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La Granja:
Revista de
Ciencias de la Vida

Editorial

Dear reader:

It is our anniversary! La Granja, The Life Sciences Journal, celebrates its 20 years. It is an excellent time to review these 20 years of work and scientific-editorial collaboration. We like to thank all people who have contributed to this project of the Salesian Polytechnic University: layout designers, translators, programmers, social network managers, among others. Additionally, those who have trusted and contributed with our journal from all over the world: authors, reviewers, guest editors and members of the Scientific Board. This scientific journal has become a community project, a virtual research society, where experts coexist with the common purpose of presenting quality scientific production. As long as the contribution is valuable, we will continue working, reason for which it is our duty and commitment to continue offering quality and access to research through La Granja.

This special issue presents the results of research that represent enriching evidence for science and knowledge. The studies carried out by the authors published in this edition have focused on the use of the biological and natural resources of our country. For this reason, Jorge Briceño and collaborators determined the effect of temperature prior to extraction on the yield and fatty acid profile of morete oil (*Mauritia flexuosa* L.F.). As such, morete is a palm tree found in the Amazon region which can be used as a replacement for conventional cooking oils, and this study highlights the use profile under the definition of processes and temperatures. On the other hand, María Ugas and collaborators, from Universidad de Pedagógica Experimental Libertador de Venezuela, quantified the CO_2 flow in the soil colonized by *Avicennia germinans*. The findings demonstrate the importance of the edaphic microbiota, as well as they show the role of mangroves as a CO_2 sink, in which the microorganisms analyzed intervene, especially because of their importance in the face of climate change conditions.

On the other hand, José Iannacone and Diego Elias Estramadoyro, from Universidad Científica del Sur, Peru, conducted a study on the ecotoxicological impact on the red Californian earthworm. The results show the impact of the use of zeta-cypermethrin, a pesticide regularly used in onion production in Lurin, Peru. Thus, an affectation to the soil biota is observed, which also benefits the absorption of nutrients to many plants. Finally, the study carried out by Jhony Mendez and collaborators, from Universidad Central del Ecuador, establishes the oenological properties of naranjilla. It shows the feasibility of the product that reaches the standards issued by the Ecuadorian normalizing entity INEN, thus opening new alternatives for crops such as naranjilla produced in the Pacto area, being an alternative to the traditional production of sugarcane in the area.

In the miscellaneous section, in the topic of Agriultural sciences, José Cedeño-Zambrano and his collaborators from Universidad del Zulia, Venezuela; Universidad Laica Eloy Alfaro, Ecuador; and Universidad Técnica de Manabí, Ecuador, present strategies for sustainable and effective fertilization of barraganete plantain. Meanwhile, from the topic of Veterinary Sciences, Wladimir Moya, from Universidad de los Lagos, Chile, shows how betany additives help to control heat stress in broiler chickens.

In the Biological Sciences, Katheryn Sacheri-Viteri and her collaborators from Universidad Espíritu Santo, present a first study on *Aspergillus* isolated from Mangrove forests, through molecular characterization. While, in the area of conservation, Sofía Crespo-Gascón and her collaborators from Universidad Técnica de Manabí, Ecuador, show the analysis of the trafficking of endangered species in Manabí, Ecuador.

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In the area of biotechnology, Lucas Pardo and his collaborators from Universidad de Guayaquil; Ecuador, and Universidad Espíritu Santo, Ecuador, show the potential as a preservative of silver nanoparticles synthesized from rosemary leaves. Finally, Xavier León and his collaborators from Universidad Central del Ecuador, Ecuador and Universidad Andina Simón Bolívar, Ecuador, present lessons learned about agroecological commercialization spaces in university environments, an important contribution in the area of sustainable development.

We are sure that these articles will contribute to their area of research, reaching more and more scientists in the region.

Sincerely,

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AGRICULTURAL SCIENCES

FERTILIZATION WITH MAGNESIUM IN 'BARRAGANETE' PLANTAIN (*MUSA* AAB) ECUADOR

FERTILIZACIÓN CON MAGNESIO EN PLÁTANO 'BARRAGANETE' (*MUSA* AAB) ECUADOR

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Abstract

Plantain is an important crop for Ecuador due to its contribution in socio-economy and food security of this country, supplying rich-energy food to most of the population; in addition, it is necessary to carry out fertilization management that allows a better use of this resource and increase the yield. Magnesium fertilization in 'Barraganete' (*Musa* AAB) plantain was evaluated in El Carmen, Manabí, Ecuador. The research was conducted in the Experimental Farm "Rio Suma", Universidad Laica Eloy Alfaro de Manabí, Extension El Carmen, located at 260 masl, average temperature of 24 °C, annual rainfall of 2 684 mm. Six levels of MgO (0, 25, 50, 75, 100, 125 kg·ha^{-1}) were applied to know its effect on morpho-physiology and plant yield. A completely randomized block design with six treatments and three replications was used; the sowing was carried out with a distance of 2.50 m × 1.80 m (2 222 plants·ha^{-1}), and the variables of vegetative growth were evaluated as repeated measures over time. The results demonstrated significant differences in all the morpho-physiological variables, which showed that fertilization with 30% MgO affected the growth of the plants; however, the reproductive variables were not affected by the applied doses; the 25 kg·ha^{-1} dose generated the best yields, agronomic efficiency and partial productivity factor.

Keywords: Morpho-physiology, yield, photosynthesis, agronomic efficiency.

Resumen

El cultivo de plátano es importante por el aporte que genera en la socio-economía y seguridad alimentaria de Ecuador, pues suministra alimentos ricos en energía a la mayor parte de la población. Además, se hace necesario realizar manejos de la fertilización que permitan una mejor utilización de este recurso e incrementar los rendimientos. Por ello, se evaluó la fertilización con magnesio en plátano 'Barraganete' (*Musa* AAB) en El Carmen, Manabí, Ecuador. La investigación se condujo en la Granja Experimental "Rio Suma", Universidad Laica Eloy Alfaro de Manabí, extensión en El Carmen, ubicada a 260 msnm, temperatura promedio de 24 °C, precipitación anual de 2 684 mm. Se aplicaron seis niveles de MgO (0, 25, 50, 75, 100, 125 kg·ha^{-1}) para conocer su efecto sobre la morfo-fisiología y el rendimiento de las plantas. Se utilizó un diseño de bloques completos al azar con seis tratamientos y tres repeticiones; la siembra se realizó con un distanciamiento de 2,50 m × 1,80 m (2 222 plantas·ha^{-1}), y las variables de crecimiento vegetativo se evaluaron como medidas repetidas en el tiempo. Los resultados demostraron diferencias en todas las variables morfo-fisiológicas, lo que demostró que la fertilización con 30% de MgO afectó el crecimiento de las plantas; no obstante, las variables reproductivas no fueron afectadas por las dosis aplicadas; la dosis de 25 kg·ha^{-1} generó los mejores rendimientos, eficiencia agronómica y factor parcial de productividad.

Palabras clave: Morfo-fisiología, rendimiento, fotosíntesis, eficiencia agronómica.

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1 Introduction

World plantain production (*Musa* sp.) during 2012-2017 was distributed in Africa (60.60 %), America (26.50 %), Asia (12.80 %) and Oceania (0.10 %), indicating the economic and nutritional importance of this crop to the population (FAOSTAT, 2018). High temperatures and relative humidity predominate in America, Asia and Africa, as well as in the subtropical and tropical areas of these continents, and plantain represents an important crop by the planted area that it represents (Ramos et al., 2016).

According to the Research Center on Sustainable Rural Development and Food Sovereignty (CEDRSSA, 2019) India is the main platain producing country with 30 477000 t, 2.67 times larger than China (11 422956 t), which is its closest competitor. In addition, six countries in Latin America stand out by their plantain production, such as Brazil (6 675100 t), Ecuador (6 282105 t), Guatemala (3 887439 t), Colombia (3 786672 t), Costa Rica (2 552822 t) and Mexico (2 229519 t).

Plantains in Ecuador represent an export item and a source of employment. Because of the importance of this crop, it is necessary to generate reliable tools for the farmer to manage the crop in an appropriate and cost-effective way (Tumbaco et al., 2015). In addition, it is important by its contribution for socio-economy and agri-food security, providing direct employment (fixed labor) and indirect employment (occasional labor and added value of products), and food to the population. According to the National Statistics and Census Institute (INEC, 2020) in 2019 there were 115069 ha planted with plantain in Ecuador as monoculture and 45 194 ha associated with other crops, and the production corresponded to 582706 and 166745 Tm, equivalent to 5064 and 3690 $kg{\cdot}ha^{-1}$, respectively. Banana and plantain exports in Ecuador accounted for \$3.27 billion, representing 17.5 % of total non-oil exports (OEC, 2017; Álvarez et al., 2020); however, the Central Bank of Ecuador (**?**) noted that the export of plantain in Ecuador during 2019 was 211732.6 Tm.

A research carried out by Molero et al. (2008), showed that the extraction of nutrients using cv. Hartón at the time of harvest was approximately N: 150; P: 60; Ca: 215; Mg: 140; Mn: 12; Fe: 5; Zn: 1.5; B: 1.25 and Cu: 0.5 $kg{\cdot}ha^{-1}{\cdot}yr^{-1}$; therefore, it was important to use restitution doses to maintain soil fertility and ensure high production. These same authors have pointed out that nitrogen, potassium and to a lesser extent magnesium in banana cultivation are the most important elements for its growth and production.

Cobeña et al. (2020) found that the nutritional elements were extracted in higher amount in plantain 'Barraganete' were K, CA, N, P and Mg, when N and K_2O were fertilized. Regarding the content of P and Mg, there was no sustained extraction behavior. Avellán et al. (2020) evaluated the export of P in 'Barraganete' plantain, finding that it presented low mobility in the soil, for this reason it was absorbing the one found around the roots, indicating that P was available and in a soluble form when fertilizing, but was fixed almost immediately, hence it was non usable for the plant; thus, P fractionning was suggested as an alternative for increasing its efficiency.

On the other hand, Mg is very important for plants. The amount of Mg contained in leaves (75 %) participated in protein synthesis and 15 to 20 % of the total Mg was found to be associated with chlorophyll pigments (White and Broadley, 2009), acting as a cofactor of enzymes involved in the metabolism and fixation of photosynthetic carbon (Hermans et al., 2013).

Between 90 and 98 % of the Mg present in soil is not available for plant absorption but it is incorporated into the crystalline structure of minerals (Senbayram et al., 2015). The usable form of Mg to be absorbed by plants is as ion Mg^{2+}, which has the smallest ionic radius, but the largest hydrated radius among cations (Maguire and Cowan, 2002). This causes a weak attachment to the negatively charged soil colloids and root cell walls, causing Mg to be easily lost (Grzebisz, 2011). At the same time, excessive fertilization with K^+ and NH_4^+ has been reported to be antagonistic with the absorption of Mg, increasing its deficiency. The deficiency of Mg increases in acid soils saturated with H^+, Al^{3+} and Mn^{2+} cations where intensive leaching occurs, especially in areas with high rainfall (Gransee and Führs, 2013).

Despite the importance of Mg^{2+} in the plant growth and development, the molecular mechanism of plant cells regulating homeostasis in Mg^{2+}, as well as the molecular mechanisms related to magnesium transport are little known. Mg^{2+} is required for adequate functioning of the many cellular activities related to chlorophyll synthesis, as the main component of chlorophyll, membrane stability and enzymatic activation (Guo et al., 2016).

On the other hand, nutrition with Mg in plants must be considered a major global problem, not only in terms of food security but also in terms of human health, since Mg is an essential nutrient in human and animal nutrition, and plants are the main source of daily intake of this element. In this regard, experiments have been carried out evaluating the effects of magnesium in plants; however, this information in plantain is scarce, particularly in Ecuador. Therefore, the aim of this research is to evaluate the response to magnesium fertilization in the plantain crop 'Barraganete' (*Musa* AAB), in El Carmen canton, Manabí, Ecuador.

2 Materials and Methods

The research was carried out from 2018 to 2019, at the Experimental Farm Rio Suma, Lay University "Eloy Alfaro" of Manabí, campus El Carmen, geographically located in the Province of Manabí, Canton El Carmen, coordinates UTM (−0.259503 S; −79.427558 0), in a humid tropical climate. The agro-ecological characteristics corresponded to altitude 260 masl, temperature of 24.15 °C, annual precipitation of 2 600 mm, relative humidity 85.6 %, sunlight 553 hours·light^{-1}·year^{-1} and evaporation 1 064 mm·year^{-1} (Climate-data.org, 2019).

The soil analysis of the study area indicated mean levels of MO (4.48 %), non-saline C.E. (0.08 ds·m^{-1}), pH of 5.7 (moderately acid). Low contents of NH_4 (11.61 ppm), P (4.56 ppm), S (2.14 ppm), Mg (0.90 meq·100 g^{-1}), base sum 8.40 meq·100 g^{-1}, Mn (9.70 ppm), and mg/K ratio (1.80), Ca mg/K (15.80). High levels of K (0.50 meq·100 g^{-1}), Ca (7.00 meq·100 g^{-1}), Cu (5.80 ppm), Fe (123.10 ppm), Zn (23.20 ppm) and Ca/mg ratio (7.78). The texture is sandy loamy (62 % sand, 28 % silt and 10 % clay) (Avellán et al., 2020). Once the culture reached the average age, soil and leaf area samples were obtained; it was also done at the end of the crop cycle. Soil samples were analyzed at the Agrolab Laboratory, located in the Water and Soil Laboratory Network of Ecuador (Relase).

The reasearch was evaluated in a first-cycle plantation of 'Barraganete' plantain (*Musa* sp. AAB), with a planting distance of 2.5 m between rows x 1.8 m between plants (4.5 m^2), for a total of 2 222 plants·ha^{-1}, corresponding to a high density management of plants. 288 plants were included in the research and were distributed in three blocks. Three-dose fractionated fertilizer applications (N-P-K and MgO) were performed. The treatments followed an experimental design in totally random blocks, with three repetitions.

Treatments were MgO fertilization at six levels (0, 25, 50, 75, 100 and 125 kg·ha^{-1}) and a standard dose of 80 kg·ha^{-1} of N, 40 kg·ha^{-1} of P_2O_5 and 150 kg·ha^{-1} de K_2O, divided into three equal parts and applied to the soil when the plant emitted leaves 12, 16 and 20. The commercial fertilizers used were urea with 46 % of N, diammonium phosphate (DAP) with 18 % of N and 46 % of P_2O_5, potassium muriate with 60 % of K_2O and magnesium oxide with 30 % of MgO.

The experimental unit consisted of 16 plants, from which four plants were selected from the central part to avoid the embroidery effect. Plants were transplanted when leaf five was obtained. The mean of the treatments were compared using Tukey test ($P<0.05$). The GLM procedure of the SAS® program, version 15.1 (Statistical Analysis System, 2020) was used for processing the data.

The morpho-physiological variables (plant height, pseudostem perimeter, number of leaves, length and width of leaf 3 and leaf area) were evaluated over time with measurements every 8 days, using the time-repeated measurement methodology through MIXED procedure (Statistical Analysis System, 2020); second-degree polynomic models were selected because these were the ones that best explained the behavior over time of the growth variables. The graphical representation of the variables was made using the SigmaPlot software.

In addition, variables related to yield were measured (number of bunches, number of fruits, length of fruits, diameter of exportable fruits, biomass of fruit and bunch, yield per hectare, soil and foliar concentration of Mg, concentration of Mg in the fruit, export of Mg in the fruit, agronomic efficiency and partial productivity factor). The leaf area was determined by the measurements obtained from the length and width of the third leaf and was calculated with the following formula:

$Leaf\,area = length \times width \times 0{,}8$ (Martínez, 1984).

Regarding the variables related to fertilization efficiency, the export of Mg was determined considering the dry biomass percentage of the fruit multiplied by the yield of the crop per hectare and multiplied by the percentage of magnesium concentration, in this way, the amount of magnesium that left the crop was calculated through the fruit, and the response was obtained in kg·ha^{-1}. Agronomic efficiency (AE) was defined as the increase in yield from fertilization per unit of the nutrient applied (Dobermann, 2007) and was calculated by considering the yield of the fertilized plots (YFP) minus the yield of the control plot (YCP), divided by the applied dose (AD), and expressed in kg of fruit·kg^{-1} of applied nutrient.

$$AE = \frac{YFP - YCP}{AD}$$

The partial productivity factor allows to evaluate the efficiency of a nutrient for the production (Bruulsema et al., 2011), and it was calculated using the formula $FPP = \frac{Y}{D}$ in which Y= yield of harvested plots (kg·ha^{-1}) and D= dose of nutrient applied (kg·ha^{-1}) (Dobermann, 2007).

3 Results and Discussion

3.1 Height of the plant and perimeter of the pseudostem

Significant differences ($P<0.01$) were found for this variable by the effect of treatments for sampling at week 10 (T25-T50; T75-T125 and T100-T125) and from week 11 to week 53 differences were between T0-T75, T0-T100, T0-T125, T50-T75 and T50-T100. In the weeks mentioned, T25 was the one that presented the highest height of the plant, reaching 3.47 m at week 53 after planting; T0 was higher than T75 and T100.

The height of plants had the same tendency for all treatments over time, adjusting to a second polynomial degree ($Y= a + bx + cx^2$), indicating that fertilization with magnesium increased the length of 'Barraganete' plantain plants with T50, T125 and T0 when compared with T75 and T100; the latter two showed similar values to T25 until week 46, and then it increased to similar values to T0, T50 and T125 (Figure 1). This second-order polynomial behavior suggests that as the emergence of the inflorescence approaches, the growth of the plant slows down until it reaches its final height.

The average height obtained in this research at 53 weeks was 3.47 m, similar to that reported by Cayón et al. (2004) which was between 3.2-3.4 m when evaluating densities and planting arrangements in a range of 1 500 to 3 000 plants·ha^{-1}, with no statistical differences between them.

Significant differences ($P<0.01$) were found for the pseudostem perimeter by effect of treatments during all the weeks where the assessments were performed. There were differences in week 10 between T0-T25; T0-T50 and T100-T125, and between weeks 11 to 53 in T0-T75, T0-T100, T0-T125, T25-T100, T25-T125, T50-T100 and T50-T125. This variable over time was adjusted to a second polynomial order ($Y= a + bx + cx^2$). There was a similar trend between treatments over time, indicating that fertilization with MgO affected the diameter of the pseudostem of 'Barraganete' plantain (Figure 2).

In this sense, it is recognized the importance of Mg in various metabolic processes and reactions in plants, particularly photosynthesis and chlorophyll content. Therefore, Cakmak and Yazici (2010) noted that plants with Mg deficiencies accumulated up to four times more sucrose when compared with those with an adequate Mg content, generating a severe inhibition of the sucrose transport in leaves to other plant-receiving organs (roots, stems, among others). Adequate nutrition with Mg during periods of carbohydrate transport from leaves to cells at other sites in the plant ensures maximum transport of carbohydrates to the recipient organs, thus promoting growth and high yields.

The overall average perimeter of the pseudostem was 52.23 cm, similar to that indicated by Barrera et al. (2011), with 52.23 cm on average for the first production cycle and 48.15 cm for the second cycle in Hartón plantain with a density of 1 111 plants·ha^{-1}; however, when applying mycorrhizae + earthworms it was 60.08 and 41.5 cm, for each production cycle, respectively, i.e., less than that obtained by Pinchao (2018) when evaluating the effect of different levels of K_2O and MgO in 'Barraganete' plantain, obtaining on average 70 cm in this same variable with a density of 2 222 plants·ha^{-1}.

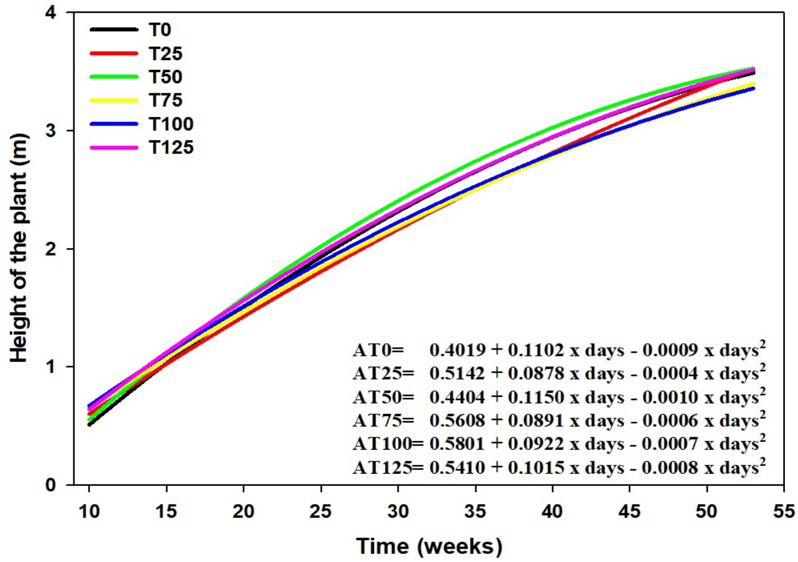

Figure 1. Height of 'Barraganete' plantain plants (*Musa* ABB), under different fertilization doses with MgO, in El Carmen, Ecuador. Data were collected every 8 days. The estimated values are presented. T0= witness (0 kg·ha^{-1} of MgO, black), T25= 25 kg·ha^{-1} of MgO (red), T50= 50 kg·ha^{-1} of MgO (green), T75= 75 kg·ha^{-1} of MgO (yellow), T100= 100 kg·ha^{-1} of MgO (blue), and T125= 125 kg·ha^{-1} of MgO (magenta).

3.2 Width, lenght and leaf area

Significant differences (P<0.01) were found for leaf width at week 10 (T0-T25, T0-T75, T0-T100, T0-T125, T25-T50, T25-T125, T50-T75, T50-T100, T50-T125, and T75-T100; for week 11 (T0-T25, T0-T100, T25-T50, T25-T75, T25-T125, T50-T100, T75-T100 and T100-125; and from week 12 to week 53 between T0-T25, T0-T100, T25-T50, T25-T75, T25-T150, T50-T100, T75-T100 and T100-T125 for the effect of treatments. There were also statistical differences (P<0.01) for the leaf lenght during the entire evaluated period between T0-T25, T25-T50, T25-T75, T25-T125, T50-T100, T75-T100 and T75-T125. The foliar area showed statistical differences in week 10 between T0-T25, T0-T50, T0-T100, T50-T100, T75-T100 and T75-T125; and between T0-T25 from week 11 to week 53 and between T25 with T50, T75, T100 and T125.

The results indicated that fertilization with MgO influenced leaf development. Both the width of leaves and the leaf area over time presented a similar trend, where T0 had the least development of leaves (Figure 3). Treatments had a second polynomial behavior degree (Y= a + bx + cx^2) for these variables, where from approximately week 30 of the development of the crop there was a tendency to increase the width of leaves between the different treatments except for T0, which maintained over time.

There was an increase in leaf width in week 53 of 1.12 with the doses 125 kg·ha^{-1} compared to T0. On average, T0 had a width of 0.68 m, while T50 and T125 had an average of 0.72 m. The development of leaves had similar behavior to that indicated by Aristizábal (2008), who mentioned that terminal leaves decreased in size during the last weeks before the emission of the inflorescence (acorn); in this investigation was observed on leaf 3 of the plant.

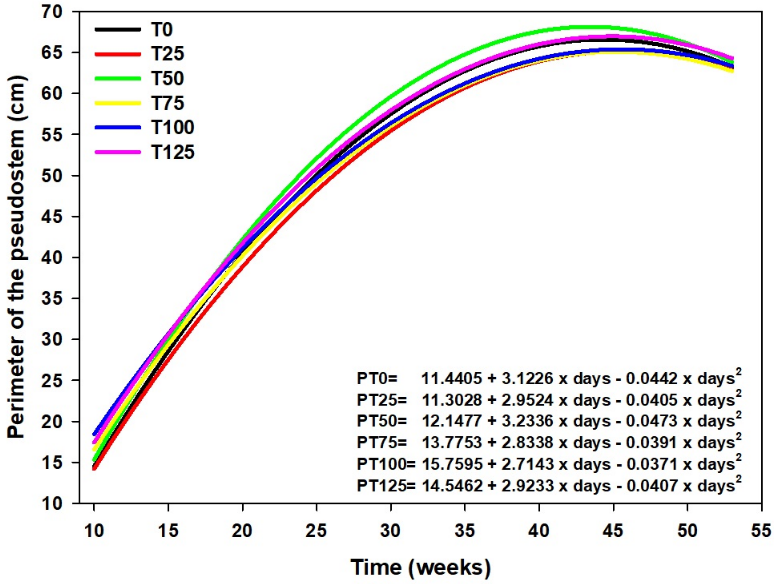

Figure 2. Perimeter of the pseudostem in 'Barraganete' plantain (*Musa* ABB), under different fertilization doses with MgO, in El Carmen, Ecuador. Data were obtained every 8 days. The estimated values are presented. T0= witness (0 kg·ha^{-1} of MgO, black), T25= 25 kg·ha^{-1} of MgO (red), T50= 50 kg·ha^{-1} of MgO (green), T75= 75 kg·ha^{-1} of MgO (yellow), T100= 100 kg·ha^{-1} of MgO (blue), and T125= 125 kg·ha^{-1} of MgO (magenta).

Regarding the length in the leaf area, it was observed in Figure 3 that from week 15 until week 30 there was a more accelerated leaf growth, on average it was 1.88 times higher in the indicated range. From week 30 to week 53, leaf growth was slower (increase of 1.04 times), showing that the length of leaves in the last weeks had a smaller slope, growth being almost horizontal. Both the width and length of leaves were evaluated on leaf 3, which has been used as a reference leaf in the crop.

According to Martínez and Cayón (2011), the logarithmic phase (vegetative phase) had a slow growth, followed by a progressive change in its speed rate, evidenced by the slope of the curve, and with exponential increase in the width and length of leaves; likewise, differentiation marked the completion of this phase. The linear phase (vegetative-reproductive phase) continued, and the plant presented accelerated and constant growth, thus stability of the curve´s slope. In this phase, the development and elongation of the floral stem was noted, and it finished with the emergence of inflorescence (flowering). The cultivation cycle completed at the beginning of the senescence phase (reproductive-productive phase), with flowering and subsequent development of the cluster; the growth rate of the plant decreased since leaves and pseudostem (sources) moved to the fruit (sump).

Leaf area dynamics over time suggested that it tended to decrease as the plant got close to inflorescence emission, which could be due to a lower leaf emission rate; reduction of leaf size, lower longevity of the last leaves emitted or the combined effect of these facts.

Figure 3 shows that T0 performed more evenly than fertilized plants with the smallest leaf area at the end of the investigation period, which was 1.19 times less than T125. This is because the lack of Mg inhibits plant growth, accelerates the aging process and generates losses in both production and crop quality (Verbruggen and Hermans, 2013), since it is involved in the photosynthesis process, so its deficiency decreased the photosynthetic rate and blocked the carbohydrate rate to the demanding organs, leading to the growth inhibition of the demanding organs and thus low productivity in many plant species (Chen et al., 2018; He et al., 2020).

Martínez and Cayón (2011) indicated that the total foliar area produced by plantain plants lose approximately 8 m^2 when they reach flowering and an additional 4 m^2 until the harvest; these losses, however, may be higher if there is not adequate management of foliar diseases or if the plant experiences water stress conditions. This means that from the dry biomass destined for the formation of leaves throughout the cycle, the plant loses 60 % in the form of non-recoverable dried leaves.

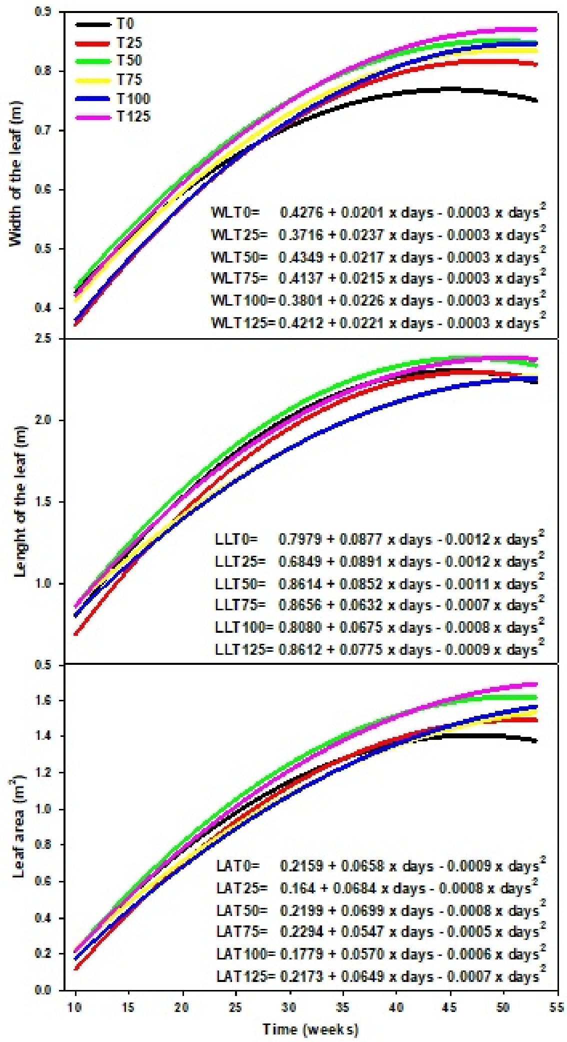

Figure 3. Width, length and leaf area of 'Barraganete' plantain (*Musa* ABB) under different fertilization doses with MgO in El Carmen, Ecuador. Data were obtained every 8 days. The estimated values are presented. T0= witness (0 kg·ha^{-1} of MgO, black), T25= 25 kg·ha^{-1} of MgO (red), T50= 50 kg·ha^{-1} of MgO (green), T75= 75 kg·ha^{-1} of MgO (yellow), T100= 100 kg·ha^{-1} of MgO (blue), and T125= 125 kg·ha^{-1} of MgO (magenta).

3.3 Number of leaves

On the other hand, there were no statistical differences ($P>0.05$) for the number of emerged leaves·plant^{-1} due to the effect of the evaluated treatments. The averages reached for the number of total leaves (38.33 leaves), the number of leaves emerging the inflorescence (12.33 functional leaves) and the time of harvest (5.67 leaves), suggest that fertilization with magnesium did not have any influence on this variable. These results were similar to those obtained by Pinchao (2018) who reported 5 leaves during the crop and 38 to 40 leaves throughout the crop cycle. Similar results were reported by Herrera and Aristizábal (2003) and Jaramillo and Aristizábal (2004) in other plantain cultivars.

According to Martínez and Cayón (2011) the plant must maintain a minimum of 8 leaves to guarantee the filling of the cluster from the emergence of the inflorescence. Aristizábal (2008) stated that while the number of functional leaves present is important, their position in the plant is much more important, since it determines their contribution to the filling of the bunch. In this regard, they observed that the foliar emission rate in Honduran Dwarf and Dominico Hartón plantain tended to decrease linearly over time, until the plant emitted the inflorescence.

3.4 Number of bunches and fruits, length and perimeter of fruits, biomass of fruits and bunches and yield·ha^{-1}

The variables number of bunches and fruits, length and perimeter of fruits, biomass of fruits and bunches and yield·ha^{-1}, all related to yield components, did not show statistical differences ($P>0.05$) with the doses of MgO applied. However, mean values were 5.44 fruits·bunch^{-1}; 26.33 exportable fruits·bunch^{-1}; fruit length of 31.44 cm; fruit perimeter of 47.17 mm; fruit biomass of 350 g; bunch biomass between 10.28 and 11.49 kg and yield between 22.98 and 25.88 t·ha^{-1}.

3.5 Agronomic efficiency (AE) and partial productivity factor (PPF)

Doses of 50 to 125 kg·ha^{-1} generated negative AE from −3.33 to −30.67 kg·ha^{-1}, while doses of 25 kg·ha^{-1} resulted in 23.95 kg·ha^{-1} (Figure 4). Negative AEs were the result of T0 (witness without MgO; 25 277.78 kg·ha^{-1}), having a higher yield than fertilized plots except for T25 (25 876.54).

The intersection of lines is considered the optimal point, where the fertilizer was mostly used by the plant to increase production. It was higher than 25 kg·ha^{-1}, with an agronomic efficiency lower than 20 kg of fruit·kg·ha^{-1} of MgO applied, suggesting the conduction of research with smaller doses and in intervals between 20 and 30 kg·ha^{-1} to determine the appropriate dose to be applied to increase the yield of the crop (Figure 4). Doses higher than 50 kg·ha^{-1} of MgO decreased agronomic efficiency under experimental conditions.

According to Avellán et al. (2020) and Cobeña et al. (2020) this could be because the absorption of nutrients, due to excess of fertilization, was higher than the one required by plants, generating a negative effect since it prevented the absorption of other nutrients that could have been present in the soil solution. This suggests a nutritional imbalance; in other words, the absorption of more MgO and the lower absorption of other nutrients, especially N and P, could contribute to lower yields.

Statistical differences ($P<0.01$) for PPF were found due to the effect of the MgO doses applied. The reported values were between 1 035.06 and 184.73 kg of fruit·kg·ha^{-1} with MgO doses 25 and 125 kg·ha^{-1} of MgO, respectively (Figure 5). In this regard, Boaretto et al. (2007) noted that as the fertilizer dose increased, the productive efficiency of the crops was lower; in other words, with low fertilization doses, the efficiency of the nutrient applied was higher.

Karley and White (2009), and White (2012) indicated that antagonisms and synergies between nutrients affecting yield are common in perennial crops such as bananas and plantains, noting that the antagonistic relationship between K, Ca and Mg has been the most studied, and concluding that when any of these nutrients is high, the content of the others decrease, thus generating a decrease in the growth and yield of plants.

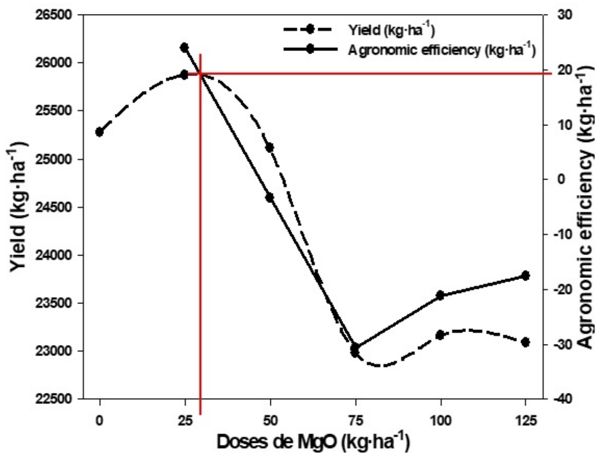

Figure 4. Yield and agronomic efficiency in 'Barraganete' plantain (*Musa* ABB), under different fertilization doses with MgO, in El Carmen, Ecuador.

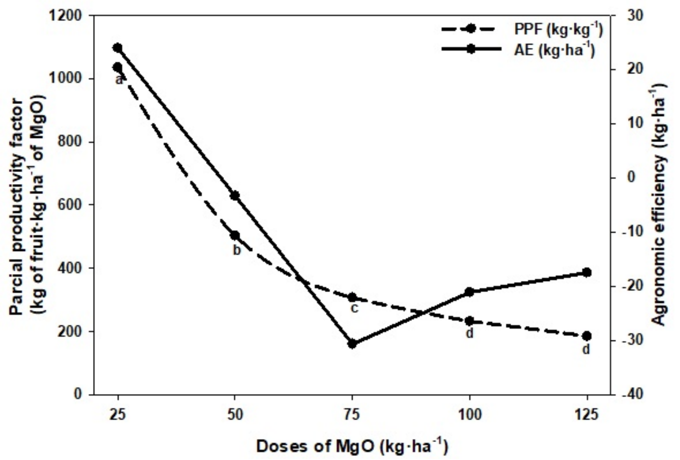

Figure 5. Partial productivity and agronomic efficiency factors in 'Barraganete' plantain (*Musa* ABB), under different fertilization doses with MgO, in El Carmen, Ecuador.

4 Conclusions

Productive variables were not influenced by the applied doses, even though the higher yield, agronomic efficiency and partial production factor were reached with 25 kg·ha^{-1} of MgO or close o it, which suggests conducting research with doses close to that value. The dynamics of the morphophysiological variables evaluated over time conform to second polynomial order equations, where the application of MgO generates differences between the doses used in 'Barraganete' plantain. The dose 50 kg·ha^{-1} of MgO showed the greatest differences between fertilization treatments with MgO.

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MOLECULAR BIOLOGY

FIRST REPORT OF TWO *ASPERGILLUS* SPECIES ISOLATED FROM MANGROVE FOREST IN ECUADOR

PRIMER ESTUDIO DE DOS ESPECIES DE *ASPERGILLUS* AISLADAS DE BOSQUES DE MANGLAR EN ECUADOR

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Abstract

Mangroves forests are located in tropical and subtropical regions. The adaptation and distribution in coastal regions is influenced by temperature, humidity, tidal and saline fluctuations; therefore, there are exposed to multiple environmental fluctuations. Mangroves are inhabited by wildlife but also is supported by a diverse community of microorganisms, including fungi. Several fungi in mangroves have multiple ecological roles as saprotrophs or as an opportunistic pathogen, many of them are also used in the industry, as the genus *Aspergillus*, that are important in biomedicine, industrial and environmental applications. In this study, we isolated species of fungi from mangrove stems and propagules. They were identified by both morphological and by its molecular characteristics. Here, we report the first isolated of *Aspergillus niger* and *Aspergillus aculeatus* from mangroves in Ecuador. Research such as these highlights the importance to determine the role of fungi in the mangrove ecosystem.

Keywords: Mangrove, *Aspergillus niger*, *Aspergillus aculeatus*, molecular characterization.

Resumen

Los bosques de manglar están distribuidos en las zonas costeras de las regiones tropicales y subtropicales de todo el mundo, siendo especies tolerantes a altas temperaturas, humedad, mareas y las fluctuaciones salinas. Por lo tanto, se ven expuestos a múltiples fluctuaciones y condiciones ambientales extremas. El ecosistema de manglar no solo es hábitat de vida silvestre, sino que también es colonizado por diversas comunidades de microorganismos, como los hongos. Varios de estos hongos tienen múltiples funciones ecológicas, ya sea saprófitos o patógenos oportunistas. Actualmente el interés de estudiar estos microorganismos radica en su potencial biotecnológico dada su capacidad para tolerar ambientes hostiles. Ejemplo de ello son algunas especies del género *Aspergillus*, las cuales son utilizadas en biomedicina, industrial y la bioremediación. En el presente estudio se aislaron e identificaron de acuerdo con sus características morfológicas y moleculares especies de hongos del género *Aspergillus*. En este estudio se reportan los primeros aislados de *Aspergillus niger* y *Aspergillus aculeatus* de manglares en Ecuador. Investigaciones como ésta resaltan la importancia de determinar el rol de los hongos en el ecosistema de manglar.

Palabras clave: Manglar, *Aspergillus niger*, *Aspergillus aculeatus*, caracterización molecular.

