotypic traits and a panel of 54,241 SNPs using next-generation sequencing. The sex chromosome (OAR) and mitochondrial-associated contig were excluded from the analysis; furthermore, 44,396 SNPs were retained after discarding missing data for individuals and SNPs, low allele frequencies, and Hardy-Weinberg equilibrium (i.e., quality control).

For the association analysis, the SNPs associated with each trait were filtered according to $P<0.001$; as a result, 7,000 significant SNPs were identified (Table 1). After ordering by lowest *P*-value and highest R^2, the 10 most significant SNPs showing the highest association with each of the eight traits were determined (Tables 2, 3, and 4) (Zhang et al. 2013), which resulted in a total of 80 SNPs for all traits. This study is the first approach in Colombia to identify candidate functional genes containing SNPs that are significantly associated with growth traits; furthermore, these SNPs may be involved in phenotypic determination. Despite the small sample size for a GWAS (Zhang et al. 2013; Peng et al. 2017), more than 10,000 SNPs were found to be significantly associated with eight growth traits (e.g., BW, AWW, WGPRE, AFW, and WGPOS), as well as traits that were

measured by ultrasonography (e.g., FT, LD, and LEA). Moreover, this research represents a pioneer study in Colombia, thereby, contributing essential information to the ovine sector in the country.

Table 1. Number of significant SNPs for each of the phenotypic traits assessed.

Phenotypic trait	SNPs*
BW	7437
AWW	1058
AFW	2872
WGPRE	1761
WGPOS	1076
FT	599
LD	147
LEA	1134

*Number of significant SNPs (P<0.001) based on the linear regression model.

The analysis indicated that chromosome OAR6 showed most significant associations (P<0.001), accounting for 12.5% of SNPs associated with AWW, WGPRE, AFW, WGPOS, FT, and LEA, followed by chromosome OAR1 with 11% of SNPs for AWW, WGPRE, AFW, FT, and LEA, and chromosome OAR5 with 9% of SNPs for BW, AWW, AFW, and WGPOS. Similarly, Zhang et al. (2013), suggested that chromosomes OAR1 and OAR3 are important for growth traits and meat production in sheep (i.e., for pre-weaning and post-weaning weight gain, daily weight gain, thoracic perimeter, shin circumference, weight at six months old, and weaning weight).

Association analysis for pre-weaning growth traits

Table 2 shows the 10 most significant SNPs associated with each pre-weaning phenotypic trait: BW, AWW, and WGPRE. For BW, annotation showed that 50% of SNPs were found in introns, 40% in the distal region, and 10% in promoters. Furthermore, six out of 10 SNPs were in five genes; however, functions were inferred for only three of these genes: LOC101107266, PAM, and GABRG2.

Association analysis for post-weaning growth traits

The 10 most significant SNPs associated with phenotypic traits AFW and WGPOS are found in Table 3. For AFW, 70% of SNPs were annotated in the distal region and 30% in introns. Five out of 10 SNPs were located in CEP135, EMCN, PRDM13, PIAS2, and SLC44A3 genes. Moreover, for WGPOS, 60% of SNPs were found in the distal region and 40% in introns. Six out of 10 SNPs were located in EFNA5, MAP3K7, CEP135, EMCN, PIAS2, and MTAP genes.

Association analysis for growth traits measured by ultrasonography

The 10 most significant SNPs associated with traits FT, LD, and LEA are shown in Table 4. For phenotypic trait FT, 80% of SNPs were annotated in the distal region, 10% in intron 2 of 20, and 10% were located downstream (1-2 Kb). Only one out of ten SNPs were located in a known gene, namely CEP135. Moreover, for LD, 80% of SNPs were annotated in the distal region and 20% in introns. Furthermore, four out of 10 SNPs were located in SUGP1, LOC101104424, PDYN, and TGFB3 genes. Finally, for LEA, 40% of SNPs were found in the distal region, 30% in introns, 20% in promoters, and 10% were located downstream. Five out of 10 SNPs were found in INPP4B, TBRG4, C17H4orf46, DDX24, and KIRREL genes.

Genomic location and importance of the most relevant SNPs

By annotating the 80 most significantly associated SNPs and mapping them to the chromosomes of the *Ovis aries* genome v3.1, eight relevant SNPs were identified on three chromosomes, namely OAR5, OAR6, and OAR23. In chromosome 5, SNPs OAR5_107968125.1 and OAR5_107977075.1 were associated with BW and located in the PAM gene (positions 99153391 bp and 99165665 bp, respectively). Furthermore, in chromosome 6, SNPs OAR6_77919148.1 (position 71481367 bp) and

Table 2. SNPs are significantly associated with BW, AWW, and WGPRE in Colombian hair sheep.

Trait	Chromosome	SNPs	Position (bp)	R^2	P	Annotation	Ensembl Gene ID	NCBI Gene
BW	3	OAR3_61737307.1	58315054	0.441	$7.18x10^{-18}$	Intron (ENSOART00000022552/ENSOARG00000020700, intron 2 of 3)	ENSOARG00000020700	LOC 101107266
BW	5	OAR5_107968125.1	99153391	0.352	$3.00x10^{-14}$	Intron (ENSOART00000020038/ENSOARG00000018405, intron 3 of 25)	ENSOARG00000018405	PAM
BW	5	OAR5_107977075.1	99165665	0.352	$3.00x10^{-14}$	Intron (ENSOART00000020038/ENSOARG00000018405, intron 3 of 25)	ENSOARG00000018405	PAM
BW	5	s11274.1	71514994	0.340	$1.28x10^{-13}$	Intergenic distal	ENSOARG00000014001	GABRG2
BW	3	OAR3_89348294.1	84390370	0.322	$1.36x10^{-12}$	Intron (ENSOART00000008654/ENSOARG00000007947, intron 4 of 4)	ENSOARG00000007947	TMEM178A
BW	17	OAR17_29260298.1	26667946	0.322	$1.36x10^{-12}$	Intergenic distal	ENSOARG00000014791	-
BW	12	OAR12_20575087.1	17694724	0.309	$1.53x10^{-12}$	Intron (ENSOART00000011686/ENSOARG00000010714, intron 17 of 74)	ENSOARG00000010714	USH2A
BW	2	OAR2_51716542.1	48200678	0.298	$2.72x10^{-11}$	Intergenic distal	ENSOARG00000021824	-
BW	3	OAR3_74952313.1	70926536	0.294	$3.84x10^{-11}$	Intergenic distal	ENSOARG00000004203	-
BW	18	s03219.1	68137231	0.288	$7.96x10^{-11}$	Promotor (<=1Kb)	ENSOARG00000013958	-
AWW	14	s01263.1	58489098	0.190	$3.79x10^{-06}$	Intergenic distal	ENSOARG00000000628	LOC101113879
AWW	20	OAR20_49893668.1	45829455	0.159	$1.06x10^{-04}$	Intron (ENSOART00000028931/ENSOARG00000026887, intron 1 of 1)	ENSOARG00000026887	-
AWW	1	OAR1_23734999.1	23651580	0.159	$1.06x10^{-04}$	Intergenic distal	ENSOARG00000004178	LOC105605154
AWW	9	s33129.1	88576469	0.156	$1.26x10^{-04}$	Intergenic distal	ENSOARG00000011632	CNGB3
AWW	5	OAR5_49721052.1	45617625	0.146	$3.65x10^{-07}$	Intergenic distal	ENSOARG00000025310	-
AWW	1	OAR1_189179554.1	175482796	0.144	$4.11x10^{-04}$	Promotor (<=1Kb)	ENSOARG00000019298	CD200
AWW	6	OAR6_12248234.1	9785894	0.143	$5.16x10^{-04}$	Intergenic distal	ENSOARG00000017991	-
AWW	26	DU261801_281.1	37119640	0.139	$6.78x10^{-04}$	Intron (ENSOART00000004427/ENSOARG00000004073, intron 2 of 15)	ENSOARG00000004073	PSD3
AWW	1	s25125.1	96847814	0.139	$6.82x10^{-04}$	Intron (ENSOART00000022309/ENSOARG00000020482, intron 4 of 46)	ENSOARG00000020482	POF4DIP
AWW	10	OAR10_59207797.1	59207797	0.138	$8.01x10^{-07}$	Intergenic distal	ENSOARG00000017151	SLITRK1
WGPRE	1	s12060.1	75971485	0.236	$3.25x10^{-08}$	Intergenic distal	ENSOARG00000017670	PLPPR4
WGPRE	23	OAR23_39144674_X.1	39144675	0.225	$9.06x10^{-08}$	Promotor (<=1Kb)	ENSOARG00000026187	-
WGPRE	4	OAR4_24114290.1	24114290	0.205	$8.60x10^{-07}$	Intron (ENSOART00000009008/ENSOARG00000008280, Intron 7 of 7)	ENSOARG00000008280	-
WGPRE	10	OAR10_91128145.1	91128145	0.205	$8.86x10^{-10}$	Intergenic distal	ENSOARG00000009132	CHAMP1
WGPRE	7	OAR7_85269064.1	85269064	0.196	$2.07x10^{-06}$	Promotor (2-3Kb)	ENSOARG00000002286	CIPC
WGPRE	8	OAR8_1452721.1	1452721	0.197	$2.13x10^{-06}$	Intergenic distal	ENSOARG00000006317	-
WGPRE	19	s52415.1	11201714	0.193	$2.86x10^{-06}$	Intron (ENSOART00000000540/ENSOARG00000000498, Intron 27 of 27)	ENSOARG00000021896	-
WGPRE	6	OAR6_77919148.1	77919148	0.192	$3.26x10^{-06}$	Intergenic distal	ENSOARG00000026595	-
WGPRE	18	OAR18_58488706.1	58488706	0.180	$1.16x10^{-05}$	Intergenic distal	ENSOARG00000024407	-
WGPRE	3	s62226.1	26419161	0.180	$1.21x10^{-05}$	Promotor (<=1Kb)	-	LOC105601856

OAR6_27552838.1 (position 24157625 bp) were located in CEP135 and EMCN genes, respectively, and were associated with AFW and WGPOS. In particular, the first of these SNP (OAR6_77919148.1) was also found associated with FT. Finally, in chromosome 23, SNP OAR23_49635171_X.1 (position 46823961 bp), located in the PIAS2 gene, was associated with traits AFW and WGPOS. Additionally, SNPs OAR5_112451694.1 and OAR8_50320412.1 were found in chromosomes OAR5 and OAR8. These SNPs were located in functional genes EFNA5 and MAP3K7, which interact in a metabolic pathway associated with WGPOS and are involved in cell signaling processes, according to the KEGG annotation.

Previous studies that have used the Illumina OvineSNPs50 BeadChip array reported several SNPs, including OAR6_41003295.1 and OAR6_42945420.1, that were found to be significantly associated with traits such as body weight (Al-Mamun et al. 2015). In this study, both of these SNPs were associated with BW. Moreover, Gholizadeh et al. (2015), reported that SNP OAR16_46544413.1 was associated with weight at six months old; similarly, this SNP was associated with AWW in this study. Additionally, SNP s52984.1 has been associated with daily weight gain and weight at six months old, while SNPs s55067.1 and s34745.1 have been associated with weaning weight (Zhang et al. 2013). Furthermore, Ai-Mamun et al. (2015) reported that SNP OAR6_40409402.1 was associated with body weight. In this study, these four SNPs were associated with AFW. Moreover, SNPs s34745.1 and s55067.1, reported by Zhang et al. (2013), were associated with pre-weaning weight gain; similar to the results of this study that showed the association between these SNPs and WGPRE. Finally, OAR16_61248510.1 has been associated with post-weaning weight gain and s52984.1 with daily weight gain and weight at six months old (Zhang et al. 2013); in this case, both SNPs were associated with WGPOS.

Among the 80 most significant SNPs (Table 2, 3, and 4), those that were associated with more than one trait were identified as the most relevant SNPs. One SNP was located near two genes (CEP135 and EMCN), one in a single gene (PIAS2), and two SNPs in the same gene (PAM). Overall, four candidate genes were detected, which could be most influential on the growth traits assessed. However, 27 additional genes were also promising, including LOC101107266, GABRG2, TMEM178A, USH2A, LOC101113879, LOC105605154, CNGB3, CD200, PSD3, PDE4DIP, SLITRK1, PLPPR4, CHAMP1, CIPC, LOC105601856, PRDM13, SLC44A3, MTAP, SUGP1, LOC101104424, PDYN, TGFB3, INPP4B, TBRG4, C17H4orf46, DDX24, and KIRREL.

SNP OAR6_77919148.1 was located on the distal region of the CEP135 (centrosomal protein 135) gene, which belongs to the family of centrosomal proteins that are an active component of the centrosome and are involved in the biogenesis of the centriole and control of cell cycle progression (Kumar et al. 2013). In humans, alterations in this gene cause reduced cell growth rates (Hussain et al. 2012). Furthermore, in *Chlamydomonas*, mutation of Bld10, an ortholog of CEP135, resulted in abnormal microtubules in interphase and the mitotic spindle; these defects occurred during cell division and significantly reduced cell growth rate (Matsuura et al. 2004). Based on this, the CEP135 gene is involved in cell growth rate and can influence AFW, WGPOS, and FT.

SNP OAR6_27552838.1 is located in the distal intergenic region of EMCN (Endomucin), which encodes a sialic-rich glycoprotein rich in type I O-glycoproteins and is specifically expressed in the venous and capillary endothelium. Recent studies suggest that this gene is a potent regulator of capillary formation from existing blood vessels, or angiogenesis (Park-Windhol et al. 2017). Furthermore, EMCN plays a key role in tissue damage and wound repair, the endometrial cycle, and the adaptation of the striated muscle to stress and exercise (Olfert et al. 2015). Therefore, there is a functional relationship between this gene and the traits assessed given the involvement of angiogenesis in the skeletal muscle and the association found here between EMCN and AFW and WGPOS.

SNPs OAR5_107968125.1 and OAR5_107977075.1 are located in introns of the PAM gene (Peptidylglycine Alpha-Amidating Monooxygenase), which catalyzes COOH-terminal amidation of peptide hormones (Traci et al. 2005). In mammals, amidation activity is presumed to be coded by a single gene product with two catalytic domains (PHM and PAL) that act sequentially to amidated peptides (Prigge et al. 2000). Amide peptides function as hormones, neuromodulators, and autocrine growth factors (Prigge et al. 2000; Merkler 1994). Therefore, this gene could be associated with BW.

Table 3. SNPs significantly associated with AFW and WGPOS in Colombian hair sheep.

