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# AGRO PRODUCTIVIDAD

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# How is the perception of the certified organic food benefits in a population from Estado de México region

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## ABSTRACT

**Objective**: To study and understand the population's perception in a region of the Estado de Mexico about the role of certified organic food in health and the environment, as well as the economic impact generated by its consumption.

**Desing/methodology/approach**: A survey was conducted among a population sample of 10 municipalities in the Estado de Mexico, which consisted of seven questions related to certified organic food.

**Results**: The population has an idea about what certified organic food is, although they confuse it with other types of food.

**Study limitations/ implications**: Results were obtained from an e-mail survey because this study was conducted during the current 2020-2021 pandemic.

**Findings/conclusion**: The information obtained suggests that the population has a great perception about the intake of certified organic food, but there is confusion about other types of food. However, the population is willing to consume them because of their potential health and environment benefits.

## INTRODUCTION

A part of the world's population is interested in producing and consuming certified organic food. However, this issue is complex to understand for several reasons, but one of the most frequent is that the concept is not properly applied, resulting in a lot of

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misinformation about these products (Gentile and Rodríguez, 2006; Cooper *et al.*, 2007; Díaz-Víquez *et al.*, 2015).

The term "organic" applies to products that have been produced based on a certification scheme with organic specifications throughout the production, storage, processing, handling and marketing stages (Mc Donnel-Bernabé *et al.*, 2008; Rembiałkowska *et al.*, 2008; Romero-Valenzuela *et al.*, 2016). In particular for plant food, the seeds must be certified; they must be approved by a certifying authority, applying the correct sowing, harvesting and post-harvesting techniques, without the application of chemical fertilizers to the soil. Finally, the food will be labeled by adding the seal provided as "Organic Food" certified by a legal and constituted authority (FAO, 2007).

Furthermore, "organic" is about a process and not a specific product, in such a way it does not mean that organic food will provide more nutrients, proteins, or that are healthier, safer, or completely natural. Rather, it refers to an adjustment of standards that include the way of producing, storing, processing, and handling (FAO, 2007).

In Mexico, organic agriculture has a small production with low capital use, so this production system can be locally adaptable. In addition, there is a great diversity of crops due to the genetic variety, in certified organic crops the use of energy external sources for harvesting is reduced and weed control is done in a traditional way (chemicals are not used). It is also relevant to state that exists a low technological dependency and few external resources for production, however an economic risk exists in the financial investment when seeking to work as an organic farmer (Brundtland, 1987; Fließbach *et al.*, 2007).

Mexico's Organic Food sector grew significantly from 2000 to 2010, positioning itself as a country with a high degree of organic farms registration (Willer *et al.*, 2012). Therefore, in 2014 the government introduced the national label for organic products to certify organic food (Sello Alimento Orgánico-SAGARPA; SAGARPA, 2016). It is essential to highlight the impact of seals on the certification for organic food produced in Mexico, since a controversy exists between a "natural" food (a product that is harvested at home or in the field) which is currently confused as "organic" food. It is therefore indispensable to identify a certified organic food, which assures the potential health benefits that can provide by being free of toxic residues from chemical compounds that are often used in harvesting and post-harvesting (Kenneth and Bugusu, 2007; Camacho *et al.*, 2001; Gobierno de México, 2018).

On the other hand, consumers consider several factors when purchasing conventional products, such as price, availability, nutritional contribution to health, and so on (Gómez *et al.*, 1999; Gómez *et al.*, 2002).

Therefore, the development of a wide, inclusive, and efficient supply and distribution system for this type of products, as well as the management of adequate information, are needed to encourage the consumers' interest in these products.

Since the broad market for certified organic products has a great potential for development, the aim of this study was to analyze the link between the economy, health and the environment according to the knowledge about the use of certified organic foods in a population sample in a particular region of the Estado de Mexico.

## MATERIALS AND METHODS

**Sample size**: To find out the population's opinion about certified organic food, a public opinion survey was randomly applied to 83 people from 10 municipalities in the Estado de Mexico (Toluca de Lerdo, San Mateo Atenco, Metepec, Lerma de Villada, Ocoyoacac, Jilotepec, Temoaya, Jiquipilco, Ecatepec de Morelos and Tejupilco).

**Application**: A survey was conducted via e-mail, mainly because this study was carried out during the pandemic season due to the recent worldwide affectation by the severe respiratory syndrome coronavirus 2 (SARS-CoV-2), therefore it was decided to apply multiple-choice questions. The questionnaire was composed by 7 questions aimed to know: a) consumption, b) purchasing establishments, c) monetary value, d) health benefits and e) environmental impact of certified organic food. At the beginning of the survey, the following description of certified organic product was placed: *The term organic applies to products that were produced based on Organic Standards throughout the production, handling, processing and marketing phase (excluding the use of chemical fertilizers) and which has been approved by a certifying authority. Organic refers to a process rather than a product* (Food and Agriculture Organization of the United Nations, 2007).

In the Google Forms survey, the participants' name, gender and age was requested, and the questions were: 1) Has your perspective of organic food concept changed based on the above definition? 2) Have you ever consumed certified organic food? 3) Do you know any establishment or online store where certified organic food can be purchased? 4) Which is that establishment or online store? 5) Do you think that the price of certified organic food is more expensive, so it probably impacts your consumption choice? 6) Do you consider that eating certified organic food is beneficial or detrimental to your health? 7) Do you consider that certified organic foods impact positively or negatively on the environment? Automatic graphs were generated with the collected results. Afterwards, the data was tabulated in Microsoft Excel/Windows 10 to visualize the responses of each participant (Table 1).

## RESULTS AND DISCUSSION

The study was conducted to determine the perception that the population has of certified organic foods and the existing association between factors that are decisive for their consumption, such as their price and the potential benefits that can have an impact on the health of the consumer and the environment. As a first step, the information was processed to obtain, on the one hand, a record of the sexes and, on the other, a record of the ages. It was found that 39% of the people were female, 59.8% were male and 1.2% of the population did not want to answer what sex they were (Figure 1). Interestingly, we would expect more women to answer the survey, but the percentage of men who answered it was higher, suggesting that even though women traditionally decide on the acquisition of food for household consumption in Mexico, it seems that a high percentage of men were interested in this topic and had an opinion about it.

The age range is from 18 years old to 53 years old (Figure 2). Which represents a broad sector of the population that may have the possibility to choose to buy food.

**Table 1**. Response options for each of the questions asked

| Questions | Answer options |
|---|---|
| 1) Has your perspective of organic food concept changed based on the above definition? | a) Yes<br>b) No |
| 2) Have your ever consumed certified organic food? | a) Yes<br>b) No<br>c) Maybe |
| 3) Do you know any establishment or online store where certified organic food can be purchased? | a) Yes<br>b) No |
| 4) Which is that establishment or online store? | a) I don't know about any establishment<br>b) Local markets<br>c) Supermarket<br>d) Internet (Amazon, Mercado libre, etc.)<br>e) Other |
| 5) Do you think that the price of certified organic food is more expensive, so it probably impacts your consumption choice? | a) Yes<br>b) No<br>c) Maybe |
| 6) Do you consider that eating certified organic food is beneficial or detrimental to you health? | a) It is health.promoting<br>b) It isn't health-promoting<br>c) It promotes or harms health according to the frequency with which it is consumed |
| 7) Do you consider that certified organic foods impact positively or negatively on the environment | a) Positive for the environment<br>b) Negative for the environment<br>c) I am unaware of the impact that certified organic products have on the environmental |

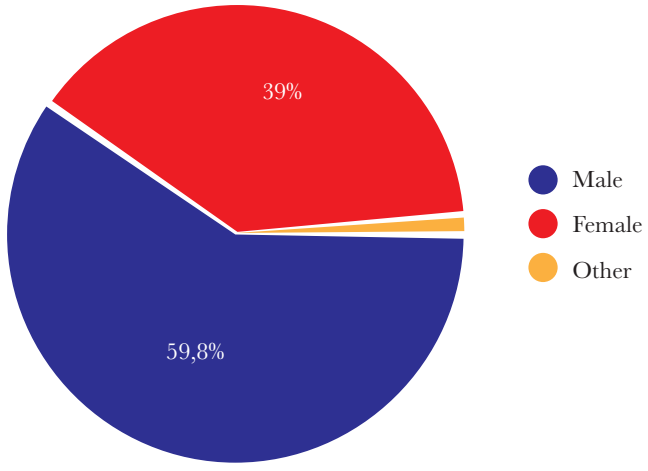


**Figure 1**. Percentage representation of registered sexes.

According to FAO's definition of organic food incorporated in the survey, it was asked if the perspective of the concept of organic food had changed. It was found that 60.2% of the interviewed population had changed their point of view, demonstrating that the main problem was to define organic food with other kinds of it. However, the remaining 39.8% of the interviewed population (Figure 3) kept the term organic food as simply grown at home, regardless of whether pesticides, pesticides, and chemical fertilizers are used, whether the seeds they use are certified or not, or even if the soil conditions and cultivation techniques are the ideal ones.

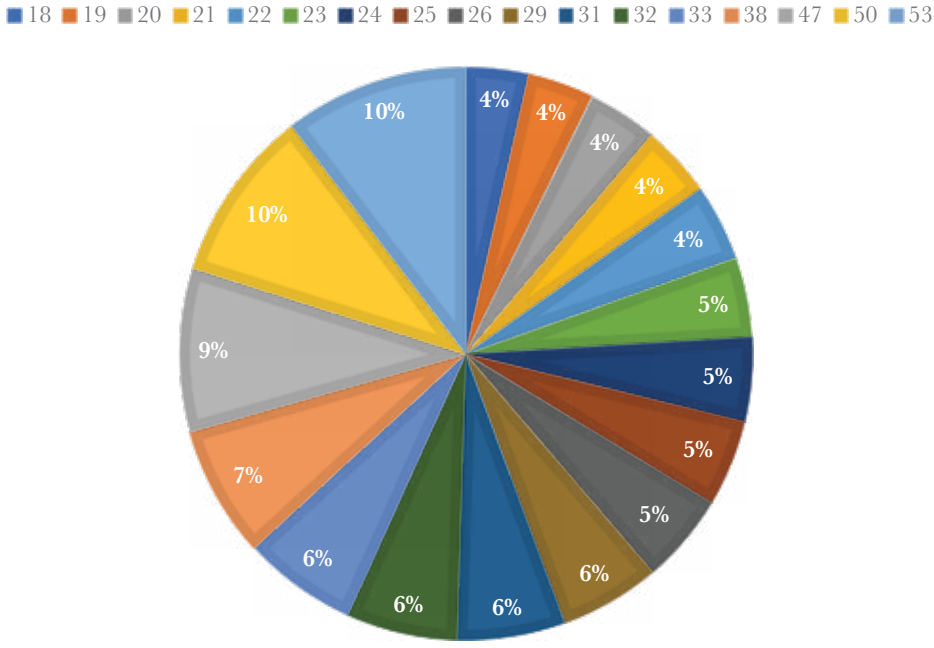


**Figure 2**. Age register of the participants.

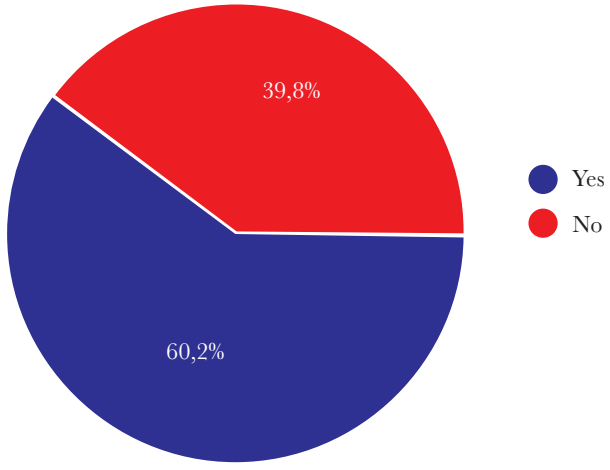


**Figure 3**. Percentage representation in relation to question number 1.

Organic agriculture is defined as an alternative production that relates environmental, social, and economic aims (Romero Valenzuela *et al.*, 2016). At the same time, it is gradually combined with the widespread acceptance of sustainable development as a concept that fully satisfies the needs of current generations without compromising the future generations' sufficiency to compensate for their own needs (Brundtland, 1987).

To know if population had consumed certified organic food, we asked the following question: Have you ever consumed certified organic food? The results showed that 42.2% considered that they had consumed certified organic food at some time, while 41% of the population interviewed stated that they had not consumed it, and a lower percentage

(16.8%) stated that they had possibly consumed it but did not realize it, since most of the times when purchasing food they do not check the labeling. These results suggest that the proportion of people who have consumed this type of food is similar to those who have not consumed it; however, the percentage of people who have consumed it could be higher if we take into account that there is a percentage who are not aware of whether they have consumed it or not (Figure 4).

The consumption of organic food is related to health care. If it is affordable to consumers, they will prefer to pay a higher price to ensure that their food is free of pesticides or toxic agents. However, the product availability in different regions of the country is a decisive consumption factor of this type of food. Although its consumption is reduced when compared against other types of food. In the market, organic food has great potential due to the nutritional benefits it can generate to the organism as well as the environmental protection.

Considering all the above, we searched if the population had enough information to purchase these foods, so we asked if they knew any establishment or online store where certified organic foods could be purchased. The results showed that 53% of the interviewed population knows the places where this type of products can be purchased, while 47% do not know where to buy them. These results showed that half of the population knows where to go while the other half does not (Figure 5).

Manufacturers and companies should publicize their products and their distribution points so the interested population could acquire them easily. Likewise, organic food demand in establishments such as supermarkets impacts the cost, which makes it harder to find in any establishment. Besides the price, the feasibility and guarantee of the product is another influential factor at the moment of purchase; therefore, the family income is also a determinant for buying this type of food, so it is not only a matter of knowledge. Generally, the population is now more concerned about the environment, so it is becoming more important to emphasize areas such as green publicity. Society's interest in organic food is increasing due to people's concern about caring for greenery

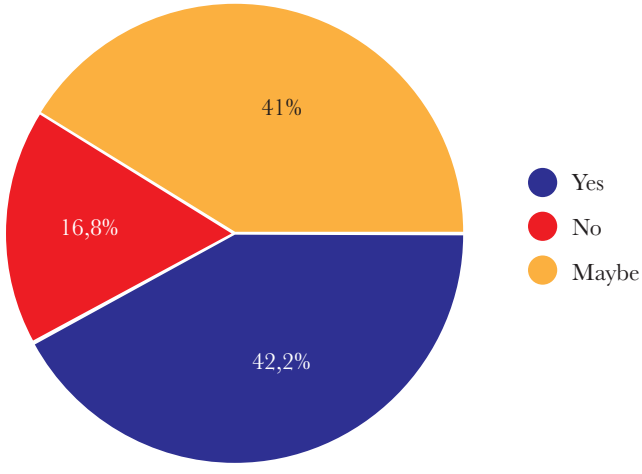


**Figure 4**. Percentage representation in relation to question number 2.

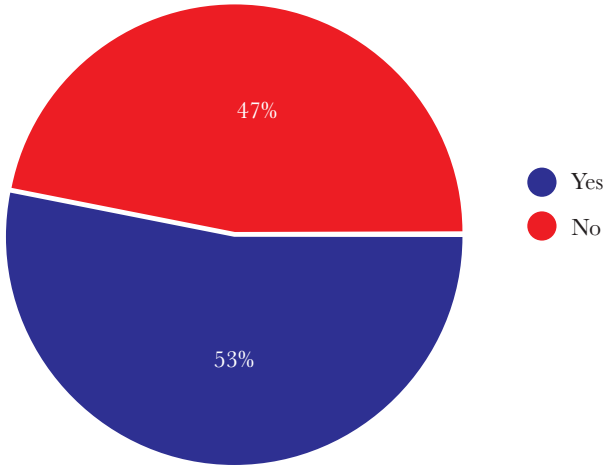


**Figure 5**. Percentage representation in relation to question number 3.

and having a deeper understanding of the damage caused to nature by the misuse of resources provided to industries and society itself (Mc Donnell, Reginald, 2008).

To deepen the knowledge about the establishments where this type of food could be purchased, it was asked: "What is the establishment or online store where you could buy certified organic food?" For this, 28.9% of the participants answered: "I do not know any establishment" and a similar percentage answered in "supermarkets" (27.7%). One-fifth of the population thought that these could be purchased through the "Internet (Amazon, Mercado Libre, etc.)" and a smaller percentage of 13.3% stated that certified organic food can be purchased in local markets, which was questionable since there are few certified organic food markets, and in the municipalities in which the survey was conducted, there is no record of the existence of the certification of these, although they may be organic food, by not having an assigned certification it cannot be said that it is a certified organic food; finally the remaining 9.6% answered that they could purchase these foods in other types of places (Figure 6).

To find out if the interviewed population had an idea of organic food's economic cost, we asked: "Do you consider that all certified organic foods are more expensive, which might influence your decision to consume it?" The results achieved showed that most of the population (63.9%) considers that the prices of certified organic foods are higher when compared to non-certified ones. These foods are more expensive than those commonly purchased, due to several reasons: 1.- The production processes require labor for each product, 2.- Since they are batches of small quantities, the cost of their mobilization increases 3.- Sale is limited since the demand for them is low. The rest of the people who answered the survey were divided into 27.7% who did not know if the prices of certified organic foods were higher or not, while the remaining 8.4% thought that they did not have higher prices compared to those they consume more frequently. However, two possible reasons to explain the answer to question 5 is that certified organic foods were probably not purchased by them, or they simply did not consider them to be higher priced (Figure 7).