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1 Introduction

Mangrove forests are estuaries located on tropical and subtropical regions; this ecosystem is characterized by its highly salinity-tolerance (Gopal and Chauhan, 2006). The distribution of mangroves is strongly influenced by temperature, humidity, water currents and variations in tide and wind flow. Altogether with the high abundance and variety of microorganisms, make it an important dynamic ecotone between the terrestrial and marine environment (Sridhar et al., 2011). Taking into account microorganisms, fungi are important decomposers of organic matter and they play a fundamental role in productivity and biodiversity of this ecosystem (Friggens et al., 2017). The fungi that inhabit mangrove forest are saprophytes, symbionts or parasites, both in filaments and in yeast (Rodríguez et al., 2013). These fungi can colonize roots, stems and branches submerged in water or can be found in the surface of the water (Li et al., 2016). The diversity of fungi depends on their metabolism, since the strata is associated with daily changes in salinity, intermittent flooding due to tide, exposure to salt fog and substrate availability (Hrudayanath et al., 2013).

Human activities constitute the main problem to mangroves, and among the main human activities are the habitat destruction, pollution and overexploitation of resources (Díaz, 2011). Many cities have settled in the nearby mangroves, which, therefore, are constantly exposed to pollution that is formed by anthropogenic activities, thus exposing them to a variety of chemicals, including heavy metals that are considered a serious problem for the mangrove ecosystem, since they accumulate on the surface of the sediments, hence increasing their concentration in the area (Fernández et al., 2014)

The most common genera of fungi isolated from *Rhizophora* spp. mangrove are *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Curvularia*, *Cylindrocephalum*, *Drechslera*, *Fusarium*, *Myrothecium*, *Nigrospora*, *Penicillium*, *Pestalotia*, *Phyllosticta*, *Trichoderma* and *Verticilium* (Sarma, 2012). However, few studies describe the role of these genera in the mangrove ecosystem. Most of the fungi identified in mangrove ecosystems are endophytes; these fungi are considered of great importance since they have been able to produce bioactive metabolites to modify the defense mechanisms of their host, allowing both to subsist in the environment (Sánchez et al., 2013). Several fungi (e.g., *Aspergillus*, *Absidia*, *Cunninghamella*, *Mucor* and *Rhizopus*) are capable of accumulating heavy metals in their cell compartments, offering an alternative in bioremediation of contaminated areas at low cost compared to tradition decontamination methods (Cardoso et al., 2010).

In many cases, mangrove forests are formed by high density natural monocultures where trees are constantly exposed to pathogens (Ramírez et al., 2006). However, phytopathogenic records in mangrove ecosystems are poorly reported (Pan et al., 2018). The majority of fungi that colonize mangroves trees are related to Ascomycota division, several parasitic species belonging to this group can produce plant death (Pan et al., 2018). Although the relationship between fungal diversity and mangroves is not clear, marine fungi are known to be responsible for the breakdown of mangrove wood in the most common species, including *Rhizophora*, which have been described to be more efficient digestible biomass than bacteria (Steinke and Jones, 1993; Kathiresan et al., 2011). Fungus *Trichoderma* and *Traustreochystrids* genus are efficient saprophytic due to enzymatic activity and potential microorganism to degraded cellulose, starch, lipid, proteins and lignin, whereas *Thichosporon*, *Fusarium* and *Aspergillus* exhibited the maximum cellulase and protease activity in the leaves of mangrove (Kathiresan et al., 2011)

Aspergillus is widely distributed in nature due to its easy dispersal strategy of its conidia and its small size; this strategy allows them to remain in the environment for a long period of time (Abarca, 2000). The fungal genus of *Aspergillus* is highly interesting, containing everything from industrial cell factories, model organisms, and human pathogens, since it has a prolific production of bioactive secondary metabolites (Kjærbølling et al., 2018). These fungi are important in the organic matter breakdown and host defense against highly pathogenic microorganism (Ramírez et al., 2006). The applications of these fungi are required to improve future omics studies (Shu-Lei et al., 2020). In the present study we present the first report of two species of *Aspergillus* isolated from mangroves in Ecuador and identified by molecular and morphologically characteristics.

2 Material and Methods

2.1 Sampling and collection area

The Parque Histórico de Guayaquil - PHG (Guayaquil Historical Park) is a remnant of mangrove forest located in Samborondón city, Ecuador. Lesions with symptoms associated with the presence of fungi in branches were identified, subsequently collected and stored in airtight bags for further analysis.

2.2 Morphological characterization of fungi samples

Morphological characterization was performed on cultures with evident generation of reproductive structure or in strains with more than 20 days of cultivation time. By the use of tape, the mycelium was separated and placed on a slide with a drop of lactophenol and sealed with a coverslip. The visualization of structures was done in a Nikon Eclipse E100 optical microscope with an integrated digital camera. In order to determine taxonomic affiliation, both, the mycobank database (*http://mycobank.org*) and taxonomic keys (de Hoog et al., 2001) were used.

2.3 Isolation and purification of fungi

The plant material was disinfected with 1 % sodium hypochlorite with repeated washings with sterile water. Fragments were subsequently cut with a sterile scalpel and deposited in SDA culture media (Saboraud Dextrose Agar, OXOID), supplemented with 15 μg/mL chloramphenicol for isolation and differentiation of fungi. Culture plates were incubated at room temperature in dark conditions until micellar growth was observed. This process was repeated until pure isolates were obtained. Samples previously isolated were inoculated in 150 mL of PW liquid culture medium (Peptone Water, CRITERION and Nutrient Broth) supplemented with 15 ng/mL chloramphenicol and incubated for 15 to 20 days until biomass appeared. Mycelium was filtered using sterile filtration units, biomass was dried at 42 °C for two days and stored at −80 °C until further analysis.

2.4 DNA extraction

Fungal material was incubated with lysozyme (10 μg/mL) at 37 °C for two hours. DNA extraction was performed using the Power Soil DNA Isolation kits (QIAGEN, Carlsbad, USA) with the following modifications: Fungi were previously dried and frozen at −80 °C and transferred to the Powerbead tubes; this material was mixed by vortex for three minutes. Subsequently, 60 μL of C1 buffer was added and a vigorous vortex was performed for three additional minutes, 20 μL of proteinase K was added and mixed with vigorous vortex for 1 minute. The mixture was centrifuged at 8,000 rpm 1 minute, the supernatant was rescued in a new eppendorf tube and 250 μL of C2 buffer was added, then an additional mixed by vortex for 5 seconds was performed and incubated at −20 °C for 5 minutes. Samples were centrifuged at room temperature at 8,000 rpm for 1 minute and the supernatant was transferred to a new tube; subsequently, 200 μL of C3 solution was added, vortexed for 1 minute and incubated at −20 °C for 5 minutes.

For DNA isolation, samples were centrifuged at 8,000 rpm for 1 minute and supernatant was transferred to a new tube with 1mL of C4 solution. The mixture passed through the columns by centrifugation at 8,000 rpm for 1 minute; thereafter, 500 μL of C5 solution was added and centrifuged at 8,000 rpm for 1 minute. For DBA elution, 100 μL ultrapure water was added to the center of the column to elute the genetic material, centrifuged at 13,000 rpm for 1 minute and stored at −20 °C until later use. DNA integrity was assessed by 1 % agarose gel electrophoresis in 1*times* TAE buffer (Tris, Acetate, EDTA) supplemented with SyBR green nucleic (Invitrogen) by comparing the intensity and molecular weight band of DNA with a 100 bp ladder (Tracklit - Invitrogen). Electrophoresis conditions were performed at 100 volts, 35 milliamps.

2.5 Amplification of the ITS-1 and ITS-2 regions by Polymerase Chain Reaction (PCR)

After DNA extraction, fungi were identified either genus or species level from the amplification of the intergenic regions of rDNA using ITS-1 (TCCGTAGGTGAACCTGCGG) and ITS-4 universal primers (TCCTCCGCTTATTGATATGC) (White et al., 1990). PCR conditions were performed in a final volume of 30 μL with final concentration of 0.2 5mM dNTPs, 2.5 mM MgCl2, 2 μM primers, 2 U taq pol and 1× PCR Buffer. The program includes denaturation at 95 °C for 5 minutes, followed by 35 cycles of 94 °C for 59 seconds, 50 °C for seconds and extension at 72 °C for 1 minute, with a final extension of 72 °C for 10 minutes.

2.6 Sequence analysis

Purified amplicons were sequenced in Macrogen (South Korea) and edited in Geneious Prime Software (version 2019.1) in order to obtain a consensus sequence. Taxonomic affiliation was confirmed by BLAST alignment algorithm (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*). Phylogenetic analysis was enhanced using Clustal W algorithm and Geneious Prime Software. A tree was generated by Neighbor Joining (Saitou and Nei, 1987) and a distance model of Kimura was used (Kimura, 1983) using MEGA 7 software, with 1,000 iteration bootstrap and eliminating gaps. The results were displayed with iTOL (Letunic and Bork, 2016). ITS sequences related to *Aspergillus* reported on NCBI database used in this research were listed in the supplementary Table 1A. The teleomorph of *Aspergillus* sp. used in this study was *Emiricella nidulans* (HQ026740.1), tree resolution was optimized using is *Aspergillus elegans* (NR077196.1 and MH992144.1), the outgroup was *Saccharomyces cerevisiae* (MG775707.1).

3 Results

Within the substrates collected in the present study, lesions allegedly caused by fungi were analyzed (Figure 1). From this plant, five strains of fungi were isolated and purified from branches and one strain isolated from propagules (Table 1). At the moment of growing in solid culture medium, white mycelia was formed, which later turned either black or grey with submerged mycelium (Figure 2 a-f). When observing the microscopic characteristics, it was determined that these isolates have smooth hyphae, with spherical vesicles covered by filiads and spherical conidia (Figure 2 h-l). Considering macro and microscopic characteristics, classical classification was performed by the use of taxonomic keys (de Hoog et al., 2001) and Mycobank database (*http://mycobank.org*). As a result, these fungi belong to *Aspergillus* genus but by ITS analysis, KCR3.2, KCR4.1sp and KCR15.1 correspond to *Aspergillus niger*, while KCR4.1.1., KCR7.2.1. and KCR14 are related to *Aspergillus aculeatus*.

Of all the 90 nucleotide sequences, 438 positions in the ITS region were used in the phylogenetic analysis. At first glance, all the species analyzed in this study belong to the Nigri section (Gams et al., 1986). The analysis is supported by a Bootstrap with 1000 iterations and by the Neighbor Joining algorithm. The tree shows well supported clades between fungi species in groups of *A. niger* and *A. tubingensis* and between *A. aculeatus* and *A. japonicus* (Figure 3). The dendrogram shows that both *A. niger* and *A. tubingensis* would be the same species, and this is also observed between *A. aculeatus* and *A. japonicus*.

As shown in Table 2, *A. niger* and *A. tubingensis* in the largest alignment with the size 3 626 085 bp are similar in 89.6 %, and between *A. aculeatus* with *A. japonicus* with the largest alignment of 3 727 362 bp, the pairwise identity is 90.9 %. This shows that they are very close to each other but also differ with members of other clades (i.e. *A. niger* with *A. japonicus* 78.3 % similar). At first glance, it can be seen phylogenetically that ITS region does not work well in the resolution of fungi groups. In order to get satisfactory results, it is necessary to complement these analyses with morphological identification and in this case, comparison between genomes.

4 Discussion

In Ecuador, the relevance of this study is related as a baseline of a mycobiome approach in mangrove fungi identification. Two species of *Aspergillus* were identified and purified, *A. niger* and *A. aculeatus*, which were previously reported in other mangroves forest in Malasya, Mexico, China and Indonesia (Sathiya et al., 2009; Lumbreras-Martínez et al., 2018; Deng et al., 2013; Li et al., 2017; Prihanto et al., 2019). Despite fungi are widely distributed in mangrove trees, and because some of them have ecological and physiological functions to increase the tolerance to biotic and abiotic stress conditions (Shu-Lei et al., 2020), the studies about diversity of fungi in mangroves are main focused in cultivable fungi but native uncultured fungus are poorly known.

Figure 1. Lesions associated with the presence of fungi in mangrove stems.

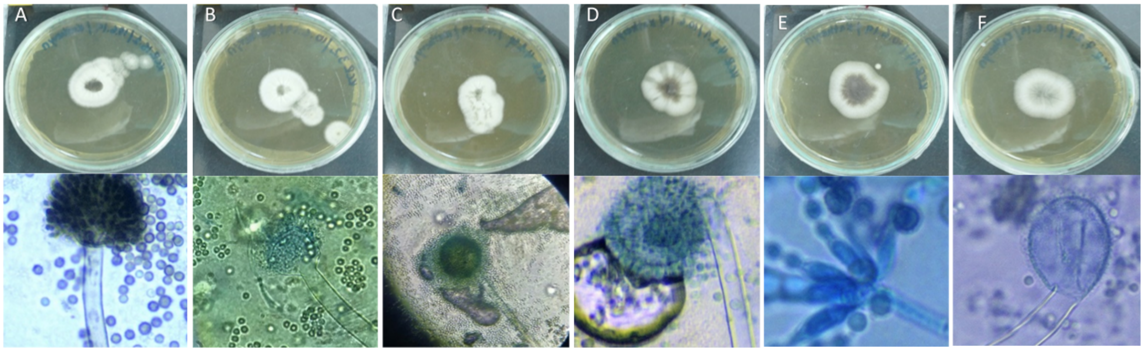

Figure 2. Morphological characteristics of fungal strains isolated from mangroves. (A-F) the top images show micellar growth in solid culture medium. A (isolated KCR 15.1); B (asylum KCR 3.2); C (isolated KCR 4.1 SP); D (isolated KCR 4.1.1); E (isolated KCR 14); F (isolated KCR 7.2.1); at the bottom image show microscopic structures of mangrove fungus isolates.

Table 1. Molecular characterization of fungus isolated from branching and propagules of mangrove

Sample	**Strain**	**Molecular Characterization**					
		Description	Max Score	Total Score	Query Cover (%)	Per. Ident (%)	E-value
Stems	KCR3.2	*Aspergillus niger*	1064	1224	100	99.83	0,0
	KCR4.1sp	*Aspergillus niger*	1048	1208	100	99.82	0,0
	KCR15.1	*Aspergillus niger*	1027	1178	100	99.82	0,0
	KCR4.1.1	*Aspergillus aculeatus*	874	874	100	100	0,0
	KCR14	*Aspergillus aculeatus*	946	946	83	99.81	0,0
Propagule	KCR7.2.1	*Aspergillus aculeatus*	1031	1031	100	100	0,0

Table 2. Genome comparison using LASTZ algorithm: Percentage values is the pairwise identity between two genome sets, numerical value is the largest sequence compare in two genome sets.

	A. niger	*A. tubingensis*	*A. aculeatus*	*A. japonicus*
A. niger	xxx	89.6 %	78.3 %	78.3 %
A. tubingensis	3 626 085	xxx	78.2 %	78.4 %
A. aculeatus	3 727 362	4 803 603	xxx	90.9 %
A. japonicus	1 444 553	4 803 603	3 727 362	xxx

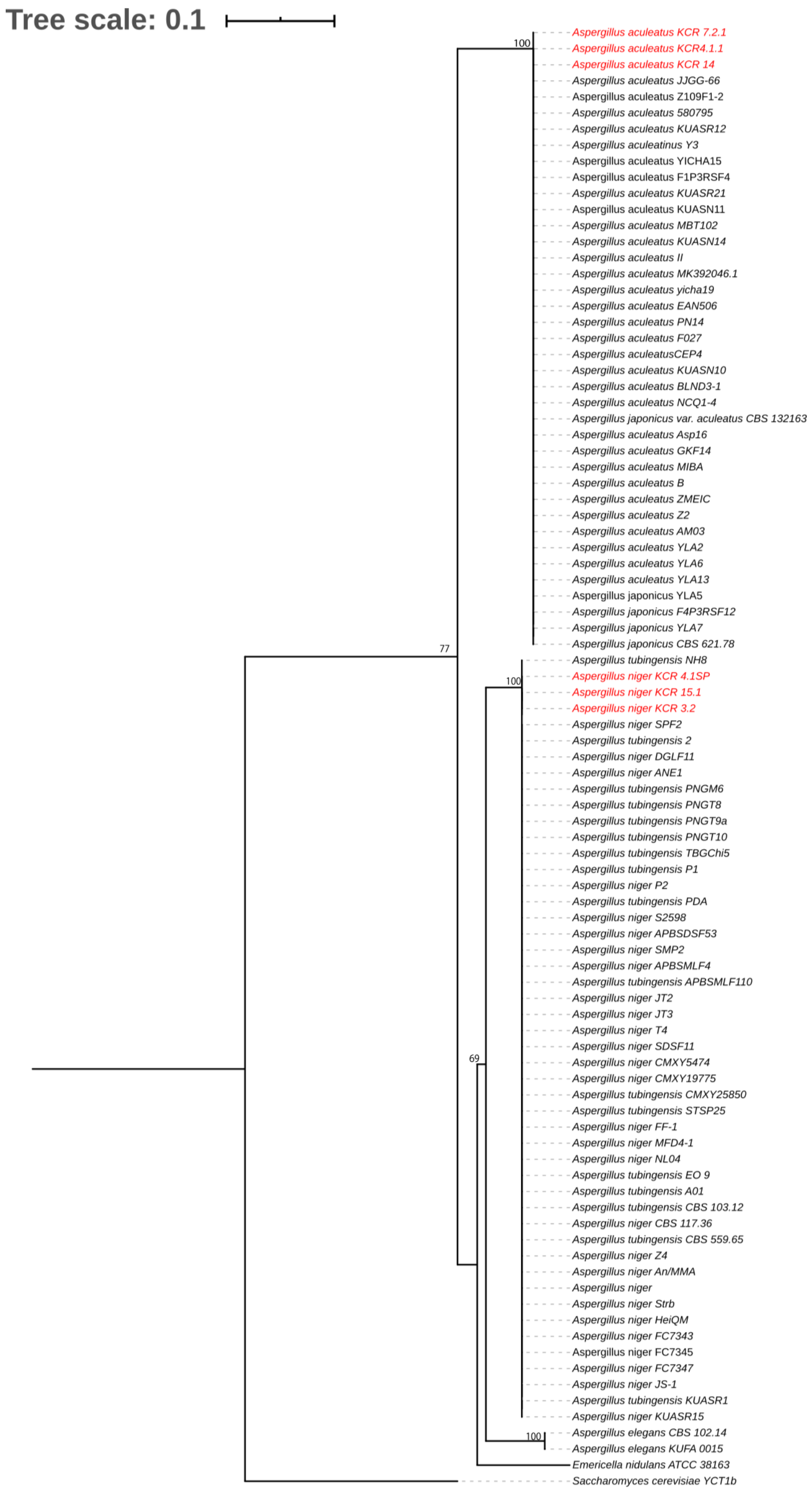

Figure 3. Phylogenetic analysis of fungi of the *Aspergillus* genus isolated from mangrove plant material. The sequences were aligned by Clustal W. The analysis was performed with a support of Bootstrap 1000 iterations and using the Neighbor Joining algorithm with the Kimura 2-parameter method. Teleomorph of *Aspergillus* is *Emiricella nidulans* (HQ026740.1) and outgroup *Saccharomyces cerevisiae* (MG775707.1) and two species of *Aspergillus elegans* (NR 077196.1, MH992144.1).

Traditionally, identification, taxonomy and classification of *Aspergillus* had been based according to morphological features (Patki et al., 2015). Nevertheless, taxonomic classification based on molecular characterization became an indispensable tool, since it allows to distinguish its phylogenetic characteristics (Samson et al., 2014). Considering previous published work about phylogenetic relationship of *Aspergillus*, it is indicated that, although the amplification of ITS regions is widely accepted as a DNA barcode for fungi in general (Conrad et al., 2012), phylogenetic analysis was not resolutive to distinguish uniseriate clades in Nigri section, especially with the aggregation of *A. niger* sequences, which could lead to misidentification of species (Abarca, 2000; Perrone et al., 2008). Our results are consistent with many of the research; the species *A. aculeatus* and *A. japonicus* were phylogenetically closed and similar *A. niger* and *A. tubingensis*. This also explains because there are minor differences between some species belonging to section Nigri, the species *A. niger sensu stricto*, *A. tubingensis*, *A. foetidus*, and *A. brasiliensis* are morphologically identical and altogether have been called *A. niger* aggregate (Munique et al., 2009). This is also consistent with Yokoyama et al. (2001) who described that most of the species of section Nigri are morphologically similar and the molecular identification by mtDNA and rDNA has been confused.

Resolutive molecular techniques as RFLP, AFLP, PFGE or Next Generation Sequencing - NGS would be greatly enhance knowledge and understanding of this fungus (Leong et al., 2006; Perrone et al., 2008; Quainoo et al., 2017). Consistently, genome comparison gives the best resolution to determine percentages of similarity between its sequences (Quainoo et al., 2017). In this study we performed the genetic similarity comparison to determine the genetic diversity between *A. niger*, *A. fumigatus* and between *A. aculeatus* and *A. japonicus*. The results showed high similarity with an average nucleotide identity (ANI) of approximately 90% of the species analyzed (Table 2). These results can be used to explain many different aspects, such as the strain background lineage, showing that this approach is highly useful for new fungal geneticists.

It has been shown that *Aspergillus* sp. is wide distributed in the environment, and it is considered as one of the most frequent phytopathogens by its ability to produce toxins that alter plants metabolisms (Pavón et al., 2012). Some of these fungi colonize roots, facilitating their growth and improving the quality of the grass. Other characteristics are solubilization of phosphorus, halotolerance, attenuation of saline stress to the plants (Li et al., 2017). *Aspergillus* is characterized by producing mycotoxins and secondary metabolites that are important in the degradation of organic matter and as defense mechanisms toward other microorganisms (Pavón et al., 2012).

The importance of this study also relies in the biotechnological capacity that both mangrove ecosystem and its microbial component can offer. One capacity is the physiological characteristic that fungi present with the production of specific protein, for example metabolites to inhibit cell growth of other microorganisms in a high salinity environment (Nicoletti et al., 2018). It has also been proven that some *Aspergillus* sp. help in bioremediation processes of environments contaminated by heavy metals, degradation of extracellular cellulose and hemicellulose (Marrero et al., 2012; Huachi et al., 2014; Araujo et al., 2016).

Regarding *A. niger*, it has been widely used to obtain enzymes for the industry such as: α-Amylase, catalase, cellulase, hemicellulase, lipase and organic acids that could be an alternative to replace petrochemicals (Patki et al., 2015; Ameen et al., 2016; Wang et al., 2016; Morthensen et al., 2017; Wang et al., 2019; Nascimento et al., 2019; Hossain et al., 2016). It is also used for production processes of citric acid that is carried out by solid state, submerged and surface fermentation (López et al., 2006). *A. niger* began being used in the biochemical fermentation industry and industrial biotechnology, since this specie of fungus produces a diverse range of proteins, enzymes and second metabolites (Cairns et al., 2018). The relevance of *A. niger* for the environment relies on its ability to bioaccumulate toxic metals such as lead, cadmium, copper and chromate (Rivera et al., 2015). Several studies have shown that *A. niger* has greater capacity to remove phenolic compounds, oil from contaminated soils and heavy metals, compared to granular activated carbon, used in several biosorption cycles (Araujo et al., 2016; Marzan et al., 2017; Villalba et al., 2018). In their study (Ghyadh et al., 2019) noted that the fungal *Aspergillus niger* showed the highest efficiency in reducing concentrations of heavy elements by 100 %. *Aspergillus aculeatus* showed high resistance to cadmium (CD) toxicity, protected the photosystem II against CD stress and increase the efficiency of photosynthesis process in perennial ryegrass. These results suggest that *A. aculeatus* could be useful to pretreating CD contaminated soils (Han et al., 2018). It has also an important effect in the attenuation of saline stress, since it produces indole-3-acetic acid and siderophores that confer tolerance to saline stress plants (Li et al., 2017). Research regarding the metabolic capacity of *A. niger* and *A. aculeatus* and their interaction with metal ions are limited (Emri et al., 2018). Although there are studies on different species of *Aspergillus* in mangroves, reports of *A. niger* and *A. aculeatus* are scarce or none. Moreover, most of the studies focus on biotechnological and bioremediation potential. *Aspergillus* species reduces high concentrations of heavy metals, but also produces a large number of mycotoxins and secondary metabolism, which may be capable of producing a bulk of bioactive compounds that are used at pharmaceutical industry (Frisvad et al., 2018; Shu-Lei et al., 2020). We need more research about *Aspergillus* species and their applications to produce different bioactives secondary metabolites.

Exploratory studies, as done in this work, allows to generate new concerns about the participation of fungi on the mangrove ecosystems. As well as assessing their possible role as protector for their hosts or determining whether they are pathogenic fungi that cause deterioration and breakdown of wood in mangroves. Briefly, this study was focused on endophytic fungus, turning it a potential candidate to the future applications for more investigations. Therefore, diversity of fungi in mangrove ecosystems are necessary worldwide, starting with the genomes sequencing, since it may represent a useful strategy for finding new metabolic pathways and, subsequently, new bioactive compounds and enzymes.

5 Conclusion

This study we report the molecular and morphological characterization of fungus isolated from mangrove belonging to clade Nigri, *Aspergillus niger* and *Aspergillus aculeatus*. This work is an effort to understanding the distribution of fungus species and highlight the importance to determine the fungus role in the mangrove ecosystem.

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Appendix

Table 1. A: List of GenBank accession number of species used in the present study.

Species	*Aspergillus aculeatus*	*Aspergillus japonicus*	*Aspergillus niger*	*Aspergillus tubingensis*
ITS sequences, accession number	MK644143.1	LC496497.1	KY082744.1	MN589663.1
	MG548756.1	MK035987.1	KY400582.1	MN239975.1
	MK371746.1	LC496498.1	KY566164.1	KY593521.1
	MN187297.1	MH861173.1	MF379661.1	KY593522.1
	MF564097.1		MG575468.1	KY593523.1
	MK271293.1		MG669185.1	KY593524.1
	MK035984.1		MG675233.1	MF143083.1
	MN187365.1		MG733652.1	MF379660.1
	MN187971.1		MG734750.1	MG279093.1
	MK886612.1		MG734751.1	MG733758.1
	MN187974.1		MG833314.1	MG991653.1
	MK911714.1		MG840739.1	MH045586.1
	MK392046.1		MG991588.1	MH398047.1
	MK418753.1		MG991627.1	MH540151.1
	MK518394.1		MH064151.1	MH854604.1
	MK559536.1		MH109325.1	MH858714.1
	MN088378.1		MH181162.1	MN187071.1
	MN173148.1		MH855726.1	
	MN186997.1		MH892847.1	
	MN396714.1		MK028957.1	
	MN509058.1		MK256745.1	
	MH865976.1		MK372989.1	
	MH656795.1		MK577432.1	
	MK713418.1		MK693450.1	
	MK733917.1		MK693453.1	
	MK788185.1		MK949087.1	
	MH892843.1		MN187307.1	
	MH892845.1			
	MK811100.1			
	LC496490.			
	LC496491.1			
	LC496492.1			

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Conservation

National illegal wildlife and threatened species trafficking: A descriptive study in Manabí (Ecuador)

Tráfico nacional de fauna silvestre y especies amenazadas: Un estudio descriptivo en Manabí (Ecuador)

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Abstract

Illegal wildlife trafficking has negative effects on biodiversity conservation at both global and local scale. Therefore, the establishment of appropriate conservation measures requires local studies that quantify this problem. The aim of this paper is to quantify and characterize the species of birds and mammals in the period 2016-2017, at Valle Alto Wildlife Rescue Center and Wildlife Refuge. The study showed that 212 specimens belonging to 41 different species were confiscated. More birds than mammals were confiscated, and a greater proportion of birds were included in a national and international threat category. A clear preference for primates, parrots and squirrels was found. Furthermore, the presence of species with a distribution range outside the study area revealed the existence of the transportation of species from other parts of the country. Although these data are only a sample of what is actually trafficked in the country, they provide an approach of the type of species that are illegally trafficked in this biodiversity hotspot.

Keywords: Illegal wildlife trafficking, endangered species, seizures, wildlife conservation, wildlife trade.

Resumen

El tráfico ilegal de vida silvestre tiene repercusiones negativas en la conservación de la biodiversidad a nivel global y también local. Por ello, el establecimiento de medidas oportunas de conservación requiere de estudios locales que cuantifiquen dicho problema. El objetivo de este trabajo fue cuantificar y caracterizar las especies de aves y mamíferos incautadas en el periodo 2016-2017, en el Centro de Rescate y Refugio de Vida Silvestre Valle Alto. El estudio mostró que 212 ejemplares pertenecientes a 41 especies diferentes fueron confiscados. Se decomisaron más aves que mamíferos y una mayor proporción de aves estaban incluidas en alguna categoría de amenaza a nivel nacional e internacional. Se encontró una clara preferencia por primates, loros y ardillas. Además, la presencia de especies con un rango de distribución fuera del área de estudio reveló la existencia del transporte de especies desde otras zonas del país. Si bien estos datos son solo una muestra de lo que realmente se trafica en el país, dan una aproximación del tipo de especies que se trafican ilegalmente en este hotspot de biodiversidad.

Palabras clave: Especies en peligro de extinción, incautaciones, conservación de vida silvestre, tráfico ilegal de fauna silvestre, comercio de vida silvestre.

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1 Introduction

Damage caused by the international illegal trade in wildlife species may represent one of the greatest threats to biodiversity conservation (Robinson and Sinovas, 2018). This problem has greatly affected Latin America for many years (Mancera and García, 2008). So much so, that in the 1960s and 70s, the Amazon basin was the main source of primate extraction for export abroad (Mittermeier et al., 1994), and in the period 2006-2012, Central and South America were, together with the Middle East, the largest importers of birds (legal and illegal trade) (Bush et al., 2014). Furthermore, a review of global trends concerning wildlife confiscation during the period 2010-2014 published by CITES shows that 32 % of the confiscated species were from wild populations in South America (D'Cruze et al., 2018). It is, therefore, possible to state that Latin America plays an important role in the legal and illegal trade of wild species, and that its countries participate as both exporters and importers (Bush et al., 2014; D'Cruze et al., 2018; Di Minin et al., 2019). Among the factors that could explain this phenomenon are poverty, the great biodiversity of the region, and socio-cultural dimensions (Duffy et al., 2016; Arroyave et al., 2020), being the root of the problem on the colonialism (Sollund and Runhovde, 2020). For example, hunting and illegal trade can become a sufficient source of income to finance subsistence expenses (Duffy et al., 2016; Rodríguez-Ríos and García, 2018).

Although few studies characterize or contribute data on wildlife trafficking by the countries in the region at the national level, it is known that illegal trade at the national level exceeds international trade in all countries (Goyes and Sollund, 2016). The specimens involved in this type of trade do not appear in the CITES data since they do not cross borders, which makes them more difficult to account for. Since Ecuador is a mega-diverse country (Mittermeier et al., 1997) with a large social inequality and a high percentage of rural populations (World Bank, 2020), it suffers the consequences of wildlife trafficking for meat consumption, trade and economic sustenance (Sinovas and Price, 2015; Ministerio del Ambiente, 2017a). According to data provided by the Ecuadorian Ministry of the Environment, a total of 1,526 specimens of birds and a total of 1,709 specimens of mammals were confiscated in the period 2003-2014 at a national level (Ministerio del Ambiente, 2012, 2014, 2015). For example, wildlife trafficking that began in the mid-1990s in Yasuni National Park tripled between 2005 and 2007, due to increased demand and improved access to remote locations (Suarez et al., 2009). However, it is important to account that the number of confiscated animals represents a low proportion of the real number of trafficked animals, since not all trafficked animals are confiscated. For instance, national confiscations in Colombia represented 1-10 % of the total number of trafficked animals during the period 1996-2004 (Mancera and García, 2008). In spite of being one of the great conservation problems (De la Torre, 2012; Tirira, 2013; Cervera et al., 2018), very few studies characterize the illegal wildlife trade in the country. The aim of this paper is to characterize the bird and mammal species that were trafficked in the province of Manabí, in the Coastal Region of Ecuador during 2016-2017, in terms of taxonomy, conservation status, original distribution range, and CITES listing. To do so, the data provided by the Valle Alto Wildlife Rescue Centre and Wildlife Refuge (the only rescue centre in the province during the study period) was used, which received all the confiscated animals from the province.

2 Material and methods

The study was carried out at the Valle Alto Wildlife Rescue Centre and Wildlife Refuge (Figure 1), located in the province of Manabí (Coordinates 1°5'37.13"S 80°16'16.57"W) in the Coastal Region of Ecuador (Zambrano et al., 2019), one of the world's biodiversity hot spots (Myers et al., 2000). This region, originally characterized by dry and humid forest, has been strongly transformed, becoming a mainly agricultural region, in which most of the forest remnants are small, fragmented patches (Rivas et al., 2020).

We employed the database of the Valle Alto Wildlife Rescue Centre and Wildlife Refuge of those bird and mammal specimens confiscated and received by the Centre during 2016-2017. The interception, confiscation and transfer to the Centre are carried out jointly by the police and the Ministry of the Environment. Once the animals arrive at the Centre, they are evaluated and remain confined until their recovery, to be released later. Those animals that cannot be released are transferred to zoos. The taxonomy status of mammal species was assigned according to Version 2020.1 of the document Mammals of Ecuador: updated checklist species (Tirira et al., 2020), and the taxonomic status of birds was assigned according to the Red List of Birds of Ecuador (Freile et al., 2019).

For these species, we considered the national level of threat according to the Red Book of Mammals of Ecuador (Tirira, 2011) and the Red List of Birds of Ecuador (Freile et al., 2019); the international level of threat according to the IUCN red list; and the original distribution (Coast, Andean, Amazon). Although this study is focus on national trade, CITES listing (https://www.cites.org/eng/app/index.php) was also included in order to get a reference about the threat of international trade for each species. We calculated absolute and relative frequencies of the number of species and individuals with respect to their distribution, order, CITES Appendix and the threat category in Ecuador and at international level. Then, we performed Fisher tests to compare proportions between birds and mammals concerning the level of threat at national and international level, CITES listing and distribution range.

Figure 1. The map shows the three main biogeographical regions of mainland Ecuador, the location of the Manabí province (yellow) and the Valle Alto Wildlife Rescue Centre and Wildlife Refuge (black spot).

3 Results

Overall, 212 animal specimens from 41 species and 14 orders were confiscated by the Valle Alto Wildlife Rescue Centre and Wildlife Refuge during 2016 and 2017. Our analysis showed that there were more birds confiscated (25 species, 147 specimens), followed by mammals (16 species, 65 individuals). From birds, the most frequent order in the confiscations was *Psittaciformes* (121 specimens, 13 species), which accounted for 82.3% of the total number of confiscated birds, being *Brotogerys pyrrhoptera* (IUCN Endangered) the most frequently confiscated species (29.9%) (Table 1). It is important to note that 39.7% of the confiscated birds were listed in one of the national threat categories and 31.3% in one of IUCN risk categories.

The most frequent confiscated mammal order was Rodentia (50.8%), mainly the species *Simosciurus stramineus* (36.9%), followed by Primates (32.3%), mainly *Saimiri cassiquiarensis* (10.8%) and *Alouatta palliata* (9.2%) (Table 2). Five species (*Choloepus hoffmanni,Alouatta palliata, Cebuella pygmaea, Cebus aequatorialis* and *Cebus capucinus*) of the sixteen mammal species were categorized as species at risk (1 CR, 2 EN and 1 VU) in Ecuador (20.2% of all mammal confiscations), and 92.3% of mammal confiscations were as Least Concern (LC) by the IUCN Red List (Table 2). It is important to highlight that one species (*Cebus aequatorialis*) was classified as Critically Endangered (CR) by the IUCN Red List.

Our study found the following differences between the birds and mammals confiscated: with regard to CITES Appendix in which the confiscated species were found, the data showed that the majority of birds (89.1%) were included in Appendix II, whereas most of the mammal species confiscated (60.1%) were not included in any Appendix, nonetheless a significant number of mammal species were included in CITES Appendix I (13.8%). As for the National risk category, a greater percentage of birds confiscated are categorized as Vulnerable in Ecuador (34.1%), while the majority of mammal species are listed in the Least Concern category (61.5%). In the case of the IUCN threat category, most of mammal species (92.3%) are included in the Least Concern category (92.3%), and an important proportion of birds (29.9%) were classified as Endangered (Table 3).

As for the distribution range, 71.4% of bird species and 64.6% of mammal species have the coast as their natural distribution range. It should be noted that 12.2% of birds and 16.9% of mammals had the Amazon as their distribution range. Most of the mammal species (91%) from Amazon were primates, and all the bird's species whose distribution range is in the Amazon belonged to the Psittaciformes order. There were not differences between mammal species and bird species concerning the distribution range in any case (Table 4).

Table 1. Bird species confiscated in Manabí (Coastal Region of Ecuador) during 2016-2017, their risk categories, and CITES Appendix listing.

Order	Scientific name	Individuals (*n*)	Relative frequency (%)	National risk category	IUCN risk category	CITES Appendix
Accipitriformes	*Buteogallus anthracinus*	1	0.7	VU	LC	II
Accipitriformes	*Rostrhamus sociabilis*	2	1.4	LC	LC	II
Anseriformes	*Dendrocygna autumnalis*	1	0.7	LC	LC	NI
Caprimulgiformes	*Steatornis caripensis*	1	0.7	LC	LC	NI
Columbiformes	*Zenaida auriculata*	4	2.7	LC	LC	NI
Columbiformes	*Zenaida meloda*	2	1.4	LC	LC	NI
Galliformes	*Ortalis erythroptera*	2	1.4	VU	VU	NI
Galliformes	*Penelope purpurascens*	3	2	VU	LC	NI
Pelecaniformes	*Ardea cocoi*	1	0.7	LC	LC	NI
Piciformes	*Pteroglossus torquatus*	1	0.7	NT	LC	NI
Psittaciformes	*Amazona amazonica*	2	1.4	LC	LC	II
Psittaciformes	*Amazona autumnalis*	11	7.5	EN	LC	II
Psittaciformes	*Amazona farinosa*	4	2.7	NT	NT	II
Psittaciformes	*Amazona ochrocephala*	1	0.7	LC	LC	II
Psittaciformes	*Ara ararauna*	3	2	NT	LC	II
Psittaciformes	*Ara macao*	1	0.7	NT	LC	I
Psittaciformes	*Ara severus*	2	1.4	LC	LC	II
Psittaciformes	*Brotogeris pyrrhoptera*	44	29.9	VU	EN	II
Psittaciformes	*Brotogeris versicolurus*	11	7.5	NE	LC	II
Psittaciformes	*Forpus coelestis*	5	3.4	LC	LC	II
Psittaciformes	*Pionus chalcopterus*	11	7.5	LC	LC	II
Psittaciformes	*Pionus menstruu*	8	5.4	LC	LC	II
Psittaciformes	*Psittacara erythrogenys*	21	14.3	NT	NT	II
Strigiformes	*Ciccaba virgata*	1	0.7	LC	LC	II
Strigiformes	*Glaucidium peruanum*	4	2.7	LC	LC	II

CR: Critically Endangered, EN: Endangered, VU: Vulnerable, NT: Near Threatened, LC: Least Concern, DD: Data Deficient, NE: Not Evaluated. NI = Not included in CITES Appendices.

Table 2. Mammal species confiscated in Manabí (Coastal Region of Ecuador) during 2016-2017, their risk categories and CITES Appendix listing.