Trait	Chromosome	SNPs	Position (pb)	R^2	*P*	Annotation	Ensembl Gene ID	NCBI Gene
AFW	6	OAR6_77919148.1	71481367	0.275	$3.66x10^{10}$	Intergenic distal	ENSOARG00000003002	CEP135
AFW	23	OAR23_39144674_X.1	37003177	0.273	$4.58x10^{10}$	Intergenic distal	ENSOARG00000022421	-
AFW	6	OAR6_27552838.1	24157625	0.256	$3.10x10^{-09}$	Intergenic distal	ENSOARG00000013694	EMCN
AFW	8	OAR8_39977285.1	37211967	0.224	$1.03x10^{-10}$	Intergenic distal	ENSOARG00000011775	PRDM13
AFW	5	OAR5_84496122_X.1	76876928	0.223	$1.24x10^{-07}$	Intergenic distal	ENSOARG00000014307	-
AFW	23	OAR23_49635171_X.1	46823961	0.223	$1.32x10^{-07}$	Intron (ENSOART00000003661/ENSOARG00000003371, intron 1 of 13)	ENSOARG00000003371	PIAS2
AFW	13	OAR13_26675629.1	24142711	0.113	$4.13x10^{-07}$	Intron (ENSOART00000003020/ENSOARG00000002785, intron 2 of 20)	ENSOARG00000021746	-
AFW	7	OAR7_104041756.1	95561909	0.211	$4.41x10^{-07}$	Intergenic distal	ENSOARG00000026731	-
AFW	7	OAR7_104050413.1	95569312	0.211	$4.41x10^{-07}$	Intron (ENSOART00000028761/ENSOARG00000026731, intron 2 of 2)	ENSOARG00000026731	-
AFW	1	s12060.1	71057388	0.210	$5.18x10^{-07}$	Intergenic distal	ENSOARG00000017408	SLC44A3
WGPOS	5	OAR5_112451694.1	103304973	0.180	$1.07x10^{-05}$	Intergenic distal	ENSOARG00000018761	EFNA5
WGPOS	20	s24831.1	11418394	0.176	$1.65x10^{-05}$	Intron (ENSOART00000016003/ENSOARG00000014698, Intron 17 of 22)	ENSOARG00000014906	-
WGPOS	5	OAR5_84496122_X.1	76876928	0.168	$3.70x10^{-05}$	Intergenic distal	ENSOARG00000014307	-
WGPOS	10	OAR10_1950751.1	4132402	0.167	$4.20x10^{-05}$	Intergenic distal	ENSOARG00000023450	-
WGPOS	8	OAR8_50320412.1	46840220	0.165	$5.09x10^{-05}$	Intron (ENSOART00000013401/ENSOARG00000012321, Intron 7 of 16)	ENSOARG00000012321	MAP3K7
WGPOS	6	OAR6_77919148.1	71481367	0.161	$7.30x10^{-05}$	Intergenic distal	ENSOARG00000003002	CEP135
WGPOS	6	OAR6_27552838.1	24157625	0.161	$7.52x10^{-05}$	Intergenic distal	ENSOARG00000013694	EMCN
WGPOS	23	OAR23_49635171_X.1	46823961	0.161	$8.41x10^{-05}$	Intron (ENSOART00000003661/ENSOARG00000003371, Intron 1 of 13)	ENSOARG00000003371	PIAS2
WGPOS	2	OAR2_96008804.1	89509922	0.159	$1.04x10^{-04}$	Intergenic distal	ENSOARG00000014411	MTAP
WGPOS	6	s48525.1	22902181	0.156	$1.30x10^{-04}$	Intron (ENSOART00000014572/ENSOARG00000013398, Intron 6 of 14)	ENSOARG00000023387	-

Table 4 . SNPs significantly associated with FT, LD, and LEA in Colombian hair sheep.

Trait	Chromosome	SNPs	Position (bp)	R^2	*P*	Annotation	Ensemble Gene ID	NCBI Gene
FT	6	s61364.1	16067116	0. 256	$3.15x10^{-09}$	Intergenic distal	ENSOARG00000006816	-
FT	6	OAR6_77919148.1	71481367	0.227	$7.28x10^{-08}$	Intergenic distal	ENSOARG00000003002	CEP135
FT	13	OAR13_26675629.1	24142711	0.195	$2.33x10^{-09}$	Intron (ENSOART00000003020/ ENSOARG00000002785, Intron 2 of 20)	ENSOARG00000021746	-
FT	6	OAR6_81305080.1	74488523	0.186	$5.88x10^{-06}$	Intergenic distal	ENSOARG00000024398	-
FT	1	OAR1_248575929.1	230581424	0.186	$6.58x10^{-06}$	Río abajo (1-2kb)	ENSOARG00000021579	-
FT	2	s37719.1	32379564	0.171	$2.7x10^{-08}$	Intergenic distal	ENSOARG00000023484	-
FT	9	OAR9_40619495.1	38690895	0.166	$5.16x10^{-05}$	Intergenic distal	ENSOARG00000026523	-
FT	2	s58833.1	101443025	0.166	$5.36x10^{-05}$	Intergenic distal	ENSOARG00000014922	-
FT	4	OAR4_6864529.1	7128966	0.163	$6.83x10^{-05}$	Intergenic distal	ENSOARG00000021732	-
FT	1	OAR1_157215006.1	145677767	0.157	$1.10x10^{-04}$	Intergenic distal	ENSOARG00000025579	-
LD	10	OAR10_42329325.1	42329325	0.232	$6.36x10^{-08}$	Intergenic distal	ENSOARG00000015005	-
LD	11	OAR11_7879331.1	7879331	0.168	$4.17x10^{-05}$	Intron (ENSOART00000008542/ ENSOARG00000007848, Intron 4 of 10)	ENSOARG00000007910	-
LD	2	OAR2_20446382.1	20446382	0.151	$2.14x10^{-04}$	Intergenic distal	ENSOARG00000025744	-
LD	5	s53828.1	3679286	0.142	$5.08x10^{-04}$	Intergenic distal	ENSOARG00000008152	SUGP1
LD	15	s40457.1	47387694	0.142	$5.21x10^{-04}$	Intergenic distal	ENSOARG00000007342	LOC101104424
LD	5	OAR5_110584928.1	110584928	0.136	$8.92x10^{-04}$	Intergenic distal	ENSOARG00000025340	-
LD	10	OAR10_68443350.1	68443350	0.127	$2.37x10^{-03}$	Intergenic distal	ENSOARG00000000563	-
LD	13	s27419.1	52439172	0.126	$2.54x10^{-03}$	Intergenic distal	ENSOARG00000007354	PDYN
LD	22	OAR22_31810853.1	31810853	0.122	$3.70x10^{-03}$	Intergenic distal	ENSOARG00000011700	-
LD	7	s52907.1	84077554	0.118	$5.52x10^{-06}$	Intron (ENSOART00000002182/ ENSOARG00000001992, Intron 28 of 32)	ENSOARG00000002050	TGFB3
LEA	14	s01263.1	55308253	0.197	$1.89x10^{-06}$	Intron (ENSOART00000014897/ ENSOARG00000013693, Intron 3 of 5)	ENSOARG00000013698	-
LEA	1	OAR1_248575929.1	230581424	0.191	$3.96x10^{-06}$	Río abajo (1-2kb)	ENSOARG00000021579	-
LEA	17	OAR17_16676148.1	15020629	0.165	$4.96x10^{-05}$	Intergenic distal	ENSOARG00000011798	INPP4B
LEA	4	s12760.1	76582310	0.163	$6.63x10^{-05}$	Promotor (1-2kb)	ENSOARG00000013838	TBRG4
LEA	6	OAR6_50229073_X.1	45303722	0.163	$7.07x10^{-05}$	Intergenic distal	ENSOARG00000008049	-
LEA	6	OAR6_12297748.1	9841765	0.160	$9.17x10^{-05}$	Intergenic distal	ENSOARG00000017991	-
LEA	17	OAR17_43310387.1	40123170	0.155	$1.36x10^{-04}$	Intron (ENSOART00000005160/ ENSOARG00000004733, Intron 18 of 20)	ENSOARG00000004417	C17H4orf46
LEA	19	OAR19_8201989.1	7898831	0.147	$3.19x10^{-04}$	Promotor (<=1kb)	ENSOARG00000026642	-
LEA	18	s50461.1	57567836	0.147	$3.54x10^{-04}$	Intron (ENSOART00000015616/ ENSOARG00000014341, Intron 3 of 7)	ENSOARG00000014341	DDX24
LEA	1	OAR1_260939703.1	106932088	0.145	$4.08x10^{-04}$	Intergenic distal	ENSOARG00000007174	KIRREL

OAR23_49635171_X.1 was found in an intron of the PIAS2 gene (Protein Inhibitor of Activated STAT 2), which belongs to the family of STAT protein inhibitors that regulate the activity of many proteins and influence diverse processes such as the immune response, cancer formation, and cell cycle progression. Furthermore, a study on *Xenopus* indicated that this gene family could play an important role in embryogenesis regulation (Burn et al. 2011). Therefore, according to the literature, PIAS2 is involved in early growth processes, and in this study, it was found in association with AFW and WGPOS, which were measured during growth and development stages and in the adult stage. These findings suggest that this gene is not only involved in early growth stages but also later periods; however, this was not conclusive.

The APC showed that the three sheep breeds were separated, the OPC_P (yellow) from the OPC_E (blue) and OPC_S (red), however, some OPC_S individuals overlapped with the OPC_E (Figure 1). Which could be used in the model to check the population stratification.

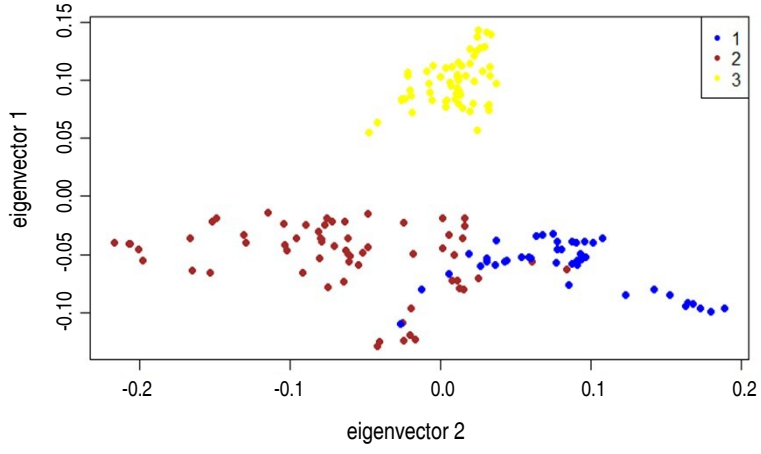

Figure 1. Principal component analysis for population stratification in three breeds of Colombian hair sheep.

MAP3K7 (Mitogen-Activated Protein Kinase Kinase Kinase 7) and EFNA5 (Efrin-A5) genes

MAP3K7 and EFNA5 genes are found in shared metabolic pathways and are involved in signaling processes. MAPK (Mitogen-Activated Protein Kinases) are members of the serine/threonine kinase protein family activated by growth and stress factors. These proteins play an important role in intracellular signal transduction, which enables the cell to integrate different extracellular signals. Therefore, MAPK participates in signaling cascades that regulate cell growth, differentiation, proliferation, and cell death (Roberts and Der 2007).

EFNA5 gene belongs to Eph receptors and their ligands (i.e., efrins), which constitute the largest family of tyrosine kinase receptors (Pasquale 2005; Kullander and Klein 2002). Additionally, this gene behaves as a chemotactic molecule that participates in the correct positioning and formation of the neuromuscular junction (Li and Johnson 2013). Accordingly, satellite cells are a resident population of stem cells of the adult skeletal muscle tissue, which is responsible for growth and regeneration. These cells generally aggregate near the ends of muscle fibers, as well as the neuromuscular junction. Li and Johnson (2013) examined the effects of EFNA5 signaling on satellite cells in bovines and found that chemokines and growth factors participate in the localization of these cells. MAP3K7 and EFNA5 genes participate in metabolic pathways involved in cellular processes; furthermore, both genes were associated with WGPOS, which was measured during the growth and development stage until the adult stage. Therefore, a coherent relationship between these genes and the growth trait was established.

Search for growth-associated genes reported in the literature

The search for CAPN1, CAST, GH, GHR, LEP, MSTN, IGF-1, FABP9, and FABP4 genes in the *Ovis aries* genome v.4.0

allowed identifying SNPs located in CAPN1, CAST, GHR, and FABP9. CAPN1 gene contained SNPs s30026.1, which showed association with traits BW, AFW, and FT ($P<0.05$). CAST gene contained two SNPs: OAR5_101792466.1 was significantly associated with traits BW, AFW, and FT, while s59216 was associated with AWW and LEA. Furthermore, there were five SNPs located in GHR, including three significantly associated SNPs, namely OAR16_34620156.1 for WGPOS, OAR16_34694443.1 for LD and LEA, and OAR16_34857607.1 for BW. Moreover, SNP OAR9_60512150.1, located in the FABP9 gene, was significantly associated with WGPRE and FT.

The annotation of the candidate genes indicated that several of these show some degree of association with cell growth, apoptosis, angiogenesis, and metabolic pathways that are directly or indirectly involved in muscle growth in different species. Therefore, several of these genes could play a similar role in sheep. The sheep genome has been poorly studied compared with other species; therefore, these results contribute to an exploratory analysis of candidate novel genes that can be used in future studies to elucidate the participation of these genes in productive traits.

CONCLUSIONS

This study is one of the first to conduct a genomic analysis of Creole hair sheep in Colombia to evaluate genomic traits associated with muscle growth traits, although the sample size is small, this is pioneering research, and its information represents a fundamental input for the country's sheep sector. In conclusion, four candidate genes, namely CEP135, EMCN, PAM, and PIAS2, were associated with eight growth traits. The CEP135 gene was the most relevant because it was associated with three of the eight traits evaluated post-weaning (AFW, WGPOS, and FT). The EMCN and PIAS2 genes were associated with the same traits (AFW and WGPOS), while the PAM gene was associated only with one preweaning growth trait (BW). It is important to highlight that the functions of these genes indicate their involvement mainly in cellular growth, apoptosis, and angiogenesis. These results can be used as a basis for future exploratory research, metabolic network analysis, and functional validation of the candidate genes.

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Evaluation of the inclusion of three oils on productive parameters and carcass characteristics in broiler

Evaluación de la inclusión de tres aceites sobre parámetros productivos y características de la canal en pollo

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ABSTRACT

An experiment was conducted to determine the effect of three oil sources with the inclusion of three levels on some performance variables, carcass quality, and broiler organs. The sources used were palm oil (*Elaeis guineensis*), chicken oil, and Sacha inchi oil (*Plukentia volubilis*). A total of 324 female Ross 308 line were assigned in a completely random design to nine treatments with six repetitions of six birds per repetition, which were fed with a diet based on corn and soybean meal containing 3, 6, and 9% inclusion of each one of the oil sources for the periods between 8 to 21 days of age and 22 to 42 days of age. Live weight and feed consumption data were recorded weekly to calculate the feed conversion ratio, considering mortality. On day 42, one female broiler from each repetition was randomly selected, weighed, and sacrificed, to obtain upgraded data characteristics from the carcass. The results suggest that there was no significant difference in animal response variables or channel quality. Regarding the carcass variables and some poultry organs, the only one affected by the source was the deposition of abdominal fat. Sacha inchi oil produced carcasses with a lower average of abdominal fat in relation to weight and slaughter, with no differences between palm and chicken oils.