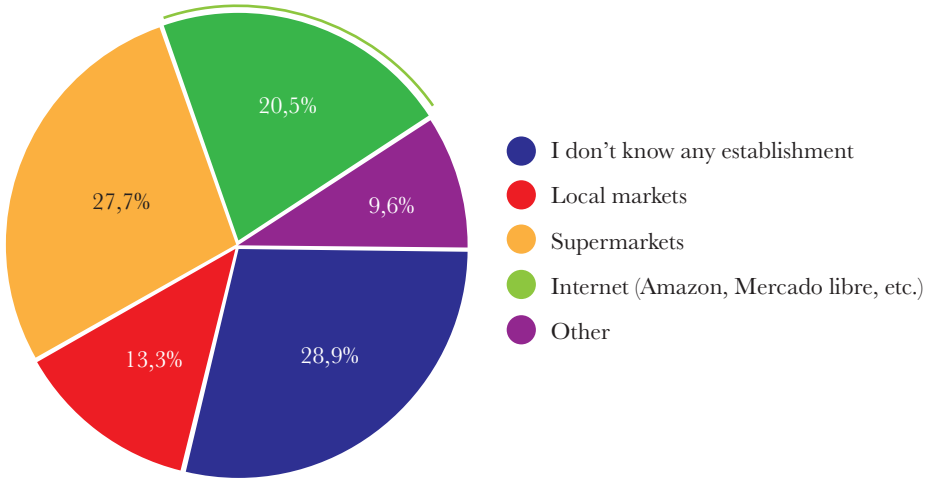


**Figure 6**. Percentage representation in relation to question number 7.

To determine whether the interviewed population has an idea about the impact that certified organic foods can cause to human health, the following question was asked: Do you consider that the consumption of certified organic foods is beneficial or detrimental to your health? The results were divided into two main groups: on the one hand, half of the population, 49.4%, indicated that the frequency of consumption of this type of food does not harm health. However, 50.6% of the population considered that they can have an adverse or detrimental effect on health, depending on the consumption frequency. To clarify this possibility, it can be exemplified with the excessive consumption of some organic fruits that could affect health because they may have a higher index of total sugars compared to the foods commonly consumed (Kowska *et al.*, 2012), impacting the consumption of a high-fructose diet, which reduces circulating concentrations of insulin, leptin and decreases postprandial suppression of ghrelin, which might promote increased caloric intake and therefore, leading to undesirable weight gain and obesity (Schaefer *et al.*, 2009). One of the major disadvantages of these foods is that since they have a

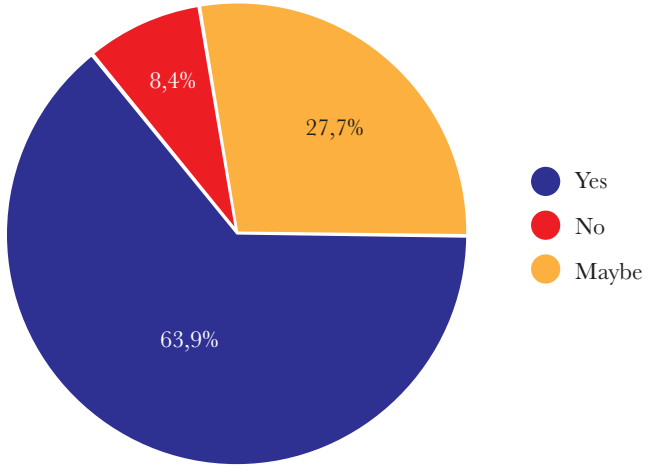


**Figure 7**. Percentage representation in relation to question number 5.

shorter shelf life, it could be consumed after the recommended consumption dates and, consequently, could lead to infections and intoxications (Figure 8).

Finally, to find out if the population knows about the impact that consuming certified organic food can have on the environment, we proceeded to ask if they consider that certified organic food has a positive or negative impact on the environment? The results obtained suggest that the majority of the population interviewed (59%) considered that the consumption of these foods is "positive for the environment", while 39.8% did not know if the consumption of these foods has a positive impact on the environment, and finally only 1.2% stated that certified organic foods have a "negative impact on the environment".

The positive environmental impact that certified organic food generates is largely since agrochemicals are not used during agriculture, thus avoiding contamination and degradation of natural resources. In this way, competent and effective products are obtained, which quality is due to the non-use of fertilizers, pesticides as well as pharmaceuticals that are usually used in common food agriculture. Some of the agricultural methods for harvesting these foods seek an ecological balance between planting and profit, since the ecosystem is the primary production input (Fliessbach *et al.*, 2001). However, despite having many points for the positive impact on the environment, it should be taken into account that most of the packaging of these foods are presented in cans, non-biodegradable plastic bags, unicel, plastic nets, polyethylene containers, among some others that continue to have a negative impact on environmental pollution, not only in the soil itself, but also in the seas, rivers, lakes and lagoons as well as in the air and in effect to the very health of living beings, since some plastics, being non-biodegradable, become non-biocompatible and therefore when integrated into organisms become toxins that are harmful to health (Chemical Society Journal, 2011). The correct management of food packaging waste should be made known to the population, to improve the quality of the environment, and when they are properly selected, they can be recycled in a much more effective and efficient way (Marsh K., 2007) (Figure 9).

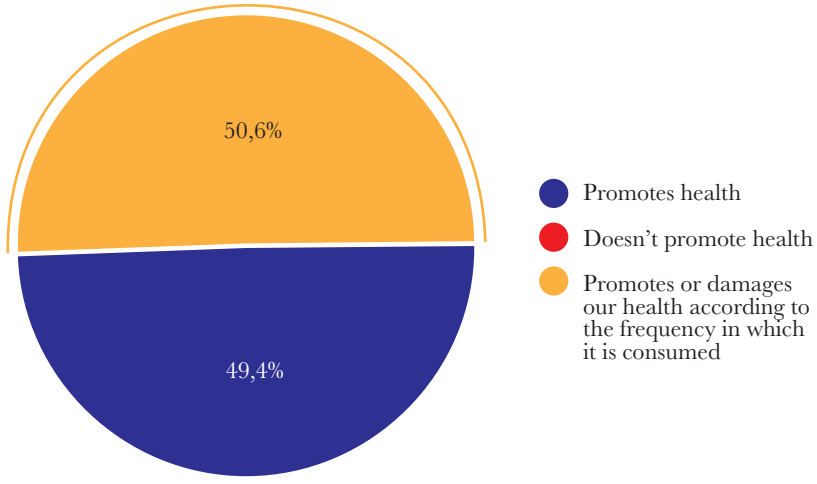


**Figure 8**. Percentage representation in relation to question number 6.

## CONCLUSIONS

Certified organic foods provide a certain guarantee to their consumers, the non-use of synthetic agrochemicals, as well as transgenic materials in their production process and techniques. Animal products ensure that aspects related to animal welfare have been respected. From the sample studied, 35 people have consumed certified organic food, however, because its price is higher than a common food, they are not affordable and in fact are rarely consumed.

Another factor is the limited availability in Mexico of organic agriculture and the accepted certifications required to guarantee the product, because of the above mentioned, the places where these products can be acquired are very scarce or totally unknown, since 24 of the people who answered the survey did not know the places where these foods are acquired. In addition, the health benefit favor or harm according to their frequent consumption; it should be clarified that not being a certified organic food will not have negative effects on the body when its consumption is exceeded, likewise it is important to know that food packaging is essential and indispensable, as it protects the food between the transformation process and how it reaches the consumer. Food packaging must be disposed in an environmentally responsible manner, so it is necessary to raise awareness when purchasing a product. Different forms of packaging should also be innovated to use less cans, unicells, bags and polyethylene.

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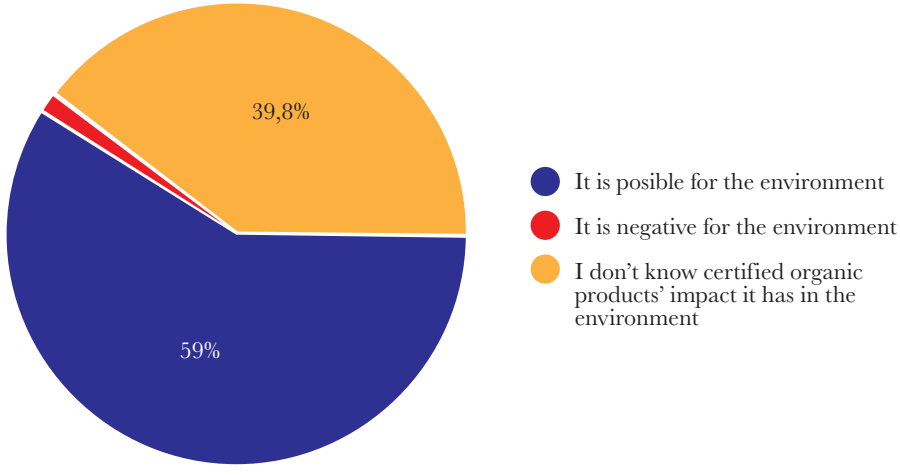


**Figure 9**. Percentage representation in relation to question number 7.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# β-lactoglobulin peptides obtained by chymotrypsin hydrolysis

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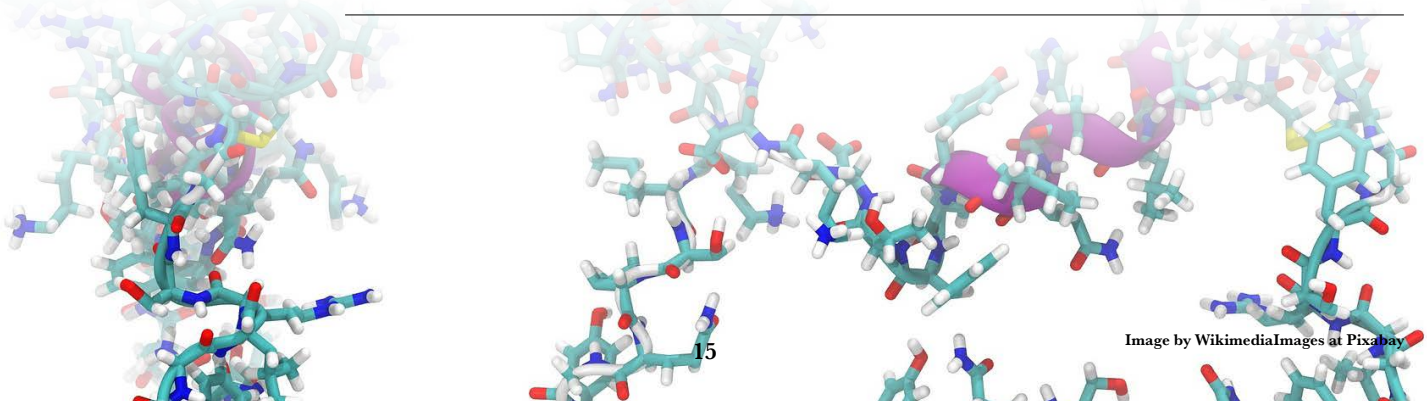
## ABSTRACT

**Objective**: Whey proteins, as β-lactoglobulin, have biological activity. Controlled hydrolysis of this protein could generate peptides with some biological function. The aim of this work was to analyze the peptides resulting from the *in vitro* hydrolysis with chymotrypsin in order to evaluate the presence of bioactive peptides.
**Design/methodology/approach**: Chymotrypsin was used in the hydrolysis of β-lactoglobulin, and its peptides were evaluated by ultrafiltration, electrophoresis, and mass spectrometry.
**Findings/conclusion**: Results showed that 2 h of chymotrypsin hydrolysis (T1) released peptides with molecular weight values of 8 and 9 KDa, while 4 h of hydrolysis (T2) produced peptides with molecular mass weight values of 7 and 5 KDa. The mass spectrometry (MALDI-TOF) showed six peaks and five of them were comparable with those obtained by *in silico* hydrolysis results (done previously by Fonseca Ayala, 2018). The identified peptides (DTDYK, DAQSAPL and LKPTPEGDL) in the fraction <1 kDa showed inhibitory activity of angiotensin converting enzyme and inhibitory activity of enzyme dipeptidyl peptidase IV according BIOPEP database. These results showed that β-lactoglobulin peptides obtained by chymotrypsin hydrolysis could have biological activity that can be used in different types of industries as pharmaceutical and food.
**Limitations on study/implications**: The *in vitro* evaluation of the biological activity of the characterized peptides is necessary.

**Key words**: β-lactoglobulin, hydrolysis, biopeptides, chymotrypsin



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## INTRODUCTION

Bioactive peptides are protein fragments that have a positive effect on body functions and health (Remanan and Wu, 2014; Sharma *et al.*, 2011), this fact has increased their scientific and commercial interest (Korhonen, 2009). These peptides are inactive within the protein, but they can be released during enzymatic hydrolysis under specific conditions in the gastrointestinal tract, therefore enzymes as pepsin, trypsin and chymotrypsin have been used. Antimicrobial, antithrombotic, antihypertensive, opioid, immunomodulatory, and antioxidant activities had been described and such activities depends on their amino acids sequence and composition (Escudero *et al.*, 2012).

$\beta$-Lactoglobulin is the main protein in whey, it has antioxidant activity and maintains retinol absorption levels in dermal cells, it also has emulsifying capacity derived from its amphiphilic structure (Pihlanto-Leppälä, 2000). Besides, bioactive peptides of this protein with different biological activities have been described such as ACE inhibitory activity (Hernández-Ledesma *et al.*, 2011; Pihlanto-Leppälä, 2000), dipeptidyl peptidase IV inhibitory activity (Girolamo *et al.*, 2008) antimicrobial capacity (Mulero *et al.*, 2011), and some peptides had been used with others showed a positive effect on metabolic syndrome (Ricci-Cabello *et al.*, 2012).

Recently, Fonseca Ayala (2018) determined the bioactivity of peptides obtained by *in silico* hydrolysis of $\beta$-Lactoglobulin with different enzymes (chymotrypsin, trypsin and pepsin) where 29 peptides were obtained by chymotrypsin hydrolysis and three of them have biological activity according BIOPEP database. Their results show that chymotrypsin hydrolysis *in vitro* could produce bioactive peptides. Therefore, the objectives of this work were to do the *in vitro* hydrolysis of $\beta$-lactoglobulin with chymotrypsin and to compare the produced peptides with those obtained by *in silico* hydrolysis.

## MATERIALS AND METHODS

### Materials

Bovine lyophilized $\beta$-lactoglobulin (purity >/=90%) (L3908 Sigma-Aldrich), and $\beta$-chymotrypsin from bovine pancreas type II (C4129 Sigma-Aldrich). All materials were reagent grade.

### Methods

**Chymotrypsin hydrolysis**. The hydrolysis was carried out according to Gillespie *et al.* (2015) with some modifications. 0.2 g of $\beta$-lactoglobulin was added to 25 ml of phosphates buffer pH 7.4 after, chymotrypsin was added in a 1:25 proportion enzyme/substrate, the reaction was done at 37 °C in agitation. Three hydrolyzed solutions were obtained, T0: without reaction time, T1: 2 h of reaction, T2: 4 h of reaction. The inactivation of the enzyme was carried out at 90 °C during 2 min. The hydrolyzed solutions were stored at freezing (−20 °C) until their analysis.

**Degree of hydrolysis**. A pH method was carried out where the samples were titrated with NaOH (0.1N) in order to maintain the optimal pH of the enzyme. The degree of hydrolysis (%DH) were calculated with Equation 1 (Adler-Nissen, 1979; Fernández and Kelly, 2016).

$$DH\,(\%) = \left[\frac{\beta \times Nb}{\alpha \times Mp \times Htot}\right] \times 100 \qquad \text{[Eq.1]}$$

where: $\beta$: (mL) volume of the NaOH solution added during the hydrolysis. *Nb*: (eq/L) is the normality of the NaOH solution. *Mp*: g of the protein in the reaction. *Htot*: (mequiv/g) total number of peptide bonds in the substrate. $\alpha$: degree of dissociation of $\beta$-amino groups released during the hydrolysis. Both, *Htot* (8.8) and $\alpha$ (1) values were obtained from the literature (Nielsen *et al.*, 2001).

**Ultrafiltration of the hydrolyzed solutions**. The hydrolyzed solutions were ultrafiltrated using a shaking chamber (Amicon 8010 Millipore) with a capacity of 10mL and a membrane filtration of 4.1 $cm^2$ of area (regenerated cellulose, Ultracel®). Membranes had a molecular scale of 5, 3 and 1KDa and were hydrated in distilled water for 2 h before the ultrafiltration. The separation was carried out at a pressure of 25 psi with $N_2$ and at a temperature of 25 °C, and in three stages: 1) The hydrolyzed solution (T1, T2) (10 mL) was put into the chamber with a 5 KDa membrane and the permeated solution was obtained, 2) this permeated solution (5 KDa) was put into the chamber with a 3 KDa membrane and the permeated solution was recovered, 3) the second permeated solution (3 KDa) was put into the chamber with a 1 KDa membrane. All permeated solutions were store at freezing (−20 °C) until their analysis.

**SDS-PAGE/ Tricine polyacrylamide Gel Electrophoresis**. a) Pre-electrophoresis sample preparation: 10 $\mu$L of sample [BSA; $\beta$-LG; 1 KDa permeated solution and unfiltered hydrolyzed solutions (T0, T1, T2) were added to 25 $\mu$L of buffer (TruPAGE 4x PCG3009, Sigma Aldrich) with 65 $\mu$L of distilled water. The mixture was stirred for 5 s until homogenized and heated at 70 °C during 10 min and cooled to −5 °C during 10 min before the analysis. The markers (Sigma S8445-10VL) (200000-6500 KDa) were prepared according to the manufacturer. b) SDS-PAGE/Tricine polyacrylamide gel Electrophoresis: The samples were analyzed by electrophoresis using a Mighty Small II SE 250 camera (Hoefer Scientific Instruments). A precast 20% to 4% gradient gel with 12 wells was used. The electrophoretic run was carried out at a voltage of 100 volts during 2 h. 10 $\mu$L of the samples and the marker were injected into each well of the gel.

Molecular weights values of the peptides of permeated solution (<1 KDa) by Mass Espectrometry (MALDI-TOF / TOF). The Autoflex Speed equipment (Bruker Daltonics) was used. The permeated solution <1 KDa of both chymotrypsin hydrolyzed solutions (T1 and T2) were dissolved in a 0.1% trifluoroacetic acid solution with three parts of acetonitrile. This mixture was prepared with a 1:1 matrix: sample ratio. The matrix was alpha-cyano-4-hydroxycinnamic acid (Sigma Aldrich). The analysis of the permeated solutions was carried out in the range between 700 and 3500 Da. The identified peaks (peptides) in the spectrogram were compared to the molecular weights values obtained by *in-silico* hydrolysis carried out by Fonseca Ayala (2018). After that, the possible bioactivity of these peptides was identified in the BIOPEP database.