Order	Scientific name	Individuals (n)	Relative Frequency (%)	National risk category	IUCN risk category	CITES Appendix
Carnivora	*Leopardus pardalis*	3	4.6	NT	LC	I
Carnivora	*Nasua nasua*	1	1.5	LC	LC	NI
Carnivora	*Potos flavus*	3	4.6	LC	LC	NI
Cingulata	*Dasypus novemcinctus*	1	1.5	LC	LC	NI
Pilosa	*Bradypus variegatus*	2	3.1	LC	LC	II
Pilosa	*Choloepus hoffmanni*	1	1.5	VU	LC	NI
Pilosa	*Tamandua tetradactyla*	1	1.5	LC	LC	NI
Primates	*Alouatta palliata*	6	9.2	EN	LC	I
Primates	*Cebuella pygmaea*	1	1.5	VU	LC	II
Primates	*Cebus aequatorialis*	4	6.2	CR	CR	II
Primates	*Cebus capucinus*	1	1.5	EN	NE	II
Primates	*Leontocebus lagonotus*	2	3.1	NT	LC	II
Primates	*Saimiri cassiquiarensis*	7	10.8	NT	LC	II
Rodentia	*Dasyprocta punctata*	6	9.2	LC	LC	NI
Rodentia	*Notosciurus granatensis*	2	3.1	LC	LC	NI
Rodentia	*Simosciurus stramineus*	24	36.9	LC	LC	NI

CR: Critically Endangered, EN: Endangered, VU: Vulnerable, NT: Near Threatened, LC: Least Concern, DD: Data Deficient, NE: Not Evaluated. NI = Not included in any CITES Appendices.

4 Discussion

This work demonstrates the existence of illegal trade of a great variety of mammal and bird species in the province of Manabí (Ecuador). A similar work performed during the same period in the neighbouring province of Guayas also showed that a great number of birds and mammals were trafficked in that region (Bazurto, 2018). Our results concur with two surveys performed in the region which showed that possession of wild animals is frequent in rural communities (Corrales, 2018; Cedeño, 2020). In consequence, illegal wildlife trade can be considered as one of the main threats for birds and mammals in Manabí, and actions are needed to reduce the demand.

It terms of legislation, for the period 2016 and 2017, it is important to note that wildlife trafficking is typified as a crime by the Article 247 of the Comprehensive Organic Criminal Code (Código Orgánico Integral Penal), which states that: "The person who hunts, fishes, captures, collects, extracts, posses, transports, traffics, benefits from, permutes or commercializes, specimens or their parts, their constituent elements, products and derivatives, of terrestrial, marine or aquatic flora or fauna, of threatened, endangered and migratory species, listed at a national level by the National Environmental Authority, in addition to international instruments or treaties ratified by the State, will be sanctioned with a custodial sentence of one to three years" (Ministerio de Justicia Derechos Humanos y Cultos, 2014). This entails that the animal specimens detained during 2016-2017 in the Manabí region were trafficked despite the law in Ecuador, which is even more dissuasive than in other neighbouring countries (e.g., Brazil) (Sollund and Runhovde, 2020), suggesting that additional measures should be established to avoid this problem. Since illegal wildlife trafficking is a complex issue involving ecological, socioeconomic, and cultural factors (Phelps et al., 2016; Biggs et al., 2017), the solution cannot be based only on legal instruments and it is necessary to develop a holistic approach to reduce the demand.

Table 3. CITES listing Appendix, and National and international risk categories of bird and mammal species confiscated in Manabí (Ecuador) during 2016-2017.

	Birds		Mammals		Fisher test
CITES appendix	*n*	%	*n*	%	P-value
I	1	0.7	9	13.8	p<0.0001
II	131	89.1	17	26.2	p<0.0001
III	0	0	0	0	–
Not included	15	10.2	39	60.1	p<0.0001
National risk categories					
CR	0	0	4	6.2	p=0.008
EN	11	7.5	7	10.8	p=0.594
VU	50	34.1	2	3.1	p<0.0001
NT	30	20	12	18.5	p=0.852
LC	45	30.6	40	61.5	p<0.0001
DD	0	0	0	0	–
NE	11	7.5	0	0	p=0.037
IUCN risk categories					
CR	0	0	4	6.2	p=0.008
EN	44	29.9	0	0	p<0.0001
VU	2	1.4	0	0	–
NT	25	17	0	0	p<0.0001
LC	76	51.7	60	92.3	p<0.0001
DD	0	0	0	0	–
NE	0	0	1	1.5	–

CR: Critically Endangered, EN: Endangered, VU: Vulnerable, NT: Near Threatened, LC: Least Concern, DD: Data Deficient, NE: Not Evaluated.
Notes: The last column shows the proportion differences according to the Fisher's exact test. The test was not performed in rows with "–" due to the low number of observations.

Table 4. Distribution Range of bird and mammal species confiscated in Manabí (Ecuador) during 2016-2017.

	Birds		Mammals		Fisher Test
Distribution Range	*n*	%	*n*	%	p-value
Amazon	18	12.2	11	16.9	p=0.389
Coast	105	71.4	42	64.6	p=0.336
Coast-Amazon	18	12.2	7	10.8	p=0.821
Coast-Andean	4	2.7	2	3.1	–
Coast-Andean-Amazon	0	0	3	4.6	–
No data	1	0.7	0	0	–
Andean-Amazon	1	0.7	0	0	–

Notes: The last column shows the proportion differences according to the Fisher's exact test. The test was not performed in rows with "–" due to the low number of observations.

According to our data, more birds were confiscated than mammals, and they had a more worrying conservation status, which entails that the illegal wildlife trade could have a greater effect on birds. This could be explained by the existing demand of birds as pets (Da Nóbrega and Pereira, 2007; Bush et al., 2014). As mentioned previously, the most frequently trafficked bird order was Psittaciformes (e.g., *Ara macao*, Figure 2a), data that coincides with reports from the Ecuadorian Ministry of the Environment for 2013, which stated that 71 % of seized birds nationwide were *Psittaciformes*; being also one of the most threatened order worldwide (Olah et al., 2016; Bird Life International, 2017). For instance, *Brotogeris pyrrhoptera* represented the 30 % of the bird confiscated, and this species is classified as Vulnerable in Ecuador and Endangered by IUCN red list. Due to the preference of this order as a pet (Romero et al., 2020) these species are highly trafficked. Indeed, the current decline of Neotropical parrot population is closely related to the local parrot trade for use as pets, in addition to their capture for international trade (Berkunsky et al., 2017). This phenomenon is indeed reflected in our study, since most of confiscated bird species were Psittaciformes, moved from the Amazon to the Coastal Region, outside their natural range distribution, which can also have a negative impact as invasive exotic species (Bush et al., 2014; Zhou et al., 2015). This is the case of *Brotogerys versicolurus*, the third most confiscated bird species in our study, native to the Amazon and which introduced the New Castle disease into Peru through the release of confiscated and infected individuals (Daut et al., 2016).

Figure 2. The scarlet macaw (*Ara macao*) (a) and the Humboldt's squirrel monkey (*Saimiri cassiquiarensis*) (b), examples of native species from the Amazon confiscated in the Coastal Region, Ecuador.

Concerning mammals, rodents were the most trafficked order, and among these squirrels were the most trafficked species. It is easy to find squirrels in international pet markets or being transported around the world (Bertolino, 2009), and it is not, therefore, surprising that it was also one of the main rodents confiscated in our study area. The second most trafficked rodent was *Dasyprocta punctata*, probably owing to the fact that it is consumed as bush meat in the region (Rodríguez-Ríos and García, 2018). After Rodentia, primates were the most seized mammal species in the region of Manabí, where they have been traditionally used as pets (De la Torre, 2012). A study about trafficking of native primates in Ecuador for 1989-2012, found that 98 % of the primates were destined to be pets (Tirira, 2013). Also, a local study with data on illegal wildlife trafficking, revealed that 42 % of the mammals subject to illegal trade were primates (De la Torre, 2012). Similarly, in 2013, a report by the Ecuadorian Ministry of the Environment stated that 30 % of confiscated mammal species were primates.

In our study, the Humboldt's Squirrel Monkey (*Saimiri cassiquiarensis*; Figure 2b), native to the Amazon region in Ecuador, was the most trafficked primate, which coincides with national data as being the most trafficked primate species during 1989-2012 (Tirira, 2013). Moreover, De la Torre (2012), in a study in the Ecuadorian Amazon, reported that 40 % of the primates confiscated by the Ministry of the Environment during the period 2008-2010 belonged to this same species. The demand for this species as pet is probably owing to its graceful appearance, small size and ability to adapt to different environments, along with a low-selective diet (De la Torre et al., 2011). Additionally, two species with natural populations in the Coastal Region, the mantled howler monkey (*Alouatta palliata*) and the white fronted capuchin (*Cebus aequatorialis*) were also confiscated during this study; *C. aequatorialis* is uncommon on the coast (Guerrero-Casado et al., 2020) and it is classified as critically endangered (CR) at national level (Tirira, 2011). In summary, our data confirm that there is a demand for primates mainly to be used as pets, thus reducing the trade of these species requires special attention.

Furthermore, we should not underestimate the fact that an important proportion of the confiscated species are classified at risk by IUCN, which suggests that the impact of national illegal trade on wild populations may be shattering. Although our study was focused on wildlife trade at a national scale, it is also important to highlight that the majority of the bird species seized are included in CITES Appendix II, which includes species that could be threatened if their international trade is not controlled. Furthermore, although most mammal species are not included in the CITES Appendices, it is worrying that 13.8 % are listed in CITES Appendix I, which includes species threatened with extinction. Reducing national illegal trade could definitely contribute to improve the conservation status of these species.

Finally, although the Coastal Region is the natural distribution range of most of the seized species, our results showed that there is also trafficking from the Amazon area (11 % of specimens), most of which were Primates and Psittaciformes. The introduction of exotic species can have negative consequences on the ecosystems of the coastal region (Bush et al., 2014), in addition to animal welfare concerns for the capturing (in the Amazon), transportation (to the coast region) and subsequent possession of specimens (Baker et al., 2013). Avoiding the movement of animals from the Amazon should be therefore considered a priority in a national strategy to reduce illegal wildlife trade.

5 Conclusión

Our data showed there is a great variety of bird and mammal species affected by illegal wildlife trafficking, many of which are threatened at both national and international level. National wildlife trade should be considered a major conservation concern, and further protection and conservation measures should be implemented to reduce the number of species subject to trade. In this regard, the objective of the National Policy for Wildlife Management (Ministerio del Ambiente, 2017b) is to allow the different governmental levels a coordinated exercise to control and monitor the use and commercialization of wildlife at a national and local level (Mestanza-Ramón et al., 2020) in order to fully reduce illegal and unsustainable wildlife trade in Ecuador. Although Ecuadorian law already considers the offence of wildlife trafficking with a custodial sentence, we believe that better controls and management information systems (e.g., improvement of data reporting) should be enforced together with the development of other complimentary actions, such as environmental education programmes with the aim of creating awareness about the negative consequences of wildlife exploitation on animal welfare, loss of biodiversity and human health. This last topic has been highlighted in the last year because of the Covid-19, and many researchers and doctors (e.g., Roe et al. (2020); Aguirre et al. (2020)) have claimed that it is necessary to reduce the illegal wildlife trade to prevent future pandemics under the one health perspective.

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Scientific paper/ Artículo científico
Biotechnology

Preparation of synthesized silver nanoparticles from extract of rosemary leaves (*Rosmarinus officinalis L.*) and its used as a perservative

Elaboración de nanopartículas de plata sintetizadas a partir de extracto de hojas de romero (*Rosmarinus officinalis L.*) y su uso como conservante

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Abstract

Nanoparticles are materials that measure from 1 to 100 nm of length. Currently, the antimicrobial property of silver nanoparticles is used by industries for the manufacture of beauty products and medicines. Nanoparticles can be synthesized from plants, algae or microorganisms, and they can also be obtained from combustion products. In this study, extracts of rosemary leaves (*Rosmarinus officinalis* L.) were used for the synthesis of silver nanoparticles (NPs-Ag) in order to produce an antimicrobial compound to be used as a fruit preservative. NPs-Ag were characterized qualitatively and quantitatively by phytochemical analysis and UV-VIS spectroscopy, showing an absorption in the range of 389-418 nm, which corresponds to their surface plasmon resonance. Furthermore, Scanning Electronic Microscopy was used to determine the size and morphology of the NPs-Ag, observing a spherical shape of 10 nm of diameter. Two bacterial strains were used in the antimicrobial tests, the Gram-negative (*Escherichia coli*) and the Gram-positive (*Staphylococcus aureus*) to verify the antimicrobial activity of the NPs-Ag. For *E. coli*, a better antibacterial activity was obtained with an inhibition halo of 3.21 mm. Subsequently, the NPs-Ag were used in apples to determine their use as a preservative, using beeswax smeared on the surface of the fruit as control, observing that synthesized NPs-Ag prolonged the maturation time of the fruits.

Keywords: Aqueous extract, silver nanoparticle, spectroscopy, rosemary.

Resumen

Las nanopartículas son materiales que pueden llegar a medir entre 1 a 100 nm de longitud, y en la actualidad la propiedad antimicrobiana de las nanopartículas de plata es aprovechada por las industrias para la fabricación de productos de belleza y medicamentos. Las nanopartículas pueden ser sintetizadas a partir de plantas, algas o microorganismos, y también pueden ser obtenidas como productos de combustión. En este estudio se utilizaron extractos de las hojas de romero (*Rosmarinus officinalis* L.) para la síntesis de nanopartículas de plata (NPs-Ag) con la finalidad de producir un compuesto antimicrobiano para usarse como conservante de frutas. Las NPs-Ag se caracterizaron cualitativa y cuantitativamente mediante análisis fitoquímicos y espectroscopia UV-VIS, presentando una absorción en el rango de 389-418 nm, que corresponde a la resonancia de su plasmón superficial. Además, se empleó la microscopía electrónica de barrido para determinar el tamaño y morfología de las NPs-Ag, observándose una forma esférica de 10 nm de diámetro. Se emplearon dos cepas bacterianas en los ensayos antimicrobianos realizados, la gramnegativa (*Escherichia coli*) y la grampositiva (*Staphylococcus aureus*) para comprobar la actividad antimicrobiana de las NPs-Ag. Para *E. coli* se obtuvo una mejor actividad antibacteriana con un halo de inhibición de 3,21 mm. Posteriormente se usaron las NPs-Ag en manzanas para determinar su uso como conservante, usando la cera de abeja como control untada en la superficie de las frutas, observándose que las nanopartículas sintetizadas alargaron el tiempo de maduración de la frutas.

Palabras clave: Espectroscopia, extracto acuoso, nanopartícula de plata, romero

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1 Introduction

Nanoparticles are considered materials with an approximate length from 1 to 100 nm (Vázquez and Blandón, 2014). Today the antimicrobial properties of Ag-NPs and other metals such as gold (Au), platinum (Pt) and palladium (Pd) are exploited by industries for ellaborating beauty products such as soap, shampoo, detergent, toothpastes, medicines, among other applications within medicine, electronics, polymers and ceramics (Anandalakshmi and Venugobal, 2017; Vera et al., 2017). Many research has been carried out on the use of nanotechnology in the food industry, and the use and application of nanoparticles has been investigated in the area of food safety and quality, packaging and development of new foods. Nanoparticles have also been used to prevent microbial contamination in packaged foods, improve the physical properties of foods, and increase the bioavailability of their components (Pardo de Santayana, 2018; Thiruvengadam et al., 2018; Ávalos et al., 2013). Currently there is no legislation regulating nanotechnology and nanomaterials in food; for this reason, they are included in the European Chemicals Regulation (REACH). The European Food Safety Authority in its publication on guidance for assessing risks posed by the use of nanotechnology and nanoscience in food recommends the need for the development, validation of methodologies and improvements in research about toxicity and genotoxicity of nanoparticles (Ávalos et al., 2013).

There are various types of metal nanoparticle synthesis such as chemical synthesis using redox reaction (reducing agent), electrochemical method, photochemical reduction and ultraviolet irradiation, and all of these use chemical reagents, consume energy and cause environmental pollution because a large amount of chemical waste is generated, having water, soil or air as its final destination (Vera et al., 2017; Chandran et al., 2006; Rodríguez et al., 2013; Torres, 2017).

Nanoparticles are currently being synthesized by green synthesis which produces less pollutant wastes compared to traditional methods. Nanoparticles are obtained by natural sources through this synthesis by changing the chemical compounds to natural extracts of plants, algae or microorganisms, being this method effective, easy and non-toxic, becoming a synthesis technique of ecological and eco-friendly nanoparticles (Flores, 2014). This non-conventional technique provides the possibility of obtaining nanoparticles of noble metals in aqueous solutions at low temperatures, using plant extracts that act as a reducing agent found in high concentrations in addition to containing natural stabilizers (Belmares et al., 2015). For this reason, to obtain nanoparticles from the green synthesis, plants are mostly used with respect to other natural sources such as algae, bacteria, yeasts, among others, because they are less toxic (Vera et al., 2017).

Nanoparticles of silver have been synthesized from the peels of some fruits or vegetables such as banana, garlic, aloe vera, basil, matico (*Buddleja globosa*), coriander and rosemary, which have bioactive components with naturally antimicrobial content. According to their properties, they are able to produce a reduction in silver (Madrid, 2017).

Rosemary (*Rosmarinus officinalis* L.) belongs to the Lamiaceae family; it is native to the Mediterranean region of Europe and is known as an aromatic and medicinal plant. This plant presents secondary metabolites and essential oils such as flavonoids, terpenes, phenolic acids, among others, and is also used as seasoning and flavoring (Purca, 2013). Rosemary is used in the food, pharmaceutical, cosmetic and other industries. According to Briones (2017), the most important by-products of rosemary are aqueous extracts and essential oils. In addition, Avila-Sosa et al. (2011) expressed that plant extracts extracted from the rosemary leaves possess antioxidant and antimicrobial compounds and properties that were used in ancient times as ancestral medicine.

The aim of this study was to extract and synthesize silver nanoparticles from the extract of rosemary leaves and to evaluate their antimicrobial activity in a traditional fruit for its conservation.

2 Materials and Methods

2.1 Obtaining of the natural extract of rosemary leaves

The following steps were followed to obtain the natural extract of the rosemary plant that favors the reduction process of silver nitrate and the formation of nanoparticles: first the rosemary plant was harvested, then the leaves that showed the best condition were selected. These were washed and disinfected with a liquid chlorine dioxide solution at 10 ppm for 10 minutes following Garmendia and Vero (2006). The reduction of the size of the leaves was then carried out using a mortar, following the methodology of Martinez et al. (2013). The extract obtained was let to boil (100 °C), boiling 100 gr of leaves with 100 ml of water for 20 to 25 minutes with constant agitation.

The sample was then filtered on paper (Whatman, 0,45 μm), and 100 gr of additional leaves were added to the broth to concentrate it to a volume of 50 ml, repeating the above extraction procedure. Finally, the final extract was filtered, allowed to cool at room temperature and then stored in a glass container. To preserve the extract, the extract was refrigerated at a temperature of 4 °C for 6 days.

The secondary metabolites were then determined by qualitative and quantitative analysis, using three types of extract with different solvents each: distiled water (EA), ethanol (EE) and acidulated water (EAC), in order to use the extract that presented the best results by its compounds and chemical properties for its use as nanoparticle. Each of these extracts had phytochemical analyses to check whether it contained functional groups with reducing chemical properties.

The latter was determined by phytochemical screening, using a successive extraction with solvents of increasing polarity, starting the analysis on the ethereal extract, followed by the alcoholic extract and finally the aqueous extract. This technique allowed identifying the secondary metabolites, using appropriate reagents (Table 1) that resulted in colored reactions or precipitation of the secondary metabolites (Amaguaña, 2018; Santorum, 2017).

Table 1. Phytochemical analysis of the aqueous, ethanolic and acidified extract of *Rosmarinus officinalis L.* used reagents and expected results.

Phyto-chemical essays	Reactive or treatment	Positive results
Saponins	Agitate	Presence of foam for 2min
Reducing compounds	Fehling Reactive	Red color and presence of reducing disaccharide sugar
Phenolic compounds	3 drops of ferric chloride solution at 5%.	Red color and presence of phenolic compounds. Dark green or blue and presence of tanins
Flavonoids	1 ml of HCl+ 1 ml H_2O + 2ml of alcohol	Red to Brown and presence of flavonoids
Alkaloids	1 drop of hydrochloric acid + 3 drops of Dragendorff reactive	Opalescence (+), defined turbidity observed (++), pellet observed (+++).
Resins	Distilled water	Pellet
Terpenoids	1 ml de chloroform + 1ml of carbon dioxide + 2 to 3 drops of concentrated H_2SO_4	Pink, green and dark green indicate positive results

2.2 Synthesis of silver nanoparticles

Silver nitrate (CAS number: 7761-88-8) with a purity of (95%) was used as a metallic precursor. For the preparation of aqueous silver nitrate ($AgNO_3$) 0.034 grams of $AgNO_3$ (1 mM) were dissolved in 200 ml of distilled water to prepare the stock solution. The rosemary leaf extract was then used in volumes of 5ml, 10ml, 20ml, 30ml, 40ml and 50ml to check the appropriate concentration, keeping the silver nitrate solution constant (5 ml). The pH of each of the prepared solutions was adjusted with sodium hydroxide (NaOH) at 0.1 N to obtain a basic pH between 8 and 10, measured with a pH meter (Thermo Scientific ™Orion Star ™A211). The temperature at which these solutions were maintained was 65 °C in constant agitation. This solution was stored at 4 °C Subsequently, the Ag-NPs was characterized by UV-VIS spectroscopy in a spectral absorption range between 350-800 nm, corresponding to the resonance of the surface plasmon of metal nanoparticles, using a spectrophotometer (Genesys 10 UV scanning). To determine the size and shape of the Ag-NPs obtained, these were observed with a scanning electron microscope (Sánchez, 2017).

2.3 Effectivity of synthetized silver nanoparticles

The antimicrobial effectiveness of Ag-NPs was measured using antimicrobial disks against the Gram-negative *E. coli* bacteria and the Gram-positive *S. aureus*. For this purpose, the inhibition halo size (mm) corresponding to the area where antimicrobial effectiveness was evidenced was considered (Bauer et al., 1966). In Petri dishes with Mueller-Hinton culture medium, a cell concentration of $1{,}5 \times 10^8$ UFC/ml of the Gram-positive and Gram-negative bacteria used was planted. Before inoculation, Petri dishes were divided into three quadrants where the pure extract, the silver nitrate solution and the synthetized nanoparticles were placed in each of them. The Antimicrobial Susceptibility Disks were then placed and the plates were incubated at 37 °C for 24 hours. Finally, bacterial inhibition halos were measured using a vernier.

2.4 Synthetized silver nanoparticles used in a fruit

To determine whether the extract of synthetized Ag-NPs can be used as a preservative, the surface of a seasonal fruit (red apple) was covered and the results were compared with bee wax. Twelve red apples (*Red delicious*) bought in the market were used, which were washed and disinfected with water containing 10 ppm of chlorine for 5 minutes. Subsequently, two apples were placed in preservative boxes that were divided into two cells. The apples of preservation box 1 were the control (white), the two apples of preservation box 2 received the nanoparticle solution with a 5:5 ratio. The two apples of preservation box 3 received the nanoparticle solution with a 5:10 ratio, and the two apples of preservation box 4 received beeswax. The two apples in preservation box 5 received beeswax plus the nanoparticle solution with a 5:5 ratio and the apples placed in preservation box 6 received beeswax and a nanoparticle solution with a 5:10 ratio (Figure 1). This study was conducted for 30 days, during this time the weight, color and maturity of the fruit were monitored.

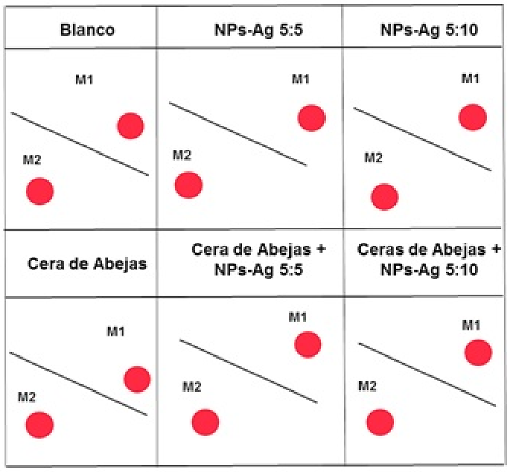

Figure 1. Graphical representation of the trial of the Ag-NPs used as a preservative (Ag-NPs= Silver Nanoparticles, M1= Apple 1 and M2= Apple 2).

3 Results and Discussion

3.1 Phytochemical characterization of rosemary leaf extract

The results obtained from the phytochemical analyzes of aqueous, acidulated and ethanolic extracts are shown in Table 2. The phytochemical characterization showed the presence of the metabolites present in the chemical composition of this plant, identifying 2844.0 mg/kg of terpenes and 24% of phenols. The presence of saponins was not evident because there was no foam formation in the sample, nor were resins observed by the aqueous extract, but there were phenolic compounds and terpenoids, as well as reducing sugar, flavonoids and alkaloids, which are considered the chemical compounds of the rosemary plant with reducing properties. This analysis was carried out to corroborate that rosemary is a plant rich in active principles, which have been widely studied. These chemical compounds act in almost all organs of the human body by possessing high percentages of essential oils whose active ingredients are flavonoids, phenolic acids, triterpenic acid and triterpenic alcohols. Rosemary leaves have a high content of Romarisnic acids and their derived rosalmarin is also present in carnosic acid, which is characterized by being unstable (Avila-Sosa et al., 2011).

As indicated by Salguero and Pilaquinga (2017), when silver nanoparticles are synthesized through the use of plant extracts, the power of the phytochemical and ethnopharmacological properties of plants is exploited. In this case, rosemary has numerous beneficial properties, along with the bactericidal property of silver nanoparticles that contributes to increasing its usefulness in the biomedical and microbiological area, without generating environmental pollution. The above may contribute to the fact that the synthesized Ag-NPs from rosemary leaf can be useful for fruit conservation.

Table 2. Phytochemical analysis of the aqueous, ethanolic and acidified extract of *Rosmarinus officinalis L.* leaves

Phytochemical essays	Aqueous extract	Ethanolic extract	Acidified extract
Saponins	Negative	Negative	Negative
Reducing compouns (Fehling reactive)	Positive	Positive	Positive
Phenolic compounds	Positive	Positive	Negative
Flavonoids	Positive	Positive	Negative
Alkaloids: (Dragendorff trial)	(+++) (++)	(+++) (+)	(+)
Resins	Negative	Negative	Negative
Terpenoids	Positive	Positive	Positive

(+) Opalescence is observed, (++) defined turbidity is observed, (+++) precipitate is observed

3.2 Synthesis of silver nanoparticles

Rosemary leaf extract was combined with silver nitrate ($AgNO_3$) for the formation of metal nanoparticles by green routes, resulting in a dark brown solution with intense spicy odor at a pH 6 and at a temperature of 65 °C, as shown in Figure 2. Out of the prepared concentrations (5:5, 5:10, 5:20, 5:30 and 5:40), the best concentration of Ag-NPs was obtained at day 3, which was when the solutions turned from yellow to dark brown. These prepared Ag-NPs solutions were characterized by UV-VIS spectroscopy and those within the manometric range were those with the ratios of 5:5, 5:10 and 5:20, as shown in Table 3. The ratios of 5:5 and 5:10 showed the best behavior because the nanosilver solution showed better stability in less time, and because its wavelengths were between the ranges of 412-418 nm. The ratio 5:5 showed a wavelength of 418 nm at day 1 and 405 nm at day 5, while in the ratio 5:10 the wavelength was 409 at day 1 and 412 at day 5.

The results obtained agree with those reported by Vera et al. (2017), who used the aqueous rosemary extract technique and the oxonitrate silver agent in their research on the synthesis of metal nanoparticles by green routes (AgNO) for the formation of Ag-NPs, estimating a 30-minute reaction time, resulting in a yellow color and confirming the formation of nanoparticles and their application as an effective antimicrobial agent.

Similarly, Salguero and Pilaquinga (2017) synthesized and characterized silver nanoparticles prepared with aqueous cilantro extract coated with Drago's blood, observing a color change from yellow to orange when 10 ml of $AgNO_3$ (10 nM) at 60 °C was mixed with 2.2 ml of the cilantro extract.

In the same way, Cardeño and Londoño (2014), used the garlic extract (*Allium sativum*) as a reducing agent, and then added an aqueous solution of $AgNO_3$ at a temperature of 50 to 60 °C, a method that allowed observing the reduction of silver ions for 30 minutes by changing the color of the solution from gold to yellow. The authors performed UV-visible measurements by using the surface plasmon resonance method to demonstrate the presence of the Ag-NPs, finding the best peaks in the prepared solutions between the bands of 400 and 470 nm, these wavelengths are similar to those reported in the present study.

Figure 2. Dark Brown colouring of Ag-NPs

As in the study carried out by Amaguaña (2018), who synthesized silver nanoparticles from the leaves of sensitiva (*Mimosa albida*), the presence of silver nanoparticles was evidenced when the coloring of the solution turned from greenish yellow to brown when the silver nitrate solution was added to it, mentioning that this brown coloring is observed due to the resonance of the superficial plasmons, which is characterized by the formation of nanoparticles.

Bijanzadeh et al. (2012) in their study on the synthesis of silver nanoparticles using chemical methods and characterizing them by UV-VIS spectroscopy observed the plasmon surface for the Ag-NPs absorption bands at wavelengths between 395 and 425 nm. Santorum (2017), in the study on the synthesis of Ag-NPs in (*Piper adumcun*) mentioned that the presence of Ag-NPs is observed during peak absorption at 400nm, and also noted that the size and shape of nanoparticles caused the absorption band to move to larger wavelengths. All these results agree with those reported in this study, where plasma resonance values between 405 and 418 were obtained for the proportions of Ag-NPs synthesized with a 5:5 and 5:10 ratio (Table 3).

Table 3. Characterization of the best proportions of Ag-NPs by UV-VIS spectroscopy

Volume ratio (ml)	Day	Wave lenght (λ)	Absorbance			Absorption factors (L/mol.cm)	Concentration (mg/l)		
5:5*	1	418	2.328	2.326	2.329	16.98	34.21	34.27	34.25
	3	405	2.362	2.363	2.362	14.12	34.72	34.75	34.76
5:10*	1	409	2.374	2.373	2.376	12.32	34.61	34.67	34.65
	3	412	2.312	2.317	2.318	14.54	34.50	34.52	34.52
5:20	1	404	2.368	2.367	2.367	16.38	34.78	34.77	34.77
	3	399	2.416	2.417	2.418	15.03	34.84	34.84	34.83

* Better synthesized ratio

Figure 3 shows that silver nanoparticles on day 1 (pH 10) showed a peak absorbance at a wavelength of 418 nm with a 5:5 ratio, on day 1 (pH 10) a peak of maximum wavelength absorbance higher than 409 nm was observed with a 5:10 ratio. On day 3, nanoparticles showed a peak of maximum wavelength absorbance higher than 405 nm with a 5:5 ratio (pH 10) and a peak of maximum wavelength absorbance higher than 412 nm was presented with a 5:10 ratio (pH 10). All absorbance peaks obtained are typical of Ag-NPs.

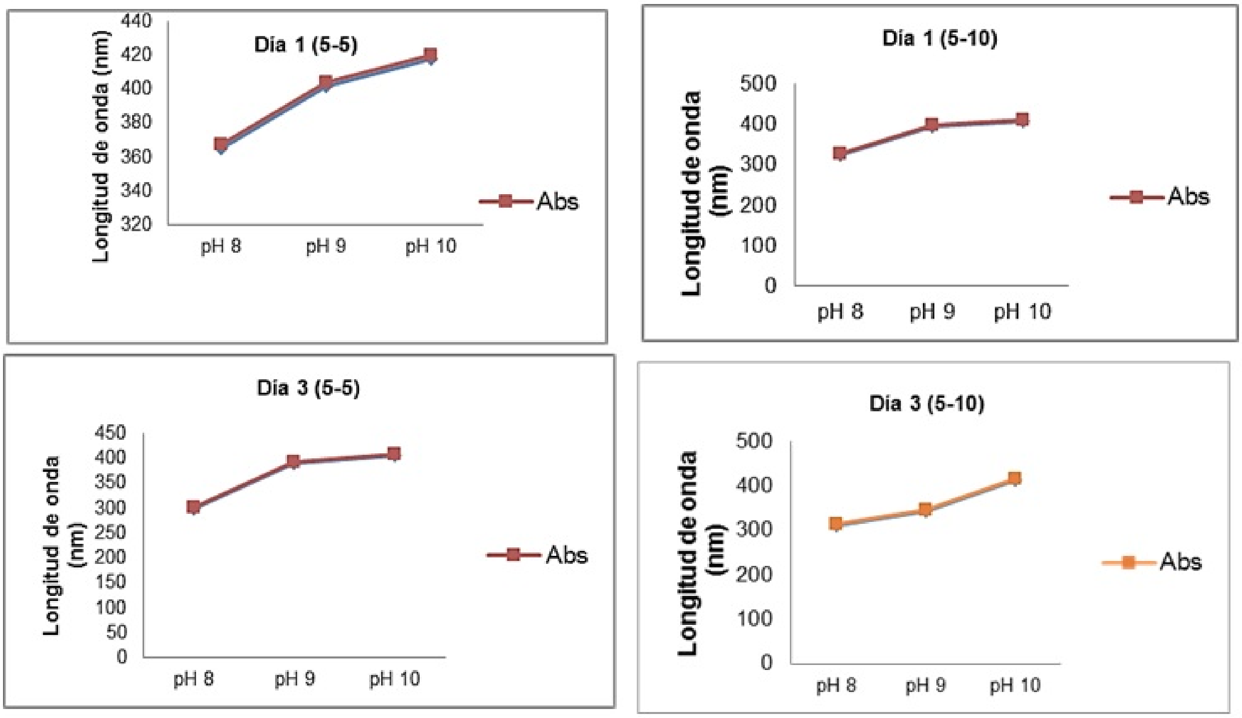

Figure 3. Graphical representation of spectrophotometric analysis during days 1 and 3 in 5-5 and 5-10 ratios

Silver nanoparticles of 5:5 and 5:10 ratios at 3 and 5 days (Figure 4) were observed on the scanning electronic microscope as bright, rounded, white spots. Ag-NPs was more detected after 3 days with a size of 10 nm in a 5:5 ratio and in smaller quantity after 5 days with a size of 10 nm in a 5:10 ratio. As Ávalos et al. (2013) the form of Ag-NPs influences antimicrobial activity. In addition, truncated triangle forms have been found to be more effective than spherical and elongated shapes, which by having more faces tend to be more active against microorganisms.

3.3 Antimicrobial effectiveness of synthesized Ag-NPs

The antimicrobial activity of silver nanoparticles for *E.coli* and *S.aureus*. bacteria was analyzed. As indicated in the methodology, petri dishes were divided into three quadrants before being inoculated with the bacteria mentioned above: one of the quadrants was placed with the pure extract, another with the silver nitrate solution, and the other with the synthesized nanoparticles. The results obtained for *E. coli* were a halo with a 2.88 mm diameter for the extract, a halo with a 1.55 mm diameter for the quadrant whose content was $AgNO_3$, a halo with a 3.21 mm diameter for Ag-NPs. For *S. aureus*, a halo of 2.12 mm, 1.30 mm and 2.18 mm was obtained for the extract, solution of $AgNO_3$ and Ag-NPs, respectively. *E. coli* bacteria presented halos with large diameter compared with *S. aureus bacteria* (Figure 5). Observing a greater antibacterial efficacy against this mmicroorganism

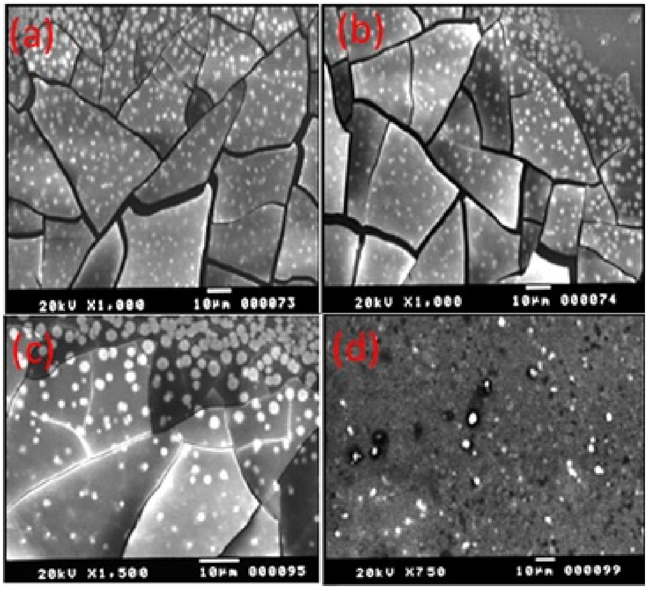

Figure 4. Observation of silver nanoparticles through the scanning electron microscope ratios of 5:5 (3 days (a)), (5 days (b)) and 5:10 (3 days (c)) and (5 days (d))

Monge (2009) said that research carried out to analyze the antimicrobial activity of silver nanoparticles synthesized for *Escherichia coli* and *Staphylococcus aureus* have reported that nanoparticles inhibit more efficiently *E. coli* bacteria. *E. coli* is a Gram-negative bacterium with a cell wall layer consisting of phospholipids and lipopolysaccharides, a cytoplasmic membrane and a thin layer of peptidoglycan, unlike Gram-positive bacteria such as *S. aureus*, which has a thick layer of peptidoglycan and plasma membrane that prevents synthesized nanoparticles from entering easily into the membrane (Villamizar and Monroy, 2015; Cruz-Monterrosa et al., 2017).In addition, Fernández (2017) also mentions that the antibacterial activity of the Ag-NPs is associated with the structural difference in the cell wall of the two bacteria. The above explains why bacterial inhibition was more efficient against *E. coli* compared to *S. aureus*. It is also important to state that bioactive compounds in rosemary leaf extract affect the cell membrane of bacteria, and cytotoxic activity directly affects the mitotic phase of Gram-positive and Gram-negative bacteria. Microorganisms such as *E. coli*, *Listeria monocitogenes*, *Salmonella spp.* and *S. aureus* are sensitive to rosemary extract components, where compounds such as caffeic acid, rosmarinic acid, carnosol, carnosolic acid and flavonoids prevail (Avila-Sosa et al., 2011; Centeno et al., 2010).

Other studies state that silver is oligodynamic because it has the ability to produce a bactericidal effect at low concentrations, as it is reactive to substances such as proteins, enzymes, DNA, RNA, among others (Monge, 2009; Nair and Laurencin, 2007). In addition, Fernández (2017) indicates that the antimicrobial activity of Ag-NPs is due to the action of silver ions that act by interfering the cellular respiration, and once inside the cell they alter their enzyme system by inhibiting their metabolism, causing the microorganism to lose all capacity to grow and reproduce, hence its death. According to Anandalakshmi and Venugobal (2017), the bactericidal action of silver nanoparticles against Gram-negative bacteria occurs when they have a size between 1 and 10 nm. In the current study, the best synthesized Ag-NPs had a size of 10nm as mentioned above.