Keywords:
Abdominal fat
Carcass quality
Inclusion level
Oil sources
Broiler chicken

RESUMEN

Se realizó un experimento para determinar el efecto de tres fuentes de aceite con tres niveles de inclusión sobre las variables de desempeño, calidad de la canal y órganos en pollos de engorde. Las fuentes usadas fueron aceite de palma (*Elaeis guineensis*), aceite de pollo y aceite de Sacha inchi (*Plukentia volubilis*). Un total de 324 hembras de la línea Ross 308, fueron asignadas en un diseño completamente al azar en nueve tratamientos con seis repeticiones y seis aves por repetición, las cuales fueron adicionados a una dieta a base de maíz y soya en harina que contenían 3, 6 y 9% de inclusión de cada una de las fuentes de aceites por un periodo entre 8 y 21 días de edad y de 22 a 42 días de edad. Los datos de peso vivo y consumo de alimento, fueron tomados semanalmente para calcular la tasa de conversión alimenticia, teniendo en cuenta la mortalidad. Sobre el día 42, una hembra de cada repetición fue pesada y sacrificada con el fin de obtener los datos de características de la canal. Los resultados sugieren que no se presentó diferencia significativa en las variables respuesta animal ni calidad de la canal. En las variables de la canal y algunos órganos de las aves la única que resultó afectada por la fuente fue la deposición de grasa abdominal. El aceite de sacha inchi generó canales con menor porcentaje de grasa abdominal con relación al peso al sacrificio, sin que se haya observado diferencia entre los aceites de palma y de pollo.

Palabras clave:
Grasa abdominal
Calidad de la canal
Nivel de inclusión
Fuentes de aceites
Pollo de engorde

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Chicken devoted to meat production has changed in the last decades at all levels. This situation involved the change of their diets' structural design, which involves, among other aspects, the inclusion of ingredients with a high concentration of digestible or available nutrients and metabolizable energy. In specialized poultry production systems, how has been guaranteed to meet the metabolizable or net energy requirements has been mainly with foods high in starch, especially cereal grains such as corn, and, partially, with oil or fat sources or the combination of both (Wiseman et al. 1986). There is no doubt that if the goals set for broiler production remain on the horizon and the decision to use cereal grains for other purposes such as alcohol production is sustained, the feeding strategy should be reviewed because to the extent that there are no of feeds with higher starch concentrations than the current ones, there will be a need to review the issue of the use oil and fats in chicken feed.

In the ninth revised edition of Nutrient Requirements of poultry, the NRC (1994) recorded as appropriate broiler starter and finisher diets based on corn, sorghum, or wheat-soybean whose fat contents were between 0 and 10%. This is perhaps the first record that provided information, although not as a requirement, on the maximum level of fat that would be appropriate in diets. This same version also identified for the first time the requirement of 1% linoleic acid for chickens up to the eighth week of life and 0.8% between weeks 8 and 18; in addition, to keeping the 1% indicated since 1971 for laying hens and broilers. After more than 45 years after these first records, it is not possible to identify, even as a recommendation, the maximum level of inclusion of fat in the chickens' diet.

The use of fats or oils in food has positive effects on broiler productivity: they improve diets acceptance and they also decrease consumption because they are sources of high energy concentration, they contribute to the homogenization of diets, reduce losses due to the selection of particles, it lowers the rate of passage of digesta through the gastrointestinal tract and improve absorption of dietary nutrients, contribute with their performance rates improvement and minimize production costs (Ayed and Attia 2015; Pesti et al. 2002). The type of fat and oil added to diets has a direct effect on the level of abdominal fat deposited and on its lipid composition (Ayed and Attia 2015). Therefore, in broilers, the type of fat used in food affects the composition of their body lipids (Scaife et al. 1994).

Fast-growing modern broiler lines also have higher levels of body fat, becoming a problem for the poultry industry. Choct (2000) reported that these lines contain between 15 to 20% fat, where about 85% is not physiologically required for body functions, leading to excess fat deposition, carcass productivity reduction, and a negative effect on consumer acceptance.

The issue of fat addition to broiler's diets is complex. Since it is not part of the fractions and nutrients that record requirements, it falls in the recommendations field and they can be based on criteria set by the animal feed industry, the meat processing industry, the consumers, and finally by the nutritionists. Moreover, the tendency has been to design diets with fat levels not higher than 3%, with limited possibilities of attempting to expand them. This study aimed to evaluate three types of oil: palm oil, in which saturated fatty acids (SFA) predominate, chicken oil, highlighted by the highest proportion of monounsaturated fatty acids (MUFA), and Sacha inchi oil, in which polyunsaturated fatty acids (PUFA) stand out, with three levels of inclusion (3, 6 and 9%) in the broiler chickens' diet between their birth and their 42 days of age. The evaluation was based on their performance rates, such as body weight, feed intake, and feed conversion, and allometric characteristics such as carcass weight, breast, leg, skin, gizzard, liver, heart, and abdominal fat both in absolute and relative values.

MATERIALS AND METHODS

The study was carried out at San Pablo Agricultural Station of the Universidad Nacional de Colombia, Medellin Headquarters, located in the municipality of Rionegro, (Antioquia, Colombia), at 2,100 meters above sea level (masl), coordinates 6°07'51.3" N and 75°27'19.1" W. According to the living zone classification system, the station is listed: as a very moisture-low montane forest living zone (bmh-MB) (Holdridge 1978), characterized by a temperature range between 12 and 18 °C and an annual rainfall of 2,280 mm. At night and early hours of the day, the relative humidity reaches approximately 75.5%. A total of 324 female Ross 308 line from a commercial incubator were used. The birds were received at 1 day of age

and were housed during the first 8 days in an experimental hatchery of 126 m^2, which was built with an east-west orientation, with natural ventilation, heating, and curtain management to guarantee a temperature of 30 °C, with a floor of cement and open water and commercial feed supply. When the birds were eight days old, they were moved to another hatchery with horizontal batteries of 0.7 m length x 0.75 m width x 0.6 m height. In the hatchery, heating and ventilation were maintained with gas brooders and managed with curtains.

Diets

During the first week of life, the birds were offered a commercial diet. From day 8 of age to day 42, birds had voluntary access to the experimental diets according to the following pattern. Two evaluation phases were established. The first one, called initiation, was with birds aged between 8 and 21 days old. And the second one, fattening, was with birds aged between 22 and 42 days old. For both phases, the diets were in the form of flour and were designed following the requirements of Rostagno et al. (2017). Except for oils, the nutritional facts of the ingredients used in these diets were taken according to the information provided by the supplier. The apparent metabolizable energy values of the oils were estimated from a trial previously performed. In each phase, nine diets were provided to account for three oils (commercial palm, commercial chicken, Sacha inchi), and three inclusion levels (3, 6, and 9%) were offered at each stage. The centesimal and nutritional composition of the diets is shown in Table 1. The diets' chemical composition analyses were carried out in the chemical analysis and bromatological laboratory at Universidad Nacional de Colombia, which is registered as a quality control laboratory before the Colombian Agricultural Institute according to registration LB0000432021 (December 7, 2021).

Table 1. Centesimal and nutritional composition of the experimental diets used in the initiation and fattening phases.

Ingredient	Initiation phase (8 to 21 days)								
	Palm oil			Chicken oil			Sacha inchi oil		
Corn	60.0	54.5	43	60.0	54.5	51.5	58.0	56.1	52.1
Soybean cake	31.5	34.0	40.0	31.5	34.0	34.0	33.5	33.5	33.5
Hemoglobin	1.0	1.0	-	1.0	1.0	1.0	1.0	-	1.0
Wheat bran	-	-	3.5	-	-	-	-	-	-
Oil	3.0	6.0	9.0	3.0	6.0	9.0	3.0	6.0	9.0
Dicalcium phosphate	1.6	-	-	-	-	-	-	-	-
Ca carbonate	1.3	-	-	-	-	-	-	-	-
Sea salt	0.3	-	-	-	-	-	-	-	-
L-Lysine HCL (99%) (Alys® 99)	0.3	-	-	-	-	-	-	-	-
DL-Methionine	0.3	-	-	-	-	-	-	-	-
L-Threonine (98.5%)	0.2	-	-	-	-	-	-	-	-
Choline chloride	0.05	-	-	-	-	-	-	-	-
Sodium bicarbonate	0.05	-	-	-	-	-	-	-	-
Premix1	0.3	-	-	-	-	-	-	-	-
Total	100	-	-	-	-	-	-	-	-

[1]Vitamin A 11,000,000 IU, Vitamin D 2,500,000, Vitamin E 12,000 mg, Vitamin K 3,000 mg, Thiamine 1,300 mg, Riboflavin 5,000 mg, Niacin 60,000 mg, Pantothenic acid 11,100 mg, Pyridoxine 2,000 mg, Biotin 50 mg, Vitamin B12 11 mg, Folic acid 600 mg, Choline chloride (60%) 500,000 mg, Zinc 76,000 mg, Manganese 76,000 mg, Copper 8,000 mg, Iron 60,000 mg, Iodine 800 mg, Selenium 300 mg, Antioxidant 100,000 mg, Maximum humidity 5%.

Table 1

Component[2,3]	Nutritional composition (Values expressed in dry matter)								
	Palm oil			Chicken oil			Sacha inchi oil		
	3.0	6.0	9.0	30	60	90	30	60	90
Moisture and other volatile matter	13.6	12.4	12.2	12.9	12.7	12.7	13.5	14.1	13
Crude protein	20.0	20.4	21.8	19.75	20.45	20.21	20.5	19.5	20.31
Crude fiber	2.50	1.50	1.72	2.53	2.55	2.50	2.60	2.56	2.49
Crude fat	5.85	8.60	11.42	5.85	8.66	11.51	5.81	8.7	11.52
Calcium	0.98	0.99	1.02	0.98	0.99	0.99	0.99	0.99	0.98
Phosphorus	0.65	0.65	0.69	0.65	0.65	0.64	0.66	0.65	0.64
EMA (kcal kg^{-1})	2.988	3.102	3.194	2.932	3.127	3.301	2.956	3.032	3.257

[2] Except for EMA, the other components are expressed in g 100 g^{-1}.
[3] The dietary EMA values were estimated from the ingredient values.

Table 1

Ingredient	Fattening phase (21 to 42 days)								
	Palm oil			Chicken oil			Sacha inchi oil		
Corn	64.3	61.3	46.8	63.0	59.0	54.3	63.3	59.0	54.3
Soybean cake	29.0	29.0	34.0	30.3	31.3	33.0	30.0	31.3	33.0
Wheat bran	-	-	6.5	-	-	-	-	-	-
Oil	3.0	6.0	9.0	3.0	6.0	9.0	3.0	6.0	9.0
Dicalcium phosphate	1.04	-	-	-	-	-	-	-	-
Ca carbonate	1.10	-	-	-	-	-	-	-	-
Sea salt	0.30	-	-	-	-	-	-	-	-
L-Lysine HCL (99%) (Alys® 99)	0.28	-	-	-	-	-	-	-	-
DL-Methionine	0.43	-	-	-	-	-	-	-	-
L-Threonine (98.5%)	0.15	-	-	-	-	-	-	-	-
Choline chloride	0.05	-	-	-	-	-	-	-	-
Sodium bicarbonate	0.05	-	-	-	-	-	-	-	-
Premix1	0.30	-	-	-	-	-	-	-	-
Total	100	-	-	-	-	-	-	-	-

[1]Vitamin A 11,000,000 IU, Vitamin D 2,500,000, Vitamin E 12,000 mg, Vitamin K 3,000 mg, Thiamine 1,300 mg, Riboflavin 5,000 mg, Niacin 60,000 mg, Pantothenic acid 11,100 mg, Pyridoxine 2,000 mg, Biotin 50 mg, Vitamin B12 11 mg, Folic acid 600 mg, Choline chloride 60% 500,000 mg, Zinc 76,000 mg, Manganese 76,000 mg, Copper 8,000 mg, Iron 60,000 mg, Iodine 800 mg, Selenium 300 mg, Antioxidant 100,000 mg, Maximum humidity 5%.

Table 1

Component[2,3]	Nutritional composition (Values expressed in dry matter)								
	Palm oil			Chicken oil			Sacha inchi oil		
	3.0	6.0	9.0	3.0	6.0	9.0	3.0	6.0	9.0
Crude protein	18.0	17.8	20.0	18.5	18.7	19.0	18.6	18.4	19.0
Crude fiber	2.5	2.5	3.0	2.5	2.5	2.5	2.5	2.5	2.5
Crude fat	6.0	8.8	11.5	6.0	8.8	11.6	6.0	8.8	11.6
Calcium	0.75	0.75	0.85	0.75	0.75	0.76	0.75	0.75	0.76
Phosphorus	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53
EMA (kcal kg^{-1})	3.098	3.341	3.242	2.972	3.183	3.339	3.021	3.084	3.289

[2] Except for EMA, the other components are expressed in g 100 g^{-1}.
[3] The dietary EMA values were estimated from the ingredient values.

Variables studied

Considering each phase, the variables studied were diet consumption, weight, and feed conversion, and for the overall period, the variables were weight and carcass yield, breast, leg, liver, heart, gizzard, wings, skin, and abdominal fat on the day of slaughter. For the calculation of diet consumption, the weekly average consumption was considered, bearing the number of dead birds during the evaluated period. For the weight variable, the birds were weighed upon arrival at the station and then every week until the end of the experiment. The live weight information was gathered from the weight of the birds of each repetition and averaged according to the number of live birds. The estimation of the feed conversion was based on the average consumption of the diet divided by the live weight during the evaluated period, without taking mortality into account. Only the live weight, consumption, and feed conversion statistical analysis are presented considering two periods: between day 21 and 42 of age and between day 8 and 42 of age.

The evaluation of carcass weight, breast, leg, liver, heart, gizzard, wings, skin, and abdominal fat was made from data obtained from a slaughtered bird by replicate (six per treatment) on day 43 of age. Slaughter was performed by the cervical dislocation method. For abdominal fat weight, all fat surrounding the gizzard, bursa of Fabricius, cloaca, and adjacent muscles were removed and weighed individually. The procedure for weighing liver, heart, gizzard, wings, and skin was the same.

For the carcass yield determination, the weight of the hot carcass without feathers, head, neck, legs, and white viscera was considered in contrast to the live weight at slaughter time. The breast and leg, liver, heart, gizzard, wings, skin weight, and abdominal fat, were also taken in contrast to the animal's live weight.

Experimental design

The experiment was planned in a 3x3 factorial arrangement with the three types of oil (palm, chicken, and Sacha inchi oil) and three inclusion levels (3, 6, and 9%) with six repetitions per treatment and six birds per replicate, having a total of 324 birds. The nine treatments were randomly distributed in the 54 cages.

Statistical analysis

Weekly weight analysis was using a linear regression model with weight at one week of age as the independent variable. Carcass variables were analyzed as absolute weight and as an average of each variable in relation to slaughter weight. Considering the absolute weight, a linear regression analysis was run in which slaughter weight was selected as the independent variable and carcass variables as the dependent ones. The bird's weight when slaughtered did not influence the abdominal fat, skin, or heart weight; therefore, the statistical analyses performed on these variables model included: this weight, the oil source effect, the level of inclusion, and the interaction between these two factors. In the analysis model for the other variables such as carcass, breast, legs, wings, gizzard, and liver weight, slaughter weight was not included. The variance analysis for each variable was performed using the General Linear Model. The average values of each treatment were compared using the SNK test. All statistical analyses were performed by means of the SAS Version 9 program.

The implemented model followed equation 1.

$$Y_{ijkl} = \mu + F_i + N_j + T_k + E_{ijkl} \quad (1)$$

Where:

Y_{ijkl}: the expected response from the experimental unit, becomes the m-th observation in the response variable.

μ: experimental media.

F_i: the i-th oil source effect.

N_j: the j-th level of oil inclusion effect.

T_k: the k-th interaction (diet) effect.

E_{ijkl}: residual error associated with the l-th experimental unit.