## RESULTS AND DISCUSSION

The chymotrypsin hydrolysis and evaluation of the degree of hydrolysis (DH) by pH-stat method were carried out simultaneously, results (Table 1) show that the DH was higher at 4 h (T2) (25.5%) than 2 h (T1) (22%), and these values are according to Tulipano *et al.* (2015) who obtained similar DH values. pH-stat method measures the protons released from the active site of chymotrypsin by their catalytic activity and indirectly determines the cleavage of the peptide bond by the number of amino groups released.

After hydrolysis, the fractions were analyzed by SDS-PAGE gel electrophoresis, the results (Figure 1 and Table 2) show that hydrolyzed fractions had different molecular

**Table 1**. Degree of Hydrolysis after 2 (T1) and 4(T2) h of hydrolysis using chymotrypsin.

| Degree Hydrolysis (%DH) | | |
|---|---|---|
| **T0** | **T1** | **T2** |
| 0 | 22 % | 25.52% |

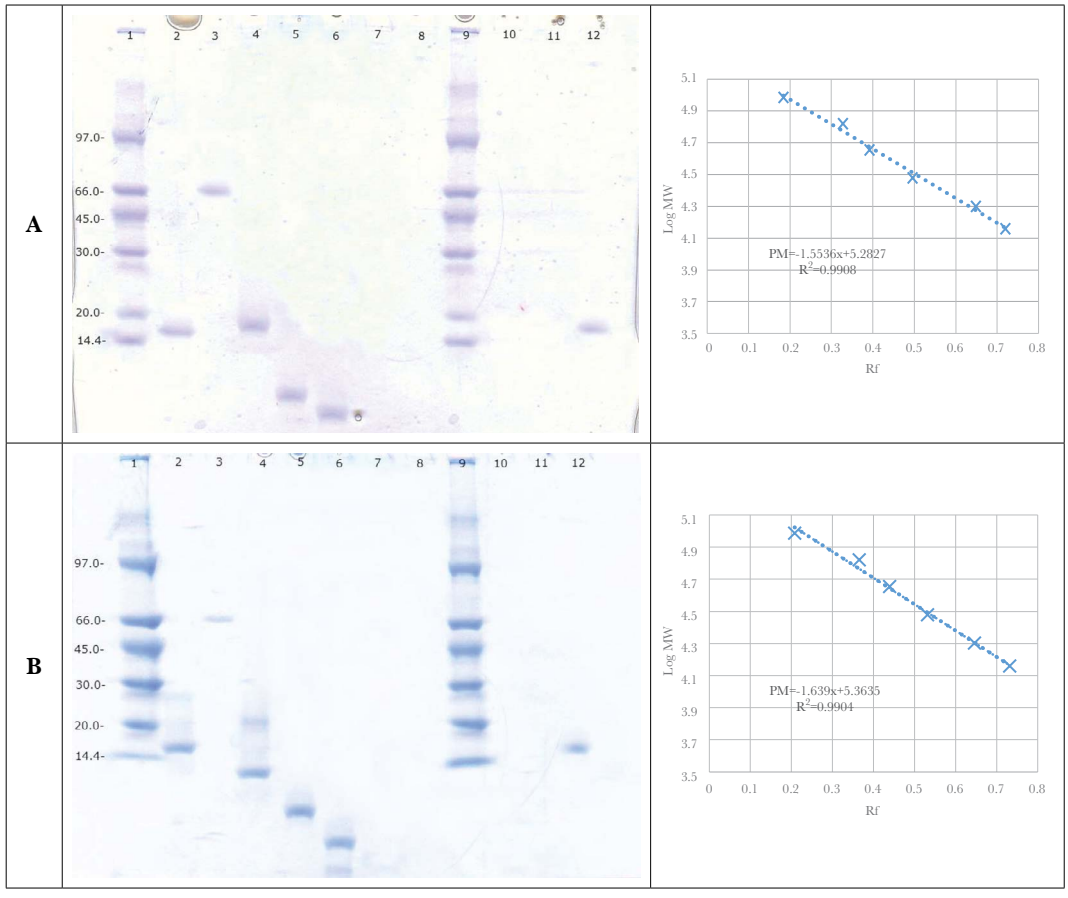


**Figure 1**. Images of electrophoresis gels of different fractions after chymotrypsin hydrolysis (A) and linear regression of the Rf *vs* Log molecular weight (LogMW) (B). 1) Low spectrum standard (97.0-14.4 KDa); 2) $\beta$-Lg (1mg/mL); 3) BSA (1mg/ml); 4) T0; 5) T1; 6) T2; 7) Permeated solution <1 KDa of T1 8); Permeated solution <1 KDa of T2; 9) Low spectrum standard (97.0-14.4 KDa); 10) Permeated solution <1 KDa of T1; 11) Permeated solution <1 KDa of T2; 12) $\beta$-Lg (1mg/L).

**Table 2**. Molecular weight values identified by the linear regression analysis of Rf and Log WM of electrophoresis gel of chymotrypsin hydrolyzed fractions.

| Samples | Gel 1 | | | | Gel 2 | |
|---|---|---|---|---|---|---|
| | Rf | Log PM | PM(Da) | Rf | Log PM | PM(Da) |
| $\beta$-Lg | 0.696 | 4.2014 | 15899.90 | 0.646 | 4.2713 | 18676.69 |
| BSA | 0.328 | 4.7731 | 59308.81 | 0.3 | 4.8563 | 71829.03 |
| T0 | 0.688 | 4.2138 | 16361.50 | 0.712 | 4.1608 | 14481.05 |
| T1 | 0.864 | 3.9404 | 8717.45 | 0.815 | 3.9853 | 9667.18 |
| T2 | 0.92 | 3.8534 | 7134.90 | 0.9 | 3.8423 | 6955.046 |
| T2.b | | | | 0.96 | 3.25 | 4950.365 |

weight values, T0 had values around 16 or 14 KDa similar to the molecular weight of $\beta$-Lg, which molecular weight was 15 KDa (Gel 1) and 18 KDa (Gel 2). T1 had fractions of 8 KDa (Gel 1) and 9 KDa (Gel 2), while T2 had fractions of 7 KDa (Gel 1 and 2) and 5 KDa (Gel 2). Also, a fraction <1 KDa was analyzed, but no bands were presented because of the gel sensibility. These results showed that as the chymotrypsin hydrolysis time increased hydrolyzed fractions had lower molecular weight values.

The portions <1 KDa of both hydrolyzed fractions (T1 and T2) were analyzed in the mass spectrometer in order to know the molecular weight of their peptides, however only the spectrogram of T1 had six identified peaks (Table 3). These molecular weight values were compared with the peptides obtained by *in silico* hydrolysis (Fonseca Ayala, 2018) but only five peptides were comparable with them. Also, three peptides (2, 5 and 6) could have bioactivity as ACE inhibitory activity and Dipeptidyl peptidase IV inhibitory activity and peptides 2, 4 and 6 could have antibacterial activity, because similar sequences were described in the BIOPEP database. However, the *in vitro* analysis of the biological activity of these peptides is necessary to corroborate these results.

**Table 3**. Molecular weight values of T1 identified by mass spectroscopy and its sequence identified by *in silico* hydrolysis.

| No. | Molecular weight values of T1 | Sequence | Sequence identified in BIOPEP database | Bioactivity reported in the BIOPEP database |
|---|---|---|---|---|
| 1 | 620.3 Da | | | |
| 2 | 643.22 Da | DTDYK | VLDTDYK | ACE inhibitor |
| | | | VLVLDTDYK | Antibacterial |
| | | | VLVLDTDYK | Dipeptidyl peptidase IV inhibitor |
| 3 | 656.85 Da | EEQCH | | |
| 4 | 686.74 Da | AASDISL | AASDISLLDAQSAPLR | Antibacterial |
| 5 | 716.80 Da | DAQSAPL | LDAQSAPLR | ACE inhibitor |
| | | | AASDISLLDAQSAPLR | Antibacterial |
| | | | DAQSAPLRVY | ACE inhibitor |
| 6 | 742.70 Da | LKPTPEGDL | LKPTPEGDL | Dipeptidyl peptidase IV inhibitor |
| | | | LKPTPEGDLEIL | Dipeptidyl peptidase IV inhibitor |

## CONCLUSIONS

Chymotrypsin hydrolysis produced fractions of 8 KDa and 9 KDa at 2 h, and 7 KDa and 5 KDa at 4 h of hydrolysis. Mass spectrometry identified six peptides in the hydrolyzed solution (T1, 2h), where five of them were comparable with peptides previously obtained by *in silico* hydrolysis. The sequences of the peptides identified (DTDYK, DAQSAPL and LKPTPEGDL) showed bioactivity according BIOPEP database.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Sheep reproductive management

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**ABSTRACT**

**Objective**: To describe some reproductive management programs that allow increasing the productive efficiency of ewes.

**Design/methodology/approach**: Description of the main hormones and their application in sheep reproductive management protocols. The subjects are reviewed based on academic references as well as on their use in sheep reproductive protocols at the Sheep and Goat Reproduction Laboratory of the Colegio de Postgraduados, Mexico.

**Results**: Hormones, socio-sexual strategies, reproductive protocols and techniques are tools that improve the reproductive efficiency of ewes during the reproductive season and seasonal anestrus.

**Study limitations/implications**: The basic techniques of reproductive management and protocols in sheep are mostly available, however, they still have room for improvement, therefore, multiple efforts involving all participants, such as the primary sector, public and private institutions, are required.

**Findings/conclusions**: Reproductive management is an important pillar for animal production; thus its implementation is fundamental to improve the reproductive and productive efficiency of a herd.

**Keywords**: Reproduction, ewe, reproductive season, seasonal anestrus.

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## INTRODUCTION

Sheep production must begin with knowledge of each breed's productive characteristics to select those that have the best productive yield according to the environmental conditions at each region, particularly of each production unit. Subsequently implementing reproductive management programs, fundamental to increase production efficiency.

Image by Hans Braxmeier at Pixabay

The objective in traditional sheep production systems is to obtain one lambing per year; however, as production systems incorporate technologies, production becomes intensive and management practices incorporate biotechnologies to manipulate reproduction, making it possible to achieve three lambings in two years or five in three.

For example, in an intensive sheep production system, the main objective is to increase their biological efficiency and economic profitability. From the reproductive point of view, they aim to increase the number of born lambs per ewe (prolificacy) and their frequency. This is a complicated task, given that many sheep breeds in the world have a seasonal reproduction rhythm (photoperiod), which is an evolutionary strategy to ensure that the offspring are born at the most adequate time of the year, mainly related to food and temperature. This evolutionary strategy naturally limits ewes' productivity, so it is necessary to implement management strategies to induce reproductive activity during the long days (seasonal anestrus) (Martin *et al.*, 2004) and improve estrus response and ovulation synchronization schemes during the reproductive season.

Thus, reproductive management consists of techniques and strategies that are applied to improve the animal's productivity and should be specific for each breed and environment to optimize the productive performance of the production unit.

**Reproductive Season**

At puberty, ewes acquire ovulating capacity (cyclically producing eggs) and manifesting estrous behavior. The reproductive activity of ewes is defined as a series of physiological events that occur during different periods throughout the year; that is, ewes have a reproductive season, alternating with an anestrus period (Figure 1).

The reproductive season is characterized by the presence of regular estrous cycles, estrous behavior and ovulation (Figure 2); in the northern hemisphere, it occurs from August to February (short days) but varies depending on breed and geographic location (latitude). The sheep estrous cycle has an average 17-day duration. The receptivity period is generally 36 to 40 h; however, it varies between breeds, prolific breeds have a longer estrous duration compared to low prolific breeds, for example, the Romanov breed has a 70 h estrous duration. The females' age is another factor that affects estrus duration; adult females have a longer estrus duration than first-time or pubescent females. The ovulation period is of between 24 and 27 h after the estrus onset (Robertson, 1977).

**Hormones in the estrous cycle**

In the estrous cycle, there is a sequence of cellular events at the ovarian level (follicular development; Figure 2) that are related to endocrine changes. Endocrine control of reproduction is exerted through different hormones secreted by the reproductive axis (hypothalamus, pituitary, ovary and uterus). For example, the ovary secretes progesterone, estradiol and inhibin, among others. The corpus luteum (CL), secretes progesterone to maintain the luteal phase during the estrous cycle or a possible gestation, inhibits new ovulation since progesterone causes an inhibition of the frequency of secretion of GnRH/LH pulses (gonadotropin-releasing hormone/luteinizing hormone; Figure 3).

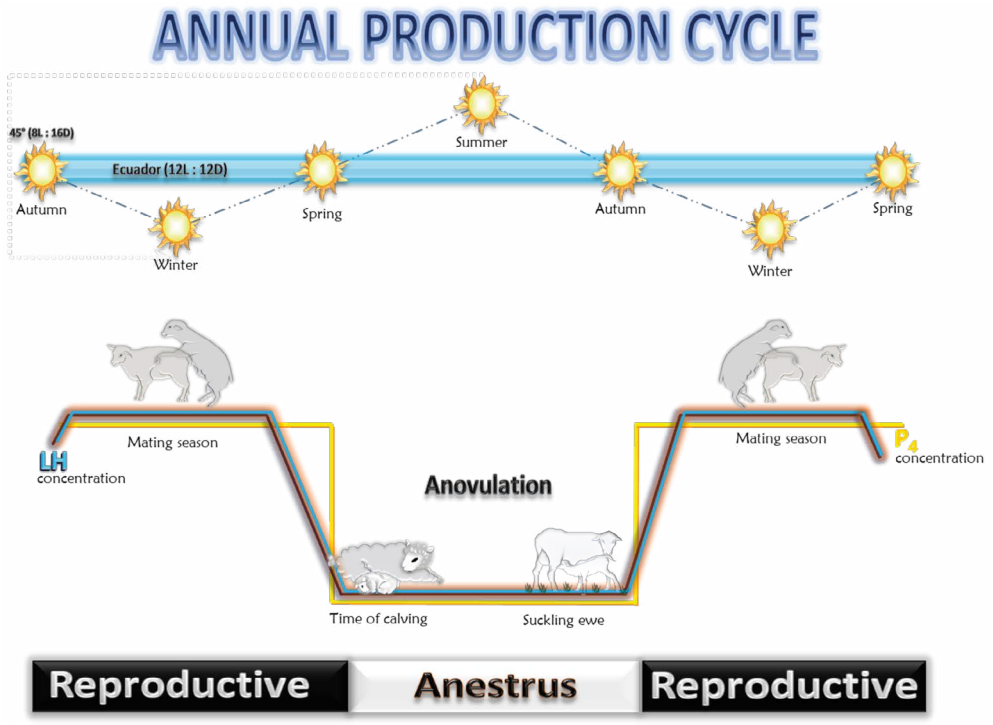


**Figure 1**. Schematic representation of the annual production cycle in sheep, showing the different stages of production in their two seasons (reproduction and anestrus) at a 45° north latitude.

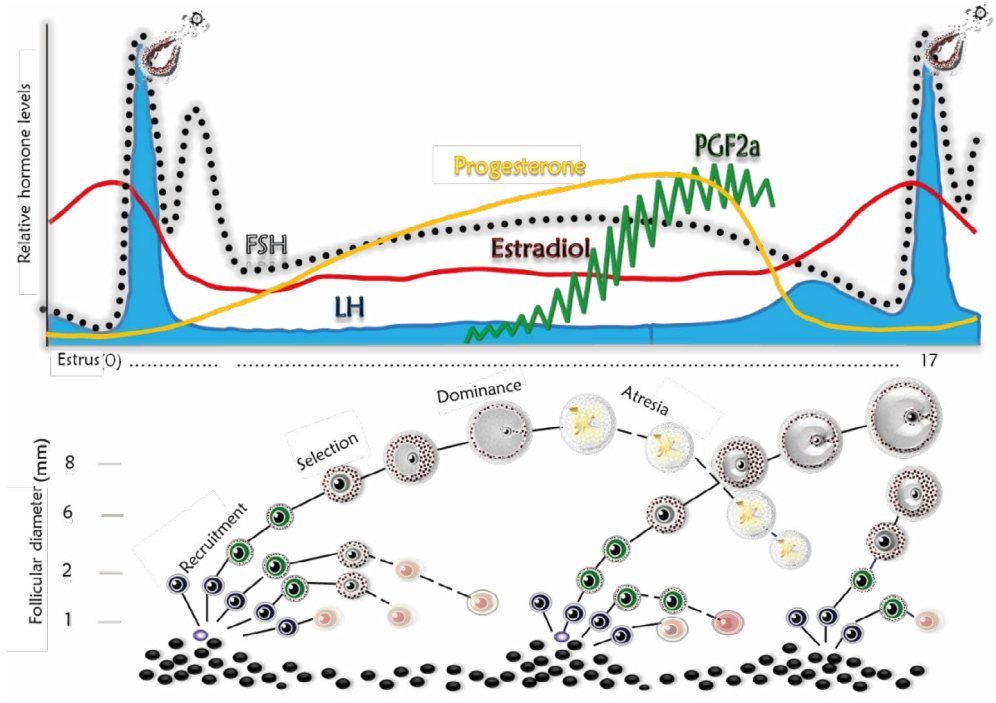


**Figure 2**. Schematic representation of hormonal profiles and follicular development during the estrous cycle of ewes. FSH (follicle stimulating hormone), LH (luteinizing hormone), PGF2a (prostaglandin F2a), Estradiol (17$\beta$E).