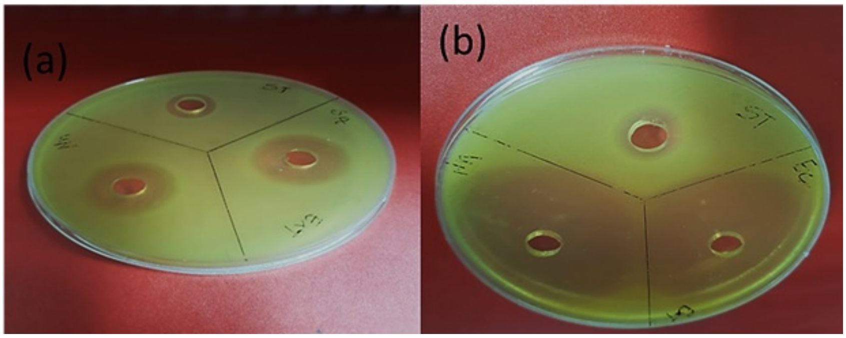

Figure 5. Antimicrobial effectiveness of silver nanoparticles against microorganisms (a) *S. aureus* and (b) *E. coli*

3.4 Aplication of silver nanoparticles in apple

The test carried out to check the effectiveness of synthesized Ag-NPs used as a preservative in red apples with bee wax, since this is normally used as a preservative because it inhibits microbial growth in fruits, showed a positive result. This is because Ag-NPs, like bee wax, delayed fruit ripening, preserving its color and initial weight from day 1 until day 30. As shown in Figure 6, apples that did not contain any preservatives changed their color, lost weight, and showed decomposition after 30 days of testing. Some studies show that nanoparticles could be applied to protect food by incorporating them into their packaging, although this possibility is still being investigated because there is no legislation on the application of Ag-NPs in food. Nanoparticles could be applied to a fruit coating or used for the manufacture of active packaging materials that protect food against pathogens (Ávalos et al., 2013; Aguilar, 2009).

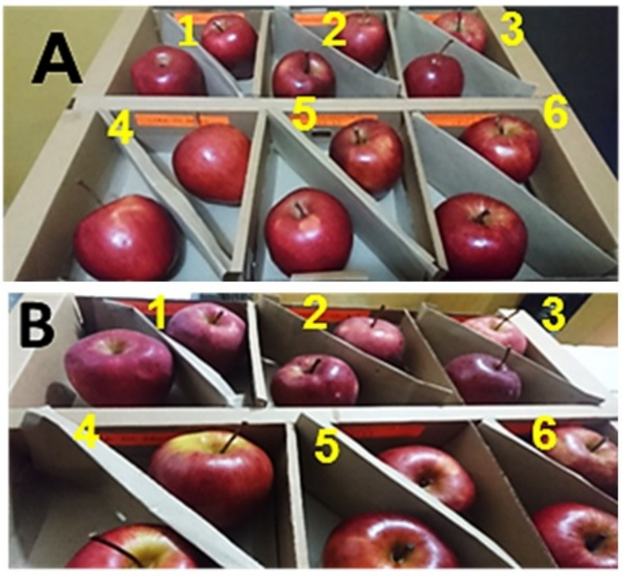

Figure 6. A. Preservative boxes with apples at the beginning of the test and B. Preservative box at 30 days (1 control, 2 Ag-NPs at 5:5, 3 Ag-NPs at 5:10, 4 bee wax, 5 bee wax and Ag-NPs at 5:5 and 6 bee wax and Ag-NPs at 5:10)

An et al. (2008), evaluated the effects of applying Ag-NPs coating on post-harvested asparagus. The coating reduced weight loss, maintained firmness, reduced chlorophyll loss and ascorbic acid formation, slowed color changes and inhibited microbial growth, increasing the post-harvest life of asparagus for 10 days.

Aguilar (2009) synthesized Ag-NPs by chemical reduction to evaluate the antifungal activity *in vitro* and *in situ* against the fungus *Colletrotrichum gloesporioides*, a fungus that causes the anthracnose of papaya. *In vitro* tests showed a fungal effect of 90 % inhibition, although papayas coated with the film containing silver nanoparticles had dark sections on the surface, affecting the appearance of the fruit.

In the study conducted by Villamizar and Monroy (2015), synthesized Ag-NPs of *Aspergilus flavus* was used for the preservation of tree tomatoes and golden berry. Fruits were packed in polyethylene bags containing Ag-NPs, and presented reduced growth of yeasts and Gram-positive and Gram-negative bacteria. In addition, Li et al. (2009) combined silver nanoparticles into a packaging containing titanium dioxide and kaolin to preserve chinese dates, demonstrating that the materials containing the packaging maintained longer the quality of the fruits than the fruits packaged in the standard container.

4 Conclusions

Silver nanoparticles were synthesized from the aqueous extract of rosemary leaves, and the qualitative and quantitative characterization of the Ag-NPs obtained by phytochemical analysis showed the presence of secondary metabolites such as phenolic acids, flavonoids, terpenoids, and reducing compounds. The color change of the rosemary leaf extract solution from yellow to dark Brown, and spicy and intense odor evidenced the formation of Ag-NPs. The characterization by UV-VIS spectrophotometry showed that ratios 5:5 and 5:10 were the best because they presented wavelengths between 212-418 nm and increased stability over time. Analysis by scanning electron microscopy showed spherical nanoparticles of 10 nm diameter. Ag-NPs showed greater bacterial inhibition against *E. coli* Gram-negative compared with bacterial inhibition presented against *S. aureus*. Gram-positive. On the other hand, Ag-NPs synthesized from rosemary leaf used as preservative in apples slowed the maturation process, maintained the initial weight, and prevented microbial contamination and fruit decomposition for 30 days.

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Sustainable Development

Agroecological marketing and training spaces: Lessons learned at Universidad Central del Ecuador

Espacios de formación y comercialización agroecológica: Lecciones aprendidas en la Universidad Central del Ecuador

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Abstract

Agroecological marketing spaces have expanded in the country, due to the importance that both consumers and producers are giving to this kind of production. However, the interaction that occurs between consumers and producers at fairs and how the latter become a pedagogical space for both of them has not been studied in depth. Therefore, the fair at Universidad Central del Ecuador was analyzed. Various research techniques were used, mainly interviews, surveys and bibliographic review on agroecology, agroecological markets, short agroecological networks and marketing. Two surveys were applied to consumers in the agro-ecological fair at Universidad Central del Ecuador. The first survey was applied to frequent consumers. The second one was applied to the same actors, once the fair was consolidated, to corroborate their preferences. This article aims to answer the following question: What have been the main lessons learned from the fair, as both a training and marketing a space for students, producers and consumers? The main conclusions show that the creation of the fair at Universidad Central represents an encounter between agroecology, education and social transformation within the academic curriculum, which also allows to identify the elements of environmental education that are implemented in the current educational project. It was also concluded that consumers prefer agroecological products for health reasons, since they consider these products are cleaner than conventional ones. Finally, regarding the challenges and limitations of the study, it is worth mentioning the need to compare similar experiences with other educational centers, to generate joint learning experiences about these fairs.

Keywords: Agroecological markets, agroecology, consumers, producers, agroecological fair, marketing spaces.

Resumen

Los espacios de comercialización agroecológica se han expandido en el país, debido a la importancia que los productores y consumidores le están dando al origen y la forma de producción de los alimentos. Sin embargo, poco se ha estudiado sobre la interacción entre consumidores y productores en las ferias, y cómo éstas se convierten en espacios de aprendizaje y comercialización para ambos actores. Por ello, se analizó la feria de la Universidad Central del Ecuador, ya que es uno de los pocos casos en el país que es impulsado por un establecimiento educativo. Para lo cual se planteó indicar las principales lecciones aprendidas de la feria, como un espacio de formación y comercialización entre estudiantes, productores y consumidores. Se emplearon diversas técnicas de recolección de datos, como entrevistas, encuestas y revisión bibliográfica sobre agroecología, mercados agroecológicos, circuitos cortos, redes y comercialización agroecológica. Se aplicaron dos encuestas a los consumidores de la feria. La primera encuesta fue realizada a los consumidores frecuentes en los primeros seis meses de funcionamiento, y la segunda encuesta después de un año, una vez que la feria estaba funcionando regularmente, para corroborar sus preferencias de consumo. Los principales resultados indican que los consumidores son en su mayoría jóvenes y mujeres, que perciben a los productos agroecológicos como más sanos y naturales, lo cual influye en sus preferencias de compra. Se recomienda para futuras investigaciones de la Universidad profundizar en los roles, conocimientos y procesos de decisión inmersos en el consumo de productos agroecológicos.

Palabras clave: Mercados agroecológicos, agroecología, consumidores, productores, feria agroecológica, espacios de comercialización.

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1 Introduction

Agro-ecological marketing has spread throughout Ecuador in the last 15 years since the emergence of the first marketing networks (Intriago and Amézcua, 2016). According to the inventory carried out in 2014 by the commercial networks office of the Ministry of Agriculture and Livestock (MAG), as part of the Alternative Marketing Circuits project (CIALCO), there were 210 marketing points nationwide, including fairs and shops. These marketing points are mainly carried out in the Highland with 154 traders, followed by the Coast with 53, the East with 18 and finally Galapagos with three traders (HEIFER, 2014). Marketing areas are generated by the consumer´s concern about the origin and the way the food is produced. At the national level, this concern has allowed the creation of various fairs, shops and other marketing forms of agro-ecological products, mainly locally (cities, neighborhoods and parishes), conducted mostly by women (Macas and Echarry, 2009) and in many cases with the support of decentralized self-government (GAD), non-governmental organizations (NGOs) and universities, which take advantage of these spaces for the training of their students, consumers and producers.

In 2017, Universidad Central del Ecuador (UCE) thought about creating an agro-ecological fair under two objectives: 1) develop a marketing space for the production of food obtained from the farms of the Faculty of Agricultural Sciences, and 2) create a training environment for its students in order to promote agro-ecological production as an alternative to conventional production. Modern conventional production is characterized by the intensive use of agrochemicals and monocultures, considered nowadays as unsustainable, in contrast to agroecological production that promotes a more diverse production without the use of agrochemicals, being more economically sustainable, environmentally friendly and healthier for humans (Rosset, 1998).

UCE fair is part of the so-called Short Marketing Circuits (CCC) or Food Circuits of Proximity (CIALP), understood as spaces of relationship between peasants or farmers, markets and territories, where products are marketed without intermediaries; these products come from nearby places and are based on the trust between producers and consumers (Ranaboldo and Arosio, 2016; Heinisch, 2017). In the case of the UCE fair, this CCC or CIALP is based on the production under the agroecology principles, understood by several authors as the management and application of ecological principles in agrosystems, which improve soil nutrient retention capacity with plants that are more resistant to pests and diseases, having a relationship between science, practice and knowledge that seek to transform the current agro-food system (Altieri, 2002, 2009a; Gliessman, 2015; Minga, 2016). It is also defined as a science and a set of practices for the construction of a healthy and sustainable agroecosystem (Castillo, 2002; Altieri and Toledo, 2011; Altieri and Nicholls, 2012).

Agro-ecological fairs are spaces of alternative marketing where the producer-consumer relationship occurs in a direct way, favoring food sovereignty, environmental sustainability and producing an equitable relationship between rurality and urbanity (Contreras Diaz et al., 2017). At these fairs, diverse products are sold and are mostly cultivated by farmers, reason for which supermarkets do not have access to this type of product (Intriago and Amézcua, 2016). These are spaces that promote fair relations between producers and consumers (Lacroix and Cheng, 2014). For this reason, agroecological fairs can be defined as marketing spaces for the production of agro-ecological products. These marketing spaces are framed in the so-called alternative food networks (RAA), the CCC or CIALP (Heinisch, 2017; Goodman and Goodman, 2009).

Agro-ecological fairs allow the small producer to increase production by offering products in the CCC or CIALP, thus increasing the economic income to the producer and at the same time guaranteeing healthier and cleaner food to consumers, providing sustainable food trading (Contreras Diaz et al., 2017). CCCs or CIALP become another option to obtain food, besides supermarkets, long agrifood chains and conventional production (Chauveau et al., 2010).

These products have a growing consumer preference in several European countries, because they are considered to have higher quality and a lower environmental impact than agro-industrial products (Renting et al., 2003). Agro-ecological fairs are also part of the RAA, which are understood as spaces in the food economy that include agroecological, fair-trade and local food; these networks are characterized by being related spatially, economically and socially (Goodman and Goodman, 2009). These marketing spaces for agro-ecological products through RAA, CCC or CIALP can generate linkages between individuals concerned about the origin of food in order to promote new forms of access to food in the future and to respond to more responsible consumption (Soler and Pérez, 2013). Thus, consumers propose that alternative networks are accompanied by training in food issues. In some cases, they have even managed to transform consumers into producers of some of their food through urban agriculture initiatives (López, 2011).

For this reason, with the aim of achieving a coherent educational process where teaching - learning is aligned with the active participation of producers and consumers, the career of Agronomic Engineering implemented the agro-ecological fair based on three fundamental foundations: 1) agroecology understood as an educational project involving the transformation of agricultural practices, making them participatory by integrating the producer with the nature and the consumer in order to make agriculture a sustainable activity in accordance with the six purposes of environmental education: awareness, knowledge, attitudes, skills, evaluation and participation, mentioned by Galiano and García (2002). 2) a liberating education based on an educational practice that cultivates, fosters and integrates human aspirations in society. 3) the transformation of society conceived as a practice of dialogue between science and knowledge, where knowledge is collective. As mentioned above, the educational process in the Faculty of Agricultural Sciences is understood as a relational process, i.e., there is a permanent dialogue between the professor and the student, assuming that this dialogue is situational, which goes beyond the simple relationship between people and becomes a relationship with the reality of a society in a historical cultural context. Therefore, methodological practice breaks the traditional conception of education in the aspect known as agricultural extension or pre-professional practice based on the techniques of green revolution.

According to Sverdlick (2007), research widens the educational process, starting from empirical knowledge that carries implicit theoretical-scientific knowledge. Hence, the academic program intertwines theory-practice, responding to society's demand for sustainable agriculture. The aim of the fair is to promote the processes that lead to the implementation of the agro-ecological model through the inclusion of environmental education in the educational project, understood as a process of relationship, environmental awareness, ecological knowledge and application of human attitudes and values with their environment, for generating processes that contribute to the sustainability of the ecosystem (Castillo, 2010). The latter is possible if the educational community feels the need to transform reality, i.e., to reach collective awareness, so that the agro-ecological model is not an imposition but an alternative to ecosystem degradation, allowing a transformative praxis.

This article aims to present the main lessons learned from the UCE fair implemented as a space for training and marketing of agroecological products among students, producers and consumers. Therefore, the objective is to analyze the main learning and results obtained at the fair since its creation, due to it is one of the few cases in the country that is driven by an educational establishment where there is an interaction that includes producers (who are at the same time students), consumers and the participation of teachers.

2 Materials and methods

Qualitative and quantitative techniques were used in combination. First, bibliographic research was carried out in various scientific journals (Jstor, Taylor & Francis, Scopus), virtual libraries (digitalia, elibro) and university repositories (UCE, Salesian Polytechnic University and Andean University). The approach used was based on agro-ecological marketing spaces for training, using the following search criteria: Ecuador, agroecology, agroecological fairs, agro-ecological markets, food sovereignty and alternative marketing networks/circuits. The result of this review generated a total of 40 sources of information, distributed in 23 articles of mainstream scientific journals, eight specific articles on the subject, seven books and two research papers (undergraduate thesis) on agroecological markets, CCC and RAA mainly. It should be mentioned that no specific information regarding agro-ecological fairs was found as training spaces.

Additional information was collected from consumers at the UCE agro-ecological fair using two surveys at different times. The first survey was applied at the beginning of the fair, i.e., between January and March 2018, and the second between October and December 2019, once the fair was running regularly, to corroborate their consumption preferences. The first survey had two sections: 1. consumer characteristics and 2. characteristics of the product demanded. This survey consisted of 88 questions that looked at aspects such as the age and gender of consumers, as well as frequencies and quantities of purchase products at the fair, and it was applied to frequent consumers of the fair. The second survey looked at complementary aspects through three questions related to the motivations for the purchase at the fair, the origin of the consumer (UCE teacher, UCE employee, student or people who are not related with UCE), as well as the degree of knowledge of what is an agroecological product. Both surveys were applied to 35 consumers in a total group of 120 average weekly consumers. Random consumers were selected during the first hour of the fair (the operating hours are: 10:00 to 13:00), because it is in this time range that there is the greatest number of consumers. It should be mentioned that in each of the surveys there was representation of the different members of the university community (teachers, administrative / workers and students), as well as people not belonging UCE.

Subsequently, meetings were held with the coordinators of the agro-ecological fairs of Quito ("La Carolina" fair, "Universidad Andina Simón Bolívar" fair, "Carcelén" fair) in order to know their experiences and compare them with UCE fair. The information of the fairs and the surveys applied, as well as the bibliographic information, work as the basis for determining the potential of agro-ecological fairs as training spaces for consumers and producers.

3 Results

The agro-ecological fair of UCE participates in the educational community, which allows the study of the different variables of agroecological production and marketing in a social and academic environment. Figure 1 shows the age groups of consumers who attend it. It was observed that the average age is 41 years, with ages ranging between 22 and 73 years. Most consumers are in the range of 40 to 70 years.

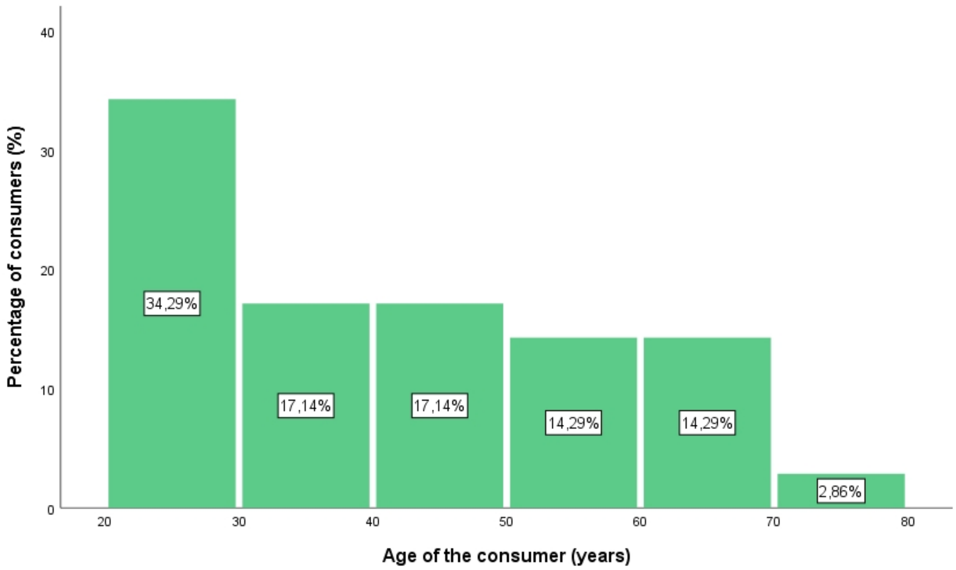

Figure 1. Age groups of consumers of the UCE fair.

Figure 2 shows the average monthly revenues of consumers, which are mostly between 500*and*1000, representing 54.29 %, followed by revenues of more than $1000 with 37.14 %, and less than $500 with 8.57 %. These results place most fairgoers in the middle class, understood as the one that has a certain degree of economic stability and can afford the basic basket (Orellana and Osorio, 2014).

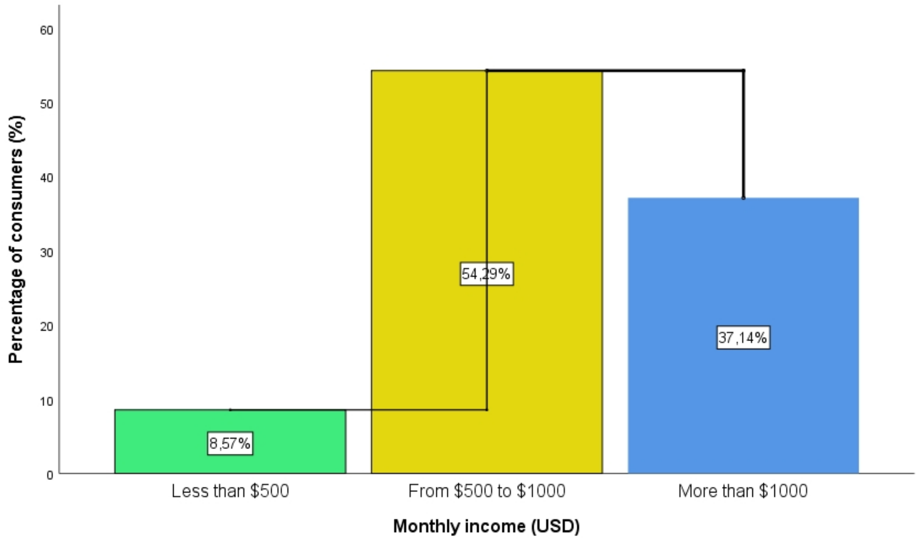

Figure 2. Average household income from consumers attending the UCE fair.

Most consumers were students and UCE administrative employees with 36.67 % and 30.00 %, respectively; also, there are external consumers who are made up of residents around the UCE with 26.67 %, and finally UCE teachers (6.67 %) as seen in Figure 3. These data indicate that students, most of whom are women 60 %, are an important player in the consumption of agroecological production on the UCE farm, which could generate a dynamic trade oriented to this group of consumers on and off the university campus. The above indicates that the fair has been a space where knowledge and experience are shared between producers and consumers, including teachers, students, workers and internal and external consumers in a responsible and healthy production. On the other hand, the results show that 71.4 % of people who attend the fair are women, probably because women pay more attention to eating healthy food from agroecological products compared to men. This result indicates that efforts to raise awareness in men is important for their more active participation in fairs, and to encourage more consumption of agro-ecological foods.

Consumers at the fair also showed a good understanding of what an agroecological product is. Figure 4 shows that 60.00 % of consumers related agroecological products to the production of chemical-free foods, 20.00 % to natural products, 13.3 % to products from a sustainable agro-economy system, and 6.67 % to products without genetic manipulation. The results in Figure 4 represent the most common definitions of an agroecological product from the consumer´s point of view. In these definitions, it is noteworthy the absence of socioeconomic elements, such as more social justice of the production or the principles of agroecology. This absence indicates that further training is needed in the social and political proposal of agroecological production in consumers, but also that current knowledge about agro-ecological product can be used to promote future marketing fairs.

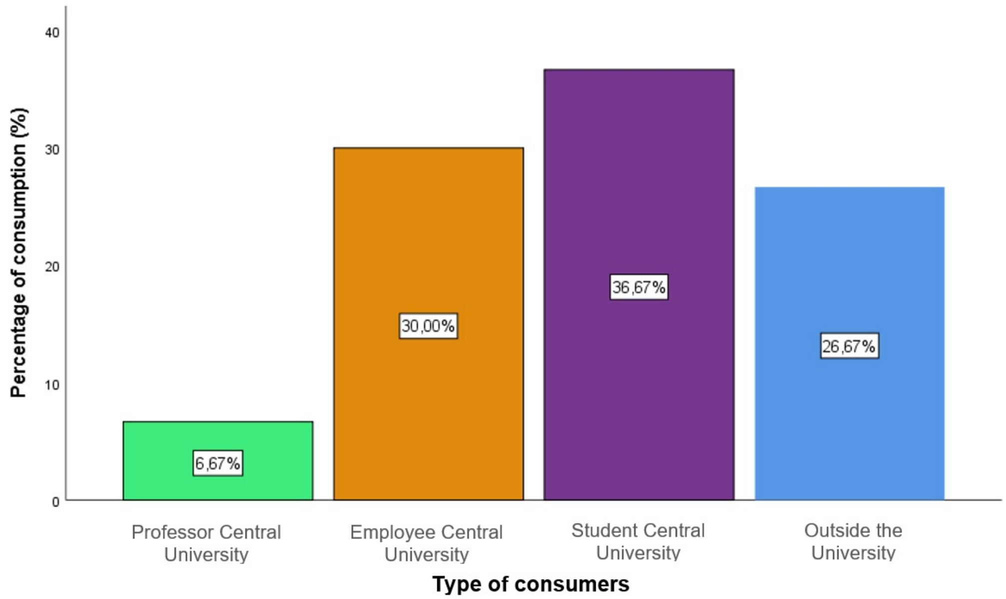

Figure 3. Average consumption of agroecological products at the UCE fair.

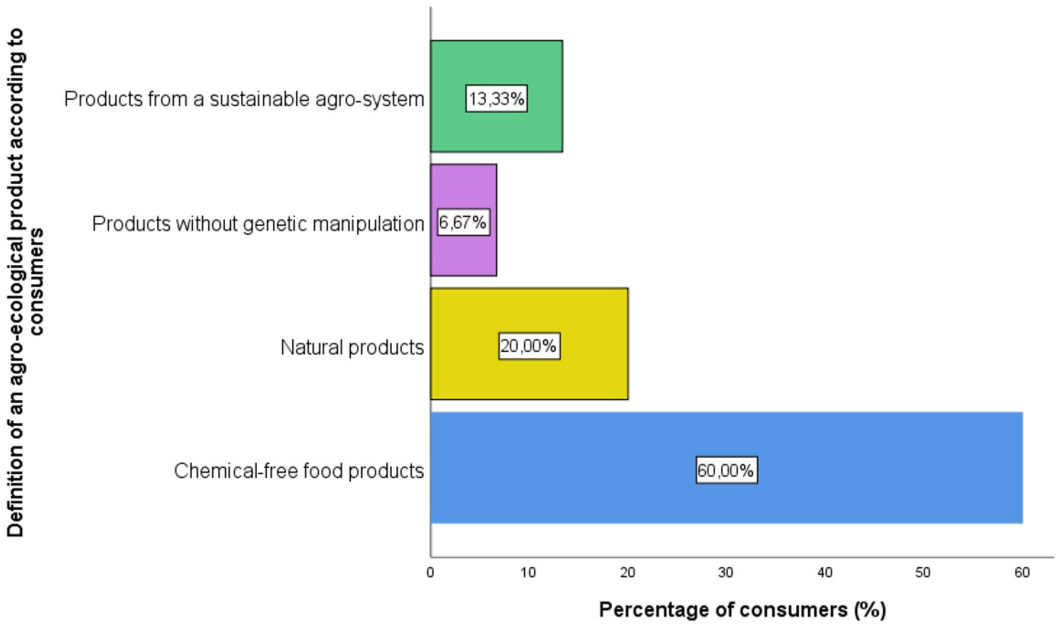

Figure 4. Average frequency of the definition of an agroecological product according to the consumers of the UCE fair.

As can be seen in Figure 5, the results show that the main motivations of consumers to buy the products at the fair are mainly because these are agroecological or organic by 50.00 %, followed by the price by 25.00 %, by health (17.50 %), other reasons (5.00 %) and because they trust UCE (2.50 %).

Regarding the purchase preference, it should be mentioned that the products generally offered at the UCE fair consist of a variety of vegetables, among which certain marker products can be mentioned, i.e., those that are demanded and have a continuous production at the Experimental Academic Center "La Tola" (CADET) as: kidney tomato, tree tomato, maize, carrot, pepper and dairies (yogurt). When the fair occurs, it finishes one the products are sold out. There are no products made with the exception of yogurt, some vegetables and fruits. Probably increasing supply and training events with consumers would be needed to encourage consumption of new products.

This exchange of knowledge and experiences on environmental education and tools for life allows students of the UCE Career of Agronomic Engineering to contribute to the construction of Ecuador's food sovereignty, in accordance with Navarrete and Madoery (2017) and in the declaration of Nyéléni Selingue (2007), which mentions that the agroecological approach is a fundamental pillar for the construction of food sovereignty. Students with the help of teachers apply the concepts of agroecology at the fair in order to achieve what Altieri (2009b) and Escandón (2012), mention about promoting biological balance, reducing pest and disease cycles, productive and economic risks and increasing yields while contributing to soil conservation and agrobiodiversity.

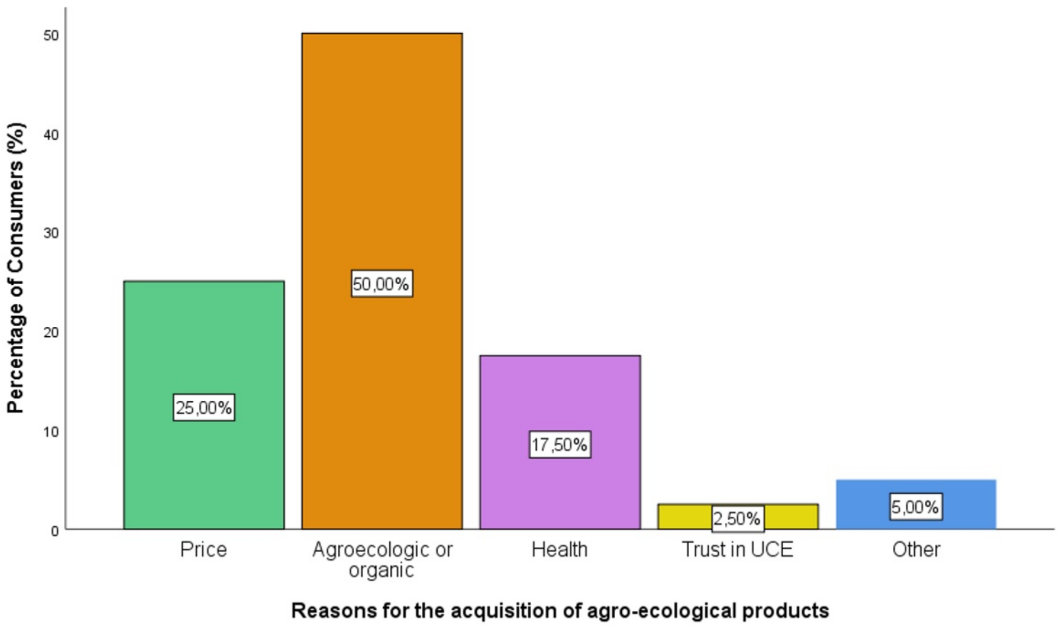

Figure 5. Motivation to buy the products at the UCE fair.

4 Discussion

The alternative from short circuits is already beginning to be strongly discussed as a way to replace the supermarket-dominated urban food distribution system, which controls the supply in cities, supported by the principles of food sovereignty that proposes access to healthier, culturally adequate and agroecologically produced food (Rosset, 2003). In Spain, for example, more than 25 % of consumers buy their food in traditional stores and other alternative stores (MAGAP, 2016), which is an important percentage that can increase with the presence of more responsible consumption groups. However, consumers' relation of agroecological products versus supply is still low, but it is growing due to increasing consumer concern about the health effects of processed food. For example, it is increasingly common to find social movements that demand food, trade and fairs in cities to have a better access to organic food or slow food, among others (Harper et al., 2009), which advocate direct supply between consumers and producers.

Currently, the city is struggling with the production model based on agrobusiness and the global agro-food system (SAG) (León-Vega, 2018). In the city, consumers play an essential role in the construction of new forms of access to food, fighting to recover spaces between producers and consumers (popular markets, agro-ecological fairs), and contribute at the same time to the construction of food sovereignty by strengthening consumer and producer rights (Harcourt, 2008). Consumer´s concern in Quito has led to the signing of the Quito Agro-Food Pact (PAQ), made up of consumers, academia, cooperation agencies and others, in order to collaborate in the formulation of public policies for food (ConQuito, 2018).

There are two legally endorsed groups of organic/agroecological food in the Metropolitan District of Quito: the Ecuadorian Corporation of Biological Farmers (PROBIO) and the Participatory Urban Agriculture Program (AGRUPAR), promoted by the Economic Promotion Agency of Quito (ConQuito) (Chamorro, 2015). On the one hand, PROBIO (2013) cited in García (2017), indicates that agroecological fairs are organized by the fair members and work under the participatory guarantee system (SPG) (ConQuito, 2016), and that bio-fairs are alternative marketing spaces where farmers offer organic products at fair price and directly to the consumer. It should be mentioned that there are more fairs in Quito, however, they are not legally registered or they are sporadic.

The production and marketing of organic/agroecological products at CCCs or CIALP in Quito have caused socioeconomic benefits to producers and consumers. Products from farmers' orchards generate savings from self-consumption and allow them to have economic income of USD 127 on average per family, as well as encouraging women's participation in the production process. Meanwhile, consumers buy healthy food at affordable prices by reducing their health expenditures, contributing to the development of fair trade and ecosystem conservation (Clavijo, 2013).

In this sense, Sotomayor (2013) says that the Agroecological Fair, Art and Culture "La Carolina" is the first fair in the country with these characteristics, created in May 2008 with the aim of selling food and crafts based on agroecological production. With regard to the consumers of this fair, Sotomayor (2013), indicates that there are three groups: frequent consumers, occasional consumers and consumers who attend for the first time. Sotomayor (2013), states that the motivations for attending the fair are due to three main factors: 1) The supply of healthy, fresh, natural, organic and agroecological products; 2) The inclusive space where all kind of people meet to talk, share and enjoy; and, 3) The consumer-producer relationship based on fair trade, solidarity economy and food sovereignty.

On the other hand, Basabe et al. (2016), indicate that the Zapallo Verde Cooperative (CZV) has been on the market for more than a decade, and its main objective is to sell its products directly to consumers in a responsible, supportive and participatory way. Products are offered according to the season and are produced under agroecological techniques. The cooperative is structured by coordinators, consumers and producers. The objective of the CZV is to haver agro-ecological production that promotes local and trade production with short circuits, leading to fair payment and direct participation of producers in the trade chain.

Likewise, Romero (2015) points out that "Plaza Civica Eloy Alfaro" fair is the first to venture in southern Quito in 2007. It consists of 20 participants from 11 orchards between 200 and 1000 m^2 that produce legumes, grains and handcrafted products. For producers, agro-ecological principles are considered as an alternative of social agriculture, which is economically equal and is sustainable over time, resulting in a qualitative change where productive units represent a space with an integral and balanced perspective. Consumers attend the fair weekly looking for organic, healthy and fresh food, and by knowing the person who produces it and how it is produced. According to Romero (2015), the fair is a social and happy space where marketing occurs in a collaborative way, without competition. In its beginnings, it had to overcome obstacles such as the perception of consumers about products that seem to be smaller and more expensive compared to conventional food; it occurred especially in the south of the city, where citizens would probably not value organic production.

Regarding the UCE agro-ecological fair, the results indicated that the most predominant age group at the fair was between 20 and 30 years, which is consistent with the age of the group with the greatest participation in the fair and which corresponds to female students. Those who attend the fair are mostly women, which may be because agroecological fairs mainly encourage the participation of women (Clavijo, 2013) since they they consider this food healthier for their families.

On the other hand, the lowest percentage of consumers at the UCE fair is for teachers, which contrasts with consumers at the Andean University fair, where most buyers were teachers and workers (Izurieta, 2018). A significant high percentage of consumers outside the University is also noteworthy, although there is not any communication and dissemination by the authorities on these fairs at UCE. According to consumers at the UCE fair, it was observed that they relate an agroecological product with natural products and the production of organic food.

As for the average income of the fair's consumers, they were in the range of USD 500 to USD 1000 per month, which is similar to consumers at the Carcelén fair (Chalá, 2017) and Andean University (Izurieta, 2018), but quite different from the Carolina fair, which has consumers with incomes over USD 1500 per month (Vasco et al., 2015), since this fair is located in a place where the socioeconomic level of inhabitants is higher than the others mentioned.

The main reasons for the acquisition of agroecological products at the UCE fair were because these are agroecological or organic products, which is a similar reason to the consumer preferences of the Carcelén fair (Chalá, 2017), probably because the two fairs are located in places where their inhabitants have a similar socio-economic situation. Finally, it is important to mention that the results of the research on the UCE agro-ecological fair will suggest the initial actions to be carried out by the authorities, with the ultimate goal of obtaining a balance between sustainable agro-ecological production for a purely academic purpose and the real requirements of consumers.

5 Conclusions

The main conclusions of this study indicate that the most representative age of people attending the fair ranges from 20 to 30 years, therefore it is young consumers who have more openness to buying agroecological products and who would represent more potential for expansion in the medium term.

Women are the ones who attend the fair the most, indicating that the presence of women should be highlighted not only in the production processes but also in the marketing processes of the fair, just as equal participation in the future should be promoted.

There is little involvement of the UCE professors, probably due to the low communication between professors and the university community about the conduction of the fair, however, "external consumers" have a significant participation.

Consumers also perceive agroecological products as healthy, natural and organic products. These findings will allow to direct future complementary research that deepens on these aspects, such as perceptions about the agroecological knowledge of people who make up the university states (students, teachers and administrative staff).

Revenue between $500 and $1000 represents the majority. However, it should not be dismissed that the largest presence at the fair is represented by students, who despite having no significant economic income are a transfer agent of knowledge acquired to the university community.

Consumers buy products at the fair mainly because they are agroecological or organic, indicating that they have good prior knowledge of this type of product.

The research carried out about the training of consumers and producers has identified the application of environmental education in the educational project and its curriculum, which is confirmed by observing the role of employees, students, professors at UCE, outside UCE and the agroecological products offered at the fair. According to their motivation to buy, it has also intrinsically allowed to observe the importance given by buyers to their food and health, which is essential for life.

The authorities of the agro-ecological fair of Universidad Central del Ecuador should propose the implementation of alternatives and new strategies of social and didactic-pedagogical inclusion based on the assessment of the surveys obtained, which allow to see this fair as a training space between consumers and producers. The above strategies are directly related to the application of environmental education, whose components are awareness and sensitivity, knowledge and understanding, attitudes, skills and participation.

The training space generated by the UCE fair, which represents the meeting point between agroecology, education and the transformation of society, should influence future lines of research and projects linked with the University's society, mainly due to the need on deepening in the roles, knowledge and decision-making processes immersed in the consumption of agroecological products, in order to encourage responsible and healthy eating.

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Veterinary Sciences

Control of Acute Thermal Stress in Broilers Ross 308 Line by Inclusion of Betaine in Drinking Water and its Economic Analysis in El Quinche Parish, Ecuador

Control del Estrés Térmico Agudo en Pollos de Engorde Línea Ross 308 mediante la Inclusión de Betaína en Agua de Bebida y su Análisis Económico en la Parroquia El Quinche, Ecuador

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Abstract

This study evaluated different concentrations of inclusion of betaine supplied in the drinking water for the control of acute heat stress in broiler chickens Ross 308 Line and its effect on the productive parameters and economic benefit in two production cycles. In two poultry houses, the experiment began with 2672 and 2304 broiler chickens that were distributed in the third week in 16 quadrants with four pseudo-replicates of 167 and 144 chickens each quadrant. Three treatments of betaine (1.5, 2 and 2.5 g/l) and one control treatment (without betaine) distributed at random were supplied in the drinking water during the last week of breeding (seven week). The supply of 1.5 g/l (T2) of betaine level showed the highest average weight (2441 ± 52.2 g) and lowest mortality (2.96 %) during the first cycle, whereas T4 exhibited highest average weight (2925 ± 60.2 g) and lowest mortality (3.43 %) during the second cycle. All treatments revealed acute thermal stress with no significant differences in body temperature. In the economic analysis T2 and T4 showed the highest net income with about 60.44 % and 67.36 % with reasonable cost-benefit ratio (1.42 and 1.93) during first and second cycle, respectively. This study suggests the supply of betaine between 1.5-2.5 g/l in the water during the last week of rearing period in Mediterranean areas along with good management practice to mitigate the acute thermal stress in commercial chicken broilers Line Ross 308.