RESULTS AND DISCUSSION

No adverse effects on the chicks associated with the conducted conditions were observed during the experiment.

Dietary consumption

Table 2 shows the results of the diet consumption analysis during the following periods: from day 8 to day 21 of age, day 21 to day 42, the accumulated from day 8 to day 42, and the estimated daily average per bird. The comparison between the intakes recorded in the experiment versus the reference ones shows that besides some exceptions, the intakes in the experiment were below the performance objectives of the genetic line; highlighting the results

regarding T4 (chicken oil at 3% inclusion), which were above those expected for the line. In the study, there was no consistent effect of the evaluated factors on consumption. For all evaluated periods, there was no oil source effect, nor the composition of its fatty acids, during the period from day 8 to day 21 of age; consumption declined with the increase in the inclusion level. In relation to the interactions between the source and the level of inclusion, it was possible to establish that the trends were similar during the period from day 21 to day 42 of age and between day 8 and day 42 of age: in both palm oil and Sacha inchi oil, the level of inclusion did not affect consumption; but regarding chicken oil, the highest consumption was achieved with 3% inclusion, with no difference between the two other levels.

Table 2. Oil source effect, inclusion level, and interaction between the source and level of dietary intake between day 8 to 21 of age, 21 to 42, cumulative between day 8 to 42, and average bird day of broilers (Values expressed in grams).

Effect	Diet consumption			Estimated daily consumption per bird from 8 to 42 days of age
	Day 8 to 21 of age	Day 21 to 42 of age	Accumulated day 8 to 42	
Oil source	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05
Palm	849.23	2997.12	3846.3	109.89
Chicken	900.87	3146.28	4047.1	115.61
Sacha inchi	890.21	2993.72	3883.9	110.96

Table 2

Inclusion level (%)	*P*<0.05	*P*>0.05	*P*>0.05	*P*>0.05
3	910.09[a]	3138.78	4048.9	115.67
6	828.68[b]	3004.65	3876.3	110.74
9	847.54[a,b]	2993.69	3825.2	110.06
Source*Level1	*P*>0.05	*P*<0.01	*P*<0.01	*P*<0.01
T1	873.5	3051.73[bc]	3925.3[bc]	112.13[bc]
T2	845.8	3060.26[bc]	3906.1[bc]	111.60[bc]
T3	828.3	2879.35[bc]	3707.7[bc]	105.95[bc]
T4	977.2	3480.93[a]	4458.1[a]	127.36[a]
T5	878.3	3131.2[b]	4009.5[b]	114.53[b]
T6	847.1	2826.71[bc]	3673.2[c]	104.95[c]
T7	879.5	2883.68[bc]	3763.2[bc]	107.51[bc]
T8	923.9	2789.60[c]	3713.5[bc]	106.10[bc]
T9	867.2	2863.88[bc]	3724.58[bc]	106.91[bc]
MSE Root	73.18	292.05	323.35	9.25
Model *P*-value	0.0361	0.0017	0.0016	0.0017
Reference value Ross AP line (2022)	959.00	3240.00	4199.00	123.50

[1]T1, T2, T3: palm oil at 3, 6 and 9%. T4, T5, T6: chicken oil at 3, 6 and 9%. T7, T8, T9: Sacha inchi oil at 3, 6 and 9%. [abc] Values with different letters in the same column differ significantly.

Body weight

Table 3 shows the results of the analyses carried out for body weight between day 8 and 21 of age, day 21 to 42, until day 42, and accumulated daily gain per bird. The estimated weight gains for females of the genetic Ross AP line (2022) for the periods between day 8 and 21 of age, 22 to 42, up to day 42 and the daily gain for the whole period provide values of 784, 1,858, 2,856 g, and

66.95 g d^{-1}. The comparison between these values with those of the study shows that there were higher values in the study when palm oil and chicken oil were used, in the three inclusion levels, and all interactions between oil source and inclusion level except for T2 and T3 (palm oil at 6 and 9%) for the period between day 8 and 21 of age and all situations for daily weight gain. In the study, the only treatment that showed higher values than those of the genetic line was T2 (6% palm oil) between days 21 and 42 of age.

The oil source affected weight gain for the periods between days 8 to 21 of age and days 21 to 42 of age. During the first period, Sacha inchi oil promoted the highest weight, but there was no difference between the other two sources. For the period from day 21 to 42 of age, Sacha inchi oil made birds with lower weights, keeping no difference between the other sources. Between days 8 to 42 of age, daily weight gain throughout the experimental period was not affected by the oil source. Ferrini et al. (2008) evaluated the effect of the 10% inclusion of different sources of fats and oils (tallow, high oleic sunflower oil, high linoleic sunflower oil, high linolenic linseed oil, and a mixture of 55% tallow, 35% linseed oil, and 10% high linoleic sunflower oil) on weight gain between day 8 and 42 of age in Ross 308-line broilers. Even though the response among the different sources changed, it was possible to establish that the lowest gains were obtained from the sunflower oil and flaxseed oil diets, while the highest gains were obtained with the mixture, having no difference from the tallow diet.

Table 3. Effect of the oil source, inclusion level, and interaction between source and inclusion level on weight gain between days 8-21, 21-42, and day 42 of age and cumulative weight gain (Values expressed in grams).

Effect	Weight gain			Daily weight gain
	Day 8 to 21	Day 21 to 42	Day 8 to 42	
Oil source	*P*<0.01	*P*<0.5	*P*>0.05	*P*>0.05
Palm	777.61^{b}	1806.83^{a}	2584.33	73.84
Chicken	798.17^{b}	1806.89^{a}	2604.89	74.42
Sacha inchi	832.22^{a}	1689.06^{b}	2521.22	72.01
Inclusion level (%)	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05
3	805.56	1785.17	2590.61	74.01
6	796.22	1761.5	2557.67	73.06
9	806.22	1756.11	2562.17	73.21
Source*Level	*P*<0.05	*P*<0.05	*P*<0.05	*P*<0.05
T1	806.83^{b}	1794.66^{a}	2601.50^{a}	74.33^{a}
T2	746.83^{c}	1886.0^{a}	2632.66^{a}	75.21^{a}
Table 3				
T3	779.16^{b,c}	1739.83^{a}	2518.83^{a,b}	71.98^{a,b}
T4	804.16^{b}	1837.0^{a}	2641.00^{a}	75.45^{a}
T5	798.66^{b}	1836.33^{a}	2635.00^{a}	75.28^{a}
T6	791.66^{b}	1747.33^{a}	2538.66^{a,b}	72.55^{a,b}
T7	805.66^{b}	1723.83^{a,b}	2529.33^{a,b}	72.26^{a,b}
T8	843.16^{a,b}	1562.16^{b}	2405.33^{b}	68.68^{b}
T9	847.83^{a}	1781.16^{a}	2629.00^{a}	75.10^{a}
MSE Root	38.02	143.48	146.92	-
Model *P*-value	0.0014	0.022	0.116	-

[1]T1, T2, T3: palm oil at 3, 6 and 9%. T4, T5, T6: chicken oil at 3, 6, and 9%; T7, T8, T9: Sacha inchi oil at 3, 6, and 9%. ^{abc} Values with different letters on the same column may differ meaningfully.

Regarding the level of inclusion of the oil sources evaluated, it has been established that there was no effect on this variable in all the periods evaluated; however, an effect on the interaction between the oil source and the level of inclusion was found. It was not possible to identify any type of trend in the effect of the interaction in the periods evaluated, suggesting that the birds' behavior had been independent with respect to the diet. Considering the above, the analysis will be focused on the period from day 8 to 42 of age and the daily weight gain of the entire experimental period. According to the results, the behavior in the three levels of inclusion of palm and chicken oils was similar, but there was a difference in the Sacha oil between 6 and 9%. If the analysis is performed by the level of inclusion at 3%, there is no difference between the three sources; at 6 and 9%, Sacha oil brought a difference in weight. Crespo and Esteve-Garcia (2001) in Ross 308 females evaluated comparing the one carried out in this study, a basal diet without added fat, and four diets with the addition of 10% of tallow (saturated fatty acids), olive oil (monounsaturated fatty acids), sunflower oil and flaxseed oil (polyunsaturated fatty acids) in a shorter period (between day 28 and 42 of age) than in this study. The results from the statistical analysis showed that the diets with the addition of different oils produced birds that weighed an average of 144 g more than those fed with the basal diet, but there was no difference between the diets with 10% fat added.

Dietary conversion

Table 4 shows the results of the analyses carried out for the diet conversion variable. The specific result of this study shows that the oil source did not affect the conversion variable, and there was only an effect of the oil inclusion level for the period between day 8 and 21 of age, and the interactions between the oil source and the inclusion level

Table 4. Effect of oil source, inclusion level, and interaction between source and inclusion level on diet conversion between days 8-21, 21-42 of age, and cumulative between days 8 and 42 of age.

Effect	Diet conversión		
	Day 8 to 21	Day 21 to 42	Day 8 to 42
Source	*P*>0.05	*P*>0.05	*P*>0.05
Palm	1.361	1.662	1.582
Chicken	1.391	1.745	1.650
Sacha inchi	1.313	1.781	1.644
Level of inclusion (%)	*P*<0.01	*P*>0.05	*P*>0.05
3	1.398[a]	1.758	1.661
6	1.376[a]	1.717	1.618
9	1.291[b]	1.713	1.597
Source*Level	*P*>0.05	*P*<0.05	*P*<0.05
T1	1.344	1.702[abc]	1.606[a]
T2	1.424	1.626[a]	1.576[a]
T3	1.315	1.658[a]	1.564[a]
T4	1.503	1.900[c]	1.796[b]
T5	1.360	1.707[abc]	1.617[a]
T6	1.310	1.629[a]	1.539[a]
T7	1.348	1.672[ab]	1.582[a]
T8	1.343	1.806[abc]	1.661[ab]
T9	1.248	1.865[a]	1.689[ab]
MSE Root	0.104	0.189	0.142
Model *P*-value	0.010	0.073	0.085

[1]T1, T2, T3: palm oil at 3, 6 and 9%. T4, T5, T6: chicken oil at 3, 6, and 9; T7, T8, T9: Sacha inchi oil at 3, 6, and 9%. [abc] Values with different letters in the same column differ significantly.

were only significant during the period between day 21 and 42 of age, including the overall period (between day 8 and 42 of age). According to the above, it was noted that the best feed conversion rate was obtained with the highest level of inclusion, without being different between the two previous levels of inclusion. As with the weight gain variable, it cannot be possible to identify any type of trend in the periods evaluated from the effect of the interaction, suggesting again that in each period there was independence in the behavior of the birds regarding the diet. For the fattening phase, from day 21 to 42 of age, there was no difference among the three levels of inclusion on the palm oil and Sacha inchi oil, but in chicken, oil the worst conversion was at 3% inclusion; in fact, the most limited conversions were obtained with T1 (3% palm oil), T4 (3% chicken oil) and T8 (6% Sacha oil), with no differences among them. At 6 and 9% there was no difference among the three oil sources. For the entire experimental period in palm and Sacha oil, there was no difference among the three inclusion levels, but for chicken oil the worst conversion was at 3% inclusion, at this level, there was no difference between palm and Sacha oils. Based on these results, it could be agreed that to improve the feed conversion rate, the use of oils with saturated and monounsaturated acids, such as palm and chicken oils, would require higher inclusion levels. Different story for polyunsaturated lipid sources such as Sacha inchi, since the conversion would be better at the inclusion level. Griffiths et al. (1977) reached different conclusions. These researchers conducted two experiments in male broilers to evaluate whether the fat sources (two mixtures of animal and vegetable fat, corn oil, and chicken oil) and the level of inclusion (at 0, 3, 6, and 9%) had an extra caloric effect and whether the system of expression of dietary energy - metabolizable or net - influenced the animal response or not. At eight weeks of age, birds fed with formulated diets based on the net energy system had carcasses with less fat and more protein.

The inclusion of corn or chicken oil, with no mixtures of animal fat and vegetable oil, produced heavier birds, without including the level of oil inclusion in the diet. In research conducted by Gaiotto et al. (2000) where soybean oil, acid soybean oil, and bovine tallow with 4% inclusion were used in broiler diets, no difference was established in consumption, weight gain, and feed conversion for the initiation stage neither for the complete cycle from day 1 to 42 of age, as well as no effect on the relative weight of abdominal fat. Several authors have evaluated the effect of oil sources or their inclusion level on the performance of poultry at different stages. For example, Pinchasov and Nir (1992) planned an experiment in which they combined different amounts of vegetable oils and tallow that created varied concentrations of polyunsaturated fatty acids (32, 48, 60, 63, and 70 g 100 g^{-1} of fat). From the performance variables evaluated (body weight at day 21 and 40 of age, weight gain between day 21 and 40 of age, feed intake, and the ratio of weight gain to diet intake), the reference between weight gain to diet intake was the only one affected linearly with the increase in the concentration of polyunsaturated fatty acids in the diet. Scaife et al. (1994) conducted a study to evaluate the effect of the inclusion of 5% of fats with different chain lengths and degrees of unsaturation (bovine tallow, soybean oil, rapeseed oil, oil of marine origin, and a mixture of 0.5:0.5 wt/wt of these fats) upon the performance (diet intake, live weight and featherless weight), carcass characteristics, lipid concentration and fatty acid composition of abdominal fat, liver and breast of male broilers between day 19 and 54 of age. Regarding the performance variables, the birds that received the diet with beef tallow consumed more and presented higher body weights and featherless weights, while with canola oil these indicators were lower. Of all diets, the one that produced the most limited feed conversion was the one with beef tallow. Several researchers found no difference in dietary intake, weight gain, and feed conversion in birds fed with different fat sources and their mixtures (Duarte et al. 2010). Works submitted by Gaiotto and his contributors in 2000, where they used different sources of oils and fats in broilers reported no difference in diet consumption between the sources used, nor for weight gain or feed conversion during the phase from day 1 to 42 of age. The comparison between a control diet with no added fat and other diets with 3% soybean or palm oil (Ayed and Attia 2015) found no difference in feed intake in the starter phase between a control diet with no added fat and other diets with the addition of 3% soybean and palm oil.

Carcass and organ variables

Tables 5 and 6 show the results of the analyses carried out on the variables associated with the carcass and some of the organs evaluated, understood as absolute weight or as the rated weight for slaughter.

According to the information in Table 5, it is possible to establish that the oil source only affected the weight of abdominal fat, and both, the level of oil inclusion and the interaction between the source and the level of inclusion did not have any influence on the variables analyzed. Regarding the influence of the source, it is clear that Sacha inchi oil (outstanding for being high in polyunsaturated fatty acids) led to lower abdominal fat weights, while for the same variable, there was no difference among the other oil sources, which were characterized by having high contents of monounsaturated (chicken oil) and saturated (palm oil) fatty acids.

Table 5. Effect of oil source, inclusion level, and interaction between source and inclusion level on the weight of some carcass and organ components of broiler chickens (Values expressed in grams).