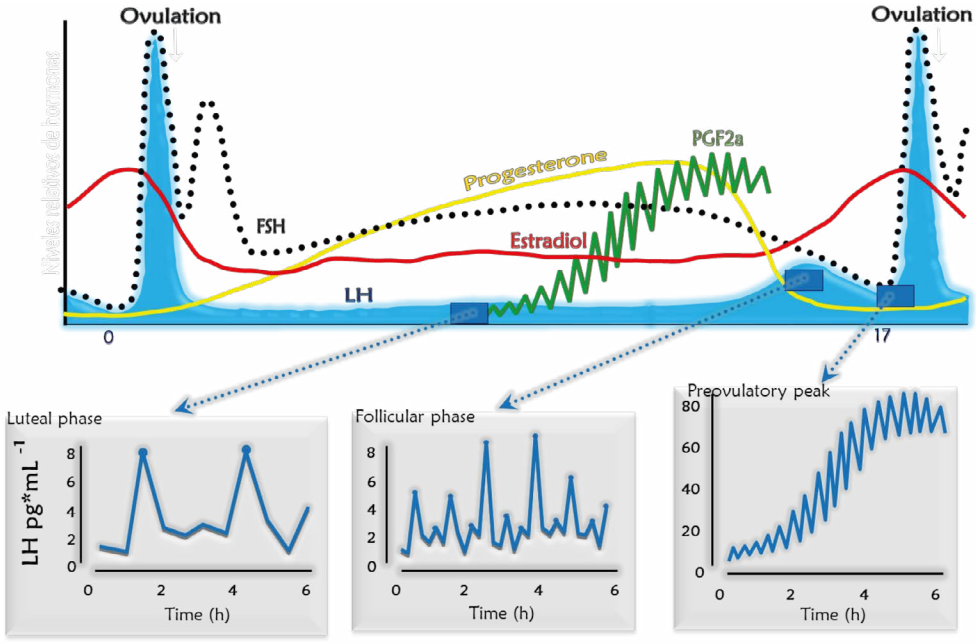


**Figure 3**. Hormonal profiles during the estrous cycle in ewes and LH secretion frequency.

FSH (follicle stimulating hormone), stimulates the follicular development responsible for producing estrogen and inhibin. Estradiol (17$\beta$E) and inhibin cause negative feedback on FSH secretion (Figure 3; Findlay and Clarke, 1987). Prostaglandin $F_2\alpha\alpha$($PGF_2\alpha$) secretion occurs in the endometrium (Inskeep and Murdoch, 1980) and causes LC lysis by inhibition of lipoprotein uptake that limits intracellular cholesterol transport in the luteal cells, which is induced by oxytocin secreted by the LC, and stimulated by estrogens (Bazer, 1989).

The progesterone secretion decrease, and increased secretion of 17$\beta$E, increases the frequency of GnRH pulse secretion, and therefore, increases the frequency of LH secretion up to one pulse every hour, causing the preovulatory LH discharge between 2 and 6 h after the onset of estrus (Figure 3). estrus manifestations occur by an increase in 17$\beta$E levels and a progesterone decrease. Ovulation produces a new CL and the previous endocrine process, which stimulates and controls follicular growth and dynamics at the ovarian level, will repeat (Figure 2) (Goodman and Hodgen, 1983) (Goodman and Hodgen, 1983).

**Seasonal anestrus**

In sheep, seasonal anestrus is characterized by the absence of estrous cycles, estrous behavior and ovulation (Figure 1). It occurs during long days when the duration of melatonin secretion is shorter; in the northern hemisphere, it occurs between February and August but varies depending on geographic location (latitude) and breed (Thiéry *et al*., 2002). During anestrus, estradiol, which is released at basal concentrations, exerts a negative feedback effect at the hypothalamus, specifically in the lateral retrochiasmatic area (nucleus A15), where it acts using dopamine as an intermediate and reduces the GnRH/LH pulses frequency (Gallegos-Sánchez *et al*., 1997) despite no estradiol receptors were found in A15 nucleus. Therefore, the exact physiological mechanism of this event

is not clear. However, there is evidence suggesting that GABA (gamma butyric acid) producing neurons present synaptic connections with dopaminergic neurons in nucleus A15 and one of the effects of GABA is to inhibit dopamine synthesis (Bogusz *et al.*, 2008), therefore, GABA secretion reduction in the A15 nucleus causes dopaminergic neurons stimulation and increases dopamine synthesis, which reduces the frequency of GnRH/LH pulse secretion.

Studies in Mexico, to establish the seasonal reproductive behavior of wool sheep, are limited. There are two methodologies to analyze the reproductive seasonality in sheep. The first, relatively simple, consists of the estrus detection, using males with apron, vasectomized, with a deviated penis, or with a testosterone implant or androgenized females. The second is to take blood samples once or twice a week and determine the progesterone concentration, concentrations greater than or equal to 1 ng $mL^{-1}$ indicate that a female is ovulating; ewes in anestrus have lower than 1 ng $mL^{-1}$ progesterone concentrations (Arroyo *et al.*, 2007).

## Reproductive Management: Induction and Synchronization of Estrus and Ovulation

There are two alternatives to improve production in the reproductive management of a flock; the first relates to the management of the reproductive efficiency of the females (fertility and prolificacy), the second, to the using reproductive biotechnologies through the synchronization and induction of estrus and ovulation, these techniques allow to increase the reproductive efficiency of the sheep.

Manipulation of the timing of estrous onset is essential for good reproductive management; it allows to determine the time of lambing, inducing ovulation during anestrus, estrous synchronizing during the reproductive season, as well as choosing and managing the parental genealogy using techniques such as artificial insemination and embryo transfer.

The ovulation synchronization and induction protocols mentioned below are used in the Sheep and Goat Reproduction Laboratory of the Colegio de Postgraduados, Mexico. Still, there are other drugs and protocols variants in the market that can be used.

### Progestogens application

Protocols for the induction and synchronization of estrus and ovulation are generally based on progestin application. Since the 1950s, various devices have been used to administrate progestogens, which have been evaluated to determine their mode of action and the time at which estrus is initiated. Progesterone or progestogens can be orally, intramuscularly, subcutaneously, or intravaginally administered. Comparative studies in ewes with the most common progestogens: FGA (fluorogestone acetate) and MAP (medroxyprogesterone acetate) have shown similar efficiency.

In ewes, progestogens reach their maximum concentration 48 h after vaginal introduction of the sponge or device and slowly decrease until the time of withdrawal. Currently, they can be maintained for 9 to 12 days in anestrus and cycling ewes (Figure 4), to overcome the duration of a possible CL in the ovary (Fukui *et al.*, 1999). If it is desired

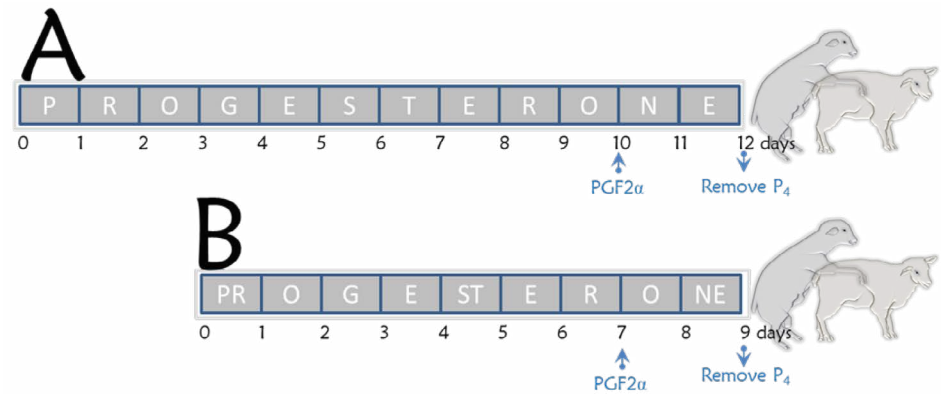


**Figure 4**. Scheme of synchronization and induction of estrus in seasonal anestrus (A) and reproductive season (B), used in the Sheep and Goat Reproduction Laboratory (LaROCa) at Colegio de Postgraduados.

to inseminate at a fixed time, it is necessary to wait for 48 to 60 h after withdrawal of the progestogen, a time that should be adjusted according to the administered drugs.

**Prostaglandin (PGF$_2\alpha$)**

Using PGF$_2\alpha$ or its analogs, as a synchronization tool, is limited to cycling ewes, as they cause CL lysis, susceptible to PGF$_2\alpha$ action five days after estrus. Estrus occurs between 36 and 144 h after PGF$_2\alpha$ administration, almost 100% of cycling ewes respond to 2 prostaglandins injections at a 9 to 12 days interval (Figure 5). For this effect, a 125 mg cloprostenol dose is effective and, in the case of natural PGF$_2\alpha$, a dose of 15 mg is recommended.

**Equine Chorionic Gonadotropin (eCG)**

eCG is a glycoprotein produced in the endometrial ridges of pregnant mares; its activity is similar to that of FSH, but it also has LH action. Its usage is mainly due to its low cost, availability in the market and its long half-life (approximately three days in the ewe), this is due to the amount of sialic acid (Schams *et al.*, 1978).

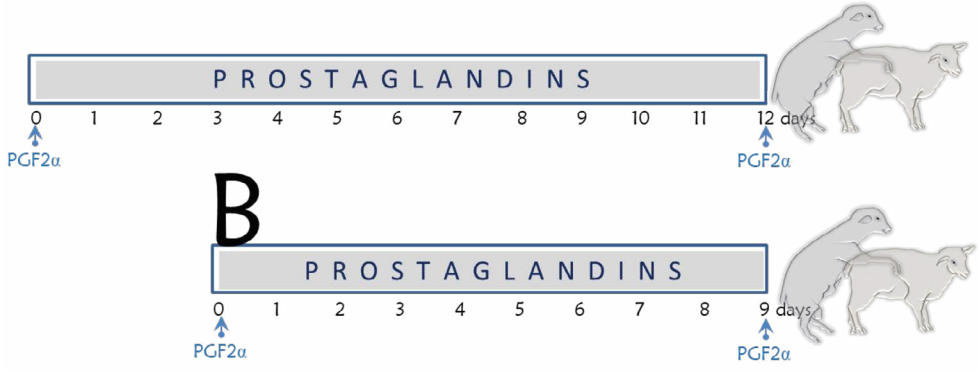


**Figure 5**. Oestrus synchronization scheme applying PGF$_2\alpha$, used in the Sheep and Goat Reproduction Laboratory (LaROCa) of the Colegio de Postgraduados.

eCG is used in several protocols for induction and estrus synchronization and ovulation; it is administered by intramuscular injection two days before or at the time of the removal of the progestogen-impregnated device. It stimulates the recruitment of small follicles, follicular development and increases the ovulatory rate, which allows estrus and ovulation to occur rapidly and homogeneously. The doses of eCG vary from 200 to 600 IU, depending on the breed, time of year, age and physiological state of the females, thus promoting the development of a greater number of follicles, increasing the ovulation rate by up to 30%. The dose of eCG is one of the most important variables in using this type of treatment; for example, the dose in wool-producing ewes is approximately (depending on the weight of the female) 500 IU and in hair ewes, it is 300 to 350 IU (Figure 6). An inadequate dose may cause a failure in the stimulation, doses higher than that recommended cause an undesired increase in the ovulation rate, and, therefore, multiple lambings and the birth of lambs with low survival capacity.

**Male effect**

Currently, and almost in all sheep production systems, the international trend is to reduce to the maximum the drug application to increase the reproductive efficiency of sheep. Therefore, new reproductive management strategies have been implemented, such as socio-sexual effects, mainly the "male effect", which has been shown to work by increasing the frequency of GnRH/LH pulse secretion (Figure 7).

Although there is controversy in the ram's ability to induce ovulation, mainly in highly seasonal breeds, such as the Suffolk, since the sudden introduction of the ram in the middle of the seasonal anestrus does not always produce the expected effect (ovulation); however, it has been shown that the lack of ram stimulation to induce ovulation under these conditions in the Suffolk breed is not due to the absence of response in the increase of pulsatile LH secretion. One of the advantages of using the male effect is that it induces an increase in pulsatile LH secretion and ovulation when females are more sensitive (Hawken *et al.*, 2005). For example, in Mexico, in ewes in anestrus (seasonal or postpartum), the sudden introduction of the male causes the resumption of ovarian activity; from the total number

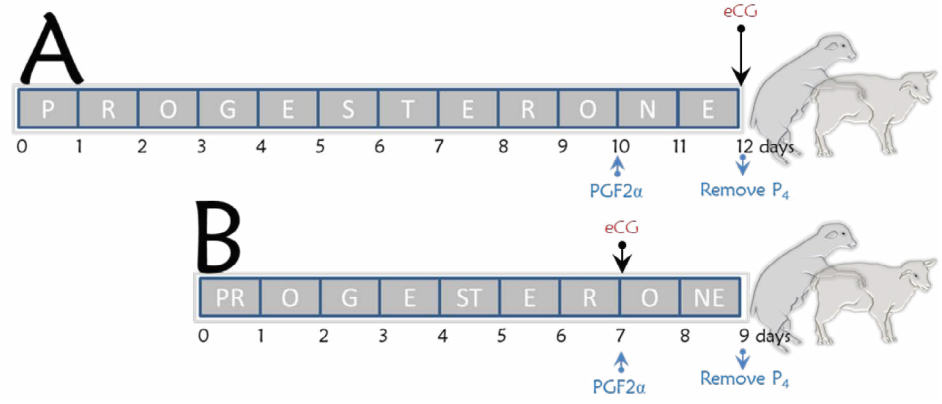


**Figure 6**. Synchronization scheme and induction of estrus using eCG, in seasonal anestrus (A) and reproductive season (B), at the Laboratory of Reproduction of Sheep and Goats (LaROCa) of the Colegio de Postgraduados.

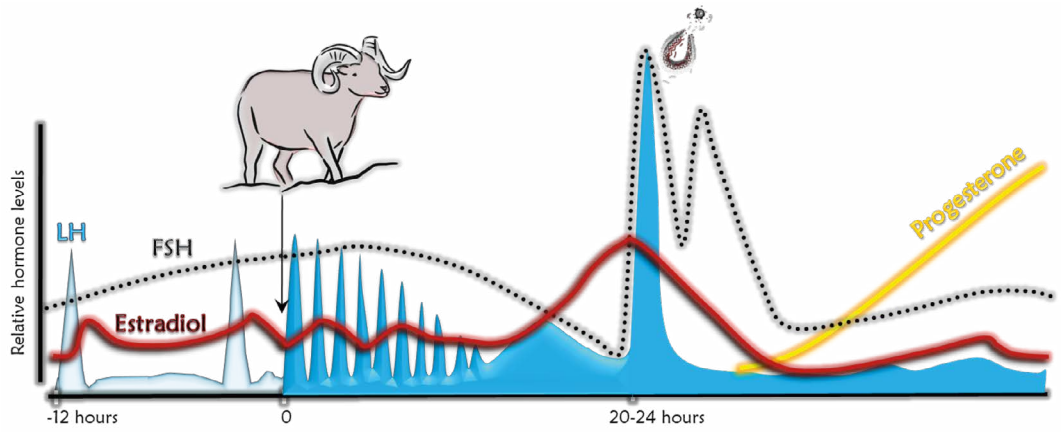


**Figure 7**. Physiological events triggered by the "male effect". Increase of the frequency of secretion of GnRH/LH pulses.

of ewes exposed to the sire, a high percentage ovulate within the first three to five days. It is suggested that to obtain a higher male effect response (stimulation of anestrus females), males should be isolated (not in contact with females) because the male should represent a "novel" stimulus.

**Pregnancy diagnosis**

The productive yields of flocks can be improved with an early pregnancy diagnosis, since it reduces the interval between lambings and facilitates the homogeneous management of animals, for example, ewes can be managed by groups, a new estrus can be induced in females that did not become pregnant and a feed supplement can be offered to the pregnant ones.

Pregnancy diagnosis is mainly done with ultrasound and a transabdominal probe; it is also possible to assess the number of embryos and the viability of the pregnancy. The diagnosis of gestation can be made from 24 days after insemination on (natural or artificial), although it is better to diagnose gestation between day 30 and 35, since the embryo presence and especially its cardiac activity increases the efficiency of the diagnosis up to 90%, reducing errors due to confusion of embryonic vesicles with intestinal loops, cross-sections of blood vessels, a localized accumulation of liquid inside the uterine horns or with possible uterine pathologies.

The assessment of the number of embryos and the separation of single, double, or multiple gestations is very useful in sheep farming. This assessment should be done by a complete sweep of the maternal genital tract, differentiating each embryo or fetus individually in the different sections of each uterine horn. The assessment of the number of embryos should be done between 50 and 55 days, where about 85% is obtained for single births and 100% for multiple births. The main errors occur by underestimation if one of the fetuses is not detected or by overestimation if the same fetus is considered as two different ones when two embryos are not simultaneously displayed on the screen (Dawson *et al.*, 1994).

## CONCLUSIONS

The techniques for artificial insemination and synchronization and induction of estrus, socio-sexual effects (male effect) and gestation diagnosis are tools that improve the reproductive efficiency of sheep, so their application is essential. It is important to mention that there are other techniques that in addition to the above mentioned, can improve sheep production, such as: *in vitro* embryo production, transgenesis and cloning.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Administration of royal jelly in estrus synchronization protocols for wool and hair sheep

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**ABSTRACT**

**Objective**: To assess the most relevant results on the usage of royal jelly in reproductive protocols of wool and hair sheep.

**Design/methodology/approach**: A review of studies referenced and published in scientific databases regard the livestock sector.

**Results**: In ewes, administration of "royal jelly" in addition to reproductive management protocols improves the response to estrus synchronization, time of onset and duration of estrus, number of large follicles, ovulatory rate and gestation rate.

**Study limitations/implications**: Royal jelly is a substance with beneficial effects on reproductive variables in ewes; however, the cost may be a limitation for its incorporation in synchronization protocols. Additionally, it is necessary to clarify the active metabolites that exert the action and the most effective route of administration.

**Findings/conclusions**: Royal jelly can be an alternative incorporated to estrus synchronization programs in ewes to substitute some hormones without decreasing reproductive variables.