Keywords: Betaine, broiler, economic analyses, productive parameters, acute thermal stress.

Resumen

Este estudio evaluó diferentes concentraciones de inclusión de betaína suministrada en el agua de bebida para el control del estrés térmico agudo en pollos de engorde de la línea Ross 308 y su efecto sobre los parámetros productivos y el beneficio económico en dos ciclos de producción. El experimento se inició en dos galpones con 2672 y 2304 aves que fueron distribuidas en la tercera semana en 16 cuadrantes con cuatro pseudo-réplicas de 167 y 144 aves en cada cuadrante. Tres tratamientos de betaína (1,5; 2 y 2,5 g/l) y un tratamiento control (sin betaína) distribuidos al azar se suministraron en el agua de bebida durante la última semana de crianza (séptima semana). El suministro de 1,5 g/l (T2) de betaína mostró un mayor peso promedio (2441 ± 52,2 g) y menor mortalidad (2,96 %) durante el primer ciclo, mientras que el T4 (2,5g/l) mostró el mayor peso promedio (2925 ± 60,2 g) y menor mortalidad (3,43 %) durante el segundo ciclo. Todos los tratamientos revelaron un estrés térmico agudo sin diferencias significativas en la temperatura corporal. En el análisis económico, los T2 y T4 mostraron los ingresos netos más altos de alrededor del 60,44 % y 67,36 %, con una relación costo-beneficio de 1,42 y 1,93 durante el primer y segundo ciclo, respectivamente. Este estudio sugiere el suministro de betaína entre 1,5-2,5 g/l en el agua de bebida durante la última semana de crianza en zonas mediterráneas junto con buenas prácticas de manejo para mitigar el estrés térmico agudo en los pollos de engorde Línea Ross 308.

Palabras clave: Betaína, broiler, análisis económico, parámetros productivos, estrés térmico agudo.

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1 Introduction

Broiler chickens are homeothermic animals because they have the ability to regulate and maintain their own internal body temperature (Araújo et al., 2015; Mascarenhas et al., 2020). This mechanism is only efficient when its internal temperature is within the thermo-neutral zone, (27.5-37.7 °C) (Ajakaiye et al., 2011; Mutibvu et al., 2017; Saeed et al., 2019). Several factors may influence the thermo-neutral zone in broilers as body weight, amount of plumage, acclimatization, and state of dehydration (Pereira and Nääs, 2008; Araújo et al., 2015; Bhadauria et al., 2017).

The resulting heat is a product of metabolism (glycolysis, Krebs cycle, phosphate derivation pathway) and muscle activity produced within the body of the animal (Zahoor et al., 2016; Lu et al., 2017; Tickle et al., 2018; Zaboli et al., 2019; Barzegar et al., 2020). Different factors can affect the amount and production of heat as physical activity, ambient temperature, circadian rhythms, among others (Syafwan et al., 2011; Lara and Rostagno, 2013; Fisinin and Kavtarashvili, 2015; Baracho et al., 2019). Therefore, to maintain body temperature within the thermo-neutral zone, broilers dissipate excess heat to the surrounding environment through cell conduction and vascular convection (Baracho et al., 2011; Da Silva et al., 2015; Nilsson et al., 2016; Nascimento et al., 2017).

Poultry farming mainly depends on climatic conditions such as temperature and humidity (Nawab et al., 2018). The internal body temperature of an adult chicken is around 40.5°C. This temperature increases as the ambient temperature rises or falls above or below the thermo-neutral zone (Aengwanich, 2007; Nascimento et al., 2011). When the core body temperature reaches its critical point (47°C), called the upper lethal temperature point, chickens may die from heat prostration (Scanes, 2016). When the balance between heat production and heat loss in the chicken's body is disturbed, heat loss is decreased while heat production is increased, resulting in the onset of heat stress (Dayyani and Bakhtiari, 2013; Lara and Rostagno, 2013; Saeed et al., 2019).

Stress can be defined as a non-specific response of the animal organism to adverse environmental conditions that produces physiological and metabolic adjustments to maintain homeostasis, generates effects on the central nervous system, the neuroendocrine system and the immune system (Lin et al., 2006; Martin et al., 2011). Heat stress negatively affects not only productive and reproductive performance but also the profitability of the poultry farmer and the welfare of chickens (Pawar et al., 2016; Alagawany et al., 2017; Ranjan et al., 2019). The increase in the cost of maintenance energy triggers heat stress in poultry (Syafwan et al., 2011; Rath et al., 2015; Shlomo, 2015; Zhang et al., 2016). On a biochemical level, an increase of up to 20 times in the breathing rate of chickens can cause greater loss of CO_2 through the lungs (Knížatová et al., 2010; Nascimento et al., 2017). This loss results, in an increase in the blood pH causing an acceleration of the acid-base balance (Olanrewaju et al., 2006; Borges et al., 2007) which ends up affecting the health, welfare and performance of the chickens.

Heat stress also causes lipid peroxidation due to excessive generation of reactive oxygen species (Akbarian et al., 2016). Antioxidant supplementation has the ability to reduce oxidative instability of proteins and lipids, and this may be associated with increased activity of cellular antioxidant enzymes (Delles et al., 2014).

Betaine can be found in different plants and animal species as a natural substance (Nudiens et al., 2001). Betaine acts in the metabolism of chickens as a donor of methyl groups for protein synthesis, nucleic acids and choline (McDevitt et al., 2000). Is a methyl derivative of glycine and a metabolite of choline degradation that acts further as an osmolite helping to maintain cellular water, ion balance, methionine conservation, and fat distribution (Eklund et al., 2005; Ratriyanto et al., 2009; Ahmed et al., 2018; Shakeri et al., 2018). Among the benefits of betaine is that can enhance water retention due to the osmotic effect increasing cell volume and hence anabolic activity, cell membrane integrity and overall performance of the chicken (Shakeri et al., 2018; Liu et al., 2019). The osmolytic property of betaine permits cellular adaptation to adverse osmotic environments noticed in hot and humid climates. As a donor of the methyl group, betaine can also replace up to 20% of the dietary methionine and up to 100% of the choline in the diets of commercial broilers, saving feed costs (Sakomura et al., 2013).

Heat stress is a major challenge to the welfare of the chickens and the profitability of the poultry farm. Therefore, growers must be aware and vigilant in managing and maintaining adequate house internal temperatures in the final weeks of rearing period, especially in areas with a Mediterranean climate during summer. Behavioral events as well as production parameters provide relevant information on the responses of poultry to heat stress conditions due to high ambient temperature and high relative humidity (Lara and Rostagno, 2013; Nyoni et al., 2019).

In Ecuador, there is a lack of scientific studies on betaine administration in drinking water to cope heat stress in chicken, their effect on production parameters and its economic benefit during hot seasons with Mediterranean climates. This study hypothesizes that the higher concentration of betaine in the drinking water, the lower mortality and lower average weight of the flock at the end of the rearing period, given a better hydration of the animals and less food intake in the hottest hours. Therefore, this study, evaluates different concentrations of inclusion of betaine supplied in the drinking water for the control of acute heat stress in chickens of the Ross 308 Line from 38 days of age and its effect on the productive parameters, as well as the economic benefit of the product applied to a Mediterranean climate zone in the Sierra region of Ecuador.

2 Methodology

2.1 Experimental site and microclimatic conditions

The research was carried out in the commune of La Victoria, belonging to the parish of El Quinche, province of Pichincha, 40 km east from the city of Quito. The study area is located at an altitude of 2619 meters above sea level (masl), with a mediterranean climate, registering average temperatures of 18°C with variations of 0.6°C throughout the year. The hottest month is September, with maximum temperatures up to 30°C and a monthly rainfall of 53 mm. The rainiest month is registered in April with 126 mm monthly average.

The experiment was conducted from August to November 2019, during the hottest season of the year. Two poultry houses with an area of 264 m^2 each, housed 2700 male birds during August to September for the first production cycle (FPC), and 2330 male birds during October to November for the second production cycle (SPC). However, the experiment initiated at the last week (seventh week) of the rearing period with 2672 and 2304 animals for the FPC and SCP, respectively, due to the normal mortality registered.

The internal temperature conditions of the houses were checked prior to the reception of the chickens, recording a temperature of 33°C each. The animals were vaccinated against Marek's disease from the hatchery and were fed with fresh, clean, commercial feed and water throughout their cycle.

2.2 Poultry management and betaine supply

At the end of the third week of life (21 days), the chickens were placed in 16 quadrants of 15 m^2 each, and evenly distributed within each poultry house to allocate 167 chickens per quadrant during the FPC and 144 chickens per quadrant during the SPC. The animals were distributed in quadrants in this week since at this age the animals are able to self-regulate their internal body temperature and not depend on external artificial heat (brooders), as well as to facilitate the management of the chickens during the experimental period.

Within each poultry house, the 16 quadrants were classified into four treatments distributed randomly with four pseudo-replications of each treatment. Each experiment consisted of a control treatment (T1: without betaine) and three treatments with different concentrations of betaine (T2: 1.5 g/l; T3: 2 g/l and T4: 2.5 g/l). Betaine was supplied in the drinkers from day 43 and 44 onwards, in the first and second house, respectively, during the hottest hours of the day (12:00 am-15:00 pm). Betaine was suspended in both houses two days prior to slaughter, to avoid the existence of residues inside the animal's body at the time of sale. Only clean water was supplied on the last day of the production cycle. The animals were in the houses until day 48 and 49 during FCP and SCP, respectively.

In broilers, glucocorticoids are produced as corticosterone, and 75% are excreted in the urine (Scanes, 2016). For this reason, a non-invasive technique was used to determine corticosterone levels avoiding generate stress in the chickens by manipulation. Fecal samples (later separation of the liquid portion of the poultry feces) were collected during the experimental period (day 43 to 46 [FCP]; day 44 to 47 [SCP]). This procedure was performed with caution during the first hours of the day (7:00 am-9:00 am) to avoid generating any type of stress (physiological or behavioral) in the animals. The samples collected for each treatment in both cycles were sent to the laboratory for subsequent analysis.

The internal body temperature was registered at day 43, 45 and 47 in both houses in 10% sample of the total chickens by each quadrant, to determine the signs of heat stress using a penetration/immersion probe. The probe was gently introduced 5 to 6 cm (depend on age and size of chickens) at the level of the terminal colon with caution to not generate stress. This procedure was performed in early hours of the morning. Finally, an economic feasibility analysis of the use of betaine in broilers was done to determine the profitability and best cost-benefit (C/B) ratio of betaine supply,

3 Statistical Analysis

Data on average weight, feed intake, weight gain per animal, and body temperature were tested for normality for the two production cycles using the Shapiro-Wilk test. If the data followed normal distribution, the ANOVA one-way test was applied to determine the differences in means between treatments and between cycles. Otherwise, a Mann-Whitney U test was used. In addition, a Multiple Linear Regression Analysis (MLRA) was done to test the hypothesis and analyze the influence of the different levels of betaine on each of the independent variables (final weight, feed consumption, weight gain and body temperature) using a least-squares approach.

Finally, a t-test was done to compare the means of final weights at each production cycle as a proxy to determine statistical differences in the C/B ratio. The most informative model was selected based on the Akaike Information Criterion (AIC). All statistical analyses were performed in version 3.4.1 of the R software (The developmet Core Team, 2017).

4 Economic Feasibility Analysis

The economic analysis was carried out for both cycles considering the final average weight of the animals, the total number of chickens for each treatment at the end of the cycle, and the price per pound in the market at that time. Only the variable costs involved during the period of the experiment (week 7) were considered in the analysis (feed and betaine cost). The net income of the poultry was determined based on the difference between the sale of the chickens (gross income) and the variable costs. Based on the net benefit, the cost-benefit relation was calculated to determine which treatment was the most adequate, with better productive parameters and better profitability.

5 Results and discussion

The results exhibited T2 (1.5 g/l) as the highest average weight during the FCP (2441 $\pm$ 52.2 g) (Table 1) with significant differences between the four treatments ($p<0.05$; n=97). During SCP, T4 (2.5 g/l) showed the highest average weight (2925 $\pm$ 60.2 g) (Table 2) with significant differences between treatments ($p<0.05$; n=83). There were significant differences between cycles, achieving a higher average weight in the SCP (2735 $\pm$ 193.21 g) than in the FCP (2315 $\pm$ 93.33 g).

During FCP and SCP, T2 showed the highest weight gain / animal / day with 66.1 $\pm$ 8,46 g and 111,3 $\pm$ 9,44 g, respectively (Table 1; Table 2) with significant differences between treatments. Significant differences were also observed between cycles, obtaining a greater weight gain in the SCP (79.7 $\pm$ 21.66 g/animal/day) than in the FCP (47.16 $\pm$ 13.18 g/animal/day). The highest feed intake during the FCP was 185.7 $\pm$ 1.21 g belonging to T4 (Table 1), with differences in all treatments except between T3 and T4 ($p=0.353$; W=244; n=97). During SCP, the highest feed intake was for T3 with an average of 179.6 $\pm$ 1.72 g / animal / day (Table 2), showing significant differences between treatments ($p<0.05$; n=83). Significant differences were observed between cycles, showing an overall average feed intake of 184.5 $\pm$ 1.76 g/animal day and 173.4 $\pm$ 4.68 g/animal/day, for FCP and SCP, respectively.

Table 1. General performance of the chickens Ross Line 308 at the end of the rearing period with the supplementation of different levels of betaine during the first cycle.

Treatment	Average weight ± SD (g)	Weight gain /animal/ day (g)	Food intake/animal/ day (g)	% Mortality	Body temperature (°C)	Corticosterone level (nmol/L)
T1 (control)	2275 ± 32.8	70.2 ±5.20	184.5 ±1.10	4.29	42.5 ±1.90	113.13 ±11.55
T2 (1.5 g/L)	2441± 52.2	66.1 ±8.46	182.3 ±1.24	2.96	43.0 ±2.62	123.10 ±5.20
T3 (2 g/L)	2213 ± 35.6	35.1 ±4.83	185.5 ±1.06	5.33	42.8 ±2.71	102.61 ±15.13
T4 (2.5 g/L)	2335 ± 48.1	50.7 ±5.96	185.7 ±1.21	4.44	43.2 ±1.89	112.64 ±18.86

Table 2. General performance of the chickens Ross Line 308 at the end of the rearing period with the supplementation of different levels of betaine during the second cycle.

Treatment	Average weight ± SD (g)	Weight gain /animal/ day (g)	Food intake/animal/ day (g)	% Mortality	Body temperature (°C)	Corticosterone level (nmol/L)
T1 (control)	2600 ± 50.4	71.6 ±5.8	174.9 ±1.6	5.2	43.7 ±1.97	116.27 ±14.9
T2 (1.5 g/L)	2903± 50.0	111.3 ±9.4	172.1 ±1.0	3.7	42.7 ±2.3	124.32 ±9.0
T3 (2 g/L)	2498 ± 41.9	55.7 ±5.0	179.6 ±1.72	7.2	43.5 ±2.2	110.56 ±15.5
T4 (2.5 g/L)	2925 ± 60.2	79.0 ±8.4	167.3 ±1.28	3.4	43.0 ±2.2	120.8 ±4.5

In the FCP, the lowest mortality at the end the rearing period was for T2 with 2,96 % (Table 1) whereas during the SCP was for T4 with 3,43 % (Table 2). No differences were observed between cycles. In total, 28 and 26 dead birds were registered in the FCP y SPC, respectively. Finally, the body temperature during the FCP showed no significant differences between treatments ($p>0.05$; n=97), recording the highest temperature for T4 with an average of 43.2 ± 1.89°C (Table 1). During the SCP, the highest temperature was for T1 with an average of 43.7 ± 1.97°C (Table 2), without showing significant differences between treatments. No significant differences were observed between cycles. It can be noted that in both cycles, the body temperatures of the chickens were less than the upper lethal temperature point (47°C).

The corticosterone analysis showed that chickens suffered acute heat stress in all treatments during the FCP (Table 1). In the SCP, the average corticosterone levels in T1 and T3 were within normal ranges (80-120 nmol/l) in spite of some animals showed more than 120 nmol/l. The T2 and T4 revealed higher average corticosterone levels with 124.3 nmol/l and 121.75 nmol/l, respectively (Table 2). No correlations between variables were observed in any of the cycles, with no problems of multicollinearity (VIF<10). The results of ARLM are summarized in Table 3 and Table 4 for FCP SCP, respectively. Data for all variables in both production cycles did not follow a normal distribution ($p<0.05$, n=97 and n=83), thus the Mann-Whitney U Test was applied to determine differences in the means between treatments and cycles.

Table 3. Multiple regression of the betaine supplementation influence in chickens Line Ross 308 at the end of rearing period during the FCP.

		Estimated	Std. Error	t-value	p-value	R^2	R^2 Adjusted	F-statistic	AIC
T1	Intercept (weight)	1243.06	320.74	3.87	0.000874 ***				235.69
	Weight gain	-0.7156	0.24	-2.92	0.008150 **	0.42	0.36	7.73	
T2	Intercept (temp)	44	0.9266	47484	<2e-16 ***				122.01
	Corticosterone (1.5 g/l)	-3.5	1.5366	-2278	0.0345 *	0.28	0.13	1.86	
T3	Intercept (weight)	2280	24.82	91861	< 2e-16 ***				231.54
	Corticosterone level (2 g/l)	-128	35.1	-3647	0.037 **	0.76	0.51	3.03	
T4	Intercept (weight gain)	330.5	226.9	1456	0.1793				
	Costicosterone level (2 g/l)	16.0	5.1	3135	0.0120*	0.82	0.55	3.04	

Hence, in the economic analysis T2, showed the best higher net income ($371.99) with the highest C/B ratio (1.42), follow by the control treatment (T1) (1.28) (Table 5). In this cycle a total of 299.8, 390 and 490.6 g of betaine were utilized for T2, T3 and T4, respectively. For the SCP, In this cycle, T4 showed the best C/B ratio (1.93), presenting a higher net income ($398.81) followed by T2 (Table 6). A total of 256.5, 327.4 and 427.6 g were consumed for T2, T3 and T4, respectively. This study evaluates different concentrations of betaine inclusion in the drinking water for the control of acute heat stress in broilers of the Ross 308 Line from 38 days age onwards and its effect on the productive parameters, and its economic benefit in dry areas of the Inter-Andean region of Ecuador. Overall, the results showed that the final weight of the broilers increased with higher level of betaine in the drinking water compared to the control treatment (T1), particularly with the addition of 1.5 g/l (T2) and 2.5 g/l (T4) at both cycles, respectively.

During the FCP, the results indicated that the weight gain showed a significant effect on the final weight of the broilers in T1 compared to the other variables (food intake, body temperature). In T2, the betaine had no significant effect with any of the variables, except with body temperature. In T3, it was observed that the inclusion of betaine of 2 g/l had a significant effect on the final weight of broilers. The inclusion of this level significantly influenced weight gain but not feed consumption. It should be noted that in the latter, the effect of betaine was obscured by the presence of the other variables in the model. This can be explained the decreased of body weight and gain weight.

Gain weight was also improved significantly with higher levels of inclusion of betaine at both cycles. These results obtained here are similar with those studies that exhibited better weight gain in broilers during heat stress period (Attia et al., 2005; Chen et al., 2018). Many other studies showed no significant effects of betaine in gain weight nor feed intake (Harms and Russell, 2002; Park and Ryu, 2010). Due to its methyl donation property, betaine could be accessible for other key functionalities such as protein synthesis and immune modulation, resulting in enhanced performance of broilers.

Several authors have found that the application of different levels of betaine (0, 0.5, 1.0 and 1.5 g/kg) in the diet have significant effects on body weight, weight gain, feed consumption and feed conversion (Awad et al., 2014). On the other hand, Nofal et al. (2015) showed that betaine levels of 0.1% and 0.2% in the diet improved body weight, weight gain, feed conversion ratio and mortality rate. Other authors have discovered that different levels of betaine in feed improve the feed conversion ratio (Tolba et al., 2007; Honarbakhsh et al., 2007; Zulkifli et al., 2004). Shaojun et al. (2015) found that 0.1%, 0.2%

and 0.4% betaine use exhibited increased in feed consumption, associated with an increased in body weight and lower feed conversion. In contrast, Sakomura et al. (2013) showed that 0.05% and 0.075% betaine supplementation did not have a significant effect on productive parameters such as feed intake and body weight gain. Likewise, El Shinnawy (2015), found that doses of 1.0, 1.5, 2.0 and 2.5 g/kg betaine in the diet indicated a significant increase in body weight and weight gain.

Table 4. Multiple regression of the betaine suministration influence in chickens Line Ross 308 at the end of rearing period during the SCP.

		Estimate	Std. Error	t-value	p-value	R^2	R^2-adjusted	F statistic
T1	Intercept (Weight gain)	1244.2	458.8	2.7	0.0161 *			
	Food Intake	-5.6	2.2	-2.4	0.0247 *	0.38	0.17	1.84
	Intercept (Food Intake)	193.1	4.4	42.9	<2e-16 ***			
	Temperature	-0.37	0.08	-4.6	0.000286 ***			
	Weight gain	-0.05	0.02	-2.4	0.024932 *			
	Corticosterone level (1.5 g/l)	1.5	0.45	3.4	0.003643 **	0.67	0.59	8.47
T2	Intercept (Temperature)	310.7	57.53	5.4	5.89e-05 ***			
	Food Intake	-1.5	0.32	-4.61	0.000286 ***			
	Weight gain	-0.09	0.04	-2.15	0.046975 *			
	Corticosterone level (1.5 g/l)	2.73	0.98	2.78	0.013171 *	0.59	0.49	5.82
T3	Intercept (Food Intake)	154.9	14.6	10.5	3.69e-09 ***			
	Weight gain	-0.009	0.003	-2.617	0.0175 *	0.61	0.57	14.6
T4	Intercept (Food Intake)	169	0.63	264.9	<2e-16 ***			
	Corticosterone level (2.5 g/l)	-2.28	0.76	-2.99	0.00772 **	0.33	0.25	4.49

According to the hypothesis of the present study showed that the higher concentration of betaine in the drinking water, the lower mortality, associated to a higher average weight, with slightly less weight gain and no mark tendency in the food intake at the end of the rearing period compared to the control treatment (T1). Surprisingly, for T4, no significant relationship was observed between the betaine level and any of the explanatory variables (weight, weight gain, feed intake, corporal temperature). Excessive inclusion of betaine in the drinking water can cause energy loss due to their excretion and increasing betaine level may reduce its efficacy.

The final weight of the herd was not compromised at the end of the productive cycle. It was assumed that during the hottest hours, the animals would reduce feed consumption; however, this only happened for SCP in which consumption slightly decreased in a relative -0.4% between the sixth and seventh week, whereas for FCP increased by 13%. There results may be reflected in corticosterone levels where animals in the second cycle showed higher average levels (117.9 nmol/l), close to the limit of the normal range (80-120 nmol/l), which would be associated with a reduction in feed intake. Several factors can explain these differences as the duration and extent of heat stress, species of broilers, growth stages, type of diets, during periods of osmotic disturbances, caused by heat stress in which betaine may protect and improve the morphological characteristics of intestinal epithelia. These results are also partially consistent with several authors (Zulkifli et al., 2004) which reported no significant effect of betaine supplementation on feed intake. However, are consistent with those obtained by Attia et al. (2005), Zhang et al. (2016), and He et al. (2015). The betaine supplementation exhibited improved growth performance with the increasing on final weight and weight gain.

Table 5. Economic feasibility analysis of the suministration of betaine in the drinking water during the first cycle.

Treatment	Average weight (g)	Average weight (pounds)	Price per pound ($)	Number of animals	Raw income ($)	Feed intake (g/animal/day)	Total intake ($)	Quantity of food (20 kg)	Unitary cost (20 kg)	Total cost ($)	Net income ($)	Cost/ benefit ratio
T1	2275	5.00	0.75	162	607.5	184.3	209.0	10.00	25	261.3	346.1	1.32
T2	2441	5.37	0.75	164	660.5	182.9	210.0	10.00	25	261.3	371.9	1.42
T3	2213	4.87	0.75	160	584.4	185.3	207.5	10.37	25	259.4	298.9	1.15
T4	2335	5.13	0.75	161	619.4	185.4	209.0	10.45	25	287.3	332.1	1.15

Table 6. Economic feasibility analysis of the suministration of betaine in the drinking water during the second cycle.

Treatment	Average weight (g)	Average weight (pounds)	Price per pound ($)	Number of animals	Raw income ($)	Feed intake (g/animal/day)	Total intake ($)	Quantity of food (20 kg)	Unitary cost (20 kg)	Total cost ($)	Net income ($)	Cost/ benefit ratio
T1	2600	5.72	0.70	137	548.5	174.9	167.8	8.39	25	209.7	338.7	1.61
T2	2903	6.38	0.70	140	625.8	172.1	168.7	8.43	25	210.9	388.9	1.84
T3	2498	5.49	0.70	134	515.4	179.6	168.4	8.42	25	210.5	278.8	1.32
T4	2925	6.43	0.70	140	630.4	168.0	164.6	8.23	25	205.8	398.8	1.93

In the economic analysis, the best treatments with higher net income and lower percentage of mortality at the end of the rearing period were levels of 1.5 g/l (T2) and 2.5 g/l (T3) of betaine for the FCP and SCP, respectively. T2 showed the best cost-benefit ratio (1.42) and T4 (1.93) during the SCP. The latter can be explained due to higher average final weight of the herd at the end of the rearing period that it reflects in a better C/B ratio. These results suggest that the C/B ratio may be influence by the final weight of the herd as well as the price in the market that will determine the rentability for the farmer. Amer et al. (2018) have found that feed intake improved growth performance with better return in the net income and C/B ratio. They suggest that the improvement in the final weight and weight gain due to betaine supplementation might be attributed to the osmolytic property of betaine that supports intestinal cell growth and enhances cell activity, resulting in improving nutrient digestibility.

6 Conclusions

The results obtained in this study suggests that supplementation of betaine in the drinking water in broiler chickens Line Ross 308, can enhance average final weight particularly with levels of 1.5 g/l that an enhance gain weight and reduce the mortality at the end of the period. Higher levels of betaine (2.5 g/l) can also improve the rentability of the farmer, nevertheless, might be depend on factors such as final weight, market price, appropriate management of the herd and suitable environment conditions of the poultry house. Finally, this study encourages the application of betaine in the drinking water in commercial chickens at small as well as large-scale productions especially in the last days of the rearing period to overcome the acute heat stress in zones with mediterranean climates. Complementary, an appropriate management along the production cycle is essential to minimize the heat stress in the animals. Practices as good ventilation inside the poultry house, adjusting bird density, ensuring availability of fresh, low-salt drinking water, serving feed at cooler times of the day or reducing the effects of excessive temperatures by separating birds by sex can help mitigate its effects.

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Quantification of the CO_2 flow in the soil colonized by *Avicennia germinans*, located in the Los Totumos, Miranda state, Venezuela

Cuantificación del flujo de CO_2 en el suelo colonizado por *Avicennia germinans*, emplazado en el humedal Laguna Grande, sector Los Totumos, estado Miranda, Venezuela

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Abstract

Mangrove ecosystems are estimated to have a high impact potential on the global carbon cycle because of their high organic matter content (Dittmar et al., 2006), and consequently a significant importance in CO_2 flow as the greenhouse gas with the greatest impact on global warming (Benavides and León, 2007; Caballero et al., 2007; CESPA, 2013). In Venezuela, however, studies focusing on this issue are scarce, hence the aim of this research was to specify significant differences in the flow of CO_2 in soil colonized by *Avicennia germinans*, as well as non-rizospheric located in Los Totumos sector. Research developed in three phases was applied: (a) fieldwork, in order to collect 120 surface soil samples (0-20 cm), (b) laboratory, with the purpose of estimating the CO2 flow from the basal breathing method (Anderson, 1982) and (c) statistical analysis, to identify significant differences based on the ANOVA and Tukey test. It is evident that there are significant differences, with flows of 7.51 mg C-CO_2 g/24h in the rhyspheric area and 1.49 mg C-CO_2 g/24h for non-rizospheric. It is concluded that: (a) microbial activity in the soil, induced by the presence of *Avicennia germinans*, is possibly contributing to its evolution, and (b) it is reaffirmed that mangrove ecosystems are a source of CO_2 and reservoir gas to the impact of climate change.

Keywords: Basal breathing, microorganisms, CO_2, mangrove, *Avicennia germinans*.

Resumen

Se estima que los ecosistemas de manglar tienen un elevado potencial en el ciclo global de carbono por su alto contenido de materia orgánica (Dittmar et al., 2006), y en consecuencia una relevante importancia en el flujo del CO_2 como el gas invernadero de mayor impacto en el calentamiento global (Benavides and León, 2007; Caballero et al., 2007; CESPA, 2013). No obstante, en Venezuela los estudios centrados en este asunto son escasos, es por ello que la presente investigación tuvo como propósito precisar diferencias significativas en el flujo de CO_2 en suelo colonizado por *Avicennia germinans*, así como no rizosférico, emplazado en el sector Los Totumos. Se asumió una investigación desarrollada en tres fases: (a) trabajo de campo, a fin de colectar 120 muestras de suelo superficial (0-20 cm), (b) laboratorio, con el propósito de estimar el flujo de CO_2 a partir del método de respiración basal (Anderson, 1982) y (c) análisis estadístico, para identificar con base en el ANOVA y prueba de Tukey, diferencias significativas. Se evidencia que existen diferencias significativas, con flujos de 7,51 mg C-CO_2 g/24h en la zona rizosférica y 1,49 mg C-CO_2 g/24h para la no rizosférica. Se concluye que: (a) la actividad microbiana en el suelo, inducida por las condiciones edáficas que genera la presencia de la *Avicennia germinans* posiblemente está contribuyendo con la evolución del mismo, y (b) se reafirma que los ecosistemas de manglar constituyen fuente de CO_2 y sumidero del gas ante el impacto del cambio climático.

Palabras clave: Respiración basal, microorganismos, CO_2, manglar, *Avicennia germinans*.

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1 Introduction

Soil is an important reservoir of carbon since around 1500 PG C (petagrams of carbon) can be found in its first meters, while it is estimated about 800 PG C in the atmosphere, and about 500 PG C in the terrestrial vegetation. However, it is in constant motion in different molecular forms (Organización de las Naciones Unidas para la Alimentación y la Agricultura, 2017).

Carbon dioxide (CO_2) is one of the organic carbon derivatives in constant interaction with the climate. In the soil-atmosphere relationship, this greenhouse gas is produced by various conditions: (a) by respiration of micro and macro organisms as well as by living roots in the rhizosphere, in addition to organic matter decomposed directly from plants or through trophic chains (Departamento de Agricultura de los Estados Unidos, 1999; Organización de las Naciones Unidas para la Alimentación y la Agricultura, 2017), and (b) absorbed into the soil from the atmosphere by plants during photosynthesis by which carbon is fixed to produce biomass, and the oxygen particle is released into the atmosphere (Visconti and De Paz, 2017).

However, this sequestration of CO_2 into the soil structure is important to the global climate cycle, as it contributes to the balance of greenhouse gases in the atmosphere. It is important to note that forests cover 29% of the planet's surface area, and the carbon found in its soils constitutes approximately 36% of the total located at one meter deep (Zambrano et al., 2004), soil respiration being the most important flow of the carbon cycle (López and Monterroso, 2020), representing approximately 75% of the total in these ecosystems (Law et al., 2001). In the case of mangroves, Moreno et al. (2002); Kauffman et al. (2013), agree that they contain the largest carbon reserves due to the development of a complex ecosystem that contributes to the capture of CO_2 in the soil.

According to Kao et al. (2010), due to the dominance of certain typical plants in wetland environments, oxygen flows from the atmosphere to the surface and subsurface area of the soil, specifically to the rhizosphere by promoting the production-oxidation of greenhouse gases; hence, the contribution and quality of organic carbon can be influenced by the forest capacity of the dominant species. It is possible that since they are located in intertidal zones, i.e., in the transition between high tide and low tide zones, these types of forests are periodically flooded by the action of the squid, so that the soil passes through dynamic oxidation-reduction processes, generating conditions conducive to the activation of microbiological processes such as nitrification, denitrification and methanogenesis, which are greenhouse gas generators that can sometimes be released into the atmosphere (Chen et al., 2016).

Sánchez et al. (2011), mention that soils of wetlands where mangroves are developed store long-term carbon since they are usually largely flooded. However, due to little recognition of these reservoirs and the impacts generated by anthropogenic actions, these have been reduced by 35% of the total. Actually, anthropogenic action related to various uses of soil combined with the logging and burning of large areas of land has generated disruption of the ecosystems that constitute reservoirs, and consequently an environmental imbalance, encouraging soils to move from sinks to sources of CO_2, worsening global warming on the planet (Sánchez et al., 2011; Visconti and De Paz, 2017; Organización de las Naciones Unidas para la Alimentación y la Agricultura, 2017).

Hence, Villalobos (2012), mention the importance of managing appropriately carbon-reserve habitats, such as mangroves, in order to know their forest capacity for the production-storage of greenhouse gases, and to contribute to the mitigation of the impacts generated to the climate system. In this regard, research has been carried out to know the forest potential of mangrove forests related to the production and sequestration of CO_2; among these Kauffman et al. (2013) and Herrera et al. (2016), mention that Mexico and Brazil are among the 4 countries with the largest mangrove reserve worldwide. Moreno et al. (2002), Lozano (2007) and Sánchez et al. (2011), agree that mangroves within coastal wetlands are an important reservoir for carbon fixation or capture.

For Costa Rica and Colombia, Yepes et al. (2016) and Villalobos (2012), respectively, claim that mangroves are part of the planet's most productive carbon-producing ecosystems, and thus constitute a key environment for global warming mitigation.

Venezuela, being in the middle of the inter-tropical zone and possessing an extensive coastline, has important mangrove communities on which various studies have been carried out aimed at recognizing their geographical distribution, as well as their structural characterization based on biology, botany and agronomy, (Pannier and Pannier, 1989; Medina and Barboza, 2003; López et al., 2011; Cumana et al., 2000; Bonilla et al., 2010; Romero and Meléndez, 2013).

Núñez et al. (2019), reported differences in the flow of CO_2 in non-rhizospheric soils for the mangrove in Boca de Uchire, located at 94.93 km southeast of the Totumos, in contrast to those dominated in surface by *Avicennia germinans* and *Conocarpus erectus*. Likewise, Sánchez et al. (2010), identified variations in the flow of CO_2 in soils colonized by *Rizhophora mangle* on Margarita Island, estimating that because climatic conditions affect the availability of salt in the soil, it also affects the flow of the gas. Studies on the quantification of organic carbon, as well as carbon dioxide flows in these ecosystems are scarce, and so far, these have not been conducted in the study area: Laguna Grande wetland, Los Totumos. This area is located at the southwest of Cabo Codera, between 10° 32′ 34″ and 10° 32′ 44″ north latitude, and between 66° 4′ 44″ and 66° 4′ 54″ west longitude, bordering the Coastal Range to the north, Buche Bay to the west and the Caribbean Sea to the south and east (Figure 1). It extends over 7.77 ha, of which the monospecific forest of *Avicennia germinans* occupies 4.42 ha, representing 56.88% of the total area (Figure 2).

According to Garrido (2017), geomorphologically the area is a semi-elongated beach-like reservoir with a length of approximately 400 meters, and has a smooth relief with a greater prominence towards the north and south ends, which mainly consists of medium-sized particles with predominance of bioclasts. The data of the Carenero and Tacarigua Mamporal climatic stations, attached to the Hydrology and Meteorology Office of the Ministry of Power for the Environment (Ministerio del Poder Popular para el Ambiente, 2013a,b), located on the same coast line without marked altitudinal and latitudinal differences compared to Los Totumos, allow to state that the area has temperatures corresponding to an isothermal regime with an estimated annual average of 26.75°C, the warmer months being April and October with 27.7°C, and the cooler months from November to March, with a registered minimum of 25.15°C. Rainfall is distributed in a unimodal regime to reach an annual amount of 1141.3 mm, the rainy period extends from June to December. Evaporation is high throughout the year, with an annual amount of approximately 1781.9 mm (Figure 3).

According to the Goldbrunner classification (Foghin, 2002), the area is located on the tropical thermal floor, with altitudes that do not exceed 10 meters above sea level. As for the edaphic features, Entisols predominates with soil characterized by presenting a little developed material with a depth lower than 10 meters, an incipient surface horizon resting on the rocky material, sandy texture, highly saline and alkaline from the Orthens suborder and the Torriorthens group (Cárdenas, 1965; Elizalde et al., 2007; Gobernación del Estado Bolivariano de Miranda, 2010).

Huber (2007) and Gobernación del Estado Bolivariano de Miranda (2010) agree that the dominant vegetation for the coastal stretch presents swords, palms, pastures and mangroves. In Los Totumos, the development of mangroves in monospecific forest of *Avicennia germinans* with closed coverage is identified. Lentino and Bruni (1994) report the mouths of the streams: The Totumos, Horno, Laguna Grande and Hoyo de la tierra, with a Uadi type regime. In addition, Lara et al. (1997) claim that the Curiepe, Capaya and Tuy are rivers flow, with permanent runoff patterns.

Because of the latter, it is assumed that the riparian and monospecific mangrove of *Avicennia germinans* located on Entisols soils of Los Totumos in Laguna Grande wetland is a space to identify locally the possible forest potential of the aforementioned mangrove species in the CO_2 flow, although it has not yet been studied to know its structural conditions, as well as its contribution to the total carbon cycle. For this reason, the aim of this research was to identify significant differences in the flow of carbon dioxide (CO_2) in soil colonized by *Avicennia germinans* as well as non-rhizospheric, from the contrast of surface samples (0-20 cm depth).