Effect	Carcass	Breast	Legs	Fat	Skin	Wings	Heart	Gizzard	Liver
Source	*P*>0.05	*P*>0.05	*P*>0.05	*P*<0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05
Palm	1925.17	750.39	549.78	54.88[a]	154.12	185.22	15.34	37.42	58.36
Chicken	1975.35	769.06	576.28	46.93[a]	149.69	186.89	15.98	37.48	56.01
Sacha inchi	1957.02	711.44	569.22	36.48[b]	147.26	195.61	15.77	36.43	57.68
Inclusion level (%)	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05
3	1974.40	756.444	527.84	48.76	153.89	202.06	15.22	38.58	59.14
6	1931.91	723.61	549.89	44.28	148.59	181.00	15.67	36.47	57.53
9	1951.23	756.44	572.72	45.28	148.59	184.67	16.21	36.29	55.38
Source*Level	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05
T1	1914.59	735.70	540.24	58.70	166.42	204.29	14.88	38.41	61.54
T2	1929.51	732.60	557.56	59.03	151.55	166.92	15.96	35.53	54.99
T3	1931.43	757.90	549.80	46.52	138.46	180.25	15.10	38.56	57.23
T4	1977.49	752.50	552.85	42.16	148.53	196.24	15.43	36.86	54.96
T5	1971.03	738.00	565.51	47.38	144.24	184.56	14.87	40.56	58.26
T6	1977.57	786.40	593.33	50.79	149.18	174.77	17.53	34.13	53.23
T7	2031.16	728.70	607.58	44.60	138.46	196.80	15.17	38.91	58.19
T8	1895.20	729.00	542.96	44.75	152.81	196.30	15.75	35.14	60.82
T9	1944.72	731.60	588.36	38.78	159.14	202.93	16.27	36.85	56.87
Slaughter weight	*P*<0.01	*P*<0.01	*P*<0.01	*P*>0.05	*P*>0.05	*P*<0.01	*P*>0.05	*P*<0.01	*P*<0.01
Root MSE	96.37	51.51	73.84	13.43	23.83	25.68	2.66	4.60	5.94
Model *P*-value	*P*<0.01	*P*<0.01	*P*<0.05	*P*<0.01	*P*>0.05	*P*<0.05	*P*>0.05	*P*<0.01	*P*<0.01

When naming some chicken organs and carcass components such as the slaughter weight rate (Table 6), the same trend is evident as in Table 5. That is, the oil source only affected the rate of abdominal fat weight. Both, the level of oil inclusion and the interaction between the source and the inclusion level, had no influence on the variables analyzed, and Sacha inchi oil produced carcasses with the lowest rate of abdominal fat in relation to slaughter weight, having no difference between the other two sources of oil evaluated.

In chicken, the content of abdominal fat is a reliable indicator for judging body fat content. Due to this relationship, it could be expected that the dietary fat effect could be reflected in both abdominal and body fat (Becker et al. 1979). This effect would not only depend on the level of oil inclusion in the diet but also the characteristics and composition of its fatty acids. So, for Crespo and Esteve-Garcia (2002), diets with a higher content of polyunsaturated fatty acids lead to greater absorption of fatty acids and energy than those containing saturated fatty acids; however, it is possible that diets with this type of fatty acids present greater oxidation, which could lead to more endogenous synthesis of these acids from carbohydrates, which would imply a higher energy cost. Therefore, for these authors, if energy retention increases

when diets with saturated and polyunsaturated fatty acids are offered, energy consumption should increase independently from fatty acid synthesis.

Although it has been stated that abdominal fat content in chicken depends on both the level of oil inclusion in the diet and the characteristics and composition of its fatty acids, literature records indicate variation in the results generated. It is important to note that most of these studies were conducted with oils other than palm, chicken, and Sacha inchi oils used in this study.

Crespo and Esteve-Garcia (2001) studied four oil sources (tallow, olive oil, sunflower oil, and linseed oil), with two

Table 6. Effect of oil source, inclusion level, and interaction between source and inclusion level on the weight of some carcass and organ components of broiler chickens (Values expressed as percentage of slaughter weight).

Effect	Carcass	Breast	Legs	Fat	Skin	Wings	Heart	Gizzard	Liver
Source	*P*>0.05	*P*>0.05	*P*>0.05	*P*<0.01	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05
Palm	68.98	26.63	19.53	1.95[a]	5.46	6.58	0.55	1.33	2.07
Chicken	70.68	27.24	20.44	1.67[a]	5.32	6.63	0.57	1.33	1.99
Sacha inchi	70.37	26.06	20.85	1.34[b]	5.40	7.17	0.58	1.33	2.13
Level of inclusion (%)	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05
3	70.54	26.56	20.11	1.72	5.40	7.10	0.54	1.36	2.08
6	69.41	26.19	20.01	1.59	5.38	6.62	0.57	1.32	2.10
9	70.09	27.18	20.69	1.64	5.40	6.67	0.59	1.31	2.00
Source*Level	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05
T1	68.55	26.45	18.92	2.05	5.93	7.28	0.52	1.37	2.18
T2	69.02	26.29	21.02	2.10	5.47	6.00	0.57	1.24	1.97
T3	69.39	27.15	19.67	1.70	5.00	6.48	0.55	1.39	2.07
T4	70.86	27.01	19.78	1.52	5.34	7.03	0.55	1.33	1.97
T5	69.98	26.60	20.22	1.65	5.16	6.57	0.52	1.43	2.06
T6	71.21	28.13	21.32	1.84	5.48	6.30	0.64	1.23	1.92
T7	60.78	25.20	19.45	2.15	4.16	6.35	0.71	1.47	2.12
T8	69.50	25.77	19.20	1.00	5.35	7.32	0.62	1.35	2.11
T9	69.69	26.27	21.10	1.39	5.72	7.24	0.58	1.32	2.04
MSE root	3.30	3.32	1.06	0.483	1.03	1.06	0.165	0.184	3.342
Model *P*-value	*P*>0.05	*P*>0.05	*P*>0.05	*P*<0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05

[1]T1, T2, T3: palm oil at 3, 6 and 9%. T4, T5, T6: chicken oil at 3, 6, and 9%; T7, T8, T9: Sacha inchi oil at 3, 6, and 9%. [a,b,c] Values with different letters in the same column differ significantly.

levels of inclusion (6 and 10%), in males from day 21 to 42 of age and females from day 21 to 49 of age. The results of the study showed that abdominal fat and cholesterol content in their legs were lower in birds fed with sunflower and linseed oils. In females, only diets with tallow or olive oil inclusion did increase abdominal fat linked to the level of inclusion. The results of the study suggested that oils with polyunsaturated fatty acids produced less abdominal fat deposition than those with saturated or monounsaturated fatty acids, becoming a similar result as the one previously found with the Sacha inchi study.

Meanwhile, Ferrini et al. (2008), in their study, established that 42-day-old chicks fed with the highest level of polyunsaturated fatty acids diet had lower fat deposition in the skin, both in absolute and relative weight, than those fed with saturated diets. Moreover, the lowest abdominal fat deposition was achieved with the linseed oil diet high in linolenic acid; these authors concluded that chickens fed with polyunsaturated fatty acids compared to those fed with saturated fatty acid diets reduced fat content in their skin and abdomen by 9 and 30%, respectively. Villaverde et al. (2006) stated that birds fed with oils with

unsaturated fatty acids such as sunflower and flaxseed decreased body fat deposition compared to those fed with saturated sources such as tallow or olive oil.

According to other studies, an effect of oil type on fat deposition in chicken has not been reported. Griffiths et al. (1977) evaluated the inclusion of 0, 3, 6, and 9% corn oil, highlighted for its polyunsaturated fatty acid content, chicken oil (monounsaturated fatty acids), and their blends in broiler diets. These researchers found no difference in dietary intake, but they found a difference in body weight, being lower for the 9% inclusion. There was a significant effect on feed conversion between 0 and 9%, but not between sources. In line with the present work, these authors reported no significant difference between the levels of inclusion in the oil sources evaluated for abdominal fat weight.

In studies conducted by Pesti et al. (2002), no differences were recorded for variables such as body weight, weight gain, feed intake, or feed conversion factor for 39-day-old broilers fed with diets containing chicken oil and palm oil both at 3 and 6% inclusion. As for abdominal fat, there was no difference associated with the level of the oil source inclusion in the diet; but they observed less abdominal fat deposition in birds with a diet containing chicken oil compared to those that received a diet with soybean oil or the mixture of these two. In this study, Sacha inchi oil produced the least amount of abdominal fat in both absolute and relative values. It can be established that oil sources with a higher level of polyunsaturated fatty acids, such as Sacha inchi oil, produce less abdominal fat compared to saturated sources such as palm oil. Opposite to this result, Scaife et al. (1994) reported no differences in the abdominal fat weight in birds that were fed between days 19 to 52 of age using diets with 5% inclusion of saturated fats such as tallow and unsaturated fats such as soybean oil, rapeseed oil, seafood oil and mixtures of these in a ratio of 50:50 and weight to weight. Duarte et al. (2010) established that bovine tallow, chicken oils, degummed soybean oil, and the mixtures of these oils with tallow and oil, provided in broiler diets, did not influence carcass yield, cuts, and the amount of abdominal fat. These results are in line with those from Crespo and Esteve-Garcia (2002) and Laral et al. (2006).

Firman et al. (2008) reported no difference in performance variables (total or phased consumption of total diet, weight gain, feed conversion), nor in the weight of the hot carcass, leg, wing, breast and abdominal fat among the fat sources evaluated (yellow fat, vegetable oil/animal fat blend, soybean oil, chicken oil, pork fat and beef tallow) provided at the same inclusion level (3%) to Cobb chickens between the day of hatch and seven weeks of age.

Laral et al. (2006) evaluated the effect of soybean degummed oil, poultry viscera oil, acid soybean oil, a mixture of 50% soybean oil and 50% viscera oil, and a mixture of 50% soybean oil and 50% acid soybean oil on the performance, the breast, leg, and carcass composition, and fatty acid profile carcass in broiler chickens. The results were consistent considering that there was no difference between the oils evaluated for carcass and cut performance, crude protein, moisture, and fat content in the carcass and the leg and breast muscles. The situation was different in the fatty acid composition of the tissues analyzed. The carcass of birds fed with viscera oil had a higher rate of monounsaturated fatty acids, and the polyunsaturated fatty acids deposition was affected by the source of oil used in the diet.

CONCLUSIONS

The results of this experiment suggest that there was no consistency in the effects of the evaluated oil source, the level of oil inclusion in the diet, and the interaction between these two sources on the variables of dietary intake, weight gain, and conversion of the diet in the periods established for the evaluation.

In the variables of the carcass and some organs of the birds, the deposition of abdominal fats was the only aspect affected by the source. Sacha inchi oil, noted for being high in polyunsaturated fatty acids, promoted carcasses with a lower rate of abdominal fat in relation to slaughter weight, with no difference between palm and chicken oils.

ETHICAL CONSIDERATIONS

All processes within the experimental analysis were carried out according to the guidelines suggested by "The international guiding principles for biomedical research involving animals" (CIMOS and ICLAS 2012). This project had the endorsement of the committee of ethics in animal experimentation of the National University of Colombia in Medellín CMED-006 as of March 17, 2016.

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Protein profiles of follicle fluid of different sizes in cows and buffaloes

Perfiles de proteínas del fluido de folículos de diferente tamaño en vacas y búfalas

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Keywords:
Bos indicus
Bubalus bubalis
Composition
Follicular liquid
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ABSTRACT

In vitro embryo production systems in buffaloes have great productive perspectives and opportunities for improvement. Among these, comparative studies with species with more significant advances in reproductive biotechnology have been developed. Accordingly, this work aimed to identify the differences in the electrophoretic profiles of proteins in the follicular fluid (FF) of cows and buffaloes and their possible association with follicle size. FF was obtained at the central abattoir in Medellín (Antioquia), Colombia, from small (<7 mm) and large (>7 mm) follicles from the ovaries of 25 cows (*Bos indicus*) and 20 buffaloes (*Bubalus bubalis*). The total protein content of the FF was quantified and subsequently depleted of albumin and immunoglobulins. Samples were subjected to denaturing electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) to determine the electrophoretic profiles using a photodocumenter. The values obtained for the relative amount of each band were compared between species and follicle sizes using the Mann-Whitney test. The results showed no significant differences in total protein concentration between the different follicle sizes and species. Further, 72.6% of the FF proteins are immunoglobulins and albumin. The profiles of small follicles (<7mm) in cows presented 19 bands and 11 in buffaloes. The molecular weight range of the bands detected was between 5 and 250 kDa. Quantitative differences of the proteins in the follicular fluids evaluated were identified. The information obtained may contribute to elucidating the physiological differences between large and small follicles but does not explain the differences between species.

Palabras clave:
Bos indicus
Bubalus bubalis
Composición
Líquido folicular
Proteínas

RESUMEN

Los sistemas de producción de embriones *in vitro* de búfalo tienen grandes perspectivas productivas y oportunidades de mejora. Entre ellas, el desarrollo de estudios comparativos con especies en las que existen mayores avances en esta biotecnología reproductiva. El objetivo del presente trabajo fue identificar diferencias en los perfiles electroforéticos del fluido folicular (FF) de diferentes tamaños en vacas y búfalas. Se obtuvo FF de folículos pequeños (<7 mm) y grandes (>7 mm), provenientes de ovarios de 25 vacas (*Bos indicus*) y 20 búfalas (*Bubalus bubalis*) colectados en la central de faenado de Medellín (Antioquia), Colombia. El contenido de proteína total del FF fue cuantificado y posteriormente se sometió a un proceso de depleción de albúmina e inmunoglobulinas. Para determinar los perfiles electroforéticos a través del análisis en un fotodocumentador, las muestras se sometieron a electroforesis desnaturalizante en geles de poliacrilamida y dodecilsulfato sódico (SDS-PAGE). Se compararon los valores obtenidos de la cantidad relativa de cada banda entre especies y tamaños de folículos, mediante la prueba de Mann-Whitney. Los resultados evidenciaron que no hubo diferencias significativas en la concentración de proteína total entre los diferentes tamaños de folículo y las especies. Además, 72,6% de las proteínas del FF evaluado son inmunoglobulinas y albúmina. Los perfiles de los folículos pequeños (<7mm) presentaron 19 bandas en las vacas y 11 en las búfalas. Las bandas detectadas estuvieron en un rango de peso molecular entre 5 y 250 kDa. Se identificaron diferencias cuantitativas de las proteínas en los fluidos foliculares evaluados. La información obtenida puede contribuir a elucidar las diferencias fisiológicas entre folículos grandes y pequeños, pero no explica las diferencias entre especies.

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As signal-transmitting and effector molecules, proteins play a leading role in any biological process, including follicular development and competent oocyte formation. During follicular development, proteins are associated with signal transmission and the induction of molecule production to express behaviors related to fertilization and early embryo development processes (Ishak et al. 2022). However, protein expression and reproductive parameters vary dramatically between species, even though they are phylogenetically close, such as cattle and buffalo, both classified as bovids. Reproductively, buffaloes have been reported to have a later puberty, shorter estrous cycles, and a longer gestational period than cows (Gimenes et al. 2015). Some proteins have been characterized and associated with these aspects (Valckx et al. 2015). However, very few studies have focused on studying them within the physiological and comparative contexts between species, allowing the proposal of theories about their role in reproductive parameters.