**Key words**: *Apis mellifera*, reproduction, reproductive management, sheep.

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## INTRODUCTION

In small ruminant reproductive management, it is possible to reduce the use of exogenous hormones by optimizing the animal's response to the environment and nutrition. These management strategies propose using knowledge and available resources (Delgadillo and Martin, 2015). In the reproductive management of sheep, it is possible to use targeted



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or strategic nutrition (Martin, 2009), with supplements containing bioactive compounds (Delgadillo and Martin, 2015) or modifying metabolic hormone concentrations (Scaramuzzi *et al*., 2006) to improve reproductive performance (Gutiérrez *et al*., 2011). In this regard, royal jelly (RJ), produced by the cephalic glands of worker bees (*Apis mellifera*), can improve reproductive response in different species (Pavel *et al*., 2011).

With the use of RJ in the reproductive management of wool ewes, positive effects are reported for reproductive variables such as estrus incidence, time of estrus onset, as well as an improvement in the percentage of gestation (Kridli *et al*., 2003; Husein and Haddad, 2006). Royal jelly administration in hair sheep at different physiological stages shows similar results. The objective of this review is to present some of the results obtained with the application of RJ in the reproductive management of wool and hair sheep.

**Royal jelly in estrus synchronization protocols in wool ewes**

Administration of RJ (250 mg $d^{-1}$) orally or intramuscularly to Awassi ewes for 12 d (during the estrus synchronization protocol), shortened the time of estrus onset (31±2.6 h), compared to ewes in the control group (45±5.4 h), and increased the number of ewes responding to synchronization to 80%, to 40% in the control group (Husein and Kridli, 2002). Kridli *et al*. (2003) report that administration of 250 g RJ $d^{-1}$, orally for 12 d, increased the percentage of ewes with signs of estrus compared to the control group (80 *vs*. 40%), in addition, the percentage of pregnant ewes also improved with RJ administration (60 *vs*. 20%).

The application of three different doses of RJ (250, 500, 750 mg $d^{-1}$) orally, during an estrus synchronization protocol (12 d), showed that there were no differences among the three different doses for the variable estrus onset. The 500 and 750 mg RJ doses improved the estrus onset (49.6±7 and 49.0±8 h), compared to ewes that did not receive RJ (59.6±7 h). The results obtained with the 500 and 750 mg RJ doses compared to the administration of a 600 IU dose of eCG showed no differences for the onset of estrus, percentage of gestation and prolificacy variables (Kridli and Al-Khetib, 2006).

When comparing the response of an estrus synchronization protocol in Awassi ewes, with the application of 12 doses of RJ of 400 mg $d^{-1}$ (orally), or the application of 500 IU of eCG, similar results were reported between both treatments for the response to synchronization, fertility or prolificacy variables, so it is proposed that RJ is an alternative for the use of eCG in wool ewes (Husein and Haddad, 2006).

Mostafa *et al*. (2008) found that RJ application for 21 d (RJ treatment initiated at 15 days postpartum) reduced days between lambing and first estrus (33.30±0.57 *vs*. 44.80±0.4 d) in Ossimi ewes. Estrus duration increased by 10 h on average in the RJ-treated ewes (41±0.42 *vs*. 31±0.31 h), possibly because RJ increases the number of growing follicles, as well as plasma estradiol levels, necessary for the ewe to show signs of estrus.

**Royal jelly in estrus synchronization protocols in hair ewes**

On a reproductive management protocol in which eCG or RJ was used in hair ewes, a shorter time to the onset of estrus was found in the eCG treated group (21.10±2.34 h), compared to the group treated with RJ (30.95±1.29 h). Ewes only synchronized with

progesterone showed signs of estrus at a longer time (36.78±2.88 h). The RJ or eCG administration did not modify the ovulation rate and percentage of gestation variables (Pérez *et al.*, 2014).

When researching whether RJ has any direct effect on follicular development, it is reported that the administration of three applications of 500 mg RJ $d^{-1}$, intramuscularly, does not modify the number of small, medium and large follicles (similar between control and RJ-treated ewes) (Figure 1). Estrus duration was longer in RJ-treated ewes (54.7±2.32 *vs.* 47.5±3.47 h) compared to those in the control group (Pérez-Ruiz *et al.*, 2015). The effect on estrus duration coincides with that reported by Mostafa *et al.* (2008) in Ossimi ewes. However, when RJ treatment is increased to seven days (500 mg $d^{-1}$) and administered intravenously, it is possible to increase the number of large follicles (>4 mm), shorten the time to estrus onset (49.08±2.09 *vs.* 54.08±1.35 h) and increase the ovulation rate (2.83±0.16 *vs.* 1.83±0.16), in Pelibuey ewes (Sosa-Pérez *et al.*, 2017).

In a reproductive management protocol excluding hormones, the administration of 1.0 g RJ on 30, 37, 44 and 51 postpartum days did not shorten the resumption of ovarian activity (evaluated by ultrasonography with the presence of corpora lutea in the ovaries) in Pelibuey ewes (Pérez-Ruiz *et al.*, 2018). In this research, in addition to the application of RJ, the suckling control strategy (30 min twice per day) was evaluated. Upon weaning, socio-sexual stimulation was initiated with the male every 12 h, and the response to estrus was evaluated, which was similar among all ewes. For the variable estrus onset, the RJ did not modify the response, but the suckling control did have a significant effect; in the continuous suckling group, estrus started in less time (3.5±.9 *vs.* 7.5±1.3 $d^{-1}$) than in the controlled suckling group. Estrus duration was significantly longer in RJ-treated ewes for both suckling modalities. The presentation of silent estrus may be a common problem in postpartum ewes, so the effect of RJ on increasing estrus duration could improve reproductive efficiency by promoting the expression of estrous behavior.

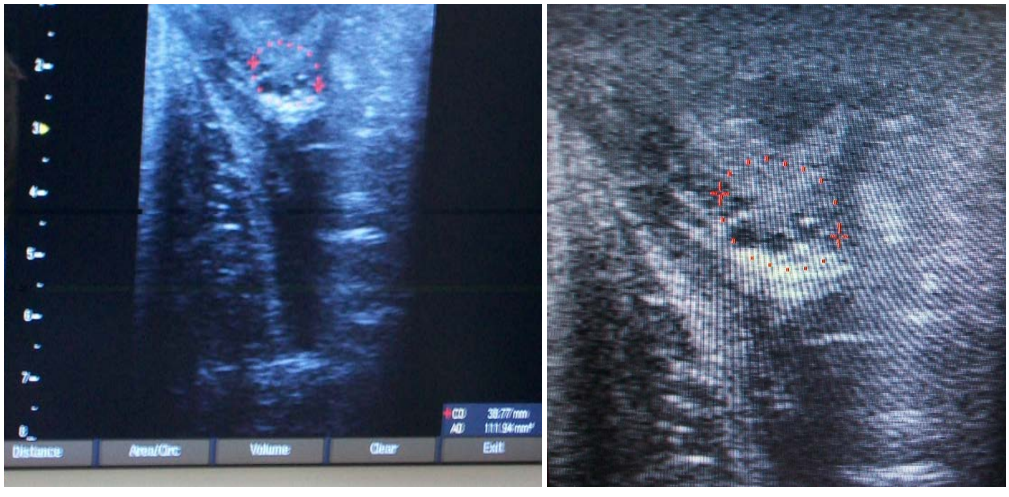


**Figure 1**. Large follicles (<5 mm) in hair sheep synchronized with CIDR® and royal jelly application.

## CONCLUSIONS

The results obtained by the administration of royal jelly in wool and hair sheep suggest a positive effect of this substance on reproductive variables such as follicular growth, estrus onset, estrus duration, ovulation rate and gestation, so it can be a natural alternative to the use of exogenous hormones, yet more research is needed to identify the metabolites that exert the action, as well as the most effective route of administration.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Evaluation of the antioxidant activity from bovine serum albumin protein fractions

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## ABSTRACT

**Objective**: Evaluate the antioxidant activity of protein fractions obtained from (bovine serum albumin) BSA protein hydrolysates.

**Design/methodology/approach**: Bioinformatics tools, such as the NCBI database, were used to search for primary sequences of BSA proteins. The methodology included a prediction of peptides with antioxidant activity through various bioinformatics servers. The antioxidant activity was determined by different methods. Statistical analysis was performed to evaluate possible significant differences using the Student Newman Keulls test for group comparison.

**Results**: Through in silica hydrolysis the following peptides were found: valine-alanine-phenylalanine (VAF), lysine-tryptophan (KW), phenylalanine-tyrosine (FY), alanine-proline (AP), among others that may have antioxidant activity. The results showed that the fraction <1 kDa hydrolyzed with chymotrypsin, this fraction showed 84% copper chelation, 61% iron chelation, while 75% inhibition of the DPPH radical. In the case of the fraction <1 kDa hydrolyzed with pepsin, it only showed 16% iron chelation, while in the other methods no value was detected.

**Study limitations/implications**: The enzyme used for enzymatic hydrolysis generates low degrees of hydrolysis and generates oligopeptide dipeptides that may not be as like some of the tested methods, in addition to the protein concentration in the fraction <1 kDa with pepsin it had very low values that could not be detected by some antioxidant methods.

**Findings/conclusions**: The antioxidant activity of the <1 kDa fraction obtained with chymotrypsin showed greater antioxidant and chelating activity, compared to the <1 kDa fraction obtained with pepsin. However, at the concentration of 2% and 5% fluctuations are observed in both fractions, because probably the composition of amino acids that is present in both fractions determines the activity in each of the tested methods.

**Keywords**: BSA, fractions, antioxidants.

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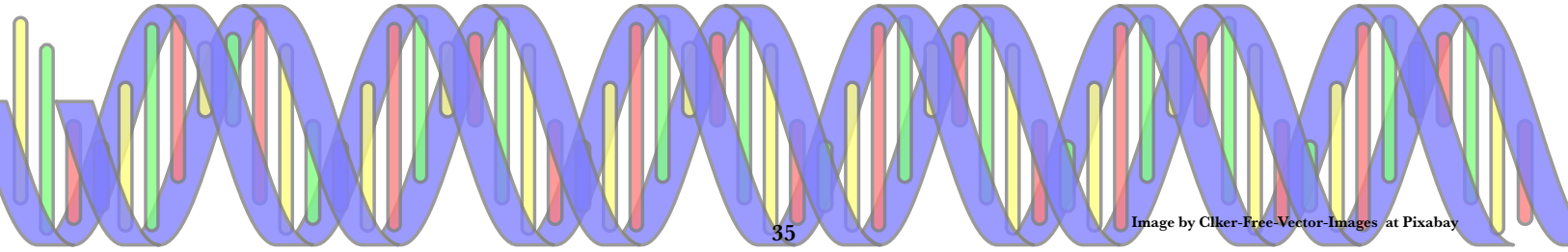
## INTRODUCTION

The increasing rise in common lifestyle-related diseases, such as cardiovascular disorders, hypertension, atherosclerosis, and diabetes, among others, has become a serious global concern. Recent advances in the field of proteomics have promoted the use



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of protein hydrolysates or bioactive peptides as a therapeutic alternative to mitigate the risk factors related to these diseases. In recent years, numerous bioactive peptides from various food sources have been isolated and characterized, which have presented various biological activities, such as antihypertensive, antioxidant, opioid, antithrombotic, immunomodulatory, antidiabetic, hyploagglutinating, hypocholesterolemic and antimicrobial, mainly. Although all are important, antioxidant activity has been one of the most studied and widely related to chronic degenerative disorders, which is based on the ability to capture free radicals, inhibition of lipid peroxidation and properties of metal ion chelation (Bhat *et al.*, 2015; Choi *et al.*, 2017; Dhaval *et al.*, 2016; Lagrange & Clark, 2019).

Proteins are high molecular weight biomolecules that represent an important source of energy and essential amino acids necessary for growth and maintenance of physiological functions. Currently, there is great interest in the identification and characterization of bioactive peptides obtained from the hydrolysis of proteins of plant and/or animal origin. The bioactivity presented by these peptides depends mainly on the composition (structure, hydrophobicity, and charge) and amino acid sequence (Saadi *et al.*, 2015). An alternative to produce bioactive peptides is enzymatic hydrolysis that under controlled conditions *in vitro* or *in vivo* and by using specific proteolytic enzymes, the desired peptides can be isolated from a complex mixture made up of amino acids, oligopeptides and peptides of different lengths (Embiriekah *et al.*, 2018; Sarmadi & Ismail, 2010). It is known that the heat treatment involved in the inactivation of the enzymes can have an important effect on the bioactivity of the peptides (O'Loughlin *et al.*, 2014). Bovine serum represents a valuable source of high-quality protein. In addition to their balanced amino acid content, bovine serum proteins have been reported to be a rich source of bioactive peptides that exert important biological and physiological effects (Ballatore *et al.*, 2020; Hernández-Ledesma *et al.*, 2014; Morris & FitzGerald, 2008) show the antioxidant and cytoprotective activity of peptides (≤3 kDa) obtained from the hydrolysis of a serum protein concentrate (WPC 35). Pasiakos, (2015) demonstrated that whey proteins have advantageous digestive and absorbent properties that facilitate the rapid, but sustained delivery of essential amino acids to the body. Bovine serum proteins are considered proteins of high biological value according to the DIAAS (Digestible Indispensable Amino Acid Score) method developed by the Food and Agriculture Organization. The main proteins that make up bovine serum are $\alpha$-lactalbumin ($\alpha$-La), $\beta$-lactoglobulin ($\beta$-Lg), immunoglobulins (Ig), albumin (BSA), lactoferrin (LF) and lactoperoxidase (LP). The presence of leucine, isoleucine, tyrosine, methionine, proline and valine in the structure of these proteins contributes greatly to their biological properties (Tovar-Jiménez *et al.*, 2017).

One of the proteins present in bovine serum that has been widely used as a standard in various scientific studies is bovine serum albumin (BSA), due to its simple structure, good stability, low cost, easy availability, and versatile ligand-binding properties (Sengupta *et al.*, 2018). BSA is a water-soluble protein that occurs naturally in milk, has a molecular weight of 66.5 kDa, an isoelectric point of 4.7 in water at 25 °C, and is made up of a polypeptide chain of 583 amino acids. It has three intrinsic fluorophores: tyrosine, tryptophan (with two residues: Trp-134 and Trp-213) and phenylalanine. The Trp-134 residue is known to be

found on the outer surface of the protein, while Trp-213 is located within the hydrophobic structure. BSA, as well as human serum albumin (HSA, with 585 amino acid residues) are non-glycosylated globular transport proteins present in serum that play an important role in the circulatory system and in some specific functions of the organism. Both proteins have a structural homology of 75.6% in their three domains and a degree of similarity of 76% in their tertiary structures (Carter *et al.*, 1994; Majorek *et al.*, 2012; Simonelli & Arancibia, 2015).

## MATERIALS AND METHODS

### Materials

Bovine serum albumin (BSA, B4287, ≥98%), pepsin from porcine gastric mucosa (P7000, powder, ≥250 units/mg solid), and $\alpha$-chymotrypsin from bovine pancreas (C4129 Type II, lyophilized powder, ≥40 units/mg protein) were obtained from Sigma Aldrich Co. LLC, St. Louis, MO, USA. All other reagents were of analytical grade.

### Enzymatic hydrolysis of BSA

Hydrolysis was carried out according to José Goulart *et al.*, (2005), Adjonu *et al.*, (2014) and Fernández Alonso, (2015) with some modifications. Briefly, 2 g of BSA to be hydrolisated with chymotrypsin, was dissolved in 40 mL of a 5% (w/v) sodium phosphate buffer solution (pH 7.8, 0.01M), while 2 g the same protein to be treated with pepsin was mixed with 40 mL of a 0.001M citrate phosphate buffer solution (pH 2). In both cases, and before starting the hydrolysis reaction, the samples without enzymes were agitated at 125 rpm (MaxQ4000, Barnstead Lab-Line, Co. USA) during 30 min at 50 °C (chymotrypsin) and 37 °C (pepsin). Afterwards, the enzymes were added in a ratio of 1:10 enzyme/substrate. The hydrolysis time was 4 h and the pH was mantained constant by the addition of NaOH (0.1M) or HCl (0.1M) for chymotrypsin and pepsin respectively. At the end of the reaction, the samples were heated at 90 °C during 3 min, then were cooled at 25 °C and were stored at −18 °C until their analysis. The pH of the hydrolyzate of pepsine was modified from 2 to 6 before the ultrafiltration because of the membrane type used.

### Ultrafiltration of the hydrolizates

Peptides were separated using a 10 mL cell with a filtration area of 4.1 $cm^2$ (Amicon 8010 Millipore, Bedford, MA, USA) and molecular cutting membranes (Ultracel®, Co. Millipore, U.S.A.) of 5, 3 and 1 kDa at a pressure of 25 psi ($N_2$) and at 25 °C following the manufacturer procedure. In all cases, the membranes were hydrated in distilled water during 2 h and were washed in the buffer solution, phosphate buffer pH 7 for chymotrypsine hydrolyzates and citrates buffer pH 2 for pepsine hydrolyzates, before their use. The filtration was done in three steps: first the hydrolizate was passed through 5 KDa membrane; this permeate was passed through 3kDa membrane and finally, the last permate was passed through 1KDa membrane. All the filtrated fractions were store at −18 °C until their analysis.

**In silico analysis**

Seed proteins sequences reported for BSA were obtained from the UniProt database (http://www.uniprot.org/) and NCBI database (https://www.ncbi.nlm.nih.gov/) with number P02769.4 for BSA.

While the development of proteolytic hydrolysis was carried out in silico, using the server for prediction of Biopep analysis. This tool was used to simulate hydrolysis to predict the possible enzymatic cleavage peptides that are released by the enzyme pepsin and chymotrypsin with which the BSA cleavages were obtained respectively. (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) (Minkiewicz *et al*., 2019).

**Antioxidant activity**

*Copper chelating activity*

The ability of BSA fractions to chelate $Cu^{2+}$ was determined according to Saiga *et al*., (2003). A volume of 200 $\mu$L of a 50mM sodium acetate buffer pH 6, 6 $\mu$L of a 4 mM pyrocatechol violet solution and 12 $\mu$L of $CuS0_4.5H_2O$ prepared a 1mg/ml, were added to 50 $\mu$L of fractions (3 mg protein /mL). Ethylenediaminetetraacetic acid (EDTA) was used as the reference molecule (1 mg/ml). Absorbance at 632 nm was measured using a microplate reader (Multiskan Spectrum, ThermoLab Systems, MA, USA). Copper chelating activity was calculated for iron, as described above.

$$\%\ Cooper\ chelating = \frac{\left(Abs_{blank} - Abs_{sample}\right)}{Abs_{blank}} \times 100$$

Where: $Abs_{blank}$=the absorbance observed for the blank, and $Abs_{sample}$=the absorbance observed for the sample.