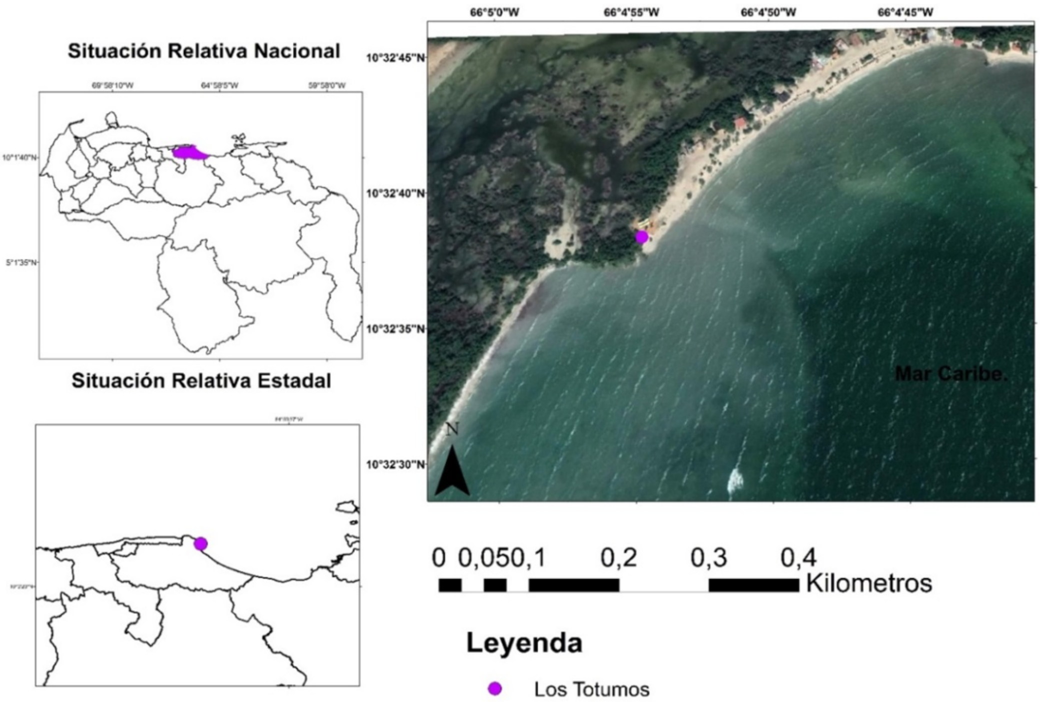

Figure 1. Location Map of Los Totumos. Elaborated using the Lansatd 8 Satellite Image. Combination of bands 321: Natural color.

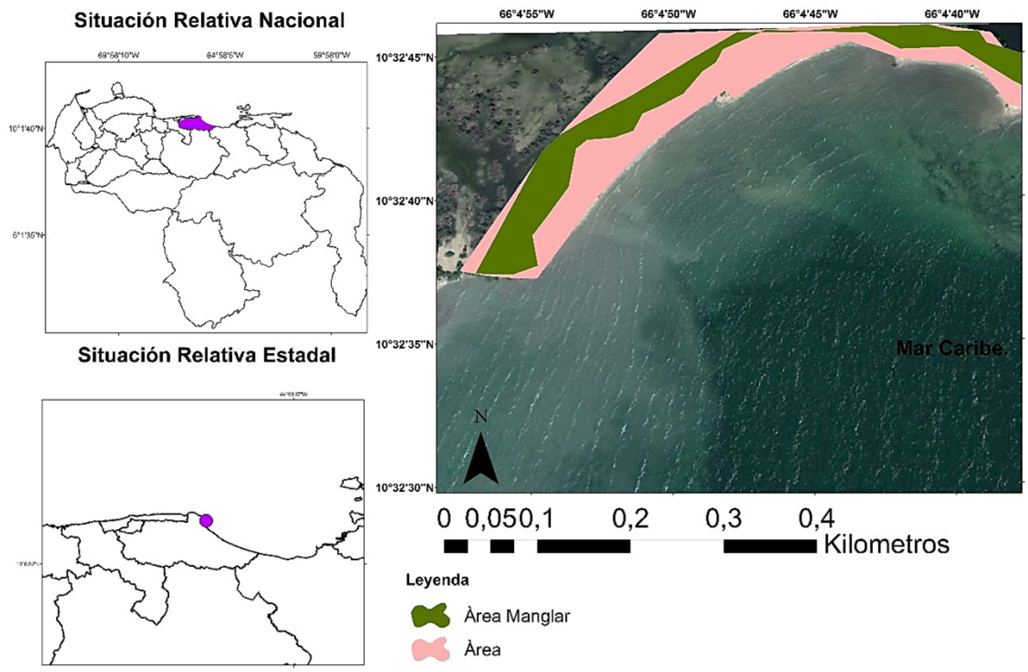

Figure 2. Map of the area covered by the forest of *Avicennia germinans* at Los Totumos, Laguna Grande wetland.

2 Materials and Methods

2.1 Field phase

Field work was carried out in May 2019, considered an active rainy period, which is an essential aspect in the collection of soil samples for the purpose of quantifying the CO_2 released. Indeed, as Luo and Zhou (2006) report, higher water availability generates an increase in the metabolic activity of roots and microorganisms, which is expressed in increased breathing. During the execution of this phase, the general observation of the area was made in order to recognize the coverage patterns of the mangrove, characterizing it as a monospecific forest of *Avicennia germinans*, as well as to identify the accessibility and homogeneity of the area, looking at areas that will visibly have the least impact associated with anthropogenic action. Based on this information, four plots of 1000 m^2 were selected, which represent approximately 10 % of the total forest area.

In order to collect the soil samples, two plots colonized by *Avicennia germinans* were selected in the mangrove area, these being considered to be those that receive the influence of the species as an intervening element in the flow of CO_2 in the soil, and two plots that even though are located in the mangrove area do not have surface vegetation, so they are considered non-rhizospheric, which for the investigation constitute the sample without intervening element.

Soil samples were collected at a surface level (0-20 cm), considering: (a) the soil in the area closest to the base of the individual tree for areas with the presence of Avicennia germinans, and (b) a distance of no less than 5 linear meters per sampling point for the area deprived of mangrove. Under the criteria described above, 120 soil samples were collected, distributed as follows: (a) 60 soil samples dominated by *Avicennia germinans*, and (b) 60 samples of non-rhizospheric or surface mangrove-free soil.

The samples were stored in dense polyethylene bags, identified with a content label with the following information: Unique identification key, coordinates of the sampling point, date and time of collection. They were preserved for their transportation to the laboratory at a temperature controlled between 4°C and 6°C.

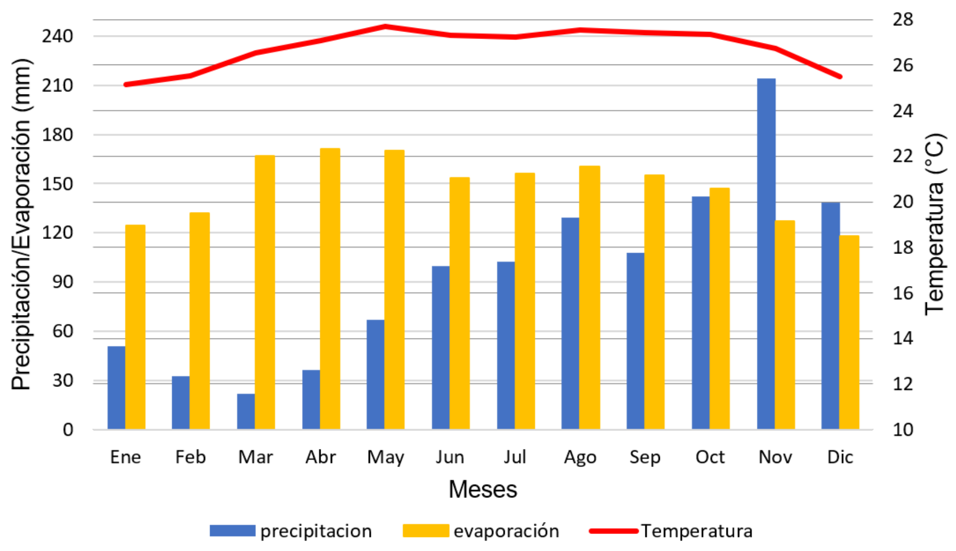

Figure 3. Climate diagram elaborated from the information in the data sheet of the stations, obtained through the Hydrology and Meteorology Office of the Ministry of Power for the Environment. Precipitation data are for Carenero station, and temperature and evaporation data for Tacarigua Mamporal station.

2.2 Laboratory phase

During the development of this phase, the CO_2 released was determined using the basal breathing method, based on the protocol reported by Anderson (1982). For this purpose, soil samples preserved at field humidity and sifted in the 10 mm sieve were pre-conditioned at room temperature (24°C) in order to achieve stabilization. Subsequently, 50 g of each soil sample was placed in 500 ml glass bottles with their lids. In addition, an alkali trap was suspended in amber glass bottles, containing 5 ml of a 0.1 mol of Sodium Hydroxide solution (NaOH). The treated samples were placed in a dark area in the laboratory. After 24 hours, the trap was removed and the solution was placed in glass bottles, adding 2 ml of barium chloride ($BaCl_2$) and 2 drops of phenolphthalein ($C_{20}H_{14}O_4$) as a pH indicator to determine the alkalinity of the solution. The absorbed CO_2 was titrated with 0.1 mol of hydrochloric acid (HCl). The result was expressed in mg C- CO_2 g/24 h. Additionally, during the execution of this procedure, jars without soil samples with alkali traps were also used in order to establish a comparison pattern.

2.3 Statistical analysis phase

To determine possible variations in the four plots under study, as well as to identify the influence of *Avicennia germinans* as a variation source in the flow of CO_2 in soil, data were statistically analyzed from the following tests: (a) the analysis of variance (ANOVA) to specify the significant differences between the means of each group of samples, corresponding to the four plots under study; and (b) the Tukey test to identify which sample groups are different and honestly significant, once significant differences have been assumed in at least one of the four sample sets.

3 Results and Discussion

Table 1 presents the minimum, maximum and average values for the flow of CO_2 (mg C-CO_2 g/24h) in the soil for the four plots under study. The estimated ANOVA per plot yielded 334.41 for test F and a probability of 6.5×10^{-57} (Table 2), which allows to assume that the average is different with 95% of reliability in at least one group of data out of the four plots under study.

Table 1. Descriptive analysis of the flow of CO_2 (mg C-CO_2 g/24h) in the soil.

Coverage	Minimum Value	Maximum Value	X	s	$O_2^{'}$
Parcel 1 (cm)	1.11	8.89	7.39	1.11	1.20
Parcel 1 (sm)	0.10	2.39	1.63	0.90	2.30
Parcel 2 (cm)	1.30	9.37	7.63	1.31	1.65
Parcel 2 (sm)	0.10	2.74	1.35	0.76	0.55

cm= with mangrove/ sm= without mangrove/ x= mean/ s= typical deviation/ $O_2^{'}$ = variance.

Table 2. Analysis of variance (ANOVA) for the four parcels under study.

Origin of variations	Suma of squares	Degrees of freedom	Averages of squares	F	Probability	Critical value of F
Between groups	1089.08	3	363.02	334.41	$6.5*10^{-57}$	2.68
Within groups	125.92	116	1.08			
Total	1215.00	119				

Tukey test showed an HSD value of 0.69 which allows to state that: (a) there are no significant differences between the weighted averages of samples for plots with Avicennia germinans, and no significant differences are identified between the weighted averages of samples for non-rhizospheric soil, and (b) there is significant difference in comparing the weighted averages of plots with surface mangrove with those that do not present it (Table 3; Figure 4). A significant difference $^{(HSD)}$ is considered if the mean difference between the sample groups is higher than the HSD value.

Table 3. Tukey test of significant difference for CO_2 flow from soil samples in the four plots under study.

HSD = 0.69	**Parcel 1 (cm)**	**Parcel 1 (sm)**	**Parcel 2 (cm)**	**Parcel 2 (sm)**
Parcel 1 cm	***	$5.76^{(HSD)}$	0.24	$6.03^{(HSD)}$
Parcel 1 sm	$5.76^{(HSD)}$	***	$6.00^{(HSD)}$	0.27
Parcel 2 cm	0.24	$6.00^{(HSD)}$	***	$6.27^{(HSD)}$
Parcel 2 sm	$6.03^{(HSD)}$	0.27	$6.27^{(HSD)}$	***

cm= with mangrove/ sm= without mangrove

According to the above analysis, it can be said that the soil covered by *Avicennia germinans* in the study area has an average CO_2 flow of 7.51 mg C-CO_2 g/24h, which is significantly different from the estimated 1.49 mg C-CO_2 g/24h for soils free of the mangrove or non-rhizospheric species. This represents a flow of 83.44% for the soil covered by mangroves, in contrast to the 16.56% estimated for the non-rhizospheric soil (Table 4; Figure 5). As already mentioned, Núñez et al. (2019), identified in Boca de Uchire, significant differences, estimating that for non-rhizospheric soil the flow is 3.74 mg C-CO_2 g/24h, while those colonized by *Avicennia germinans* register 10.61 mg C-CO2 g/24h, and *Conocarpus erectus* 13.88 mg C-CO_2 g/24h.

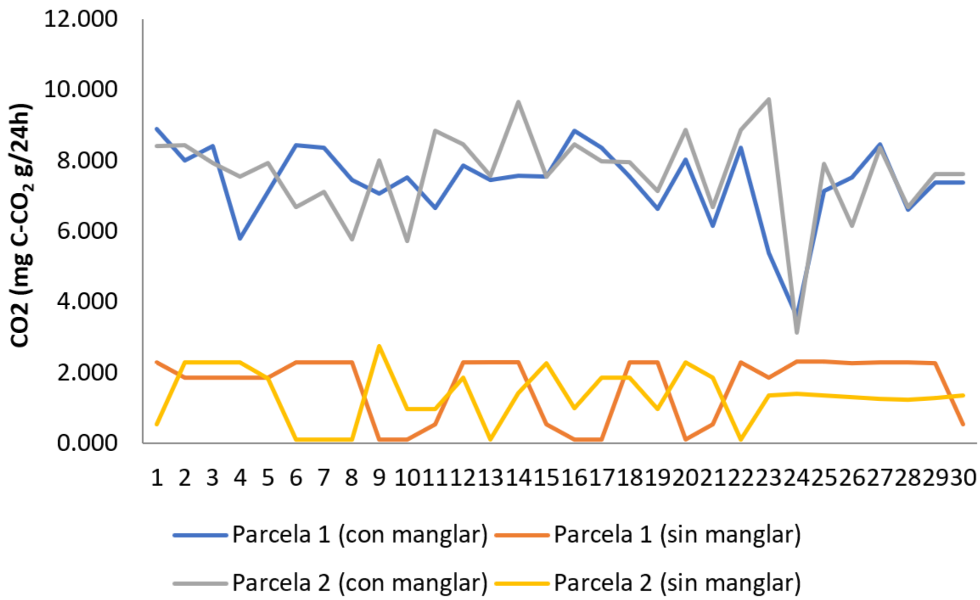

Figure 4. Distribution of CO_2 flow (mg C-CO_2 g/24h) in the soil by plot and coverage.

It is possible that the lower value of CO_2 flow in the soil colonized by *Avicennia germinans* in Los Totumos sector, compared with that reported for the same mangrove species in Boca de Uchire is linked to edaphic salinity conditions, because excess of this component not only affects the structural development of plants (Cintron et al., 1978), but limits their forest potential as a CO_2 sink (Mitra et al., 2004).

In fact, Sánchez et al. (2010) identified variations in CO_2 flow in soils colonized by *Rizhophora mangle* associated with sodium fluctuations by climatic periods, registering for highly saline soil: 21.1 ± 18.9 mg C-CO_2 g/24h (dry period), 25.0 ± 12.3 mg C-CO_2 g/24h (rainy period) and 31.9 ±18.5 (transition period). Although these are different mangrove species, it is possible that the high salinity condition for the soil of Los Totumos is an influential factor in the flow of CO_2.

Singh et al. (2010), report that slow rates of soil respiration can be considered as indicators of limited biological activity, stress generated by inadequate use, climatic disturbances, or even resource deficits. According to Carrero and García (2009), these mangroves have been cut off for conducting engineering works for tourist and commercial purposes, generating some negative effects. Therefore, it is considered that anthropogenic intervention in the area has generated disturbance conditions to the ecological balance of the mangrove, affecting the structural development of the forest, and limiting forest potential in the capture and storage of CO_2 in the soil.

Table 4. Distribution percentage of CO_2 flow (mg C-CO_2 g/24h) in the soil by coverage.

Coverage	CO_2 average	%
With Mangrove	7.51	83.44
Without Mangrove	1.49	16.56

Regarding oxygen penetration in the soil in mangrove areas, Olguín et al. (2007), report that it is limited to the first centimeters of the soil profile, because the near-surface groundwater level enhances the reduction conditions, causing bacterial respiration to use NO_3, MnO_2, $FeOH_2$, SO_4 and CO_2 in the anaerobic zone as final electron acceptors. Hence, the contribution of CO2 to the anaerobic areas of the soil, possibly caused by the respiration of mangrove roots, generates conditions for the existence of anaerobic bacteria responsible for the decomposition of organic matter into methane, by giving this area the sink condition of these greenhouse gases.

In the oxygenation zone, CO_2 intake is possibly caused by the respiration of aerobic microorganisms that inhabit the rhizosphere, constituting an indirect indicator of microbial activity. In effect, Karmarkar (1982) reports that such biological activity linked to the proliferation of microorganisms favors the accumulation and decomposition of organic residues provided by the mangrove, in the period when the soil remains low in water.

However, even in periods of flooding, Mitsch and Gosselink (2000) say that such water flows help to reduce the reduction conditions in the surface area of the soil, because these sheets provide dissolved oxygen, which favors microbial activity and the consequent release of CO_2. This CO_2 released is generally transformed by photosynthetic bacteria into carbohydrates used by plants. Additionally, the microorganisms that generate it are essential for the decomposition of organic matter into labile humus, beneficial for the development of plants by its contribution of macronutrients such as nitrogen, necessary for the succulence and greenery of leaves.

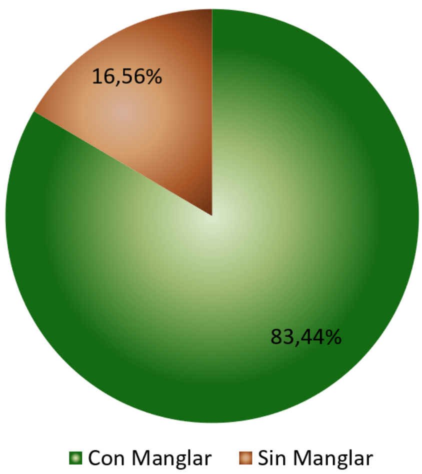

Figure 5. Distribution percentage of CO_2 flow (mg C-CO_2 g/24h) in soil by coverage.

Indeed, Hesse (1961) and González et al. (2016), state that *Avicennia germinans* requires soils with at least 0.4% of nitrogen for its development. In this sense, Holguín et al. (2007), say that the proliferation of microorganisms leads to an increase in the rate of biological nitrogen fixation, contributing from 40 to 60 % of the requirements of the mangrove ecosystem.

The development of these forests generates a positive balance for the climate system, since as mentioned by Canadell et al. (1995), they present a high potential in the sequestration of carbon in the soil, in response to the increase in atmospheric CO_2. Hence, Zhong and Qiguo (2001) affirmed that these soils should be considered as natural regulators of atmospheric CO_2 concentration and flow. Indeed, Bouillon2008, stated that these forests directly capture 4996 g of CO_2 m^{-2} per year.

Based on the latter, the surface dominance of mangrove species contributes to the increase of CO_2 in the soil, and additionally, it is inferred that such variation may have as a conditioning element the edaphic potentialities of the colonizing species, along with climatic, edaphic and anthropogenic variables.

In addition, the microbial activity associated with the presence of *Avicennia germinans* in Entisols soils such as the one in Los Totumos can be interpreted as an indicator of its incipient pedogenetic development, since the bacteria activate the humification process, which allows assuming that these naturally mineral soils are added by organic matter transformation and organic compounds that progressively increase aeration, cation exchange capacity and cause improvements in structure.

4 Conclusions

Based on the results of this research, it is concluded that a monospecific forest of *Avicennia germinans* is developed in the area of study, covering approximately 56 % of the total area of Los Totumos.

Similarly, the coverage of this mangrove species contributes to the development of microbial activity in the surface area of Entisols soil, which is inferred from the estimated significant differences in respiration of samples corresponding to rhizosferic and non-rhizosferic zones. In fact, 7.51 mg C-CO_2 g/24h was estimated in the area colonized by the abovementioned mangrove species, while the estimated breathing was 1.49 mg C-CO_2 g/24h in the non-rhizospheric zone without surface vegetation. This represents a slightly more than 500 % increase in the flow of CO_2, associated with the presence of mangrove in the surface area of the soil. Also, the soil microbial activity induced by edaphic conditions that generates the presence of *Avicennia germinans* is possibly contributing to the evolution of the soil. Finally, it is reaffirmed that mangrove ecosystems are a source of CO_2 generation, so its study should contribute to generating information that allows the decision-making on its management in order to mitigate the emission of this greenhouse gas into the atmosphere.

It is necessary to continue conducting studies in the above-mentioned mangrove to deepen the analysis of the possible relationships between its structural conditions, the soil properties in which it is located and the total carbon flow.

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Effect of the temperature prior to extraction on the yield and fatty acid profile of morete oil (*Mauritia flexuosa* L.f.)

Efecto de la temperatura previa a la extracción en el rendimiento y perfil de ácidos grasos del aceite de Morete (*Mauritia flexuosa* L.f.)

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Abstract

Morete (*Mauritia fleuxosa* L. f.) is a palm from the Amazon that produces a fruit with a pleasant taste, good nutritional value and a high oil content. The aim of the present research was to study the effect of the heat treatment of the different parts of morete on the performance and fatty acids profile of oil obtained by pressing. A randomized complete block design with three replicates was applied, combining the use of pulp and pulp with fruit rind under different heating conditions before pressing: 45°*C* for 30 min, 65°*C* for 20 min and 85°*C* for 10 min. Also, the oxidative stability of oil was measured using the Oxitest Reactor. Oils with different content of saturated, monounsaturated, and polyunsaturated fatty acids were obtained with oleic acid prevalence. The best treatment was using pulp heated at 85°*C* for 10 min and pressing, founding a yield of 56.77% oil with 79.80% oleic acid, and oxidation stability of the oil 14.5 months at 21°*C* was determined, which is the average temperature of the city of El Puyo, Ecuador, where morete was collected. On the other hand, the effect of this temperature prior to extraction on the content of bioactive compounds and the possibility of oxidation of fats must be evaluated. In conclusion, morete is a good source of oil and the heat treatment technology will allow improving extraction, industrializing, and offering an alternative oil for food.

Keywords: Vegetable oil, oxidative stability, oil extraction, acid number, induction period, amazon palm tree.

Resumen

El morete (*Mauritia fleuxosa* L. f.) es una palmera de la Amazonía que produce un fruto de agradable sabor, buen valor nutricional y alto contenido de aceite. El objetivo de la presente investigación fue estudiar el efecto del tratamiento térmico de las diferentes partes del morete en el rendimiento y perfil de ácidos grasos del aceite obtenido por prensado. Se aplicó un diseño de bloques completos al azar con tres réplicas, combinando el uso de pulpa y pulpa con corteza del fruto en diferentes condiciones de calentamiento antes del prensado: 45°*C* por 30 min, 65°*C* por 20 min y 85°*C* por 10 min. También, se determinó la estabilidad oxidativa del aceite empleando un Reactor Oxitest. Se obtuvieron aceites con diferentes contenidos de ácidos grasos saturados, monoinsaturados y poliinsaturados, prevaleciendo el ácido oleico. El mejor tratamiento fue empleando la pulpa calentada a 85°*C* por 10 min y prensada, encontrándose un rendimiento de 56,77 % de aceite con 79,80 % de ácido oleico, y una estabilidad a la oxidación de 14,5 meses a 21°*C*, temperatura promedio de la ciudad de El Puyo, Ecuador, sitio donde se recolectó el morete. Por otro lado, se debe evaluar el efecto de esta temperatura previa a la extracción sobre el contenido de compuestos bioactivos y la posibilidad de oxidación de las grasas. En conclusión, el morete es una fuente de aceite y la tecnología de tratamiento con calor permitirá mejorar la extracción e industrialización, y ofrecer un aceite alternativo para la alimentación.

Palabras clave: Aceite vegetal, estabilidad oxidativa, extracción de aceite, índice de acidez, periodo de inducción, palmera amazónica.

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1 Introduction

Vegetable oils are a source of saturated, monounsaturated or polyunsaturated fatty acids, which play an important role in human nutrition (Kumar et al., 2016). Therefore, there is interest in new sources of edible oils, such as those coming from fruits and seeds of plants that possess nutritionally important oils (Amri et al., 2017), among these sources is morete fruit, which has an oil with a high content of monounsaturated fatty acids, superior to that of olive oil (Milanez et al., 2016).

Morete (*Mauritius flexuosa* L. f.) is a palm tree that is found in the Amazon region, it has different names depending on the country in which it grows, such as: morete in Ecuador, moriche in Colombia and Venezuela, aguaje in Peru, real palm in Bolivia and burití in Brazil (Restrepo et al., 2016). It is a monic and arborescent palm with stems higher than 25 meters high and diameter between 30 to 60 cm. It grows in the Amazonian forests on poorly drained soils, marshes and alluvial plains (Montúfar and Brokamp, 2011). Its fruit is a source of food for birds, some species of mammals and native peoples, and is used to produce juices, creams, jellies, jams and oil (Pereira et al., 2016). This palm tree has economic and social importance in the countries where it grows (Bataglion et al., 2015; Forero-Doria et al., 2016).

There are three ecotypes of the palm, three varieties are mentioned by the color of the fruit: (a) yellow or posheco when the mesocarp is yellow, (b) colored when the outer part is red, and (c) shambo when the mesocarp is red (Vásquez et al., 2010). The fruits are from 6 to 7 cm, with brown-reddish color when they reach maturity, with a weight of 50 g. It is formed of an exocarp with imbricated scales and a fleshy mesocarp, and the seed has a subglobous form with a homogeneous endosperm (Trujillo-Gonzalez et al., 2011). Mesocarp is edible, rich in bioactive compounds such as vitamins, antioxidants, unsaturated oils and dietary fiber (Cruz et al., 2020; Rudke et al., 2019). The oil in the bite has a large amount of oleic acid 79.33 % compared to the other fatty acids of its composition (Cândido and Silva, 2017) and the nutritional value of the oil can vary according to the season and the extraction process (Aquino et al., 2012).

The oils used in food are sensitive to oxidation, resulting in stale odors, unpleasant flavors, discoloration and their service life is reduced (Corbu et al., 2020), hence it is important to know the oxidative stability of oils. Its determination is carried out under normal storage conditions, using the peroxide index and accelerated methods using equipment such as Rancimat or Oxitest Reactor (Rodríguez et al., 2015; Caruso et al., 2017). The Oxitest reactor is an instrument for predicting oxidation stability in solid or liquid samples and is a fast and ecological alternative compared to the Rancimat method (Tinello et al., 2018). Oxitest subjects the sample to an environment of oxidative stress at high temperature and high oxygen pressure; the drop in oxygen pressure within the oxidation chambers depends on the composition of the food and is expressed as an induction period (IP), which is the time required to obtain the starting point of lipid oxidation (Riciputi and Caboni, 2017).

Mechanical pressing is generally used extracting oil in rural areas, because it has a low initial investment and does not require highly trained personnel to operate the equipment (Nde and Foncha, 2020), because of the latter, the objective was to study the effect of heat treatment in the different pre-extraction phases of morete on the yield and fatty acid profile of the oil obtained by pressing in order to get a technology that allows to be used in the rural communities where these palms are cultivated.

2 Materials and Methods

2.1 Fruits

Morete fruits (*Mauritius flexuosa* L. f.) of the yellow pulp posheco ecotype, acquired at Los Bananas market, located in the Mariscal area of El Puyo, province of Pastaza, Ecuador, were used.

2.2 Extraction of morete oil by pressing

The process was based on the extraction methodology of olive oil described by (Moreno and López, 2017) with modifications, and described below: morete fruits were cleaned and washed with running water to remove impurities; then the bark, seed and the pulp were manually separated.

In order to improve the extraction of the oil, the pulp and bark were subjected to thermal treatment before pressing, for which 400 g of the different parts of morete were heated in a stainless-steel container using an IKA heating plate. Temperature and warm-up time conditions are shown in Table 1 and were designed according to Adrianzén et al. (2011) and Tambunan et al. (2012). A fully randomized block design was used in the treatment of the parts of morete and in triplicate.

The heat-treated sample was ground using a blender for 3 minutes, then the oil was obtained with a manual Al-Equip press (Figure 1). The extracted oil was set aside in separating funnels in order to separate the water from the oil by difference in density. Finally, the separated oil was filtered with a grade 1 Whatman qualitative filter paper to remove solid waste and obtain an oil without any particles. The oils obtained were packed in amber glass bottles and stored in environment conditions until the next day for their analysis.

Table 1. Treatments for the extraction of mulberry oil.

Experiment, T	Parts of the fruit	Pre-treatment	
		Temperature, $^{\circ}C$	Time, minutes
T1	Pulp with shell	45	30
T2	Pulp with shell	65	20
T3	Pulp with shell	85	10
T4	Pulp	45	30
T5	Pulp	65	20
T6	Pulp	85	10

Figure 1. Manual press oil extractor

2.3 Analysis of morete fruits

Size and weight

Morete fruits were measured to determine their length (cm) from the base to the apex and the diameter (cm) in the central part using a vernier caliper. The masses of the fruits, shell, pulp and seed were determined on an analytical scale according to the method described by Quispe et al. (2009).

Humidity

It was analyzed by weight loss, Official Method 930.15 (AOAC, 2002). 5 g of sample were weighed in previously dried and tarted porcelain capsules, then were carried to a drying oven at 105 $^{\circ}C$ until a constant weight was achieved, then were placed in a desiccator for cooling. Humidity loss of samples was determined as a percentage.

Fat

It was determined using a solvent for continuous extraction of fat from samples, Official Method 920.39 (AOAC, 2002). 3 g of ground, dry sample were weighed in a cellulose cartridge and 100 mL of hexane were added in a previously heavy glass; both containers were placed on a Goldfish Velp Scientifica machine to extract the fat for 4 hours. The cup was removed with the fat to evaporate the solvent in a stove at 105 $^{\circ}C$ for 5 hours and was set aside in a desiccator at room temperature before weighing it. In the end, the percentage of fat in the sample was determined by weight difference.

2.4 Analysis of oils extracted by pressing

Oil extraction performance per pressing

It was determined by the ratio of the extracted oil weight, when 400 g of morete are subjected to the pressing and the weight of the fat contained in the sample. The fat of morete was determined by the Official Method 920.39 (AOAC, 2002). The oil extraction performance was expressed as an oil percentage extracted in each treatment using equation 1.

$$\text{Yield}\,(\%) = \frac{\text{Weight of oil extracted from morete}}{\text{Weight of fat contained in morete}} * 100\,\% \quad (1)$$

Acidity index

It was determined by base acid titration, Official Method No 940.28 (AOAC, 2002). It was based on the determination of free acids, for which the oil sample was dissolved in ethanol and the free fatty acids were evaluated by an ethanolic solution of sodium hydroxide 0.1M using phenolphthalein as a visual indicator. The acidity index was determined by equation 2 and was expressed as a percentage in oleic acid.

$$OI = \frac{M * V * M_{NaOH}}{10 * m} \quad (2)$$

Where:

OI = oil acidity index (% oleic acid)
M = molecular mass of oleic acid (282 g/mol)
V = Volume of hydroxide solution consumed at titration in cm^3
M_{NaOH} = Molarity of sodium hydroxide solution determined daily against a primary standard.
m = mass of the analyzed sample expressed in grams
10 = conversion factor to percentage

2.5 Fatty acid profile of oils

Oil extraction of morete

The different parts of morete were dried at 80 $^{\circ}C$ for 24 h, then were ground in a porcelain mortar and the oil was extracted in a Soxhlet. 5 g of the sample were weighed on the cellulose cartridge, 200 mL of hexane were placed on the balloon and brought to boiling for 6 hours. In the end, the solvent was recovered by evaporation at 50 $^{\circ}C$ in a rotary evaporator selecta brand to obtain solvent-free oil. The oils extracted from the different parts of the fruit were filtered and stored in amber bottles until their analysis.

Esterification of the fatty acids of different morete oils

From 0.020 to 0.025 g of the oil extracted from each sample were placed in test tubes with 15 ml thread, 2 mL of KOH 0.5 M prepared in methanol were added. The tubes were closed and taken to the water bath for 10 min. Then the tubes were set aside in environment conditions and 1 mL of HCl in methanol (1:4 v/v) was added. They were re-capped and taken to water bath at 50 °C for 25 minutes. The tubes were re-cooled and 3 mL of distilled water with 10 mL of hexane were added to each tube, stirred and set aside for 24 hours. Finally, fatty acid methyl esters were collected from the upper layer of the tubes and placed in amber bottles (Carrillo et al., 2018).

Chromatographic analysis

Esterified fatty acids were characterized by an Agilent Technology 7980A gas chromatograph, coupled with an MSD 5977 mass spectrometer. Separations of esters were performed on Agilent Technologies' HP-88 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium was used as carrier gas at 0.7 mL/min^{-1}, the injector temperatures, GC-MS interface and MSD source were 250, 300 and 230 °C, respectively. The following oven temperature program was used: (a) initial temperature of 80 °C, (b) ramp 10 °C/min up to 120 °C, (c) ramp 20 °C/min up to 140 °C, (d) ramp 2 °C/min up to 200 °C. It was maintained for 10 min and finally (e) ramp 5 °C/min to 240 °C and it was maintained for 4 min. The mass detector operated in full scan mode, with the m/z range being 50 to 500. Additionally, 0.2 μl of ester samples were injected using splitless injection mode. A reference material consisting of 37 methyl esters of Supelco fatty acids (Component Fame Mix) was used to verify their identification and quantification; quantification was carried out by integrating the peak areas resulting from chromatographic analysis and expressed as the mean value of fatty acid ± standard deviation of three injections of each oil

2.6 Oxidative stability of morete oil

Oxidative stability was evaluated for the oil obtained from the best treatment, for this purpose an Oxitest reactor of Velp Scientifica (Usmate, Milan, Italy) was used, following the method described by Caruso et al. (2017). 6 g of oil were weighed on each of the titanium plates of the equipment and placed in each oxidation chamber. The following oxidation temperatures were selected: 80, 90 and 100 °C and grade 5 oxygen was used with a pressure of 6 bar to enter the Oxitest reactor. The instrument includes the OXISoft software used to calculate the Induction Period (IP) in hours and minutes for each selected temperature of the experiment, the calculation is done automatically by a graphical method. IP values are expressed as oxidation stability time, and correspond to the pressure drop due to sample oxidation.

With the IP value and the temperature, a graph was done representing the Ln(IP) on the axis of the orders and the oxidation temperature in the abscissa in Celsius degrees. The line equation (Equation 3) is then estimated using the least squares method. Finally, this equation extrapolated the desired temperature to estimate the IP value. For this study, 21 °C was used, which is the average temperature of the city of El Puyo in Ecuador, where morete is produced and the extracted oil will be stored.

$$y = mx + b \tag{3}$$

Where:

y : Natural logarithm of the induction period, Ln(IP) in hours.
x : Oxidation temperature: 80, 90 and 100 °C.
m : slope of the regression line.
b : intercept of the regression line.

2.7 Statistical analysis

Tables with their respective means and standard deviations were elaborated with the results obtained from the physico-chemical characteristics of morete, oil extraction yield, acidity index and fatty acid profile. The Statgraphics Centurion XVI statistical package was used to perform the statistical analysis using ANOVA and the significant differences were calculated with Tukey test for 95% confidence.

3 Results and Discussion

3.1 Physico-chemical characteristics of the fruit

Figure 2 shows the typical morete fruit used for this study and the oil obtained. Table 2 shows the results of the characterization of the morete fruit, and it was found that it has dimensions consistent with those reported by Mendieta-Aguilar et al. (2015).

Additionally, it was obtained that the selected morete has on average 56.23% of comparable humidity reported by Sandri et al. (2017) and Darnet et al. (2011) 59.11 and 50.5 %, respectively.

The fruit is made up of pulp, seed and peel, and in this investigation pulp and pulp with shell were used for the treatments indicated in Table 1. It was found that the mass of the fruit corresponds to one fifth of the pulp, one fifth of the shell and three fifths of the seed.

The highest percentage of fat calculated according to 100 g of the part of the fruit considered was found in pulp and pulp with shell (Table 2) and was higher than the 19.0 % reported by Darnet et al. (2011); therefore, these parts of the fruit were considered for the extraction of oil.

The differences in fat content with those obtained in this study are because the composition of the fruits is influenced by altitude, temperature, rain and soil, because they control the availability of nutrients to the plants (Nascimento-Silva et al., 2020).

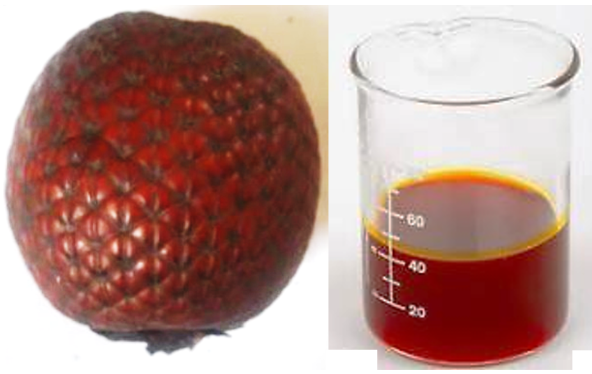

Figure 2. Morete fruit and morete oil obtained in this study

Table 2. Characterization of morete fruit.

	Length, cm	Diameter, cm	Weight, g
Morete fruit (n=20)	5.47 ± 0.15	4.59 ± 0.18	51.83 ± 0.31
Mendieta-Aguilar et al. (2015)	5 – 7	4 – 5	–
Part of the fruit	Percentage in fruit	Fat content[2] (g)	
Pulp[1]	20.19 ± 0.46	26.01 ± 0.84	
Shell	19.43 ± 0.73	14.62 ± 0.20	
Seed[1]	59.92 ± 0.31	4.15 ± 0.05	
Pulp with shell[1]	39.62 ± 0.43	22.06 ± 0.64	
Darnet et al. (2011)	–	19.0	

[1] The mean and uncertainty are indicated as the standard deviation for n = 3.

[2] Per 100 g of the parts of the fruit.

3.2 Yield and acidity of morete oil

It was considered as the best treatment that allows the highest oil extraction yield with the greatest amount of oleic acid. The variance analysis for the yield on the extraction of morete oil indicates that there are significant differences ($P<0.05$) between the treatments, obtaining the double extraction yield in the T6 experiment in relation to the T1 treatment (14.76 g of oil extracted in relation to 100 g of morete pulp). Figure 3 illustrates the evolution of the extraction yield of morete oil for each of the treatments.

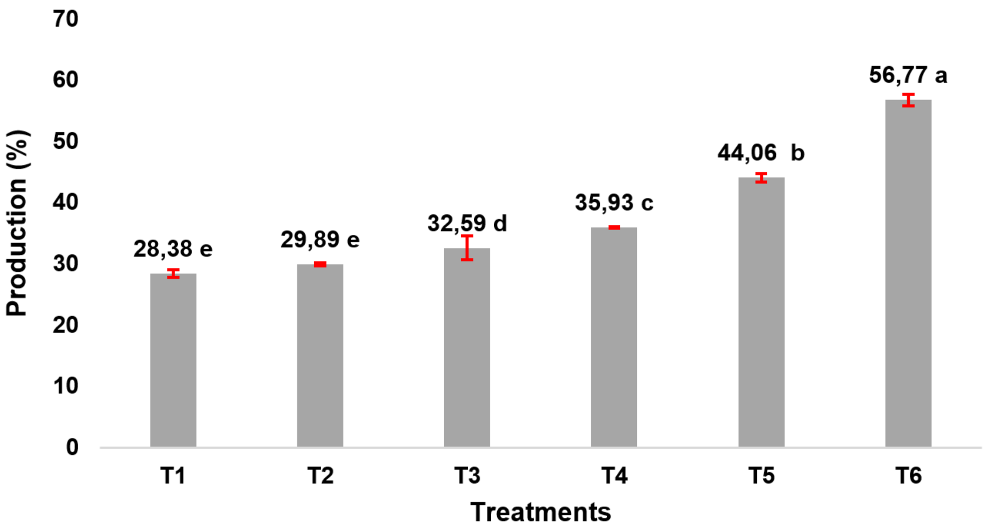

Figure 3. Evolution of the oil extraction yield of the different treatments. Different letters in the graph indicate significant differences ($P<0.05$)

It was found that the oil extraction percentage is directly proportional to the heating temperature of the pulp, and it decreases when the pulp with shell is used in the pre-treatment before pressing. The pre-extraction heat supply allows proteins from the cell walls to clot, making them permeable to the passage of oil during pressing, also decreasing the viscosity of the oil, which facilitates the extraction process (Adrianzén et al., 2011). In addition, it may inactivate enzymes responsible for oil deterioration, as indicated by Onyebuchi (2013), who notes that enzymes such as peroxidase drastically decreases activity to 80 $°C$ in seed oils. On the other hand, the stability of the bioactive compounds should be evaluated in order to know the effect of the heat presented in each of the treatments.