In this sense, there are several proteins in follicular fluid (FF) that are related to aspects such as nuclear and cytoplasmic maturation of the oocyte, parameters that are specific to each species and may vary according to the time of the estrous cycle (Filipiak et al. 2016). Evaluating these variations is the basis for searching for potential oocyte and follicle quality markers. The ovary has two functions: 1) an endocrine function associated with the production of steroidal hormones and proteins and 2) another related to the production of gametes. The second is carried out by two mechanisms: oogenesis and folliculogenesis (Valckx et al. 2015). Folliculogenesis begins during fetal life, becoming active around day 140 of gestation in cattle. The follicle grows from 25-30 µm to reaching about 15 mm at ovulation in a continuous growth process. However, not all of them grow. Most of them suffer from atresia during their reproductive life. The follicle is composed of the oocyte and the granulosa cells surrounding it; during development, these multiply and become the cells that produce the FF, which will be accumulated in a structure called antrum (Fair 2003). Follicular deviation has been defined as the moment during the growth of a follicle cohort when one acquires more LH receptors. This follicle continues its development towards ovulation; meanwhile, all others within the cohort that have not been chosen regress. Studies in buffalo have shown that follicular deviation occurs when follicles are around 7 mm. It is assumed that this size may define two moments during development and, thus, the physiology of the follicle (Gimenes et al. 2011).

The FF is an exudate of blood plasma modified by the metabolic activity of granulosa cells and possesses different proteins, glycoproteins, glycosaminoglycans, and steroids (Gordon 2003). The composition is variable and depends on the physiological state of the follicle and the time of the estrous cycle of an individual. Its composition has been reported as being affected by environmental conditions and the age of the animal (Iwata 2017). In the FF, there are proteins such as albumin, polypeptides, and lysosomal enzymes, in addition to ions, ascorbic acid, and steroids, including estradiol and progesterone. Furthermore, there are also gonadotropins, such as LH, FSH, alpha and dimeric inhibins (between 34 kDa and >160 kDa), prolactin, high-density proteins, glycosaminoglycans, and growth factors (Neira-Rivera et al. 2020). They all compose a complex mechanism that influences follicular dynamics and contributes to oocyte maturation and growth. Schweigert et al. (2006) found in human beings some differences between FF proteins and serum proteins associated with the reproductive process, mainly because some are synthesized by the interaction between follicle components, including theca cells, granulosa, and the oocyte itself. Moreover, the authors found peptides between 6.9 and 13.8 kDA in higher concentrations in FF than in serum. Albumin and immunoglobulins, predominantly IgG, have been identified as the most abundant proteins in FF from humans, pigs, canines (Fahiminiya et al. 2010), and horses (Fahiminiya et al. 2011).

In this context, the knowledge derived from the study of FF proteins can contribute to the development of embryo production systems in species and, in this case, to the development of buffalo production systems. This study aimed to evaluate if there are differences in FF protein profiles in cow and buffalo follicles before and after the follicular deviation, as a contribution to the knowledge of the species and to the improvement of *in vitro* embryo production programs, especially in buffaloes.

MATERIALS AND METHODS

Animals and sampling

For this study, 25 ovaries from *Bos indicus* cows and 20 from *Bubalus bubalis* buffaloes were collected from a slaughterhouse in Medellín, Antioquia Department,

Colombia. The follicles of each ovary were measured with a graduated ruler and classified according to their size as large or small, i.e., larger, or smaller than 7 mm. After measurement, the FF was aspirated with an 18-gauge needle attached to a 10 mL syringe. In large follicles, the volume obtained was sufficient for analysis, while in small ones, it was necessary to make a pool of aspirated follicles (3 to 5). The FF obtained was centrifuged at 13,000 rpm for 30 min at 4 °C, and phenylmethylsulfonyl fluoride (PMSF) dissolved in dimethyl sulfoxide (DMSO) was added as a protease inhibitor. FF samples were stored at -20 °C for subsequent analysis.

Protein quantification

The total protein concentration in FF was determined through the Bradford method (Bradford 1976) in 96-well Elisa microplates. The measurement was performed at 620 nm in a Biotec Cytation Elx 800 spectrophotometer. Then, 200 µL of FF samples were used to remove albumin and immunoglobulins, and the ProteoPrep Blue Albumin and IgG Depletion Kit from Sigma (Catalog No PROTBA-1KT) was used, according to the manufacturer's instructions.

Electrophoresis in denaturing gels

For denaturing electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), 7 cm mini gels with an acrylamide/bis-acrylamide concentration of 15% were used according to the Laemmli method (Laemmli 1970). The weight marker Precision Plus Protein™ Dual Xtra from BioRad (Bio-Rad), with a range between 2 to 250 kDa, was used. Electrophoresis was performed in a Mini Protean II chamber (Bio-Rad) with a constant voltage of 140 V for 75 min to determine the molecular weight of the protein bands. The gels were developed by staining with 0.025% Coomassie Brilliant Blue for 2 h, 10% acetic acid, and 30% methanol destaining solution. The gels were digitized in an Image Gel Doc™ documenter (Bio-Rad). They were analyzed with the Image Lab software (Bio-Rad) to determine the molecular weights by comparison with the weight marker and the relative amounts of the bands evidenced through the measurement of the optical density in pixels obtained from the area and depth of each band.

Statistical analysis

Descriptive species and follicle size statistics were applied to protein concentration values obtained and compared using the Wilcoxon and Mann-Whitney test, with a *P*-value $P<0.05$ considered significant. All analyses were carried out with the statistical program R Studio.

RESULTS AND DISCUSSION

Follicle fluid was collected from the ovaries of 25 bovines and 20 buffaloes. Between 10 and 500 µL/ovary of FF were obtained. It was classified by the size of the follicle from which they were obtained. Finally, FF from 13 buffalo samples (five from large and eight from small follicles) and 16 cow samples (eight from large and eight from small follicles) were analyzed. The initial protein concentration and the one after removing albumins and immunoglobulins (depletion) were determined. Total protein concentration in FF from small and large follicles was 53.82±21.79 mg μL^{-1} for bovine follicles and 57.2±19.74 mg μL^{-1} in buffalo follicles. There were no significant differences in total protein concentration between follicles of the same size or species or follicles of different sizes for both species ($P>0.05$) (Figure 1A).

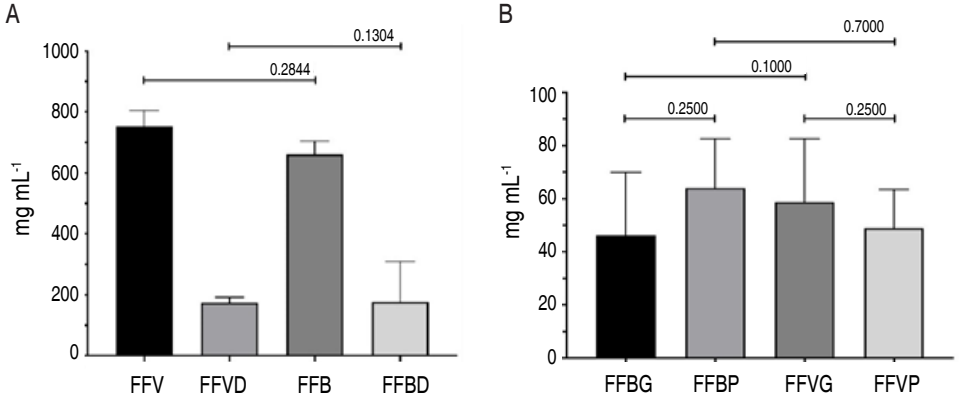

Figure 1. A. Total protein from cow (FFV) and buffalo (FFB) follicle fluid before (original) and after depletion (FFVD, FFBD, respectively). B. Total protein concentration in cows and buffaloes from big (>7 mm; FFVG, FFBG, respectively) and small follicles (<7 mm; FFVP, FFBP, respectively).

In all samples, after the depletion of the most abundant proteins (albumin and immunoglobulins), the total protein concentration decreased by about 70% (Figure 1B). This was done to find protein bands with less concentration that were not detected when most proteins were not eliminated. However, there was no difference (P>0.05) in the number of detected bands among the samples to which the removal procedure was performed.

The analysis of the electrophoretic profiles shows that the undepleted FF samples from cows showed a total of 19 protein bands in the small follicles (maximum number of bands), while the buffalo FF samples had a maximum of 11 bands. Large FF from undepleted cows presented a total of 11 protein bands (maximum number of bands), while buffalo FF samples had a maximum of 13 bands. The detected bands had a molecular weight range between 5 and 250 kDa (Figure 2).

All the band proteins were grouped according to their molecular weight before depletion. Buffaloes showed more bands than cows (24 vs. 31). However, there were more bands in the cow samples (32) than in those of buffaloes (22) after the depletion procedure was performed.

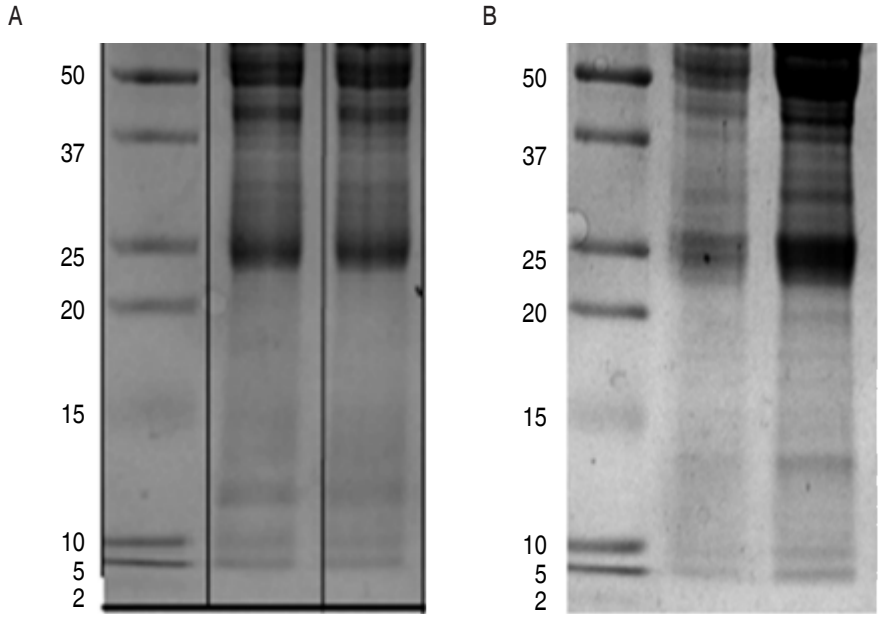

Figure 2. Electrophoretic profile of follicle fluid proteins without depletion in A. cow ovaries and B. Buffalo ovaries. Line 1 (left): Molecular weight marker (kDa); line 2 (center): Large follicles (>7 mm); line 3 (right): Small follicles (<7 mm).

Moreover, in all the evaluated groups, the highest amount of protein in FF corresponded to the group of less than 40 kDa, specifically in the 13/21 (cows) and 15/25 (buffaloes) groups without depletion, and in the 23/32 (cows) and 13/22 (buffaloes) groups that were depleted (Table 1 and Figure 3).

Table 2 shows the number of proteins grouped by molecular weight within the categories analyzed. The qualitative results (relative % proportion of number of bands) did not coincide with the quantitative results (proportion of each band within the total). Proteins smaller than 20 kDa were not so abundant. In contrast, those between 20 and 6.0 kDa were more abundant.

Concerning protein concentration in FF of buffaloes, the results obtained in this study (57.2±19.74 µg μL^{-1}) are lower than those reported by Behera et al. (2016) in buffaloes in India (61.5 µg μL^{-1}) without alterations in their estrous cycle. In Colombia, Neira-Rivera et al. (2020) reported that in cows, the average total protein concentration of FF (<3 mm) was 60.6±16.6 µg dl^{-1}, specifically 50.6±21.0 µg dl^{-1} in follicles with a size between 3 to 6 mm, and 53.4±16.3 µg dl^{-1} in follicles of more than 6 mm. These values are similar to those obtained in this study.

In contrast, Shabankareh et al. (2013) reported a decrease in total protein concentration as follicle size increases

Table 1. Number of bands grouped by molecular weight in buffaloes and cows.

Band N	> 100 kDa n (weight kDa)	80 to 99 kDa n (weight kDa)	60 to 79 kDa n (weight kDa)	40 to 59kDa n (weight kDa)	20 to 39 kDa n (weight kDa)	< 20 kDa n (weight kDa)
Cow, w/depletion N=21	2 (250, 190)	2 (75, 86)	0	4 (59, 47, 44, 41)	5 (35, 32, 28, 33, 26)	8 (17, 13, 11, 8, 18, 14, 13, 10)
Buffalo, w/depletion N=25	4 (190, 104, 216, 131)	2 (75, 76)	1 (64)	3 (52, 43, 44)	7 (38, 31, 25, 35, 33, 28, 21)	8 (19, 16, 13, 9, 19, 18, 14, 9)
Cow, Depleted N=32	2 (168, 250)	1 (82)	1 (76)	5 (58, 45, 43, 40, 48)	11 (35, 32, 27, 23, 21, 33, 26, 24,23, 21, 21a)	12 (17, 14, 12, 10,18, 16, 12, 10,7, 6, 6a, 5)
Buffalo, Depleted N=22	3 (194, 182, 102)	1 (84)	2 (60, 76)	3 (45, 41, 43)	6 (39, 33, 35, 23, 30, 25)	7 (19, 18, 13, 19, 17, 13, 10)

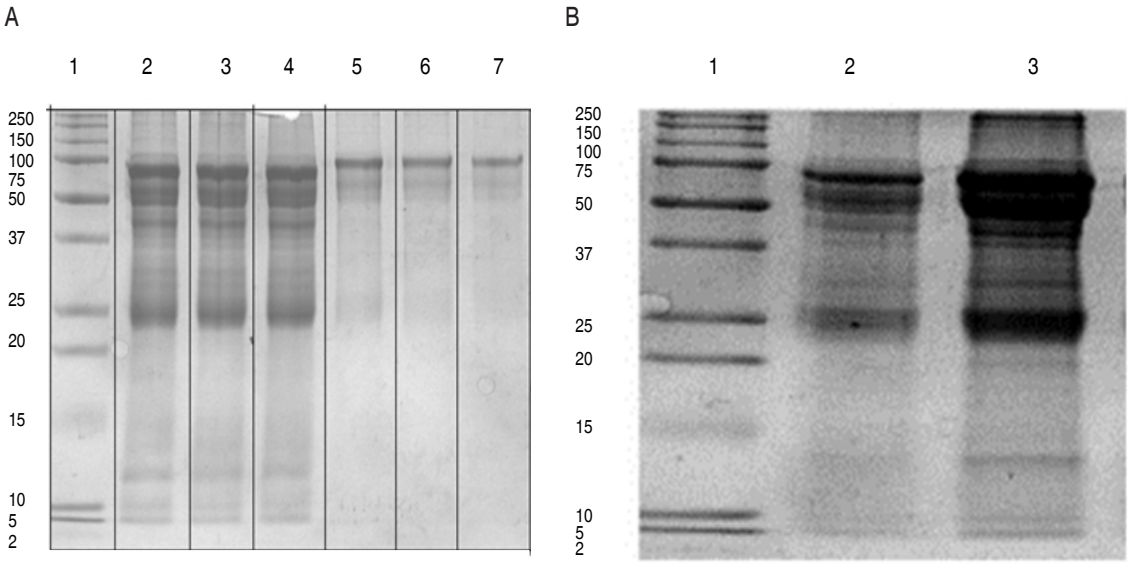

Figure 3. Comparison of uni-dimensional electrophoretic profiles of follicle fluid proteins with and without depletion. A. Cows: Line 1 Molecular Marker (MW) (kDa); lines 2 and 3>7 mm; line 4 <7 mm undepleted; lines 5 and 6>7 mm; line 7<7 mm depleted. B. Buffaloes: Line 1 MW (kDa); line 2>7 mm depleted; line 3 undepleted.

in cows, suggesting that the cause was a dilution of the proteins due to the increase in follicle fluid volume. Other authors have reported that in buffaloes, there are no significant differences in the amount of follicle protein between cyclic (0.49±1.07 µg μL^{-1}) and acyclic animals (0.6±0.28 µg μL^{-1}).