*Iron Chelating Activity*

The ability of BSA fractions to chelate $Fe^{2+}$ was measured by the method of Saiga, Tanabe, & Nishimura, (2003). A volume of 30 $\mu$L of a 2 mM ferrous chloride solution and 150 $\mu$L of 100 mM acetate buffer at pH 4.9 were added to 50 $\mu$L of BSA fractions (3 mg protein/mL). After 30 min of incubation at room temperature, the reaction was inhibited by the addition of 12.5 $\mu$L of a 40 mM ferrozine solution. Ethylenediaminetetraacetic acid (EDTA) was used as the reference molecule (1 mg/ml). After 10 min, the absorbance was measured at 560 nm by using a microplate reader (Multiskan Spectrum, Thermo Lab Systems, MA, USA). A decrease in absorbance corresponds to an increase in iron chelating capacity. The ability of the samples to chelate ferrous ion $Fe^{2+}$ is defined as follows:

$$\%\ Iron\ chelating = \frac{\left(Abs_{blank} - Abs_{sample}\right)}{Abs_{blank}} \times 100$$

Where: $Abs_{blank}$=the absorbance observed for the blank, and $Abs_{sample}$=the absorbance observed for the sample.

*Evaluation of radical scavenging activity of the hydroxyl* $(-OH)$

The hydroxyl radical scavenging capacity was measured using modified method as described previously de Avellar *et al.*, (2004). 50 $\mu$L of a 0.75 mM 1, 10-phenanthroline and 25 $\mu$L of a 0.75 mM $FeSO_4$, 150 $\mu$L of phosphate buffer pH 7.4 and 50 $\mu$L of $H_2O_2$ 0.1% (v/v %) were added to 50 $\mu$L of fractions (3 mg protein/mL). Ascorbic acid was used as a positive control (1 mg/mL). The mixture was incubated at 37 °C for 60 min, and the absorbance was measured at 536 nm in a microplate reader (Multiskan Spectrum, ThermoLab Systems). The % hydroxyl radical scavenging activity were determined using the following equation:

$$\%\ OH\ radical\ scavenging\ activity = \frac{\left(A_s - A_1\right)}{\left(A_0 - A_1\right)} \times 100$$

where: $A_s$ it is the absorbance of the sample; $A_1$, is the absorbance of the control (distilled water instead of the sample); and $A_0$, is the absorbance of the blank solution containing 1, 10- phenantroline and $FeSO_4$.

*Evaluation of the scavenging activity of the superoxide radical* $(O_2^-)$

Scavenging activity of the superoxide radical $(O_2^-)$ for the BSA fractions was generated from the pyrogallol autooxidation reaction according to the method of Udenigwe and Aluko, (2012). 50 $\mu$L of BSA fractions (3 mg/mL) were mixed with 80 $\mu$L of 50 mM Tris-HCl-EDTA buffer pH 8.3 in a 96-well microplate followed by the addition of 40 $\mu$L of 1.5 mM pyrogallol dissolved in 10 mM HCL. The scavenging activity of the superoxide radical $(O_2^-)$ induced by pyrogallol was measured as the absorbance at 420 nm at room temperature. Scavenging activity of the superoxide radical $(O_2^-)$ was calculated using the following equation:

$$\%\ Scavenging\ activity\ of\ O_2^- = \frac{\left(Abs_{blank} - Abs_{sample}\right)}{Abs_{blank}} \times 100$$

The Tris-HCl buffer solution was used as blank ($Abs_{blank}$) and the absorbance of the samples with pyrogallol and Tris-HCl-EDTA buffer ($Abs_{sample}$).

## RESULTS AND DISCUSSION

The peptide fractions obtained from the protein hydrolysates obtained with pepsin and chymotrypsin, presented a protein content that ranged between 14.39-6.34 mg/mL and 106-18.86 mg/mL respectively. The protein content decreased proportionally to the

molecular weight cutoff of the membranes used in the separation of the protein hydrolyzed fractions, observing the highest amount of protein in the 5-10kDa fraction and the lowest in the <1kDa fraction.

To determine the percentage of amino acids according to their polarity, the number of dipeptides, tripeptides, and free amino acids, as well as the fragments that probably may have some biological activity, the in-silico hydrolysis of bovine serum albumin (BSA) in the BIOPEP database, using pepsin and chymotrypsin as proteolytic enzymes individually. In Figure 1 the analysis showed that in the case of hydrolysis with pepsin, a higher proportion of peptides with more than 5 amino acids is obtained, while only 11 free amino acids and 12 tripeptides are observed, of which only one VAF is the showing angiotensin converting enzyme inhibitory activity. In the case of hydrolysis with chymotrypsin, a greater number of free amino acids and dipeptides are obtained that show antihypertensive and antioxidant activity. It should be mentioned that the number of peptides and their length will depend on the activity and specificity of the enzyme. In the case of pepsin, it acts on peptide bonds of amino acids with hydrophobic side chains, while chymotrypsin acts on residues of aromatic amino acids and Leu (Damodaran & Parkin, 2017).

## Antioxidant activity

*Copper quelating activity*

The hydrolysate obtained from hydrolysis with chymotrypsin was subjected to ultrafiltration to obtain peptides of different molecular weight ranging from fractions

***In silico* hydrolysis with pepsin**

MKWVTF - ISL - L - L - L - F - SSAYSRGVF - RRDTHKSEIAHRF - KDL - GEEHF - KGL - VL - IAF - SQYL - QQCPF - DEHVKL - VNEL - TEF - AKTCVADESHAGCEKSL - HTL - F - GDEL - CKVASL - RETYGDMADCCEKQEPERNECF - L - SHKDDSPDL - PKKPDPNTL - CDEF - KADEKKF - WGKYL - YEIARRHPYF - YAPEL - L - YYANKYNGVF - QECCQAEDKGACL - L - PKIETMREKVL - TSSARQRL - RCASIQKF - GERAL - KAWSVARL - SQKF - PKAEF - VEVTKL - VTDL - TKVHKECCHGDL - L - ECADDRADL - AKYICDNQDTISSKL - KECCDKPL - L - EKSHCIAEVEKDAIPENL - PPL - TADF - AEDKDVCKNYQEAKDAF - L - GSF - L - YEYSRRHPEYAVSVL - L - RL - AKEYEATL - EECCAKDDPHACYSTVF - DKL - KHL - VDEPQNL - IKQNCDQF - EKL - GEYGF - QNAL - IVRYTRKVPQVSTPTL - VEVSRSL - GKVGTRCCTKPESERMPCTEDYL - SL - **IL** - NRL - CVL - HEKTPVSEKVTKCCTESL - VNRRPCF - SAL - TPDETYVPKAF - DEKL - F - **TF** - HADICTL - PDTEKQIKKQTAL - VEL - L - KHKPKATEEQTVMENF - **VAF** - VDKCCAADDKEACF - AVEGPKL - VVSTQTAL - A

***In silico* hydrolysis with chymotrypsin**

M - **KW** - VTFISL - L - L - L - FSSAY - SRGVFRRDTHKSE - IAHRFKDL - **GE** - E - HFKGL - VL - IAFSQ - Y - L - Q - Q - CP - FDE - HVKL - VN - E - L - TE - FAKTCVADE - SHAGCE - KSL - HTL - FGDE - L - CKVASL - RE - TY - GDM - ADCCE - KQ - E - P - E - RN - E - CFL - SHKDDSP - DL - P - KKP - DP - N - TL - CDE - FKADE - KKFW - GKY - L - Y - E - IARRHP - Y - **FY** - **AP** - E - L - L - Y - Y - AN - **KY** - N - GVFQ - E - CCQ - AE - DKGACL - L - P - KIE - TM - RE - KVL - TSSARQ - RL - RCASIQ - KFGE - RAL - KAW - SVARL - SQ - KFP - KAE - FVE - VTKL - VTDL - TKVHKE - CCHGDL - L - E - CADDRADL - AKY - ICDN - Q - DTISSKL - **KE** - CCDKP - L - L - E - KSHCIAE - **VE** - KDAIP - E - N - L - P - P - L - TADFAE - DKDVCKN - Y - Q - E - AKDAFL - GSFL - Y - E - Y - SRRHP - E - Y - AVSVL - L - RL - AKE - Y - E - ATL - E - E - CCAKDDP - HACY - STVFDKL - KHL - VDE - P - Q - N - L - IKQ - N - CDQ - FE - KL - **GE** - Y - GFQ - N - AL - IVRY - TRKVP - Q - VSTP - TL - **VE** - VSRSL - GKVGTRCCTKP - E - SE - RM - P - CTE - **DY** - L - SL - **IL** - N - RL - CVL - HE - KTP - VSE - KVTKCCTE - SL - VN - RRP - CFSAL - **TP** - DE - TY - **VP** - KAFDE - KL - FTFHADICTL - P - DTE - KQ - IKKQ - TAL - **VE** - L - L - KHKP - KATE - E - Q - TVM - E - N - FVAFVDKCCAADDKE - ACFAVE - GP - KL - VVSTQ - TAL - A

**Figure 1**. In silico hydrolysis of BSA with pepsin and chymotrypsin individually. The fragments in bold are those that show antioxidant activity in each of the hydrolysis.

>10 kDa, <10 kDa, <5 kDa, <3 kDa and <1 kDa. Once the fractions were obtained, the copper chelation test was carried out (Figure 2a), where it was observed that the 5, 3 and 1 kDa fractions showed the highest chelation of this metal, which allows to elucidate that these peptides can trap said metal and with this stop oxidation reactions in the body. The percentage of chelation of these last three fractions is greater than 80%, so it gives a relationship with the amount of short chain peptides obtained in enzymatic hydrolysis. However, the fractions greater than 5kDa showed less chelating activity, this inhibition being around 20%. Also, the fraction <10 KDa showed the lowest activity with only 7% inhibition. On the other hand, the fractions obtained from the pepsin hydrolysate (Figure 2b), yielded values below 30% of metal chelation, being below the values obtained with chymotrypsin. Metal chelation measures the degree of protection against oxidation reactions, which are catalyzed by transition metals such as $Cu^{2+}$ and $Fe^{2+}$ (Saiga *et al.*, 2003) and which can catalyze the generation of reactive oxygen species such as the hydroxyl radical (•OH) and the superoxide anion $O_2^-$ (Stohs, 1995). These *in vivo* oxidation reactions are apparently involved in the pathogenesis of neurodegenerative diseases (Mandel *et al.*, 2006). Copper chelating peptides can prevent the oxidative activity of this metal by chelating it. Therefore, they can be useful, not only preventing the oxidative activity of this metal that can damage the cells of the luminal space of the stomach, but also, they can prevent the oxidation of LDL induced by copper, if they reach the bloodstream (Burkitt, 2001). Copper chelating peptides, being rich in His, have been shown to prevent the oxidative activity of this metal. The imidazole ring of this residue is directly involved in bonding with copper. On the other hand, it has also been observed that these peptides are rich in Arg and although this amino acid lacks chelating properties, it can favor the union of the peptide with the metal ion (Megías *et al.*, 2008).

The fractions obtained from the chymotrypsin hydrolysate (Figure 3a), showed activity to chelate iron, ranging between 40 and 70% activity. In this way, the fraction <10 kDa and the fraction <5 kDa, showed the highest values, 67.91±0.16 and 68.83±0.02 respectively. On the other hand, in the fractions obtained from the pepsin hydrolysate

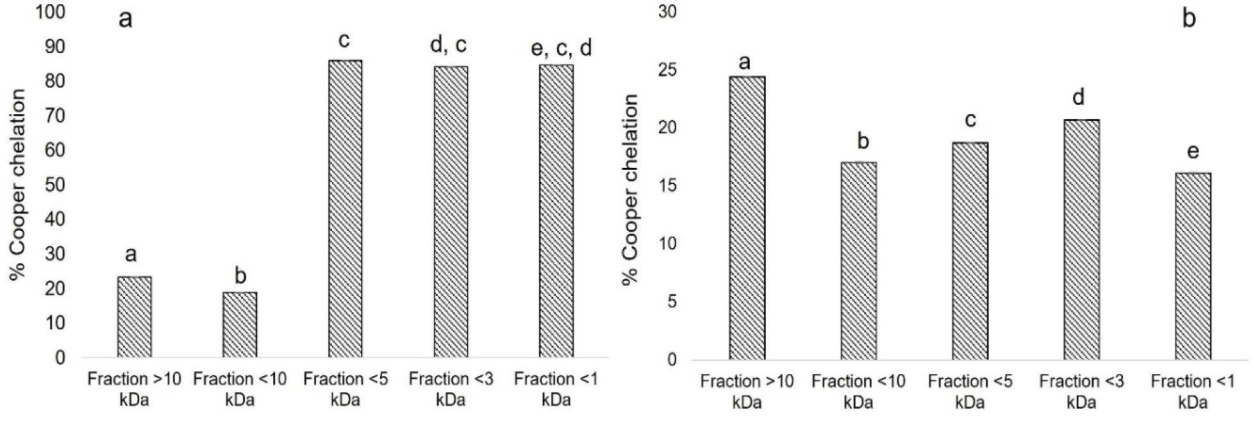


**Figure 2**. Cooper chelating activity in BSA protein fractions, obtained by hydrolysis with chymotrypsin a) and pepsin b). The results represent the mean of three independent determinations. Different letters indicate significant difference ($p<0.05$). Unifactorial ANOVA, group comparison by Student Newman Keuls (SNK).

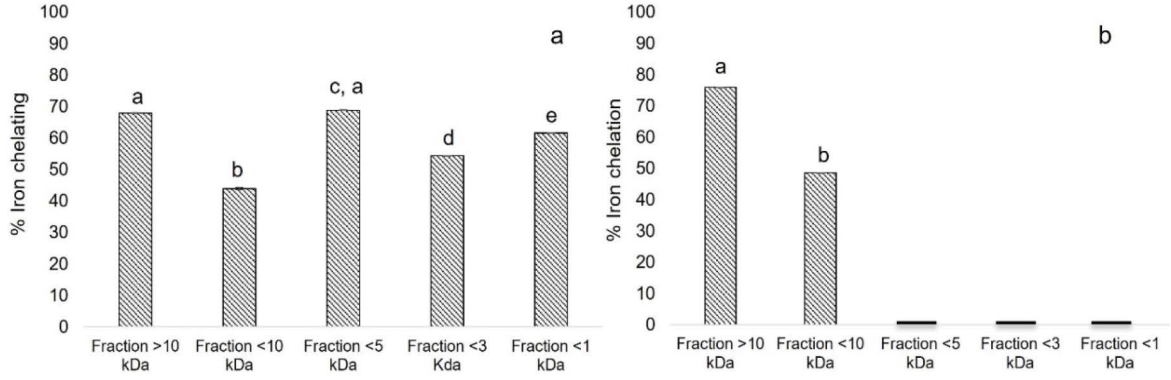


**Figure 3**. Iron chelating activity in BSA protein fractions, obtained by hydrolysis with chymotrypsin a) and pepsin b). The results represent the mean of three independent determinations. Different letters indicate significant difference ($p<0.05$). Unifactorial ANOVA, group comparison by Student Newman Keuls (SNK).

(Figure 3b), only the fraction >10 kDa and <10 kDa, showed activity to chelate, in the case of the remaining fractions no activity was detected, this probably because the protein concentration was very low, and therefore the amount of amino acids present did not manage to trap the metal.

Chelation of this metal appears to be at least partially responsible for the antioxidant activity that has been found in several amino acids, including Tyr, Met, His, Lys, Arg, and Trp (Huang *et al.*, 2010). Furthermore, iron chelation by His, Glu, Asp, and Cys shows results in iron absorption and can also lead to reduction of ferric to ferrous ion (Storcksdieck Genannt Bonsmann *et al.*, 2007).

The scavenging activity of the $SO^{-2}$ radical of the fractions obtained from the hydrolysate with chymotrypsin and pepsin is observed in Figure 4a and 4b respectively. The protein fractions obtained from the chymotrypsin hydrolysate showed a better ability to inhibit the $SO^{-2}$ radical except for the <1kDa fraction that showed no activity. In the case of the fractions obtained from the pepsin hydrolysate, all the fractions except for the fraction <5 kDa and <1 kDa showed no activity.

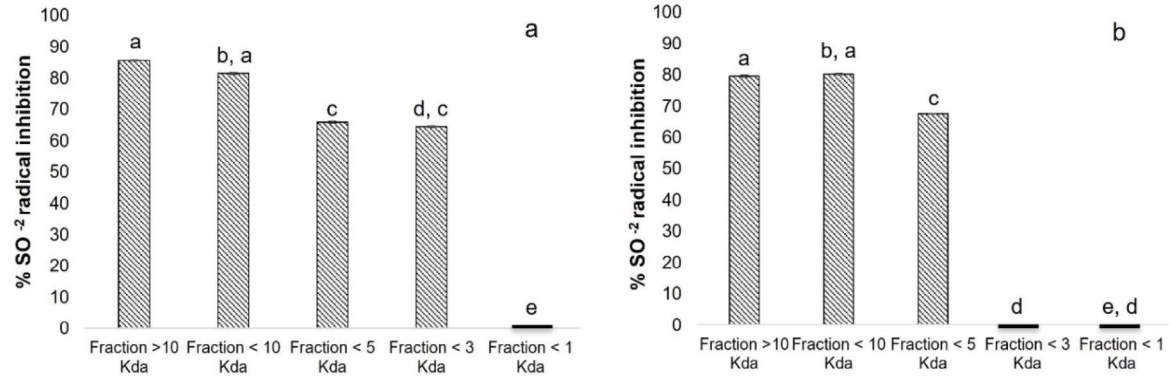


**Figure 4**. % $SO^{-2}$ radical inhibition in BSA protein fractions, obtained by hydrolysis with chymotrypsin a) and pepsin b). The results represent the mean of three independent determinations. Different letters indicate significant difference ($p<0.05$). Unifactorial ANOVA, group comparison by Student Newman Keuls (SNK).