The thermal process in the different parts of morete fruit affects the acidity present in the extracted oils (Figure 4), being the one with the lowest acidity when using only morete pulp and the lowest heating temperature (45 $°C$) before starting the pressing for oil extraction; on the other hand, the acidity is higher when higher temperatures are used or when pulp with shell is used for the extraction. However, the acidity percentage of oils obtained from the different treatments is in the range of virgin olive oil, Standard 29:2012 (INEN, 2012), which states that they must have between 0.8 and 2% acidity, lower percentages than those reported by Vásquez et al. (2010), who indicate an acidity value of 2.69% in Peruvian morete oil. Reboredo-Rodríguez et al. (2016) mention that low temperatures reduce the oxidation rate of the oil and cold extraction has less acidity.

3.3 Fatty acid profile of morete oil of the treatments

Ten fatty acids were identified in the lipid profiles of oils, except in experiment T1, where lauric acid, a saturated fatty acid with a content of 1.35% was also found. In another study, Cruz et al. (2020) reported 0.03% of lauric acid in Brazilian morete oil.

For all treatments, high oleic acid (omega 9) content was found to be close to 80% and no significant differences were found between the experiments. In addition, linoleic acid (omega 6) was identified from 1.39% to 1.73% and α-linolenic acid (omega 3) from 0.70% to 1.17%. These results are comparable to those reported by Vásquez et al. (2010) in morete cultivated in Peru, reporting values of 75.6% oleic acid, 2.19% linoleic acid and 0.82% α-linolenic acid.

The oleic acid of morete oil has comparable values to those of the olive oil produced in the region of Extremadura, Spain, where Martínez et al. (2014) report values between 68.82 and 79.30%. In addition, they were superior to those obtained from olives grown in Turkey and Argentina reported by Ghanbari et al. (2019) and Rondanini et al. (2011), whose content was between 68.64 and 70.56% and 51.8% to 71.9% of oleic acid, respectively.

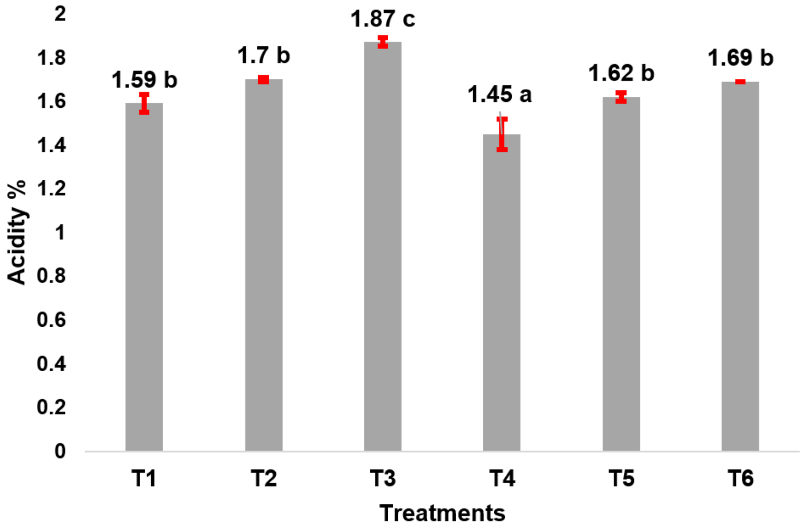

Figure 4. Acidity percentage of oils extracted from the different treatments. Different letters in the graph indicate significant differences (P<0.05)

Other minority fatty acids with less than 0.3% were found: myristic acid, pentadecanoic acid, palmitoleic acid, margaric acid, arachidic acid, and gadoleic acid, followed by stearic acid from 0.75% to 1.51%. Finally, of the saturated fatty acids, palmitic acid proved to be more abundant (16.55% in T5), however, it is less than the mentioned by Cruz et al. (2020); Restrepo et al. (2016) and Vásquez et al. (2010).

Table 3 summarizes the fatty acid profile of oils extracted from morete in each of the different treatments compared to the results of other authors. The best treatment selected was the one that allowed a maximum oil yield with the greatest amount of oleic acid, because in all treatments the oleic acid content was close to 80% with no significant differences with $\alpha = 0.05$. the best result was the one obtained with T6, since it has a higher extraction yield.

The nutritional value with respect to unsaturated fatty acids from treatment T6 was: 79.80% of Omega 9; 1.39% of Omega 6 and 0.78% of Omega 3. On the other hand, the content of saturated, monounsaturated and polyunsaturated fatty acids was 17.62%, 80.20% and 2.17%, respectively.

Pereira et al. (2016) mention that oils rich in monounsaturated and polyunsaturated fatty acids have been associated with a decreased risk of cardiovascular disease, hence associating this reduction with the anti-inflammatory effect of fatty acids. Freitas et al. (2017) indicate that oleic acid is considered essential for its beneficial properties in reducing oxidation of LDL cholesterol; in addition, it is a precursor in the production of most other polyunsaturated fatty acids and hormones.

Figure 5 shows the diagram proposed for the extraction of morete oil with the T6 treatment by handmade pressing with previous heat treatment of morete pulp.

Table 3. Fatty acid profile of morete oil extracted in the different treatments and morete oils of Colombia, Peru and Brazil.

Fatty acid	Lipid number	T1* (Pulp with shell at 45 °C)	T2* (Pulp with shell at 65 °C)	T3* (Pulp with shell at 85 °C)	T4* (Pulp at 45 °C)	T5* (Pulp at 65 °C)	T6* (Pulp at 85 °C)	Colombian oil (Restrepo et al., 2016)	Peruvian oil (Vásquez et al., 2010)	Brazilian oil (Cruz et al., 2020)
Lauric acid	C12:0	1.35 ± 0.09	–	–	–	–	–	–	–	0.03
Myristic acid	C14:0	0.07 ± 0.01 ^{b}	–	0.04 ± 0.01 ^{b}	0.02 ± 0.01 ^{a}	0.03 ± 0.01 ^{ab}	0.02 ± 0.01 ^{a}	0.06 ± 0.01	–	0.12
Pentadecanoic acid	C15:0	0.05 ± 0.01 ^{a}	0.07 ± 0.01 ^{b}	0.05 ± 0.01 ^{ab}	0.08 ± 0.02 ^{b}	0.05 ± 0.01 ^{ab}	0.03 ± 0.01 ^{a}	–	–	0.07
Palmitic acid	C16:0	15.08 ± 0.39 ^{a}	16.05 ± 0.26 ^{b}	16.55 ± 0.33 ^{b}	16.05 ± 0.38 ^{b}	15.93 ± 0.22 ^{b}	15.96 ± 0.24 ^{b}	21.27 ± 0.80	19.61 ± 0.41	22.18
Palmitoleic acid	C16:1	0.06 ± 0.01 ^{c}	0.05 ± 0.01 ^{c}	0.04 ± 0.01 ^{c}	0.07 ± 0.01 ^{c}	0.12 ± 0.01 ^{b}	0.16 ± 0.05 ^{a}	0.29 ± 0.06	0.15 ± 0.01	0.15
Margaric acid	C17:0	0.05 ± 0.01 ^{ab}	0.04 ± 0.01 ^{a}	0.03 ± 0.01 ^{a}	0.05 ± 0.01 ^{ab}	0.07 ± 0.01 ^{b}	0.05 ± 0.01 ^{ab}	–	–	0.12
Stearic acid	C18:0	0.79 ± 0.04 ^{a}	0.75 ± 0.03 ^{a}	0.82 ± 0.03 ^{a}	0.82 ± 0.03 ^{a}	1.27 ± 0.03 ^{b}	1.51 ± 0.25 ^{b}	4.19 ± 0.04	1.57 ± 0.02	2.51
Oleic acid	C18:1 n-9	79.47 ± 0.39 ^{a}	80.09 ± 0.47 ^{a}	79.91 ± 0.40 ^{a}	79.83 ± 0.37 ^{a}	79.85 ± 0.35 ^{a}	79.80 ± 0.64 ^{a}	68.69 ± 1.60	75.6 ± 0.31	72.23
Linoleic acid	C18:2 n-6	1.73 ± 0.07 ^{a}	1.69 ± 0.14 ^{ab}	1.61 ± 0.10 ^{ab}	1.65 ± 0.07 ^{ab}	1.62 ± 0.10 ^{ab}	1.39 ± 0.12 ^{b}	2.05 ± 0.08	2.19 ± 0.25	0.51
α-linolenic acid	C18:3 n-3	1.12 ± 0.16 ^{a}	0.98 ± 0.10 ^{ab}	0.70 ± 0.03 ^{b}	1.17 ± 0.04 ^{a}	0.78 ± 0.05 ^{b}	0.78 ± 0.17 ^{b}	0.87 ± 0.03	0.82 ± 0.06	1.15
Arachidic acid	C20:0	0.06 ± 0.02 ^{b}	0.03 ± 0.01 ^{a}	0.04 ± 0.01 ^{a}	0.04 ± 0.01 ^{a}	0.05 ± 0.01 ^{ab}	0.05 ± 0.02 ^{ab}	–	–	0.16
Gadoleic acid	C20:1 n-9	0.16 ± 0.01 ^{a}	0.23 ± 0.04 ^{a}	0.20 ± 0.07 ^{a}	0.23 ± 0.10 ^{a}	0.24 ± 0.06 a	0.30 ± 0.07 ^{a}	–	–	0.58
Saturated fatty acids		17.46 ± 0.55	16.94 ± 0.24	17.53 ± 0.32	17.05 ± 0.39	17.39 ± 0.24	17.62 ± 0.42	25.52	21.18	25.19
Monounsaturated fatty acids		79.68 ± 0.40	80.38 ± 0.42	80.15 ± 0.36	80.13 ± 0.38	80.21 ± 0.38	80.20 ± 0.64	68.98	75.75	72.96
Polyunsaturated fatty acids		2.85 ± 0.15	2.67 ± 0.18	2.31 ± 0.07	2.82 ± 0.05	2.40 ± 0.15	2.17 ± 0.29	2.92	3.01	1.66

*The mean and uncertainty are indicated as the standard deviation for n = 3.
Different letters in rows indicate significant difference ($P<0.05$)

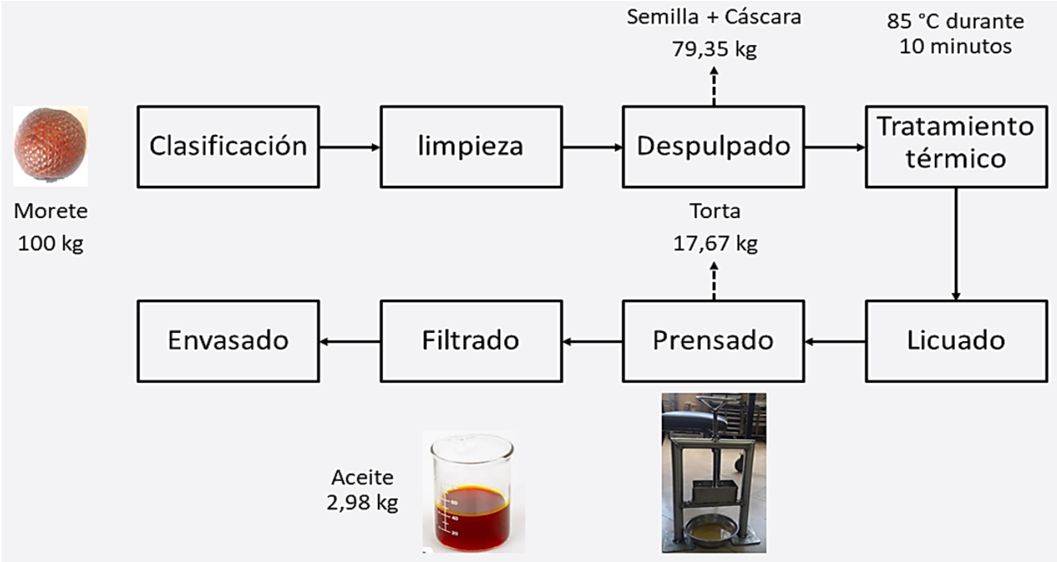

Figure 5. Proposed diagram for the extraction of Morete oil.

3.4 Oxidative stability of morete oil

This study did not evaluate the influence of thermal treatment on the potential loss of bioactive compounds from extracted oil; however, it was observed that the variation in the acidity of the oil in all treatments remained below the standard 29:2012 (INEN, 2012).

The oil obtained from the best treatment was subjected to accelerated oxidation conditions using Oxitest equipment. For each of the temperatures selected in the oxidation chamber of the equipment, the IP value (Table 4) expressed in hours and minutes was determined.

Table 4. Values of the IP induction period (hours) at different temperatures.

Temperature (°C) in the oxidation chamber of the Oxitest	IP (hours) in morete oil of the best treatment
80	37.58 ± 0.27
90	17.55 ± 0.23
100	5.77 ± 0.11

Average and uncertainty are indicated as standard deviation for n = 3

With the values found in Table 4, the IP value (Ln IP) is transformed from minutes to hours and then to natural logarithm; then equation 3 is estimated as described in section 2.6. The Ln IP was plotted against the temperature of the oxidation chamber and equation 3 was found by linear regression, obtaining the expression $y = -0.09425x + 11.22917$.

Equation 3 was extrapolated, for which 21 °C was replaced as the x value representing the average temperature of the city of El Puyo. The IP value was then cleared and the corresponding change from units to months was made and T6 was achieved to have an oxidative stability of 14.45 months when stored at 21 °C. Landeo (2019) used the peroxide index to estimate the stability time of morete oil grown in Peru, and reported 7.5 months at 18 °C storage; this shelf life is lower than the determined in this work.

Morete oil has high oleic acid content with values close to olive oil, which could have a useful life compared to olive oil. Irigaray et al. (2016) indicate that Uruguay's extra virgin olive oil has a useful life of 12 to 18 months of storage in bottles at room temperature. Martínez-Robinson et al. (2019) note that the oxidation stability of oils provides a good estimate of susceptibility to self-oxidation, leading to their aging and deterioration of their quality.

4 Conclusions

In the samples studied, it was found that the fat content in all treatments was higher in the pulp, followed by the bark and the seed. It was established that the best extraction process of morete oil was using pulp by pressing after treatment at 85 $^{\circ}C$ for 10 minutes. The morete oil obtained under the best conditions used in this work proved to be a source of oleic acid, with a useful life compared to that reported for extra virgin olive oil.

Oxitest equipment was used to evaluate the oxidative stability of the oil extracted from morete under accelerated conditions. An equation was obtained to estimate the induction period and, consequently, the life time of the morete oil at any storage temperature.

The proposed extraction technology made it possible to obtain 2.98 kg of oil for every 100 kg of morete; thus, producers of this fruit could move toward industrialization and offer a new type of oil which is high in oleic acid.

Acknowledgments

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Special Issue / Edición Especial

Natural Resources Sciences

ABYA YALA UNIVERSIDAD POLITÉCNICA SALESIANA

Ecotoxicological impact of three pesticides on *Eisenia fetida* (Californian red worm) on *Allium cepa* (onion) crop in the district of Lurín, Lima, Peru

Impacto Ecotoxicológico de tres plaguicidas sobre *Eisenia fetida* (lombriz roja californiana) en el cultivo de *Allium cepa* (cebolla) en el distrito de Lurín, Lima, Perú

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Abstract

The research evaluated the impact of three pesticides on *Eisenia fetida* in the cultivation of *Allium cepa* (onion) in the district of Lurín, Lima, Peru. The mean lethal dose values (LD_{50}) at 14 days, evasion percentage and food activity were evaluated as well as the exposure ratio of the toxic (TER) and the environmental risk coefficient (RQ) on pesticides zeta-cypermethrin, pendimethalin and profenofos based on ecotoxicological tests with *E. fetida*. The LD_{50} values at an exposure time at 14 d for the three pesticides were: zeta-cypermethrin>profenofos>pendimethalin. A relationship with the dose of pesticides close to the LD_{50} was calculated for the evasion and for food activity. TER value for the three pesticides was calculated which indicated no ecotoxicological impact for *E. fetida*. However, in the CR value, there were high risk values in the total, where zeta-cypermethrin indicated 92.77% of the environmental risk, while pendimethalin presented the lowest environmental risk with 0.09%. Within Peruvian legislation, these pesticides are still allowed to be used; hence, based on this research, it is suggested that zeta-cypermethrin be evaluated in more detail, because it was the only pesticide that presented a significant CR. In conclusion, zeta-cypermethrin causes an environmental impact on the onion crop in Lurin.

Keywords: Environmental risk, *Eisenia fetida*, DL_{50}, TER, RQ, Zeta-cipermetrin.

Resumen

La investigación evaluó el impacto de tres plaguicidas sobre *Eisenia fetida* en el cultivo de *Allium cepa* (cebolla) en el distrito de Lurín, Lima, Perú. Se calcularon los valores de dosis letal media (DL_{50}) a los 14 días, porcentaje de evasión y actividad alimentaria, y posteriormente la proporción de exposición del tóxico (TER) y el coeficiente de riesgo ambiental (CR) para los plaguicidas zeta-cipermetrina, pendimetalina y profenofos en base a ensayos ecotoxicológicos con *E. fetida*. Los valores de DL_{50} a un tiempo de exposición a los 14 días para los tres plaguicidas fueron: zeta-cipermetrina >profenofos >pendimetalina. Se calculó una relación con la dosis de los plaguicidas cercanos a la DL_{50} para la evasión y para la actividad alimentaria. El valor TER para los tres plaguicidas fue calculado e indicó que no hay impacto ecotoxicológico para *E. fetida*. Sin embargo, en el valor de CR se presentaron valores de riesgo alto en el total, donde la zeta-cipermetrina indicó el 92,77 % del riesgo ambiental, mientras que la pendimetalina presentó el menor riesgo ambiental con 0,09 %. Dentro de la legislación peruana, estos plaguicidas siguen siendo permitidos, por lo cual, en base a esta investigación, se recomienda que se evalúe a más detalle la zeta-cipermetrina, debido a que fue el único plaguicida que presentó un CR significativo. En conclusión, la zeta-cipermetrina ocasiona impacto ambiental en el cultivo de cebolla del distrito de Lurín.

Palabras clave: Riesgo ambiental, *Eisenia fetida*, DL_{50}, TER, CR, Zeta-cipermetrina.

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1 Introduction

There is interest in controlling insects and other disease vectors, as happens with the threat to food and other agricultural products (Ferrer, 2003). FAO (2003) states that "pesticides are any element or combination intended to prevent, suppress or manage any pest, including vectors that affect humans or animals". In Peru, until 2017, 428 active ingredients were reported to the National Agricultural Health Service (SENASA) (Cruz, 2017). The Law N° 0020-2013-AG-SENASA-DIAIA, presents a list of pesticides registered, canceled and prohibited in the country, including those studied in this work.

The pyrethroid group has been used since the 70s for agricultural activities, including zeta-cypermethrin, and are used for agricultural activities (Lao et al., 2012). Pendimethalin is an herbicide from the dinitroanilines family used in pre-planting, pre-emergence of grass and early post-emergence for crops (Ponz, 2010). Profenofos is a pesticide belonging to the group of phosphorus that operates with the contact (Reddy and Rao, 2008). The species *Eisenia fetida* (known as California red worms) is one of the organisms that plays a beneficial role to the soil. Giménez et al. (2004) highlight the importance of *E. fetida* in the integration and putrefaction of organic matter, the recycling of nutrients and soil conservation, being beneficial indicators for assessing soil pollution (Wen et al., 2020).

The district of Lurín, Lima, Peru, has very fertile soils that provide food to the markets of Metropolitan Lima. Moreno and Huerse (2010), mention that onion, from the point of view of its gross economic value contributed to the basin, is an important crop and it must be considered when conducting a phytosanitary evaluation. To determine the impact of the three pesticides, it is important to link *E. fetida* responses, using relevant toxicity parameters such as biological and behavioral activity. The bioassays employ laboratory-acclimatized, guidance organisms, and these include an initial view for pesticide impact assessment (Piola, 2011; Tian et al., 2018; Sotelo-Vásquez and Iannacone, 2019; Aparecida-Giordani et al., 2020).

For this reason, the aim of this research is to evaluate the impact of the three most commonly used pesticides on *A. cepa* culture, and ecotoxicological tests were conducted to find DL_{50} (mean lethal dose), CR (risk coefficient) and TER (individual toxic exposure) used in *E. fetida*.

2 Materials and methods

2.1 *Eisenia fetida*

Adult earthworms (*E. fetida*) were obtained from the National Agrarian University La Molina (UNALM) and transferred to the Laboratory of Animal Ecology and Biodiversity (LEBA) of the National University Federico Villarreal (UNFV), Lima, Peru. The adaptation was carried out in a 15 L container at 19 $\pm$ 2$^{\circ}C$ and a period of 16 h of light and 8 h of darkness. For the worm tests, a weight range of 200-600 mg was considered (Organization for Economic Co-operation and Development, 1984).

2.2 Artificial Soil

Artificial soil was prepared according to Organization for Economic Co-operation and Development (2004) standard with some modifications described in Romero and Cantú (2008). The components of artificial soil were: 70% fine sand, 20% clay and 10% moss. Prior to the beginning of the tests, the moisture content was adjusted to 50% using the Avalos-Ruiz and Iannacone (2020) method.

2.3 Pesticides

Pesticides used were zeta-cypermethrin from Furia brand with a formulation of 180 $g \cdot L^{-1}$ with concentrations of 30 $mg \cdot kg^{-1}$, 60 $mg \cdot kg^{-1}$, 120 $mg \cdot kg^{-1}$, 240 $mg \cdot kg^{-1}$ and 480 $mg \cdot kg^{-1}$; profenofos from the Selecrom brand with a formulation of 500 $g \cdot L^{-1}$ with concentrations of 625 $mg \cdot kg^{-1}$, 1 250 $mg \cdot kg^{-1}$, 2 500 $mg \cdot kg^{-1}$, 5 000 $mg \cdot kg^{-1}$ and 10 000 $mg \cdot kg^{-1}$; and pendimethalin from the Arrow brand with a formulation of 400 $g \cdot L^{-1}$ with concentrations of 2 666, 67 $mg \cdot kg^{-1}$, 5 333.33 $mg \cdot kg^{-1}$,10 667.67 $mg \cdot kg^{-1}$, 21 333.33 $mg \cdot kg^{-1}$ and 42 667.67 $mg \cdot kg^{-1}$.

2.4 Ecotoxicity tests

2.4.1 Mortality and other parameters

All tests were performed with four replications and adjusted to 50% of humidity for the tests. To validate the test, it was considered as a principle that the control mortality does not exceed 10 %. The protocol of Avalos-Ruiz and Iannacone (2020) was used for this test, which consisted of measuring physical and chemical indicators such as temperature, humidity, pH and organic matter, as well as mortality for the determination of DL_{50} at 14 days of exposure, and average weights at the beginning and end of the test at 14 days (Hulbert et al., 2020; Avalos-Ruiz and Iannacone, 2020). All worms that were not found at the moment were recorded as dead; it was decided to measure their progeny (eggs and young offspring) present in artificial soil, but none of them was evident in the treatments of the three pesticides.

2.4.2 Evasion

This test used the protocol of García-Santos and Keller-Forrer (2011). Transparent, disposable rectangular 1000 mL flexo-lid containers were used where the treated floor was placed on one side and the control was placed on the other. Later, adult worms were placed on the dividing line of both soils and allowed to penetrate them (McGuirk et al., 2020). After three days, the partitions were reinserted and the number of worms on each side was counted. Zeta-cypermethrin doses of a formulation of 180 $g \cdot L^{-1}$ were 15 $mg \cdot kg^{-1}$ and 30 $mg \cdot kg^{-1}$, for profenofos of a formulation of 50 $g \cdot L^{-1}$ were 312.5 $mg \cdot kg^{-1}$ and 625 $mg \cdot kg^{-1}$, and pendimethalin of a formulation of 400 $g \cdot L^{-1}$ with doses of 1 333.33 $mg \cdot kg^{-1}$ and 2 666.67 $mg \cdot kg^{-1}$. The Equation 1 from De Silva and Van Gestel (2009) was used to determine evasion.

$$NR(E) = \frac{C - T}{N} \times 100 \tag{1}$$

NR represents evasion (percentage), *C* represents total *E. fetida* in the control soil, *T* represents total *E. fetida* in the impacted soil, and *N* represents total *E. fetida* at the beginning of the test (Alves et al., 2013).

2.4.3 Food Activity (AA)

Acrylic sheets of 100 mm long, 10 mm wide and 0.1 cm thick were used to evaluate food activity. These sheets had 16 holes of 0.1 cm of diameter and were filled with a bait substance using a combination consisting of cellulose carbohydrate (69 %), wheat bran (30 %) and active carbon (1 %) (Van Gestel and Weeks, 2004). The trial was performed for three days, then leaves were removed, and the number of holes without bait in the leaves of each treatment were counted. AA was determined on the basis of the average proportion of holes that were completely or partially empty per concentration (Piola, 2011).

2.5 Statistical Data Management

DL_{50s}, DL_{50s}-inferior and DL_{50s}-superior were determined with Excel-Probit-2016 calculator (Raj Mekapogu, 2016). SPSS 25.0 statistical program was used to define the homocesticity of the variances, the normality of the data and the type of tests, like ANOVA or non-parametric tests, depending on the case, at a significance level of 0.05. A Chi-square test (χ^2). was performed for the evasion and food activity tests.

2.6 Environmental Impact Assessment (EIA)

To determine environmental impact assessment (EIA), parameters were identified to find potential threats to *E. fetida* and the agroecosystem. Two approaches to environmental impact assessment were used; the first was based on a calculation of TER for *E. fetida*, while the second CR for each local sample (Pivato et al., 2017; Wee and Aris, 2017; Avalos-Ruiz and Iannacone, 2020).

2.6.1 Exhibition

The data collected from the literature was analyzed. The controlled environmental dose (MECs) of pesticide residue in agricultural soil was used to represent the PECs (projected environmental dose in soil) (Vašíčková et al., 2019).

2.6.2 Impact

Environmental impact was evaluated using data found from DL_{50} and PNEC (dose without ecological effect). The PNEC value was found using a factor of 1 000 for short-term assays. For pesticides with a Kow greater than 2, the value of its DL_{50} was divided by 2 as proposed in (European Commission, 2019) based on the Equation 2.

$$PNEC = \frac{DL_{50}}{1000} \tag{2}$$

2.6.3 Risk

The environmental risk for each of the three pesticides was initially found using the criteria based on TER values. According to the European Commission mentioned by Hartnik et al. (2008), if the value of acute TER is less than 10, no authorization should be given for the use of pesticides. The acute TER value was found with the Equation 3.

$$TER_{species} = \frac{DL_{50}/PNEC_{\text{species}}}{MEC_{\text{max or mean}}} \quad (3)$$

The CR was classified using the equation 4 into four levels: 1. Zero risk ($CR < 0,01$), 2. Low risk $(0,01 \geq 0,1)$ 3. Medium risk $(0,1 \leq CR < 1)$ and high risk ($CR \geq 1$) Sánchez-Bayo et al. (2002). The sum of the CR of the three pesticides allowed to determine the total environmental risk from the application of zeta-cypermethrin, profenofos and pendimethalin; finally, the contribution of each of the three pesticides was quantified.

$$CR = \frac{MEC_{soil}}{PNEC_{mss}} \quad (4)$$

3 Results

The acute toxicity of zeta-cypermethrin on *E. fetida* was known at 14 days of exposure (Table 1). The DL_{50} at 14 d of exposure was 48.26 $mg \cdot kg^{-1}$. Regarding the average of weights, a marked decrease was observed when the dose was increased. Acute toxicity of profenofos on *E. fetida* was established at 14 days of exposure (Table 2). DL_{50} at 14 d of exposure was 1250 $mg \cdot kg^{-1}$. Regarding the average of weights, a significant decrease was observed.

Acute toxicity of pendimethalin on *E. fetida* was found at 14 d of exposure (Table 3). The DL_{50} at 14 d of exposure was 3 771.23 $mg \cdot kg^{-1}$. Regarding the average of weights, a significant decrease was observed. Table 4 shows the evasion percentage of the three pesticides at 60%, 40% and 80% in the doses of 30 $mg \cdot kg^{-1}$, 625 $mg \cdot kg^{-1}$ and 2 666.67 $mg \cdot kg^{-1}$, respectively. The Chi-square analysis (χ^2) indicates that a connection was found between evasion and the presence of the pesticide for both doses of zeta-cypermethrin, profenofos and pendimethalin, excepting the dose 312.5 $mg \cdot kg^{-1}$ of profenofos and 1 333.33 mg of pendimethalin.

The data found in this test (Table 5) show that the percentage of pesticide food activity at 3 d of exposure has values of 33.33%; 29.12% and 32.66% in dose concentrations of 30 $mg \cdot kg^{-1}$, 625 $mg \cdot kg^{-1}$ and 2 666.67 $mg \cdot kg^{-1}$, respectively. The Chi-square analysis (χ^2) indicates that there is a connection between food activity and the presence of the pesticide for zeta-cypermethrin, profenofos and pendimethalin at the doses already mentioned. TER values (Table 6) were obtained, which were 482.6, 12 500 and 377 123 $mg \cdot kg^{-1}$. On the other hand, CRs of each pesticide were 1.04, 0.08 and 0.001. It is shown that CR t is higher than 1, representing a high environmental impact. The contribution of zeta-cypermethrin was 92.77%, followed by profenofos with 7.14% and pendimethalin with 0.09%.

4 Discussion

4.1 Acute Ecotoxicity and Other Important Parameters

E. fetida study showed effects on mortality and weight reduction for zeta-cypermethrin (DL_{50} = 48.26 $mg \cdot kg^{-1}$). Similar results were found in the Czech Pesticide Database (PPDB, 2019) showing a DL_{50} value of 37.50 $mg \cdot kg^{-1}$. Junquera (2011) mentions that cypermethrin is also known as zeta-cypermethrin. It allows to be contrasted with other studies such as Hartnik et al. (2008) who obtained an alpha-cypermethrin value of 762 $mg \cdot kg^{-1}$. Zhou et al. (2008, 2011) showed values of 84.14 $mg \cdot kg^{-1}$ and 86.04 $mg \cdot kg^{-1}$, respectively.

Organic matter (OM) has the characteristic of accumulating zeta-cypermethrin, and earthworms make it more available through its cuticle or during feeding (Styrishave et al., 2010). Based on this, the amount of organic matter in the study substrate was initially found at 5.42%, so there is not much absorption of the contaminant. This fact also occurs in the study of Hartnik et al. (2008), since its soil presented 2.2% of organic matter where the compound was available in its mineral particles and/or water; therefore, there is a higher risk of the pesticide to be present in worms. Pesticides can be retained by organic matter of the soil and may be degraded by organisms found in the organic matter, which will depend not only on the properties of pesticides, but also on the nature and concentration of pesticides in the organic matter (Araneda et al., 2016).

Table 1. Mortality and average weights of *Eisenia fetida* exposed to zeta-cypermethrin at 14 d of exposure.

Eisenia fetida			
Doses (mg·kg^{-1})	**Acute ecotoxicological effect of zeta-cypermethrin (% M)** 14 d (± DE)	**Average Weights (g)**	
		0 d ± DE)	14 d (± DE)
Control	0.00 (± 0.00)a	2.89 (± 0.06)a	3.18 (± 0.05)a
30	20.00 (± 0.71)b	2.55 (± 0.07)b	1.72 (± 0.15)b
60	65.00 (± 1.25)c	2.42 (± 0.06)b	1.10 (± 0.17)c
120	100.00 (± 0.00)d	2.51 (± 0.07)b	0.00 (± 0.00)d
240	100.00 (± 0.00)d	2.40 (± 0.04)b	0.00 (± 0.00)d
480	100.00 (± 0.00)d	2.31 (± 0.04)b	0.00 (± 0.00)d
DL_{50}	48.26	N.C	N.C
DL_{50}-low	35.70	N.C	N.C
DL_{50}-upper	65.24	N.C	N.C
PNEC	0.04826	N.C	N.C
KW	22.78	14.6	22.34
Sig	0.00	0.01	0.00
Levene test	6.4	0.41	8.07
Sig	0.00	0.84	0.00
SW	0.76	0.94	0.79
Sig	0.00	0.14	0.00

% M: % mortality. DE: Standard Deviation. NC: Not applicable. DL_{50}: Mean lethal dose. DL_{50}-Low: DL_{50} (Low Limit). DL_{50}-Upper: DL_{50} (Upper Limit).
PNEC: Dose without ecological effect. KW: Kruskall–Wallis statistician. Levene test: Statistical to evaluate homocesticity of variances.
SW: Shapiro–Wilks Statistician: Test to evaluate normality.
Equal lowercase letters in the same column indicate that mortality (%) is statistically similar according to Tukey's multiple comparison test.

Other variables that determine the presence of zeta-cypermethrin are water solubility and octanol/water distribution coefficient (Kow). Both variables correlate significantly with the mobility of insecticides in the soil (Somasundaram et al., 1991). Zeta-cypermethrin has a Kow log of 5.5, which indicates that it is relatively lipophilic, and therefore its infiltration potential is low in agricultural soils due to its low solubility in water and adsorption to the soil (Sakata et al., 1986). This was evidenced in the work of Hulbert et al. (2020), where a higher number of alive *E. fetida* was found on the surface compared to a greater depth.

According to Wang et al. (2012), the reduced toxicity of alpha-cypermethrin is due to its accelerated metabolic process, because it can be metabolized prior to reaching the central nervous system. There was clear evasion so that this dose increases as in the food activity. This behavior is observed by the presence of chemoreceptors of *E. fetida* on its body surface (Zhou et al., 2007). On the other hand, profenofos obtained a DL_{50} mortality of 1 250 $mg \cdot kg^{-1}$ and weight reduction 14 days after the test. Other investigations such as Bart et al. (2018) showed values such as 127 $mg \cdot kg^{-1}$. Harnpicharnchai et al. (2013) reports that the average value of profenofos in soil is 0.041 $mg \cdot kg^{-1}$ in summer, while it is 0.016 $mg \cdot kg^{-1}$ in winter.

Regarding soil evasion with profenofos, it was observed that there was only a relation with the dose of 625 $mg \cdot kg^{-1}$. Chakra and Rao (2008) mention that it may be because earthworms are affected by pesticides through skin contact or through contaminated soil residues. Mainly because these toxins pass through the skin and reach the celomic fluid to be transported by the body.

Organic matter is essential for the feeding of worms. In the study conducted by Gómez et al. (1999), the influence of profenofos was investigated in *Azospirillum brasilense* cells, and they found that it significantly reduced nitrogen fixation in the soil, which may be related to the amount of organic matter available and which may affect food activity as observed at 625 $mg \cdot kg^{-1}$, where food activity was dose-related. A decrease in weight was observed with pendimethalin with respect to the increase in pesticide concentrations and a DL_{50} of 3 771.23

$mg \cdot kg^{-1}$. In the same database (PPDB, 2019) a value of $DL_{50} > 1000\ mg \cdot kg^{-1}$ was found. In the study by Mosqueda et al. (2019) it is mentioned that the permitted range of worms is 3 545.96 $mg \cdot kg^{-1}$ for the pesticide Prowl H_2O, being close to the value obtained in this study as well as the one of Traoré et al. (2018) with a value of 3 555.96 $mg \cdot kg^{-1}$.

Table 2. Mortality and average weights of Eisenia fetida exposed to profenofos at 14 d exposure.

Eisenia fetida			
Doses (mg·kg^{-1})	**Acute ecotoxicological effect of profenophos (% M)** 14 d (± DE)	**Average Weights (g)**	
		0 d ± DE)	14 d (± DE)
Control	0.00 (± 0.00)a	2.89 (± 0.06)a	3.18 (± 0.05)a
625	25.00 (± 1.56)b	2.22 (± 0.09)b	1.61 (± 0.39)ab
1250	50.00 (± 1.83)c	2.23 (± 0.04)b	1.17 (± 0.47)b
2500	100.00 (± 0.00)d	2.19 (± 0.08)b	0.00 (± 0.00)c
5000	100.00 (± 0.00)d	2.27 (± 0.08)b	0.00 (± 0.00)c
10000	100.00 (± 0.00)d	2.13 (± 0.05)b	0.00 (± 0.00)c
DL_{50}	1250	N.C	N.C
DL_{50}-low	810.30	N.C	N.C
DL_{50}-upper	1928.30	N.C	N.C
PNEC	0.8103	N.C	N.C
KW	21.94	11.39	22.03
Sig	0.00	0.04	0.00
Levene test	10.69	0.54	6.65
Sig	0.00	0.75	0.00
SW	0.73	0.84	0.76
Sig	0.00	0.14	0.00

% M: % mortality. DE: Standard Deviation. NC: Not applicable. DL_{50}: Mean lethal dose. DL_{50}-Low: DL_{50} (Low Limit). DL_{50}-Upper: DL_{50} (Upper Limit).
PNEC: Dose without ecological effect. KW: Kruskall–Wallis statistician. Levene test: Statistical to evaluate homocesticity of variances.
SW: Shapiro–Wilks Statistician: Test to evaluate normality.
Equal lowercase letters in the same column indicate that mortality (%) is statistically similar according to Tukey's multiple comparison test.

In relation to the evasion and food activity, it would be related to their availability as they could be degrading with temperature increase and humidity decrease, and therefore would be less available. These results are according to the study conducted by Zimdahl et al. (1984), where the degradation of pendimethalin was higher than 30°C, decreasing as well as soil moisture. The same would happen in the ecotoxicological test where the average temperature ranged from 20.6 to 20.7°C and the humidity decreased from 75% to 25%, being more available in the soil and affecting *E. fetida*.

4.2 Environmental Impact Assessment

Regarding zeta-cypermethrin, TER was 482.6 which indicated low toxicity with respect to *E. fetida*. Considering the European Community legislation as a reference, it would not have an impact on the environment since it is less than 10, thus it does not represent a risk to *E. fetida*. A TER of 1 500 was determined for alpha-cypermethrin, as cited by Hartnik et al. (2008); while the CR obtained a value of 1.04, which is considered a high environmental risk. Contrary to TER, the CR approach is used in this study to identify the most sensitive organism and to assess the toxicity of the combination of the three pesticides used in onion cultivation; however, the environmental impact of earthworms in soil may be similar in other crops where zeta-cypermethrin, profenofos and pendimethalin are used.