After depletion, 72.3% of the total proteins present in the FF were immunoglobulins and albumin, with no statistical differences between species or the follicular sizes evaluated. Fahiminiya et al. (2010) reported that some previously undetected proteins were observed after albumin depletion. Therefore, they were not analyzed, and the authors suggested a possible bias in the analysis. Furthermore, these are associated with specific events of follicular development and their physiological role, i.e., oxidative stress and as scavengers of reactive oxygen species. Figure 1 shows that in the depleted samples,

there is a decrease in the number of bands between 3 and 75 kDa, which are albumin and immunoglobulins.

For protein banding patterns, more protein bands were identified in buffalo FF samples than in cow FF before depletion (25 vs. 21). However, after depletion, 34 protein bands were found in FF from cows vs. 22 from buffaloes. Neira-Rivera et al. (2020) found 25 bands in cows, a lower number than reported in this study.

When the bands were grouped by molecular weight, 76.4% in cows and 60% in buffaloes correspond to proteins below 40 kDa (Table 2), maintaining the same ratio after depletion with 71.8 and 59.0%, respectively. Similar results can be observed when comparisons are made with data grouped by follicle size (Table 2). Notably, the relative number of bands is higher in those with molecular weights higher than 40 kDa. Fu et al. (2016) reported that the bands identified by SDS-PAGE in FF from swamp buffaloes were between 10 and 200 kDa. They managed to identify 363 proteins, of which 153 were related to some metabolic or signaling pathway. Additionally, they found 11 proteins with differential expression between large and small follicles involved in inhibiting serine and threonine proteases, oxidation, and the complement cascade. Subsequently, the authors identified some candidate proteins as molecular markers of follicle quality. Finally, the same authors compared the proteins identified in the buffalo FF with those of human serum and found that of 349 proteins identified in the buffalo FF, 217 were shared with human serum, and 132 were exclusive to the FF.

Table 2. Effect of the species and depletion procedure on the protein proportion in follicle fluid of cows (V) and buffaloes (B).

Band Proportion (kDa)	More than 100 (kD)	80 to 99 (kD)	60 to 79 (kD)	40 to 59 (kD)	20 to 39 (kD)	Less than 20 n-(kD)
BIG FOLLICLE (> 7 mm)						
w/depletion V	0.43	0	19.88	21.81	25.69	33.32
w/depletion B	6.12	0	31.59	28.04	32.64	1.61
depleted V	6.49	13.32	0	26.19	50.71	3.18
depleted B	13.46	0	11.90	21.60	29.76	23.25
SMALL FOLLICLE (< 7 mm)						
w/depletion V	1.56	31.21	0	42.05	22.08	3.08
w/depletion B	13.42	0	17.50	16.41	37.50	16.06
depleted V	1.67	0	9.72	12.55	41.11	35.42
depleted B	5.74	14.44	0	34.67	31.39	13.71

This work found differences between the number of proteins identified in the species studied and the size of the follicle from the samples. Since follicular development has the same pattern, it can be affirmed that the species studied perform the same function with different proteins and that these are responsible for the differences in follicular development or preparation for ovulation.

Since the follicle structure acts as a barrier to the diffusion of proteins larger than 100 kDa, it is possible that those found in the FF do not necessarily reflect what is happening in the bloodstream, allowing the granulosa cells to exert their function in follicle development. This has been evidenced by reports describing that the protein concentration can be up to to twice as high in healthy follicles compared to atretic follicles (Clarke et al. 2006).

Several proteins have been identified in the FF, such as follicle-stimulating hormone (30 kD), inhibin (32 kDa), some growth factors (<30 kDa), transforming factor β (7.5 kDa), fibroblast growth factors (16-17 kDa), uterine serpins (52 kDa), albumin (62-69 kDa), immunoglobulins

with their two heavy chains (50-70 kDa) and two light chains (23 kDa), transferrin (78-80 kDa), complement factors (31.8 kDa), gelsolin (80 kDa), arsenic methyl transferase (42.5 kDa), vitamin D (54.9 kDa), gelatinase B (91.5 kDa), lactotransferrin (78.05), and osteopontin (45 kDa) (Neira-Rivera et al. 2020). All these proteins perform some function in the development of the follicle, ovulation, or the atresia process. Many may be included in the protein bands found in this research, but this needs to be confirmed in future works using two-dimensional gel electrophoresis and mass spectrometry. It can be evidenced that the proteins of low molecular weight are those most associated with reproductive processes, while those of higher molecular weight correspond to the basal functions of the organism.

Regarding the effect of FF proteins on reproductive function, Ghosh et al. (2005) immunized goats with protein fractions larger than 30 kDa in buffalo FF and observed a lengthening of the cycle and a delay in the estrus onset. Another aspect associated with the functions of the proteins identified in human FF, which may have a plasma origin, is that they are related to inflammation, reaffirming the theory that it assimilates the ovulatory process into an inflammatory process (Zamah et al. 2015).

CONCLUSIONS

The diversity observed among species may reflect the significant number of possibilities that nature has to exert a function and events associated with follicle growth and their consequences on oocyte maturation. This forces researchers to be careful when extrapolating results and protocols from one species to another. Quantitative and qualitative differences exist in the proteins present in the follicular fluid evaluated from cows and buffaloes, both classified as Bovidae, and their follicle sizes (large and small). This information can contribute to the explanation of the physiological differences between both species.

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Impact of light conditions on the early development of *Cinchona officinalis*

Impacto de las condiciones lumínicas en el desarrollo temprano de *Cinchona officinalis*

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Keywords:
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Forest massif
Forest plantation
Site conditions

ABSTRACT

Cinchona officinalis, a native plant species known in Peru as the cinchona tree, has civic importance because it represents the plant wealth of this nation and medicinal importance since it was for more than 300 years the only cure for malaria, and is currently threatened. The aim was to determine the effect of light conditions on the percentage of mortality, height increase, and diameter increase of *Cinchona officinalis*. In the community of La Cascarilla (5°39'48.87 "S 78°54'35.24 "W), two treatments were installed, forest massif (FM) and enrichment bands (EB). For each treatment, three replicates and 16 plants per replicate were used. Monthly evaluations were carried out in which the number of dead plants, plant height, and diameter were recorded. These parameters were correlated with precipitation, temperature, and photoperiod records of the study area. The results showed that mortality in FM was 27.1% higher than that reported in EB, while the increase in height and diameter increment in the FM plot was 45.5 and 25.1% higher than that obtained in EB. In addition, a negative correlation was observed between the percentage of mortality and precipitation (r_s=-0.54), and between the increase in height and diameter increment with the maximum temperature (r_s=-0.73 and r_s=-0.60, respectively) for the FM treatment, while for the EB treatment, there was a negative correlation between the increase in height and precipitation (r_s=-0.55) and a positive correlation between the diameter increment and the minimum temperature (r_s=0.53). In general, shaded conditions allow a higher survival rate at the cost of reducing height and diameter increment.

Palabras clave:
Árbol de la quina
Fajas de enriquecimiento
Macizo forestal
Plantación forestal
Condiciones de sitio

RESUMEN

Cinchona officinalis, es una especie vegetal nativa conocida en Perú como árbol de la quina, tiene una importancia cívica porque representa la riqueza vegetal de esta nación y una importancia medicinal puesto que fue por más de 300 años la única cura para la malaria, y actualmente se encuentra amenazada. El objetivo fue determinar el efecto de las condiciones de luz sobre el porcentaje de mortalidad, incremento en altura e incremento diamétrico de *Cinchona officinalis*. En la comunidad de La Cascarilla (5°39'48,87"S 78°54' 35,24"O) se instaló dos tratamientos, mediante macizo forestal (FM) y fajas de enriquecimiento (EB). Para cada tratamiento se empleó tres repeticiones y 16 plantas por repetición. Se realizaron evaluaciones mensuales en las que se registró el número de plantas muertas, altura de la planta y diámetro. Estos parámetros se correlacionaron con registros de precipitación, temperatura y fotoperíodo de la zona de estudio. Los resultados mostraron que la mortandad en FM fue un 27,1% superior a lo reportado en EB, mientras que el incremento en altura e incremento diamétrico en la parcela FM fue un 45,5 y 25,1% superior a lo obtenido en EB. Además, se observó una correlación negativa entre el porcentaje de mortandad y la precipitación (r_s=-0,54), y entre el incremento en altura e incremento diamétrico con la temperatura máxima (r_s=-0,73 y r_s=-0,60, respectivamente) para el tratamiento FM, mientras que para el tratamiento EB, se observó que existe correlación negativa entre el incremento en altura con la precipitación (r_s=-0,55) y existe una correlación positiva entre el incremento diamétrico y la temperatura mínima (r_s=0,53). En general, las condiciones de sombra permiten una mayor tasa de sobrevivencia a costa de reducir su incremento en altura y diámetro.

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Peru is one of the most megadiverse countries on the planet (Rodríguez and Young 2000), which is home to several very important medicinal and food plants (De-la-Cruz et al. 2007), among them is the cinchona tree (*Cinchona officinalis*), which has great importance for the country since it represents its plant wealth (Álvarez 2013); in addition, *C. officinalis* has medicinal importance because it contains alkaloids that were used for more than 300 years as the only cure for malaria (Bharadwaj et al. 2018). Cinchona alkaloids are considered to be the most influential tree bark-derived medicine in human history (Prendergast and Dolley 2001).

Different studies report that *C. officinalis* requires specific conditions to develop, in addition to the fact that its distribution range is limited (Armijos-González and Pérez-Ruiz 2016). In Peru, *C. officinalis* is found in Andean Forest areas, specifically in the Cajamarca and Piura regions (Huamán et al. 2019). As of 2023, the habitat of *C. officinalis* is being degraded by various factors, most notably agriculture, and cattle ranching (Fernandez et al. 2022; Huamán et al. 2019); in addition, *C. officinalis* has little natural regeneration due to its low germination capacity (Valdiviezo et al. 2018), these conditions make it difficult to locate large populations in natural forests (Huamán et al. 2019), leading to the prioritization of the conservation and recovery of *C. officinalis* in Peru (Albán-Castillo et al. 2020).

Similarly, to other crops, *C. officinalis* depends on growing conditions around the plant and efficient light capture (Asare et al. 2017). This second factor has a direct effect on the primary metabolism of plants influencing: morphology, cell growth, and resource allocation (Bastías and Corelli-Grappadelli 2012); in addition, light properties have a direct effect on the production of secondary metabolites (Sivakumar et al. 2017). Light is a very necessary abiotic resource for the growth and development of plant species (Wang et al. 2019), low light conditions can limit the synthesis of some defenses by limiting carbon uptake (Calder et al. 2011). In addition, shaded conditions can generate metabolic changes and imbalances in the photosynthesis process, which implies limiting plant growth (Huang et al. 2018).

However, there are other studies indicating that shade-grown crops tend to reduce the negative impacts generated by high temperatures (Mensah et al. 2022) because they reduce radiation load, leaf temperature, and water stress (Fernández-Milmanda and Ballaré 2021). In addition, shade can even improve photosynthetic efficiency under high-temperature conditions (Mensah et al. 2022). In addition, shade goes so far as to reduce soil temperature during the day (Aguiar et al. 2019) and contributes to the improvement of physiological performance (Mensah et al. 2022). The effects on the physiology of shade on *C. officinalis* are still unclear so the line of research should be pursued. For example, it has been reported that, in agroforestry systems, shade levels between 30 and 50% increase yields in cocoa plantations (Asare et al. 2017).

Under this context, this research aimed to determine the effect of light conditions on the percentage of mortality, height increase, and diameter increase of *C. officinalis* plants installed in the final field using a forest massif (FM) and enrichment bands (EB).

MATERIALS AND METHODS

Study area

The research was carried out from October 2021 to April 2022. The study area is located at 2,000 m (Figure 1), 1.5 km northwest of the community La Cascarilla (5°40'21.03"S and 78°53'53.90"W), province of Jaén, Peru. The annual precipitation is 1,730 mm, the minimum temperature is 13 °C, and the maximum is 20.5 °C (Fernandez and Huaccha 2022).

Two 25 x 25 m plots were installed, one plot was installed in the absence of shade which was called “forest massif (FM)” and the other plot was installed under the forest canopy which was called “enrichment belts (EB)”. The plants were planted at a 3 x 3 m distance. The holes were 30 x 30 x 30 cm. Each plot consisted of three blocks of 16 plants per block.

Measurements

To determine the performance of *C. officinalis* plants in the two planting conditions, monthly monitoring was carried out for six months to record the number of dead plants, plant height (from the ground to the apex of the plants), and diameter (measured with a digital vernier at ground level).

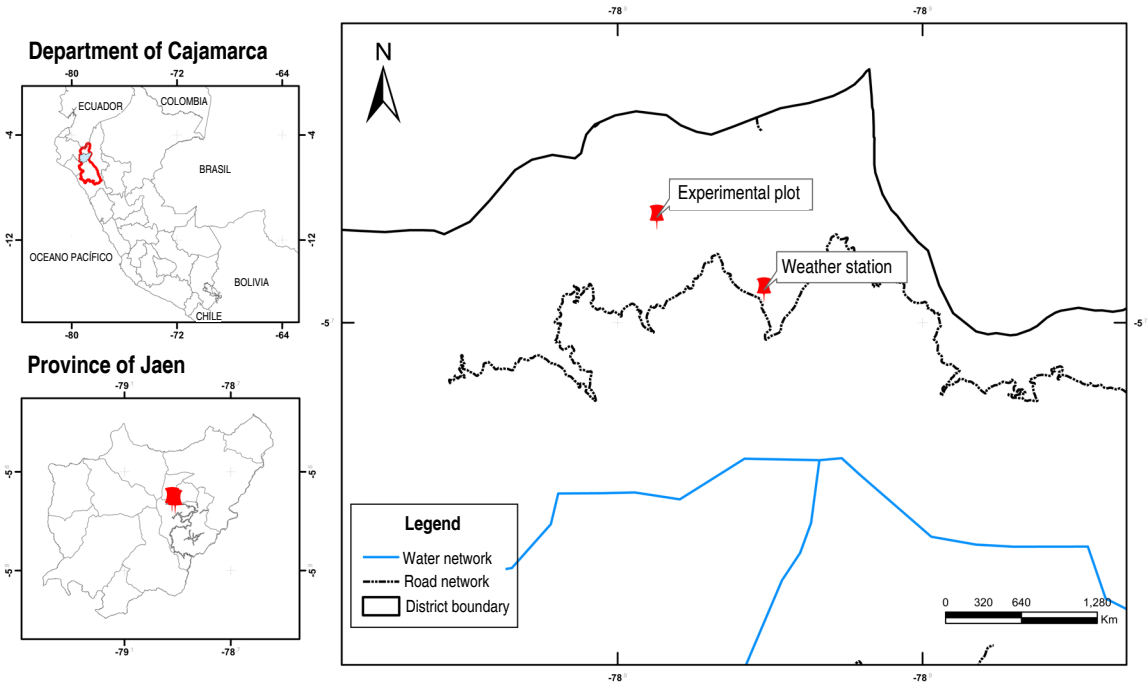

Figure 1. Location of the experimental plot.