The trapping activity of the −OH radical of the fractions obtained from the hydrolysate with chymotrypsin and pepsin is observed in Figure 5a and 5b respectively. The protein fractions obtained from the chymotrypsin hydrolysate showed a better ability to inhibit the −OH radical except for the <1kDa fraction that showed no activity. On the other hand, the fractions obtained with pepsin also showed radical trapping activity, however the percentage of inhibition in all the fractions was around 50%, this being half of what was obtained with the fractions obtained with chymotrypsin. In addition, it was also observed that the fraction <1 kDa did not show activity.

On the other hand, Ballatore *et al.*, (2020), demonstrated that the protein fractions (<3 kDa) of whey obtained by hydrolysis with trypsin show $SO^{-2}$ and −OH scavenging activity, which compared with our results the fraction <3 kDa showed the same activity but only with the fraction obtained with chymotrypsin. Peng *et al.*, (2010), determined that the fractions between 0.1-2.8 kDa fractionation with a Sephadex G-10 gel filtration column obtained from whey protein isolate hydrolyzed with alcalase for 5 h, showed strongest free radical scavenging effects, which was evidenced by the electron spin resonance (ESR) of 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH) and of scavenging hydroxyl (OH) and superoxide radicals. This activity is important to determine since the superoxide anion is normally formed in cellular oxidation reactions. Although it cannot directly initiate lipid oxidation, the superoxide radical can produce hydrogen peroxide and hydroxyl radical, through dismutation and other types of reactions (Dorman *et al.*, 2004).

As it has been shown above, superoxide can participate in many important epigenetic processes including DNA methylation/demethylation, histone methylation/demethylation, and histone acetylation/deacetylation. Therefore, the disruption of superoxide balance might stimulate dangerous changes in these processes. As it has been discussed above, the effects of superoxide on epigenetic processes might be more prominent ones in pathologic states characterized by the enhanced levels of ROS such as cancer, aging, cardiovascular diseases, and diabetes mellitus (Afanas'ev, 2015).

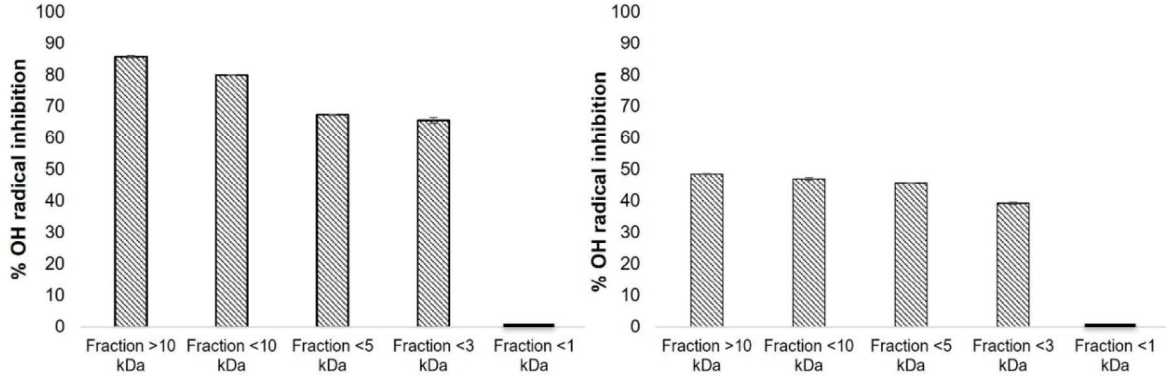


**Figure 4**. % OH radical inhibition in BSA protein fractions, obtained by hydrolysis with chymotrypsin a) and pepsin b). The results represent the mean of three independent determinations. Different letters indicate significant difference ($p<0.05$). Unifactorial ANOVA, group comparison by Student Newman Keuls (SNK).

## CONCLUSIONS

The antioxidant activity present in the protein fractions of BSA hydrolysed with chymotrypsin and pepsin can be attributed at least in part to their ability to trap radicals, this due to the presence of various amino acids or short peptides in the range of <10 kDa to <3 kDa that showed the extinction of radicals, playing an important role in the general antioxidant effect of the protein fractions. Much more research is needed to isolate the individual peptides responsible for the antioxidant activity of BSA, as well as to identify the amino acid sequence, which will allow a better structure-functionality relationship of the peptides.

## ACKNOWLEDGEMENTS

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Physical-chemical properties and microstructural characterization of traditional mexican chili (*Capsicum annuum* L.) powders

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## ABSTRACT

**Objective**: Evaluate the physical-chemical properties and characterize the microstructure of four varieties of traditional Mexican chili (*Capsicum annuum* L.) powders: "Arbol", "Guajillo", "Piquin" and "Mole ranchero" (Ancho chili).

**Design/methodology/approach**: Physical-chemical properties of chili powders were evaluated by means of moisture content, particle size, aerated and tapped bulk density, Carr index, Hausner ratio, angle of repose (flow properties), capsaicin, and carotenoids content. Microstructure of samples was characterized by Confocal Laser Scanning Microscopy and Scanning Electron Microscopy. ANOVA analysis and Tukey test were performed to evaluate the significant statistical difference between samples at 95% of confidence level.

**Results**: "Arbol", "Guajillo", "Piquin" and "Mole Ranchero" chili powders presented a cohesive behavior respect to its flow properties related to aerated and tapped bulk density, angle of repose, Carr Index, and Hausner ratio values under moisture content between 6.59-14.48 g $H_2O$/100g d.s. "Arbol" and "Piquin" chili powders presented the higher capsaicin content, while "Guajillo" and "Mole ranchero" showed the higher carotenoids content. FTIR spectra confirmed the presence of secondary amide, phenolic groups, alkanes, and aliphatic chains that belong to capsaicin structure at specific absorption bands. Microstructure of chili powders presented particles with surface imperfections as cracks and dents, and smooth surface that influence physical-chemical and flowability properties.

**Limitations on study/implications**: Hight moisture content affect the physical-chemical properties, flowability and microstructure of traditional Mexican chili powders.

**Findings/conclusions**: Moisture content between 6.59 and 14.48 g $H_2O$/100g d.s. influences the physical-chemical properties, flowability and microstructure of traditional Mexican chili powders. To improve physical-chemical properties and flowability behavior of chili powders is required that moisture content be lower than 6.59 $H_2O$/100g d.s.

**Keywords**: Mexican chili powders, physical-chemical properties, flowability, capsaicin, carotenoids, microstructure.

## INTRODUCTION

The chili (*Capsicum annuum* L.) (Solanaceae) is native of America, is consumed as vegetable, spice, and as source of vitamins A, C and E, carotenoids, and capsaicin which produce the characteristic pungency of *Capsicum* species. The chili in Mexico is usually processed in dried form to conserve its color and flavor. Dehydrated chili is employed in instant soups, frozen pizzas, salad dressings and a great variety of sauces and foods. During the dehydration process, water content and water activity are partially or totally reduced and, consequently, microbial growth and the enzymatic activity are limited, yielding an extended shelf-life of the product, beside of protect the bioactive compounds and conserve the physical-chemical properties of chili-based products (Domian & Poszytek, 2005; De Marino *et al.*, 2006; Cisneros-Pineda *et al.*, 2010; Yaldiz *et al.*, 2010; Valdez-Fragoso *et al.*, 2013). Processing and handling of powders have increasingly in the food and pharmaceutical industries. Knowledge of food powder properties has grown to the extent that increases product value, and processes complexity as well as development of new formulations. In this sense, flow properties are very important in many unitary operations which involve powders flowability, such as: pneumatic transport from specific equipment or silo, mixing and packaging. Flowability is affected by many factors such as particle size, shape, density, chemical composition, and moisture content. Powder properties are commonly determined under loading conditions of gravity, by means of angle of repose, standardized flow rate, aerated and tapped bulk density values, beside of Carr Index and Hausner ratio (Zou and Brusewitz, 2002; Thalberg *et al.*, 2004; Cavalcante-Alves *et al.*, 2008; Emery *et al.*, 2009; Perea-Flores *et al.*, 2010). Density is a basic property for materials and industrial processes characterization, storage selection, packaging, and distribution conditions. Aerated and tapped bulk density are generally used to characterize final products obtained by mean of milling or drying, weight estimation to fill containers and to relate the powder density before and after compression (Abdullah & Geldart, 1999; Barbosa-Cánovas *et al.*, 2005; Fitzpatrick, 2005). Angle of repose is the steepest angle of descent relative to the horizontal plane to which a material can be piled without slumping. This parameter indicates that interparticulate friction has been used to characterize flow behaviour of the powder and granular materials respect to flowability (Frączec *et al.*, 2007; Xinde *et al.*, 2007; Ileleji & Zhou, 2008). Powder compressibility is commonly used as a flowability indicator and is often expressed using the Hausner ratio, by means of aerated/tapped bulk density ratio and is appropriate to estimate cohesion in powders. In addition, the Carr index is other compressibility indicator to evaluate powder flow properties (Thalberg *et al.*, 2004; Barbosa-Cánovas & Juliano, 2005; Cavalvante-Alves *et al.*, 2008; Khandai *et al.*, 2014). Nowadays, the study of food powder microstructure is relevant and has grown in parallel with the development of microscopy and image processing techniques (Wang, 2006; Pérez-Alonso *et al.*, 2009). Some methods analyze food microstructure and correlate it with chemical composition and properties: Light Microscopy (LM), Scanning Electron Microscopy (SEM), and Confocal Laser Scanning Microscopy (CLSM), covering the whole dimensional scale from microstructural to macrostructural level (Kim *et al.*, 2009; Perea-Flores *et al.*, 2010). Therefore, the aim of this research was evaluating the physical-

chemical properties and characterize the microstructure of four traditional Mexican chili powders: "Árbol", "Guajillo", "Piquin" and "Mole ranchero" (Figure 1).

## MATERIALS AND METHODS

**Samples**: Four samples of traditional Mexican chili powders: "Arbol", "Guajillo", "Piquin" and "Mole ranchero" (Ancho chili), were purchased in a local market from Toluca, State of Mexico, Mexico. Capsicum oleoresin standard was provided by Sensient Colors Company, S.A. de C.V. (Lerma, State of Mexico, Mexico). All reagents used in the study were purchased from Sigma-Aldrich, S.A. de C.V. (Toluca, State of Mexico, Mexico).


**Figure 1**. Traditional mexican chili powders (*Capsicum annuum* L.) Source: Created by the authors.

**Aerated and tapped bulk density**: samples were gently poured into a 100 mL graduate cylinder. Aerated bulk density ($\rho_a$) was calculated as the ratio between the weights (g) of the sample contained in the cylinder and the filled volume (100 mL). Tapped bulk density ($\rho_b$) was estimated by tapping the cylinder (100 times) until no measurable change in volume was noticed (León-Martínez *et al.*, 2010; Gallo *et al.*, 2011).

**Particle size**: was determined using a particle size analyzer Malvernsizer 2000 (Malvern Instruments, Ltd., Malvern, Worcetershire, UK). The average particle size was obtained using the software Malvernsizer 5.6 integrated to the equipment (Pérez-Alonso *et al.*, 2009).

**Moisture content**: was determined according to AOAC method (2005).

**Angle of repose**: was determined by pouring a pre-defined mass of 50 g of chili powder sample through a funnel located at a fixed height on a graph paper flat horizontal surface and measuring powder conical pile height ($h$) and radius ($r$) formed. The tangent of the angle of repose is given by the $h/r$ ratio (Gallo *et al.*, 2011).

**Carr index and Hauser ratio**: were evaluated by means of the relationship between aerated bulk density and tapped bulk density, using equations (1)-(2) (Ganesan *et al.*, 2008; Gallo *et al.*, 2011):

$$CI\% = \left[\frac{(\rho_b)-(\rho_a)}{\rho_b}\right]\times 100 \quad (1)$$

$$HR = \left[\frac{(\rho_b)}{(\rho_a)}\right] \quad (2)$$

*CI*% is the Carr index and *HR* is the Hausner ratio, ($\rho_a$) is the aerated bulk density in g/mL and ($\rho_b$) is the tapped bulk density (g/mL).

**Capsaicin content**: 0.1 g of sample and 0.025 g for capsicum oleoresin standard, were extracted during 4 hours at 25 °C with 50 mL of acetone, with a slight modification respect to Hornero & Mínguez spectrophotometric method (2001). Absorbance was measured in a UV spectrophotometer GENESYS2-UV/visible (Spectronic, Rochester, NY, USA) at $\lambda = 460$ nm and using acetone as blank (Hornero & Mínguez, 2001; Braga & Oliveira, 2007).

**Carotenoids content**: determined according to Hornero & Mínguez (2001) spectrophotometric method with a slight modification. Absorbance measurements were made in a UV spectrophotometer GENESYS 2-UV/visible (Spectronic, Rochester, NY, USA) at $\lambda = 472$ and 508 nm. To obtain isochromic fraction and total carotenoids content, the following equations (3)-(5) were used:

$$CR = \left[\frac{(A_{508})\times(2144)-(A_{472})\times(403.3)}{270.9}\right](\mu g / mL) \quad (3)$$

$$CY = \left[ \frac{\left(A_{472}\right) \times \left(1724.3\right) - \left(A_{508}\right) \times \left(403.3\right)}{270.9} \right] \left(\mu g / mL\right) \quad (4)$$

$$CT = CR + CY \left(\mu g / mL\right) \quad (5)$$

$A_{508}$ is the absorbance of samples at $\lambda = 508$ nm, $A_{472}$ is the absorbance of samples at $\lambda = 472$ nm of the samples *CR* represents the red isochromatic fraction content, *CY* represents the yellow isochromatic fraction content, and *CT* represents the total carotenoids content.

**Extractable color** (**ASTA units**): were determined according to ASTA 20.1 method. The absorbance of each sample was measured against acetone blank at $\lambda = 460$ nm in a UV spectrophotometer GENESYS 2-UV/visible (Spectronic, Rochester, NY, USA). Extractable colour values were expressed in ASTA units and calculated by the Ec (6) (Topuz *et al.*, 2009; Rascón *et al.*, 2011):

$$ASTA\ units = \left[ \frac{A \times 164 \times If}{W_{sample}} \right] \quad (6)$$

*A* is the absorbance of sample at $I = 460$ nm, *If* is the deviation factor of the spectrophotometer and *W* is the sample weight (g) in dry basis.

**FTIR Spectroscopy**: chemical groups associated to capsaicin and carotenoids were identified by FTIR Spectroscopy, using a Micro-Raman Spectrometer (Lab RAM HR800, Horiba Jobin-Yvon, France) coupled to Fourier Transform Infrared Spectroscopy and a Charge Detector, using an Attenuated Total Reflectance objective (ATR-FTIR) with 36x magnification. Spectra were acquired from 4000 to 400 $cm^{-1}$ and the baseline spectra adjusted with Origin Pro 8.0 software (Origin Lab Corporation, MA, USA) and compared with capsicum oleoresin standard spectrum.

**Scanning Electron Microscopy** (**SEM**): microstructure was characterized using a Dual Beam SEM Microscope model (Quanta 3D, FEG, FEI, Holland). Samples were fixed with double-sided carbon adhesive tape on aluminum stubs and directly observed at 15 kV, using a low vacuum secondary electron detector to minimize charging. Micrographs were acquired at 250x and 1000x (Quintanilla-Carvajal *et al.*, 2011; De la Rosa-Millán *et al.*, 2014).

**Confocal Laser Scanning Microscopy** (**CLSM**): samples were characterized with CLSM equipment (CLSM 710 NLO, Carl Zeiss, Germany) using a plan-Apochromat 40x/1.3 oil DIC M27 objective, and were excited at $\lambda = 405$, 488 and 561 nm. Autofluorescence intensity measurement was performed using the software ZEN coupled to the equipment. Were acquired 3D images of samples (x, y and z) in sections of different focal planes (Quintanilla-Carvajal *et al.*, 2011).

**Statistical analysis**: ANOVA and Tukey test were performed to evaluate the significant difference between samples at 95% of confidence level.

## RESULTS AND DISCUSION

**Aerated and tapped bulk density**: Density is influenced by particle size; at lower particle size, aerated and tapped bulk density increase because there is more surface contact area available for cohesive and frictional forces to resist flowability and the influence of compaction capability and the shape of particles of chili powders, this behavior is showed in "Guajillo", "Piquin" and "Mole ranchero", meanwhile "Arbol" chili exhibit the higher particle size and the lower density values (Table 1). "Mole ranchero" showed the highest aerated and tapped bulk density values. In the other hand, particle size and shape influence the aerated and tapped bulk densitiy due to the compaction capability that powders present by the presence of surface imperfections as cracks and dents that "Arbol", "Guajillo" and "Piquin" presented and by the smooth surfaces that "Mole Ranchero" presented.

**Angle of repose**: to classify flowability of powders, Gallo *et al.* (2013), give a classification between 25° and 30° as an excellent flowing and greater than 31° as poor flowing, while Santomaso *et al.* (2003), give values between 30° and 45° to powders that free flowing and values between 45° and 60° to powders fairly to free flowing. "Arbol"chilli", "Piquin" and "Mole ranchero" showed a behavior near to free flowing, while "Guajillo" showed a behavior fairly to free flowing, and these values indicate that chili powders are cohesive and tend to form agglomerates (Table 1, Figure 2). Angle of repose involves a compaction and tapping process, which produce a little deformation of agglomerates and reflect the surface properties, including the degree of agglomerates, particle morphology, friction, and cohesive forces between particles, whereby the angle of repose could be used as a flowability index of chili powders. Figure 2 illustrates the flow pattern of the conical pile formed to measure angle of repose from traditional mexican chili powders.