Table 3. Mortality and average weights of *Eisenia fetida* exposed to pendimethalin at 14 d of exposure.

	Eisenia fetida		
Doses (mg·kg^{-1})	**Acute ecotoxicological effect of pendimethalin (% M)** 14 d (± DE)	**Average Weights (g)**	
		0 d ± DE)	14 d (± DE)
Control	0.00 (± 0.00)a	2.89 (± 0.06)a	3.18 (± 0.05)a
2 666.67	42.50 (± 0.85)b	2.17 (± 0.09)b	1.25 (± 0.15)b
5 333.33	57.50 (± 1.10)c	2.15 (± 0.07)b	0.92 (± 0.22)b
10 666.67	100.00 (± 0.00)d	2.32 (± 0.12)b	0.00 (± 0.00)c
21 333.33	100.00 (± 0.00)d	2.22 (± 0.09)b	0.00 (± 0.00)c
42 666.67	100.00 (± 0.00)d	2.16 (± 0.06)b	0.00 (± 0.00)c
DL_{50}	3771.23	N.C	N.C
DL_{50}-low	1866.24	N.C	N.C
DL_{50}-upper	7620.78	N.C	N.C
PNEC	3.77123	N.C	N.C
KW	22.19	10.66	22.21
Sig	0.00	0.06	0.00
Levene test	9.00	0.42	8.82
Sig	0.00	0.83	0.00
SW	0.78	mn1c|0.84	0.75
Sig	0.00	0.14	0.00

% M: % mortality. DE: Standard Deviation. NC: Not applicable. DL_{50}: Mean lethal dose. DL_{50}-Low: DL_{50} (Low Limit). DL_{50}-Upper: DL_{50} (Upper Limit).
PNEC: Dose without ecological effect. KW: Kruskall–Wallis statistician. Levene test: Statistical to evaluate homocesticity of variances.
SW: Shapiro–Wilks Statistician: Test to evaluate normality.
Equal lowercase letters in the same column indicate that mortality (%) is statistically similar according to Tukey's multiple comparison test.

Table 4. Evasion effect of zeta-cypermethrin, profenofos and pendimethalin on *Eisenia fetida* at 3 d of exposure.

Pesticides	***Eisenia fetida***				
	Evasion Effect	**Parameters**			
	Doses (mg· kg^{-1})	Without Toxic (%)	With Toxic (%)	χ^2	Significance
Zeta-Cypermethrin	15	45	55	4.27	0.04
	30	60	40	7.91	0.01
Profenofos	312.5	5	95	0.05	0.82
	625	60	40	7.91	0.01
Pendimethalin	1 333.33	40	60	3.33	0.07
	2 666.67	80	20	15.24	0.00

Statistical χ^2: Chi Square.

TER in profenofos was 12 500, higher than that of zeta-cypermethrin. Vašíčková et al. (2019) define TER as an approximation that characterizes the risk of an independent compound and gives a general estimate of the environmental impact for each species in the soil. CR is applied since its exposure is divided depending on its toxicity, as in the case of profenofos, which represented a low risk with a value of 0.08. Residues of profenofos in the soil indicate environmental concerns, such as adverse effects on crops and migration to groundwater (He et al., 2010; Bedi et al., 2015; Gonzales-Condori et al., 2020).

Finally, pendimethalin proved to have lower TER and CR values in the range of environmental impact in the different classifications with values of 377 123 and 0.001, yielding zero risk values in both cases. Goto and Sudo (2018) suggest that the simple lipid content of pendimethalin and trifluralin was not the only factor affecting bioaccumulation potential, but differences in the composition of lipids could generate variability in bioconcentration in biological organisms (Van der Heijden and Jonker, 2011).

Table 5. Effect of food activity of zeta-cypermethrin, profenofos and pendimethalin on *Eisenia fetida* at 3 d of exposure.

Pesticides	***Eisenia fetida***			
	Evasion Effect	**Parameters**		
	Doses (mg·kg^{-1})	Food Activity (%)	χ^2	Significance
Zeta-Cypermethrin	15	54.19	0.76	0.39
	30	33.33	10.97	0.00
Profenofos	312.5	39.65	3.63	0.06
	625	29.12	16.28	0.00
Pendimethalin	1 333.33	49.28	0.05	0.83
	2 666.67	32.66	10.86	0.00

Statistical χ^2: Chi Square.

Table 6. Environmental impact values of zeta-cypermethrin, profenofos and pendimethalin on *Eisenia fetida*.

Parameters	**Zeta-Cypermethrin**	**Profenofos**	**Pendimethalin**
MEC or PEC (mg·kg^{-1})	0,05 (BASF, 2014)	0,1 (Van den Brink et al., 2003)	0,005 (Vašíčková et al., 2019)
(DL_{50})	48.26	1 250	3 771.23
PNEC (DL_{50})	0.04826	1.25	3.77123
TER (DL_{50})	482.6	12 500	377 123
CR (DL_{50})	1.04	0.08	1
Contribution (%)	92.77	7.14	0.09
CR t (DL_{50})		1121	

MED: Controlled environmental dose of pesticide. PEC: Predicted environmental dose of the soil. PNEC: Dose without ecological effect. TER: Toxicity exposure ratio. CR: Environmental risk coefficient. CR t: The total environmental risk coefficient of a studied pesticide area. Contribution: contribution % to the environmental risk per pesticide.

5 Conclusions

The data found from DL_{50}-14d show that the ecotoxicity level based on mortality and reduction in *E. fetida* weight from the most used pesticides in onion cultivation in the district of Lurín, Lima, Peru, was below the recommended doses for pest control by manufacturers, except zeta-cypermethrin. Evidence of evasion and food activity with *E. fetida* was associated with the dose closest to DL_{50}. TER value for the three zeta-cypermethrin, profenofos and pendimethalin was calculated, and it did not have any environmental impact on *E. fetida*. However, high-risk values were presented in the total CR, where zeta-cypermethrin contributed with the highest value, profenofos showed low risk and pendimethalin did not show risk for onion cultivation in Lurín. In Peru, RD N° 0020-2013-AG-SENASA-DIAIA shows that these three pesticides are still registered and can be used; so, based on this research, it is recommended that the impact on other soil organisms be evaluated more deeply, especially zeta-cypermethrin in chronic reproductive trials on *E. fetida*, because it was the only pesticide that presented an environmental risk.

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Study of the oenological properties of naranjilla for gastronomy uses, Pacto-Pichincha province

Estudio de las propiedades enológicas de la naranjilla con fines gastronómicos en Pacto-Pichincha

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Abstract

The present study sought to provide an economic alternative to the inhabitants of the Pacto parish, as an initiative for production, postharvest optimization and industrialization of the local fruit growing area, also considering the tourist potential of the area, where wine tourism represents an opportunity for the area. The aim was to find the technical viability for the elaboration of wine from naranjilla. The applied research approach was mixed, and an exhaustive literature review was made on the winemaking techniques. The test evaluated number of sugars in the must (18, 21 and 24 ° Brix), addition of water (4:1) (Ma) and whole must (Mo). Yield, fermentation time, organoleptic properties of the final product (color, smell and flavor) were determined using a 5-point hedonic scale and physicochemical characteristics of wines with better acceptance. Commercial Saccharomyces cerevisiae was used as inoculum. The average yield for Ma was 70.6% w/w and Mo 45.0% w/w, fermentation was observed up to 288 hours. The addition of water adversely affected the taste; however, the general organoleptic evaluation had a weighting between 3 and 4 out of 5 points. Wines with the best acceptance were Mo24 and Ma21, adhering to the requirements of the Ecuadorian Technical Standard for fruit wine, characterized as semi-sweet. It is necessary to work on the improvement and transfer of the established technical process, especially to obtain higher yields, decrease acidity and incorporate new studies with tropical fruits for the benefit of agriculture, gastronomy, tourism and local development.

Keywords: *Solanum quitoense Lam*, lulo, tourism, fruit wine.

Resumen

El presente estudio buscó brindar una alternativa económica a los pobladores de la parroquia Pacto, como iniciativa a la producción, optimización en postcosecha e industrialización del área frutícola local, considerando además las potencialidades turísticas de la zona, donde el enoturismo representa una oportunidad. El objetivo planteado fue encontrar la viabilidad técnica para la elaboración de vino a partir de la naranjilla. El enfoque de investigación aplicado fue de carácter mixto, y se hizo una exhaustiva revisión bibliográfica sobre las técnicas de elaboración del vino. El ensayo evaluó la cantidad de azúcares en el mosto (18, 21 y 24 °Brix), adición de agua (4:1) (Ma) y mosto íntegro (Mo). Se determinó el rendimiento, el tiempo de fermentación, y las propiedades organolépticas del producto final (color, olor y sabor) mediante una escala hedónica de 5 puntos y características fisicoquímicas de vinos con mejor aceptación. Como inóculo se utilizó *Saccharomyces cerevisiae* comercial. El rendimiento promedio para Ma fue 70,6% p/p y Mo 45,0% p/p, y se observó fermentación hasta 288 horas. La adición de agua afectó negativamente al sabor; sin embargo, la valoración organoléptica general tuvo una ponderación entre 3 y 4 de 5 puntos. Los vinos con mejor aceptación fueron Mo24 y Ma21, apegados a requisitos de la Norma Técnica Ecuatoriana para vino de frutas, caracterizándose como semidulce. Es necesario trabajar en la mejora y la transferencia del proceso técnico establecido, especialmente para obtener mayores rendimientos, disminución de la acidez e incorporar nuevos estudios con frutas tropicales en beneficio del agro, gastronomía, turismo y desarrollo local.

Palabras clave: *Solanum quitoense Lam*, lulo, turismo, vino de fruta.

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1 Introduction

The humid subtropical zone of Ecuador allows to have biodiversity of crops, especially fruit trees between native crops and those crops that have adapted easily to the micro region of the Chocó Andino in various areas (Endara, 2017), as is the case of the northeastern area of Pacto parish, known as an area of greater fruit biodiversity and agro-productive potential (GAD parroquial de Pacto, 2012). The diverse production, high annual yields and the lack of industrialization processes of fruit in this area generates instability in the commercialization of these products, causing economic losses for the farmers and their easy decoupling of this activity, as well as their migration to the city.

Taking advantage of the nutritional characteristics of natural or processed fruits, not only improve the quality of the diet but they are also essential to maintain health (Swami and Divate, 2014), because they are source of vitamins, minerals, carbohydrates and active biological compounds. Various studies show that fermentation of fruits and vegetables can be a viable technique to create new processed products, with modified chemical and organoleptic physical characteristics with great acceptance by the consumer (Swami and Divate, 2014). The product obtained is called fruit wine (Fracassetti et al., 2019), specifying the fruit used for its production (Matei, 2017).

Excepting grapes, fruits that have been investigated for the production of wine are apples, apricots, berries, cherries, plums, strawberries, oranges, mangoes, bananas, pineapples, pears, papayas, jaca juice, melons (Veeranjaneya and Reddy, 2009; Swami and Divate, 2014; Ogodo et al., 2015; Fracassetti et al., 2019); optimization process for mulberry wine (Wang et al., 2013); from cocoa pulp (Dias et al., 2007); sour sop (Ho et al., 2020); coconut with bee honey (Balogu and Towobola, 2017), among others; in addition to fermentations with mixtures between fruits and grape must. As a result, a variety of alcoholic beverages can be classified as natural wine (9 - 14 % alcohol), dessert wines and appetizer wines (15 - 21 % alcohol), among others. Wine varieties will depend on the region and climate for the fruits produced, types of fermentation and additives included to improve flavor (Matei, 2017). Better organoleptic characteristics and wine yields can be established by studying different strains of fermenting microorganisms (Veeranjaneya and Reddy, 2009; Ogodo et al., 2015; Baidya et al., 2016; Fracassetti et al., 2019).

Among the fruit production of Pacto stands out the cultivation of lulo (*Solanum quitoense* Lam.), which is a hybrid that has been the most cultivated nationally (60 % of the total) (Revelo and Sandoval, 2003), but its price is low on the market and its use depends on the preference of the consumer, being the highest consumption for the so-called "common" varieties (Guayasamín, 2015). Generally, people like this fruit by its citrus flavor (Gancel et al., 2008; Loizzo et al., 2019), giving it different gastronomic uses due to its bioactive compounds like polyphenols, with antioxidant capacity superior to Kiwi, melon or watermelon, among others (Acosta et al., 2009), especially in overmature-maturity phase (Cerón et al., 2010). Currently, research for the production of wine from lulo is limited, with only one online study carried out by (Granados et al., 2013) for a wine snack.

Because of the latter, this work aims to find the technical feasibility for the elaboration of wine from lulo cultivated at Pacto parish, as an initiative to the production, post harvest optimization and industrialization of the local fruit area, also considering the tourist potential of the area, where wine tourism represents an opportunity (Montaner Montejano, 1996) and (Tresserras et al., 2011), in which the idea of wine tourism combines oenology with gastronomy to guide the view toward places of culinary and oenological culture, where these aspects are important when deciding on a tourist trip. Similarly, the aim is to evaluate the performance of the final product obtained by conditioning the must to different amounts of fermentable sugars and dilution with water, the organoleptic characteristics (taste, color, odor) in treatments with better acceptance and to analyze the physico-chemical properties established by the Ecuadorian regulations in force for fruit wine.

2 Materials and Methods

2.1 Raw material

50 kilogramos (kg) of *S. quitoense* Lam. fruits were obtained from farms in the parish of Pacto, Qui-

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to, province of Pichincha and were taken to the bromatology laboratory of the Faculty of Agricultural Sciences of the Central University of Ecuador. Healthy fruits were chosen regardless their size, in maturity state 4 and 5 (yellow-orange color in fruit = 75 and 100%, respectively) according to (Andrade et al., 2015), subsequently, cleaning and washing was carried out.

To obtain the must, the fruit was crushed and the pulp was separated by sieving, leaving bark and seeds as residue, as shown in Figure 1.

2.2 Initial characterization of the must

pH, titratable acidity and total dissolved solids or brix degrees (°Bx) were analyzed. All determinations were made in triplicate.

2.3 pH

20 mL of homogenized filtered must were added, which were previously in a 25 m beaker and then the pH reading was carried out with a digital potentiometer horiba – Laqua brand, equipped with a glass electrode and calibrated previously with standard solutions 4.0; 7.0 and 10.0 pH at 20 °C.

2.4 Total soluble solids

Using a dropper, 4 drops of must were added to the optical reader of a digital refractometer, Milwaukeebrand (reading 0 to 85 °Bx) previously soaked with distiled water, and °Bx was read.

2.5 Titratable acidity

In a 50 mL precipitation vessel, 5 mL of must was weighed, a similar amount of distiled water was added and placed in a magnetic stirring system. A potentiometric titration was performed with a sodium hydroxide solution (NaOH) of normal concentration equal to 0.0445, the volume of the titrant obtained (graduated buret ± 0.05 ml) was recorded at a pH reading equal to 8.3 (digital potentiometer brand Horiba – Laqua). The titratable acidity value was expressed as a percentage of citric acid.

2.6 Fermentation of the must

Orange juice must (1 kg/bottle) was placed in 18 amber glass bottles of 4 L capacity, with two thin plastic hoses for gas evacuation and sampling. Then, the must of 9 containers was diluted with 250 mL of water (Ma) (ratio 4:1), while the rest remained untouched (Mo). Additionally, both the Ma and Mo musts were adjusted with sucrose to a final concentration of 18, 21 and 24 °Bx. The calculation was performed using the following equation and verified with the same procedure used to determine the soluble solids.

$$Grams\,of\,sugar = \frac{(final\,°Brix - initial°Brix) \times Grams\,of\,initial\,must}{100 - final\,°Brix}$$

Once the initial conditions of the must were established, 6 treatments were obtained (Table 1) with three replications, obtaining a total of 18 experimental units.

In each experimental unit (1 kg of must) 50 mg of potassium metabisulfite was added, it was stirred and set aside for 20 minutes. Subsequently, 1 g of dry granulated commercial yeast *Saccharomyces cerevisiae* (live cells > 85%) was inoculated, which was activated for 15 minutes in 10 mL of a sucrose solution (1.7% p/p) at a temperature of 38 °C according to the manufacturer's recommendation; in addition, 20 mg ammonium phosphate was added.

All the bottles were stirred, capped and placed on a shelf at random with the tip of one of the hoses on a water container and the other sealed hermetically. The test was covered with black plastic and kept for 480 hours (h) at room temperature.

Table 1. Treatments for wine making from naranjilla juice (*Solanum quitoense* Lam.).

Treatments	Combination
1	Mo18
2	Mo21
3	Mo24
4	Ma18
5	Ma21
6	Ma24

Mo= whole must
Ma= must with water in 4:1 relation
18, 21, 24= different concentrations of soluble solids (°Brix) of the must

2.7 Fermentation dynamics of soluble solids

To observe the fermentation dynamics of sugar, the amount of °Bx remaining within 72, 168, 288 and 480 hours was followed. For this, 2 mL of fermented product was extracted. The °Bx analysis was performed in triplicate, similar to that described for the initial must.

2.8 Decanting and clarification

After the fermentation time, the fermented product of each treatment was transferred with the aid of the sampling hose, avoiding dragging sediments (decanting 1) and was set aside for 12 hours. A second decanting was performed and flavored-free gelatin (0.08 g/L) was added, it was homogenized and maintained for 24 hours at 8 °C without moving it. Then, the precipitates were separated by filtration (Whatman N° 1 qualitative filter paper and vacuum filtration system), packed in 750 mL wine bottles with potassium metabisulphite (100 mg/L) added and sealed.

2.9 Sensory evaluation

The evaluation of the acceptance degree of the final product (naranjilla wine) was determined by sensorial-descriptive analysis (Pszczólkowski and Ceppi De Lecco, 2016) of color-limpidity, odor and taste-tact-end of mouth by 20 untrained judges but who normally taste fermented drinks. The evaluation initiated with Mo treatments and the following day Ma was used. For the test, 3 wine glasses with 5 ml of wine, coded according to °Bx and a sheet with a 5-point hedonic scale (Table 2), were presented to each panelist for the assessment of each sensory characteristic.

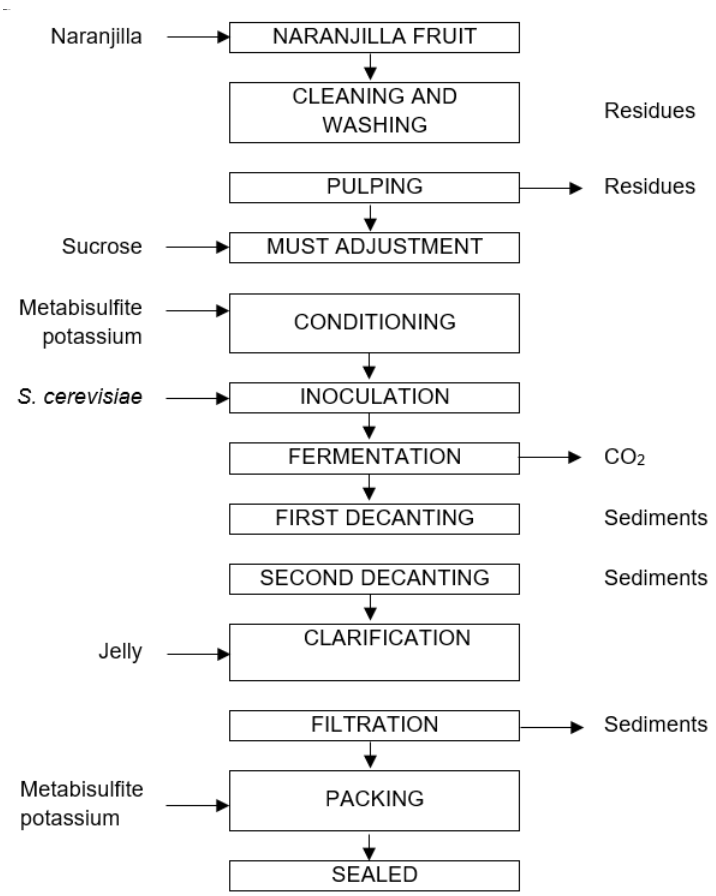

Figure 1. Flowchart for the elaboration of naranjilla wine (*Solanum quitoense* Lam.)

Table 2. Hedonic scale for the evaluation of color, flavor and smell of naranjilla wine (*Solanum quitoense* Lam.).

Perception	Weighting
I like it very much	5
I like it	4
I don't like it or dislike it	3
I dislike it	2
I dislike it very much	1

2.10 Statistical analysis

The effect of water to must dilution and °Bx on wine yield and acceptance degree was determined by a 2 × 3 multifactorial analysis (must:water and full must × °Bx). Mean separation in both cases was performed using Tukey ($p < 0.05$).

2.11 Physico-chemical evaluation of the final product (naranjilla wine)

Wines with the highest acceptance were physically evaluated to verify compliance with the requirements established by the Ecuadorian Technical Standard (INEN, 2016) for fruit wine. The parameters analyzed were alcoholic degree, total acidity (tartaric acid) and volatile acidity, total sulfur dioxide, methanol and total sugars. All the analyses were carried out in the laboratory of the Public Service Agency (OSP) of the Faculty of Chemical Sciences of the Central University of Ecuador.

3 Results and Discussion

3.1 Characterization of naranjilla must

Table 3 shows the pH, titratable acidity and °Bx values of *S. quitoense* Lam. must found in this study and also in literature reports from previous research on this fruit. The pH of the must is similar to the one found in other investigations; however, titratable acidity and soluble solids were lower. This variation may be related to factors specific to the cultivation where the research and the maturity of the fruit were carried out (González et al., 2014).

The pH of the must (3.13) was within the range (3.0 to 4.0) that according to (Dias et al., 2007; Swami and Divate, 2014) is considered suitable for good fermentation depending on the fruit and inoculum; in addition, the pH value found guarantees an acidic medium to yeasts and metabolite yield (Matei, 2017), so no adjustments were necessary (Coronel, 2008).

The titratable acidity showed to be relatively high in relation to other fruits used for the elaboration of wine, such as strawberries, blackberries, plums, apples, among others, where the value does not exceed 1 % (Matei, 2017); therefore the same author recommends its regulation with chemical compounds such as calcium carbonate to avoid sour flavors in the final product. However, this treatment was not conducted in this research because it was necessary to evaluate characteristics of naranjilla wine with its natural properties.

Furthermore, the °Bx found in the must (6.3) were not suitable for alcoholic fermentation, where the range fluctuates between 16 to 20 °Bx (Coronel, 2008), hence adjustments were needed by adding sucrose to complete the proposed values to be evaluated.

3.2 Fermentation dynamics

Figures 2a and 2b show the sugar fermentation dynamics of naranjilla must in Mo and Ma treatments, respectively, based on the °Bx determined during the process. A significant reduction in °Bx was observed up to 288 h in all treatments; however, from that time the concentration of °Bx remained constant until 480 h except for Ma24 which showed a slight decrease. All this indicates that there would be no need to ferment *S. quitoense* Lam. must for a time greater than 288 h for wine production, at a temperature between 16 to 19 °C which corresponded to fluctuation during the process.

In addition, it was observed that the final °Bx was greater than 6 %, which vary in relation to the initial °Bx, which demonstrates the cessation of fermentation of sugars without their entire consumption. This fact may be related to the inactivation of yeasts by the effect of alcohol concentration and nutrient balance (Swami and Divate, 2014).

Table 3. Physico-chemical characteristics of naranjilla must (*Solanum quitoense* Lam.) presented in the literature.

Parameter	Value found	Other reports		
		(Acosta et al., 2009)	(Brito et al., 2012)	(González et al., 2014)
pH	3.13	3.20 ± 0.04	3.00	2.89–2.94
Titrable acidity (gram of citric acid/100 g)	2.02	2.63 ± 0.07	2.56	3.78–3.21
Soluble solids (°Brix)	6.3	9.1 ± 0.5	10.8	6.58–9.04

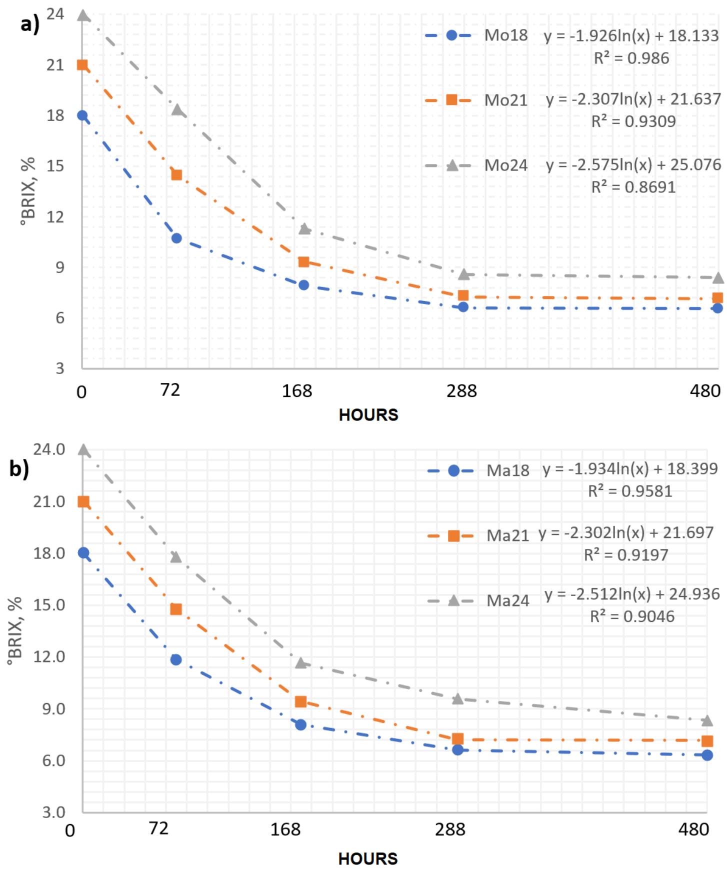

Figure 2. Dynamics of the fermentation of sugars in the production of naranjilla wine (*Solanum quitoense* Lam.) (a) Treatments without having added water to the must, (b) Once water was added to the must (ratio 1:4)

3.3 Yield of the final product (naranjilla wine)

Adding water to the naranjilla must before fermentation resulted in more wine (70.6%p/p), compared to the non diluted must that showed a yield of 45.0%p/p (Figure 3). But in addition to improving the yield with the amount of water to be added, juice can be easily extracted from some fruits and can decrease strong astringent flavors or acidity (Matei, 2017). In addition, it was observed that the percentage of sugars also influences the yield of wine, where the highest quantity was obtained at 21 and 24 °Bx with 58.8 and 61.1%, respectively. In general, yields found are similar to studies on other fruits, such as mango wine that reports yields up to 60% but with incorporation of pectinases that improve fermentation conditions (Veeranjaneya and Reddy, 2009).

The high amount of waste obtained in the first decanting, and the low yield in must without water at 18 °Bx allow considering the use of enzymes during maceration of the must (Romero, 2008) as an alternative for wine of *Solanum quitoense* Lam.

Figure 4 shows the yield of wine with respect to treatments. The results corroborate the above (Figure 3), where Ma in the different concentrations of °Bx shows better yields than Mo; however, in Mo as °Bx increases, more wine was obtained.

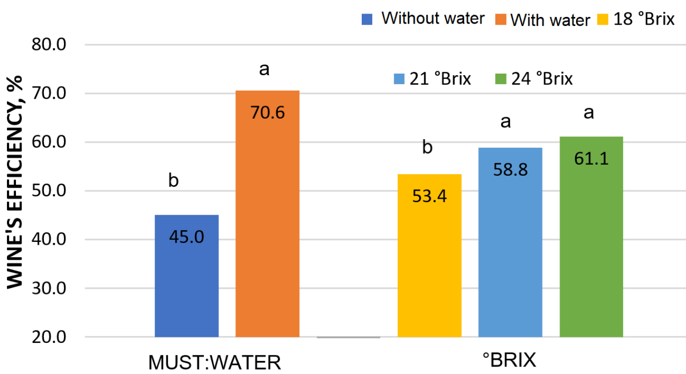

Figure 3. Effect of the addition of water and °Brix on the yield of naranjilla wine (*Solanum quitoense* Lam.). (Distinct letters show statistical differences, Tukey $p < 0.05$)

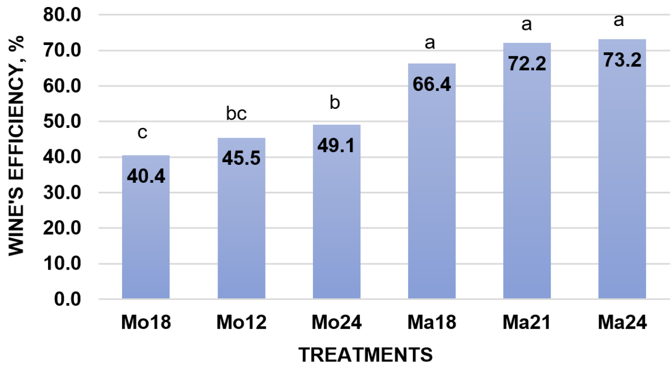

Figure 4. Yield of naranjilla wine (*Solanum quitoense* Lam.) according to the treatments. (Distinct letters show statistical differences, Tukey $p < 0.05$)

3.4 Sensory evaluation of naranjilla wine

It was found that the addition of water to naranjilla must negatively affects the taste of wine (Figure 5a), as well as the color and smell; although according to the established hedonic scale, the average wine taste rating is "I don't like or dislike", unlike the color and smell that obtained "I like it" on wine without water. These results would demonstrate a decrease in aromatic compounds in the beverage, as found by (Dias et al., 2007) where the addition of water decreased the flavor of cocoa-based wine.

Also, it was found that °Bx statistically influence the smell of the final product, where wines with 21 and 24 °Bx reach "moderately acceptable" to the consumer as opposed to 18 °Bx, while the color and taste of wine does not vary at different initial °Bx in the must with average ratings of "I like it" and "I do not like or dislike", respectively. In general, the low level of acceptance of taste was related to observations noted by some tasters at the feeling of acidity in the wine, related with the initial acidity of the must, which was not adjusted in this study.

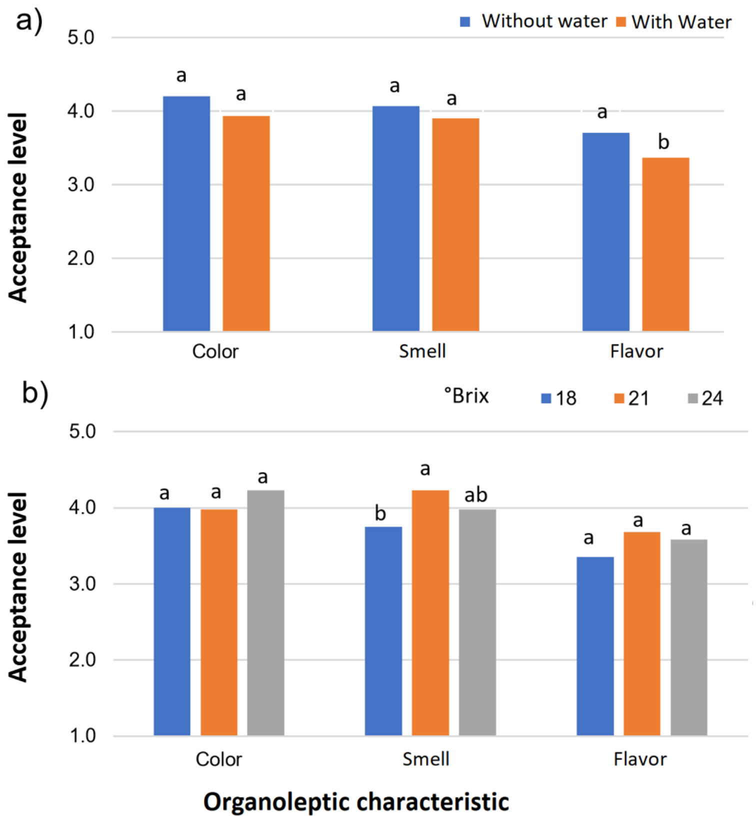

Figure 5. Level of acceptance of naranjilla wine (*Solanum quitoense* Lam.) according to color, smell and flavor, (a) Valuation according to the addition of water, (b) °Brix. (Distinct letters show statistical differences, Tukey $p < 0.05$)

An interaction was found (Figure 6) between the addition of water and 24 °Bx on the taste of wine, showing a deterioration in this attribute under these initial conditions of the must, generating a feeling of "moderate dislike" unlike the taste of wine without addition and with the same °Bx amount that "moderately likes". This effect may be related to cessation of fermentation by alcohol concentration or decreased nutrients as the source of nitrogen (Swami and Divate, 2014), with subsequent lysis of yeasts and dissolved cell wall components, as noted in (Vasantha et al., 2017). This fact was corroborated by the high turbidity presented in the wine that after the filtration treatment had organic residues that caused unpleasant taste, which could indicate that adding water should not only increase sugars, but also nutrients to achieve stable cell growth.

Table 4 shows the acceptance of the consumed naranjilla wine with respect to the sensory attributes evaluated and generally in the different treatments. No statistical differences were found for color and smell, but for general taste and acceptance with the same classification ranges. Thus, wines with greater acceptance were in Mo24 and Ma21 treatments, and for Ma24; while the rest remained in the same category at intermediate level.

According to the hedonic scale proposed for the assessment of wine acceptability, treatments Mo24 and Ma21 had values higher than 4 for almost all sensory characteristics, excepting the flavor of Ma21 which obtained an average of 3.9. This shows that the acceptance of wine produced under these conditions is in a weighting of "I like it"; similarly, the general acceptability in the other treatments shows that *S. quitoense* Lam. can be a fruit with great potential for wine making, because its consumption does not produce feelings of displeasure; however, more works should be conducted for Mo24 and Ma21 to improve the yield and decrease of acidity, which is the factor that influences taste.

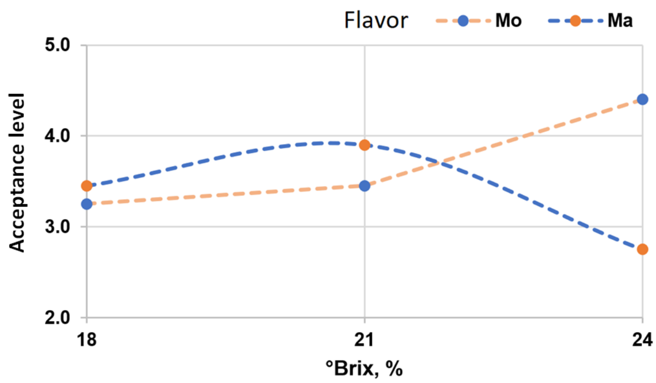

Figure 6. Interaction between the addition of water to the must and °Brix on the flavor of naranjilla wine (*Solanum quitoense* Lam.).

Table 4. Sensory evaluation of naranjilla wine (*Solanum quitoense* Lam.) according to treatments.

Treatment	Color	Smell	Flavor	General Acceptance
Mo18	4.1^{a}	3.8^{a}	3.3^{bc}	3.7^{bc}
Mo21	4.0^{a}	4.3^{a}	3.5^{bc}	3.9^{bc}
Mo24	4.6^{a}	4.2^{a}	4.4^{a}	4.4^{a}
Ma18	4.0^{a}	3.8^{a}	3.5^{bc}	3.7^{bc}
Ma21	4.0^{a}	4.2^{a}	3.9^{ab}	4.0^{ab}
Ma24	3.9^{a}	3.8^{a}	2.8^{c}	3.5^{c}

Means in the same column with distinct letters are significantly different ($p < 0.05$) according to Tukey.

3.5 Physico-chemical analysis of the final product (naranjilla wine)

According to the sensory analysis (Table 4), wines with the highest acceptance (Mo24 and Ma21) had a physico-chemical analysis (Table 5) to verify compliance with the requirements of NTE INEN 374-3 for fruit wine.

The results showed that the alcohol percentage in wines was 15%v/v in Mo24 and 13%v/v in Ma21, values that would be directly related to the initial concentration of fermented sugar in must (Matei, 2017). These concentrations are accepted by the NTE INEN which does not specify a maximum in the alcoholic degree. According to the classification of fruit wines described by (Joshi and Attri, 2005; Swami and Divate, 2014) the product obtained in treatments Mo24 would be called a dessert wine and aperitif, and wine Ma21 as a natural wine. The total acidity expressed as tartaric acid was 23.2 and 19.4 g/L for Mo24 and Ma21, respectively, showing that the wine obtained was acid, which is consistent with the observations found during the testing of the initial acidity of the must, which was not adjusted. This process was omitted to evaluate a procedure that does not imply greater use of economic resources, but considers the technicality, such as the addition of acidity deregulators, use of yeast varieties that allow to minimize this parameter (Joshi and Attri, 2005) or the use of naranjilla in combination with other fruit. The low volatile acidity found 0.18 g/L in Mo24 and 0.09 g/L in Ma21 represents adequate fermentation in both treatments and the organoleptic characteristics of wine are not altered by acetic acid. The concentration of total sulfur dioxide was 17.9 and 21.8 g/L in Mo24 and Ma21, respectively, values that are within the permitted range, and the quantities found are low and will not influence the sensory characteristics of wine (Dias et al., 2007). The non-detection of methanol ensures the fermentation quality of naranjilla wine under the established conditions. Finally, according to the amount of total sugar found in treatments Mo24 (34.9 g/L) and Ma21 (30.5 g/L), the established standard categorizes these fruit wines as semi-sweet.

Table 5. Physico-chemical characteristics of naranjilla wine in treatments with better acceptance-requirements of the Ecuadorian Technical Standard for Fruit Wine.

PARAMETERS	UNIT	NARANJILLA WINE		REQUIREMENTS*	
		Mo24	Ma21	Minimum	Maximum
Alcoholic degree at 20 °C	% (v/v)	15.0	13.0	6.0	–
Total acid (tartaric acid)	g/L	23.2	19.4	3.5	–
Volatile acid (acetic acid)	g/L	0.18	0.09	–	1.5
Sulfide total sulphur dioxide	mg/L	17.9	21.8	–	400.0
Methanol	mg/L	0.01	0.01	–	1000.0
Total sugar	g/L	34.9	30.5	25.0 (semi-sweet)	50.0

Mo24= naranjilla wine from must without added water and 24 °brix
Ma21= naranjilla wine from must with added water (4:1) and 21 °brix
*NTE INEN 374-3 Alcoholic beverage requirements. Fruit wine.

4 Conclusions

A viable technique was established for the production of semi-sweet wine from naranjilla, since good acceptance was demonstrated in relation to the sensory characteristics, and the wine obtained meets all the requirements established in the corresponding Ecuadorian Technical Standard. Hence, this fruit is proposed in enology on a small or medium scale, because of its low cost, availability and pleasant aromas, which would greatly help to link new economic activities, generating opportunities for territorial development by the possibility of increasing tourism due to the conjunction of wine production with gastronomy.

It is necessary to work on the improvement and transfer of the established technical process to obtain higher yields, decrease acidity and conduct new studies with tropical fruits and integrate them into the development of oenological proposals with the participation of the community and visitors.

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