Abiotic factors

To relate the growth variables of *C. officinalis* with some abiotic factors, photoperiod data extracted from the National Oceanic and Atmospheric Administration (NOAA), precipitation (mm), maximum, minimum, and mean temperature (°C) collected from the meteorological station La Cascarilla (5°40'18.3'' S and 78°53'51.6'' W) belonging to the National Service of Meteorology and Hydrology (SENAMHI) of Peru, located at 1.5 km southeast in a straight line from the study area.

Data analysis

Normality (Shapiro Wilk) and homogeneity of variances (Levene) were verified, and then regression analysis was performed to determine the relationship between mortality, height increment, and diameter increment concerning the number of days after planting in the final field of *C. officinalis* plants. Pearson's correlation test was used to relate the growth variables (mortality, height increment, and diameter increment) with temperature, precipitation, and photoperiod. The mean of each growth variable was used with the mean of the average temperature, minimum, maximum, monthly precipitation, and photoperiod for the months of study, using Rstudio software.

RESULTS AND DISCUSSION

The soil characterization analysis showed that the pH presented similar values in the two experimental plots, qualified in both cases as strongly acidic. The percentage content of organic matter (OM), phosphorus (P), potassium (K), and magnesium (Mg) was higher in the EB plot, while the cation exchange capacity (CEC), calcium (Ca), and sodium (Na) presented higher values in the FM plot (Table 1). Finally, the textural classification for EB was sandy loam, while for FM it was sandy clay loam.

The average temperature during the study presented the highest value for November 2021 (16.1 °C) and the lowest value was recorded in February and March 2022 (15.1 °C), the relative humidity was around 87% and the highest precipitation was recorded at March 2022 (415.6 mm) and the lowest value was recorded in April 2022 (149.5 mm) (Figure 2).

Table 1. Characterization of the soils in the two experimental plots.

Plot	pH	OM (%)	P (ppm)	K (ppm)	CEC	Ca^{+2}	Mg^{+2}	Na^{+}
EB	4.67	12.61	10.40	265.00	22.40	1.33	1.13	0.60
FM	4.96	6.51	3.10	66.00	24.48	3.06	1.03	0.77

*OM: organic matter, *CEC: cation exchange capacity.

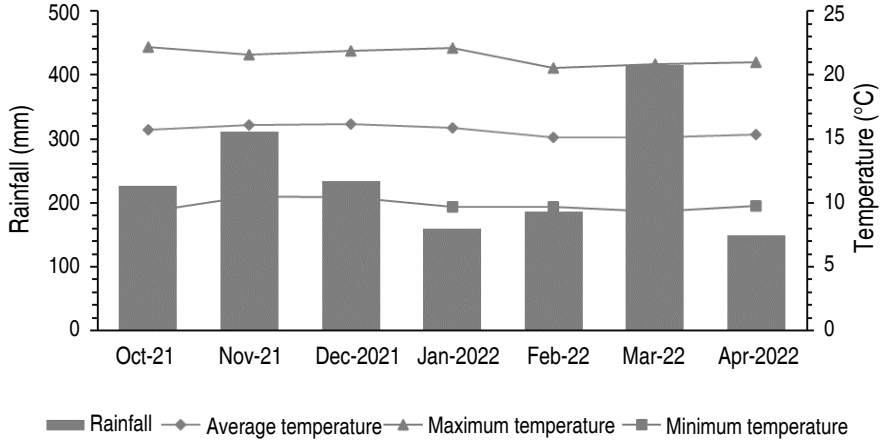

Figure 2. Precipitation (mm) and mean annual temperature (°C) of the study area, from October 2021 to April 2022.

Figure 2 shows that in the plot without shade, the mortality of *C. officinalis* plants began on day 90 after installation in the final field and reached 87.5% at 180 days (Figure 3A), in the case of height increase (Figure 3B), at 180 days after planting, *C. officinalis* plants increased 5.44 cm concerning the average height at the beginning of the trial, likewise, 0.59 mm of diameter increase was observed (Figure 3C) with respect to the average diameter at the beginning of the experiment. In addition, it was observed that in the plot installed under the enrichment bands modality, the mortality of *C. officinalis* plants began on day 30 after installation in the final field and reached 60. In the case of height increase (Figure 3E), at 180 days after sowing, *C. officinalis* plants increased by 3.52 cm with respect to the average height at the beginning of the trial, and 0.43 mm of diameter increase was observed (Figure 3F) with respect to the average diameter at the beginning of the experiment.

When comparing the results of both plots, there was a significant difference in the percentage of mortality, in the EB plot the mortality was 27.1% less than in the FM plot (Figure 4A); however, the increase in height (Figure 4B) and diameter increase (Figure 4C) in the FM plot was 45.5 and 25.1% higher than the results obtained in the EB plot, respectively.

Plant growth and development are affected by different environmental factors; among them, light plays a very important role in the overall performance of a plantation (Ghorbel et al. 2023; Kishore et al. 2021). Light availability has effects on biomass accumulation by controlling photosynthetic rate; in addition, it indirectly regulates biomass distribution by influencing leaf growth behavior, stem thickness, and immanent root length (Fernández-Milmanda and Ballaré 2021; Yin et al. 2023). The results of this research show that in the FM plot, the diameter increase was 45.5% higher than that obtained in the EB plot. Several studies reveal this effect, in which plants in shaded conditions tend to reduce their diameter increment (Yan et al. 2010), compared to sun-exposed plants that usually choose to shift their growth potential to the root system in order to increase water and nutrient uptake (Giday et al. 2019).

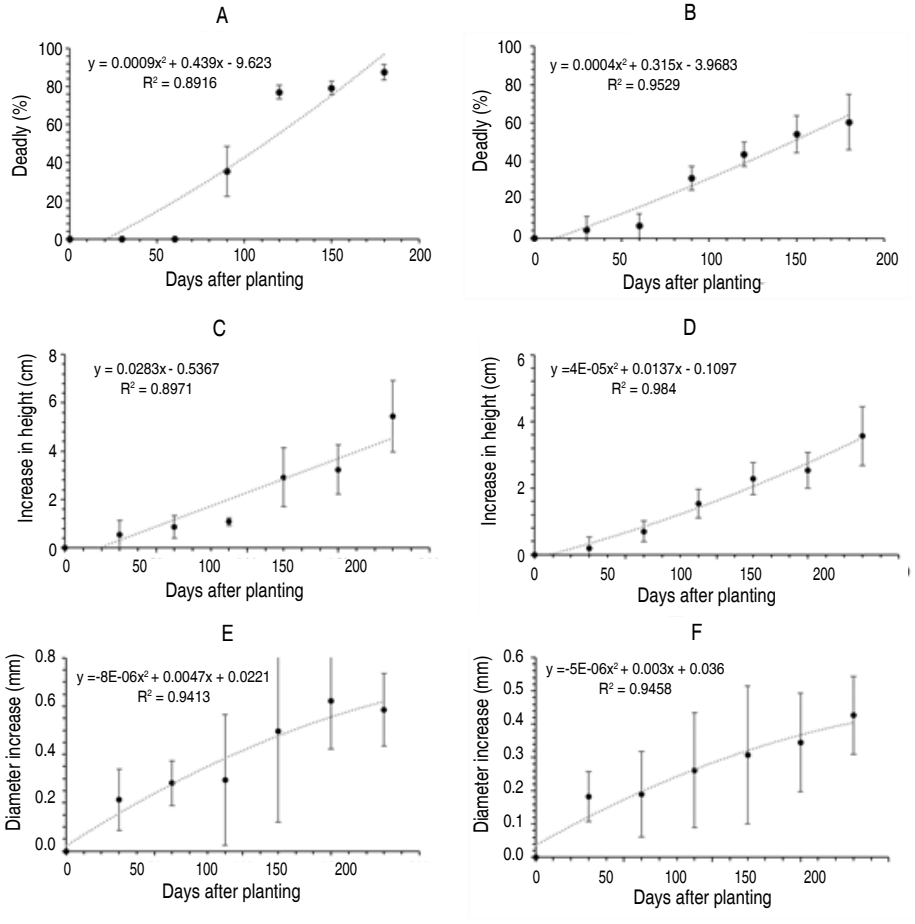

Figure 3. Growth parameters evaluated in *C. officinalis* plants planted as forest clumps. A. Percentage of mortality, B. Height increment (cm), and C. Diametric increment (mm) and by enrichment bands. D. Percentage of mortality, E. Height increment (cm), and F. Diametric increment (mm).

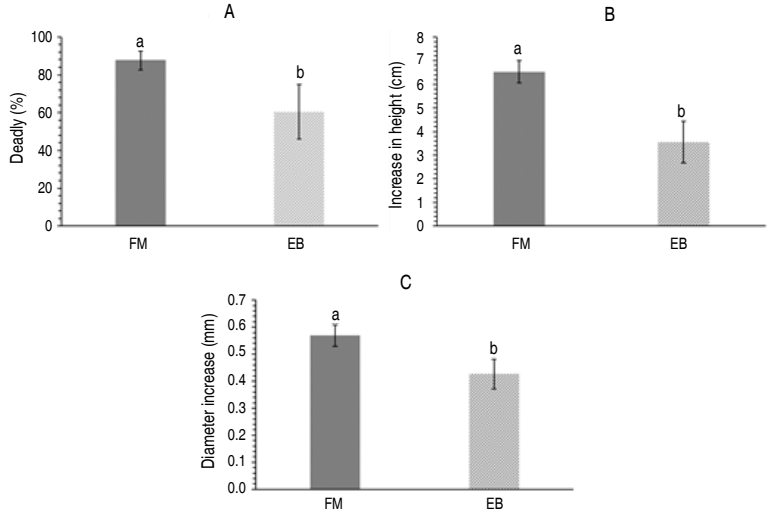

Figure 4. Cumulative growth parameters at the end of the trial in *C. officinalis* plants from the FM and EB plots. A. Percentage of cumulative mortality, B. Height increment (cm), and C. Diametric increment (mm). Different lowercase letters indicate significant differences.

In addition, it could be directly associated with the amount of photosynthetically active radiation (PAR) incident on *C. officinalis* plants, although it was not measured in this research, several studies indicate that PAR radiation tends to decrease in shaded conditions (Liu et al. 2012). In addition, the increase in height in the plot exposed to the sun was 25.1% higher than that obtained in the plot installed using enrichment bands, although this is a contradictory result with respect to other studies that report that shaded conditions tend to promote an increase in height in order to reach a greater amount of solar radiation (Fernández-Milmanda and Ballaré 2021) this could be explained by the amount of PAR radiation received in the FM plot which was supposed to be higher than that received in the EB plot and consequently, *C. officinalis* plants installed in direct sun exposure grew taller than plants planted under canopy shade (Zhang et al. 2018).

For the FM plot, the results indicate that there is a negative correlation between the percentage of mortality and precipitation, and between the increase in height and diameter increment with the maximum temperature (Figure 5A), while for the EB plot, there is a negative correlation between the increase in height and precipitation and a positive correlation between the diameter increment and the minimum temperature (Figure 5B).

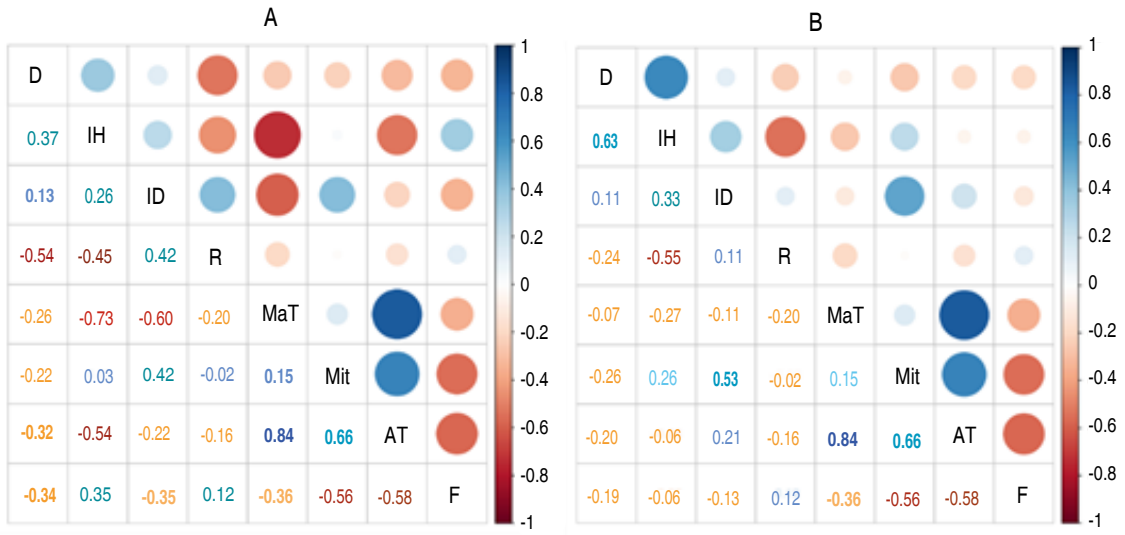

Figure 5. Correlation matrix between growth variables evaluated in *C. officinalis* plants planted in forest massif (A) and enrichment bands (B). The abbreviations D, IH, ID, R, MaT, MiT, AT, and F stand for mortality, height increment, diameter increment, precipitation, maximum temperature, minimum temperature, average temperature, and photoperiod, respectively.

In the FM plot, mortality was 27.1% higher than in the EB plot, and Figure 4A shows that there is a negative correlation between the percentage of mortality and precipitation, i.e., the lower the precipitation, the higher the percentage of mortality in the FM plot. The EB plot has allowed the plants not to be exposed to high temperatures due to the shade of the canopy (Mensah et al. 2023); in addition, the shade decreases evapotranspiration and therefore it is to be expected that there is a higher moisture content in the soil, and given that *C. officinalis* needs humid conditions for its development (Huamán et al. 2019) this environment would provide conditions for this species to thrive. In addition, the higher mortality rate in the plot exposed directly to the sun is probably due to a decrease in the photosynthetic capacity of *C. officinalis* plants (Mensah et al. 2022). Finally, this result could be associated with the higher organic matter content found in the EB plot, because this parameter acts as a water regulator and allows a greater number of microorganisms to develop, including mycorrhizae (Jia et al. 2023) whose positive effect on the growth of *C. officinalis* was reported by Fernandez-Zarate et al. (2022).

CONCLUSIONS

This study showed, for the first time, the effects of shade conditions on the survival and increase in height and diameter of *C. officinalis* plants installed in the definitive field. In general, shaded conditions decreased the mortality rate by 27%; however, the diameter and height increase were lower when plants were planted under a canopy. These results suggest that during the first year of installation of *C. officinalis* plants in the final field, they should be shaded to ensure their establishment. In the future, it is necessary to know the behavior of this species under different percentages of shade to determine if there are the same results as those reported in this research; in addition, the relationship between the growth parameters of *C. officinalis* with the quality and quantity of light could be determined. It is also necessary to establish permanent monitoring plots in order to evaluate the long-term behavior of this species.

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