**Carr index and Hausner ratio**: these parameters express the compaction and compressibility capabilities related to frictional forces between powder particles. Gallo *et al.* (2013) give Carr index values between 10 and 25 to identify powders with excellent and acceptable flowability. Santomaso *et al.* (2003) give Hausner ratio values between 1 and 1.25 to identify powders with excellent and near to free flowing, while values between 1.25 and

**Table 1**. Physical-chemical properties of traditional mexican chili powders.

| Samples | Moisture content ($gH_2O/100g$ d.s) | Particle size $d_{4,3}$ ($\mu m$) | Aerated bulk density ($\rho_a$) (g/mL) | Tapped bulk density ($\rho_b$) (g/mL) | Carr index (au)* | Angle of repose $\alpha_r$ (°) | Hausner ratio (au)* |
|---|---|---|---|---|---|---|---|
| Arbol chilli | $7.92\pm0.10^a$ | $643.68\pm0.00^a$ | $0.40\pm0.00^a$ | $0.57\pm0.01^a$ | $29.82\pm0.95^a$ | $37.9\pm0.09^a$ | $1.43\pm0.01^a$ |
| Guajillo chilli | $9.58\pm0.10^b$ | $330.99\pm0.00^b$ | $0.42\pm0.02^b$ | $0.61\pm0.00^b$ | $31.14\pm0.00^b$ | $46.68\pm0.08^b$ | $1.45\pm0.00^b$ |
| Piquin chilli | $6.59\pm0.10^c$ | $247.49\pm0.00^c$ | $0.44\pm0.02^c$ | $0.69\pm0.01^c$ | $36.23\pm1.00^c$ | $44.31\pm0.40^c$ | $1.56\pm0.02^c$ |
| Mole ranchero | $14.48\pm0.10^d$ | $342.53\pm0.00^d$ | $0.52\pm0.05^d$ | $0.77\pm0.01^d$ | $32.46\pm0.01^d$ | $41.20\pm0.04^d$ | $1.48\pm0.01^d$ |

*(au): adimensional units.
The values with different letter in the same column present a significant difference between every sample.


**Figure 2**. Angle of repose evaluated from traditional mexican chilli powders (*Capsicum annuum* L.) Source: created by the authors

1.4 indicate a behavior fairly to free-flowing powders. "Arbol", "Guajillo", "Piquin" and "Mole ranchero" showed a cohesive behavior fairly to free flow with significant difference between Carr index and Hausner ratio values. "Mole ranchero" is a very cohesive powder due to its higher moisture content of 14 g$H_2O$/100g d.s. respect to "Arbol", "Guajillo" and "Piquin" chili at moisture contents of 7.92, 9.58 and 6.59g $H_2O$/100g d.s. respectively.

**Chemical group's composition**: FTIR spectra of chili powders presented in Figure 3, showed a characteristic band of absorption of axial deformation of C-H aromatic and aliphatic chains at 2900 $cm^{-1}$, a band of asymmetric and symmetric axial deformation of C-O-C bonds at 1690 $cm^{-1}$, a band at 1652 $cm^{-1}$ associated to a secondary amide, a band of axial deformation of C=C of the double ring at 1590 $cm^{-1}$, and a band of absorption of axial deformation of C=O bonds at 1158 $cm^{-1}$ related to a phenolic group. These results are agreed with capsicum oleoresin standard spectra and with the FTIR spectra reported for chili samples by Toshimasa *et al.* (2003) and De Marino *et al.* (2006), which confirms the chemical groups related to capsaicin molecule and carotenoids structure.

**Capsaicin and carotenoids content**: results are shown in Table 2. "Piquin" and "Arbol" presented the higher capsaicin content, meanwhile "Guajillo" and "Mole ranchero" showed the lower capsaicin content, respect to the capsicum oleoresin standard content. In the other hand, "Guajillo" and "Mole ranchero" presented the higher carotenoids content respect to the capsicum oleoresin standard content. "Guajillo" showed the higher carotenoids content and a lower capsaicin content, while "Piquin chili" presented the higher capsaicin content and the lower carotenoids content. "Guajillo" and "Mole Ranchero" presented the highest red fraction, yellow fraction, and total fraction of carotenoids, and the lowest capsaicin content. Experimental values obtained are in accordance with the values reported in chili samples by Rodríguez-Maturino *et al.* (2012).

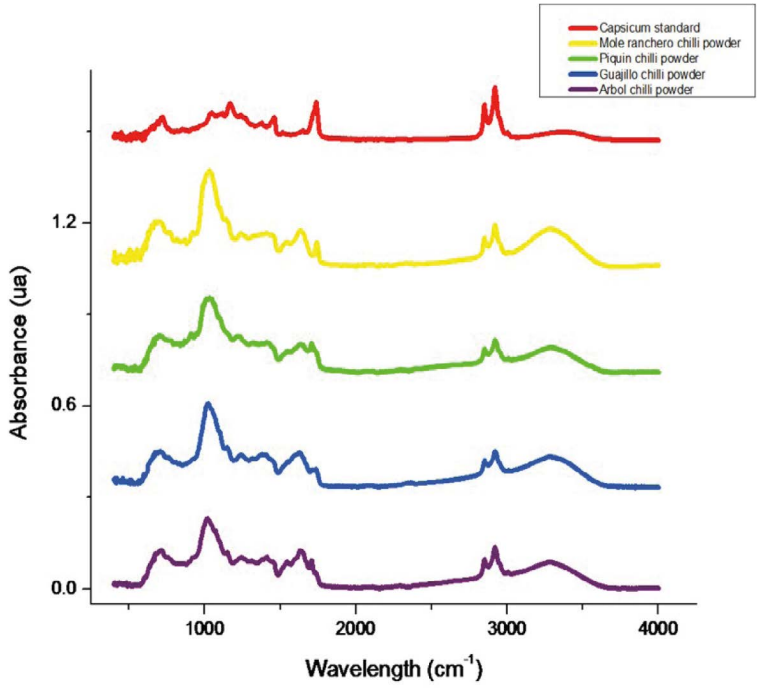


**Figure 3**. FTIR spectra of traditional mexican chilli powders (*Capsicum annuum* L.) Source: created by the authors.

**Extractable color (ASTA units)**: "Guajillo" and "Mole Ranchero" showed the higher extractable color value, while "Arbol" and "Piquin" showed the lower extractable color values. All samples were compared with capsicum oleoresin standard extractable color values (Table 2). The significant difference of extractable color in the samples is related to the Red (CR) and Yellow (CY) fractions of carotenoids, where "Guajillo" and "Mole Ranchero" presented the higher red and yellow fractions of carotenoids, while "Arbol" and "Piquin" presented the lower red and yellow fraction (CR). Results obtained are agreed with the values reported by Rodríguez-Maturino *et al.* (2012).

**Microstructure**: "Arbol", "Guajillo" and "Piquin" presented particles with surface imperfections as cracks and dents, while "Mole Ranchero" showed a smooth surface, which

**Table 2**. Capsaicin, carotenoids, and color content of traditional mexican chilli powders.

| Parameters | Capsaicin | Carotenoids | | | Color |
|---|---|---|---|---|---|
| **Samples** | **Concentration (mg/L)** | **Red fraction CR ($\mu$g/mL)** | **Yellow fraction CY ($\mu$g/mL)** | **Total fraction CT=CR+CY ($\mu$g/mL)** | **ASTA units (au)*** |
| Arbol chilli | 97.28±0.18$^{a}$ | 197.73±0.00$^{a}$ | 260.10±0.00$^{a}$ | 457.84±0.00$^{a}$ | 279.48±0.00$^{a}$ |
| Guajillo chilli | 18.67±0.00$^{b}$ | 846.44±0.00$^{b}$ | 1324.71±0.00$^{b}$ | 2171.15±0.00$^{b}$ | 1256.92±0.01$^{b}$ |
| Piquin chilli | 121.95±0.00$^{c}$ | 117.07±0.00$^{c}$ | 155.26±0.00$^{c}$ | 272.33±0.00$^{c}$ | 192.30±0.00$^{c}$ |
| Mole ranchero | 46.23±0.00$^{d}$ | 316.94±0.01$^{d}$ | 427.43±0.01$^{d}$ | 744.37±0.01$^{d}$ | 476.41±0.01$^{d}$ |
| Capsicum std | 136.38±0.00$^{e}$ | 794.14±0.00$^{e}$ | 1477.24±0.00$^{e}$ | 2271.38±0.00$^{e}$ | 1405.64±0.00$^{e}$ |

*(au): adimensional units
The values with different letter in the same column present a significant difference between every sample.

can be observed in Figure 4. The 3D CLSM images showed the autofluorescence (green and red) identified in traditional Mexican chili powders, which is related to capsaicin and carotenoids compounds (Vazquez-Gutiérrez *et al.*, 2011), where the red fluorescence is associated to capsaicin content at $\lambda$=460nm.

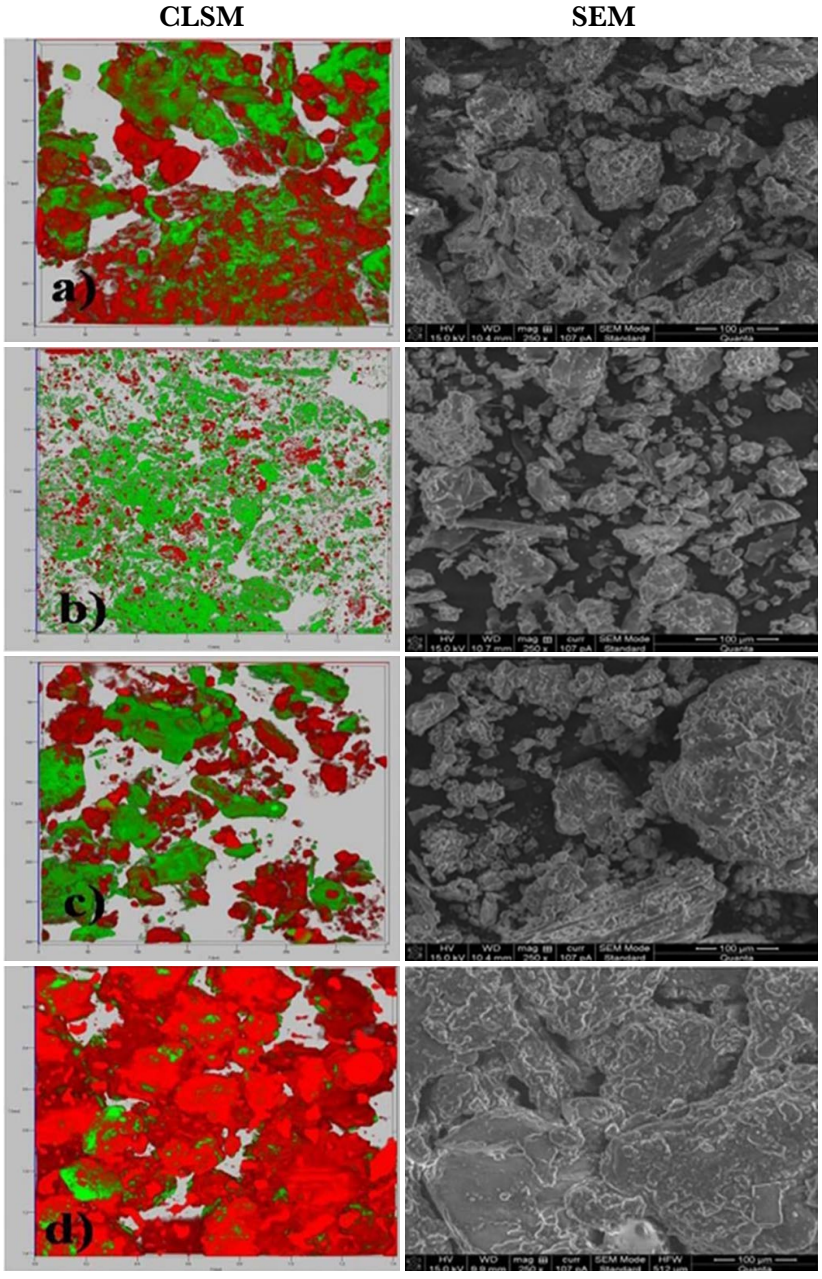


**Figure 4**. Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM) micrographs of traditional mexican chilli powders. a) Arbol chilli, b) Guajillo chilli, c) Piquin Chilli and c) Mole ranchero. Source: created by the authors.

## CONCLUSIONS

"Arbol", "Guajillo", "Piquin" and "Mole Ranchero" presented a cohesive behavior respect to its physical-chemical properties and flowability. "Arbol" and "Piquin" present the higher capsaicin content while "Guajillo" and "Mole ranchero" showed the higher carotenoids content. FTIR spectra confirmed the presence of secondary amide, phenolic groups, alkanes, and aliphatic chains that belong to capsaicin structure from chili. Microstructure of chili powders presented particles with surface imperfections as cracks and dents, and smooth surface that influence physical-chemical properties. Moisture content between 6.59 and 14.48 g $H_2O$/100g d.s. influences the physical-chemical properties, flowability behavior and microstructure of traditional Mexican chili powders. To improve physical-chemical properties and flowability behavior of chili powders is required that moisture content be lower than 6.59 g $H_2O$/100g d.s.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Biorational and conventional insecticides efficacy to control thrips (*Frankliniella occidentalis* Perg.) on strawberries (*Fragaria×ananassa* Duch.) at Morelos state, Mexico

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## ABSTRACT

**Objective**: To evaluate the insecticidal effectiveness of biorational and conventional products as alternatives to control thrips and their phytotoxic effects in strawberry cultivation.

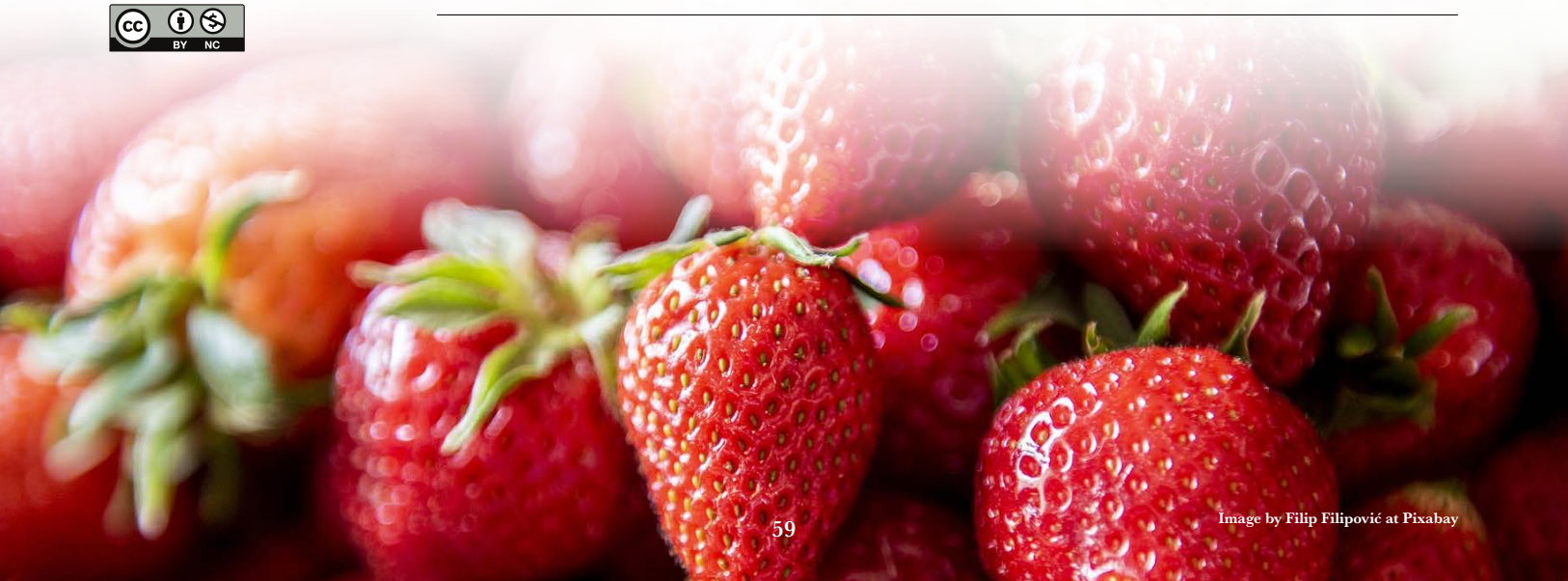
**Design/methodology/approach**: The research was carried out in strawberry cv. Camino Real during its flowering stage - fruiting established in open ground; A completely randomized experimental design with seven treatments and four replications was used. The treatments evaluated were: T1: neem oil extract, T2: paraffin oil, T3: garlic extract + hot chili pepper + cinnamon, T4: spinosad, T5: imidacloprid + lambda cyhalothrin, T6: bifenthrin, and T7: control. Applications were made weekly and the mortality evaluation was carried out by counting the number of live thrips per flower. With the obtained data, an analysis of variances and a mean comparison test (Tukey, $p \leq 0.05$) were performed.

**Results**: Significant differences between treatments could be identified ($p \leq 0.05$); imidacloprid + lambda cyhalothrin and bifenthrin caused the highest mortality of thrips with 92 and 93% efficacy, respectively. Spinosad obtained good results, ranking as the second-best option with 90% control efficiencies; neem extract stood out as an excellent biorational pest management alternative with 77% control efficacy in its last evaluation. No phytotoxic effects were observed from any of the treatments on the crop.

**Limitations on study/implications**: It is important to continue the study in the laboratory to obtain the LD50 and LD90 of the management alternatives, as well as an MRL analysis of the molecules used.

**Findings/conclusions**: The proposed protocol evidenced the efficacy of biorational and ecological thrips control treatments in intensive horticultural systems.

**Keywords**: ecological management, extract, identification, neem, spinosad.



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## INTRODUCTION

In Mexico, the strawberry (*Fragaria×ananassa*) cultivation began around the middle of the last century and in 1950 began to be exported to the United States. In the country, strawberries are one of the most important productive chains, due to the production value estimated at more than 100 million dollars every year (SIAP, 2019), as well as being an important source of employment in their production regions. Its planted area is more than 7 thousand hectares, although in Mexico the crop occupies only 1% of the area dedicated to agriculture. It is important for the generation of foreign exchange because it is an export product. In Mexico, the states of Michoacán, Baja California and Guanajuato generate more than 90% of total production.

The strawberry crop presents multiple phytosanitary constraints among which pests such as red spider mite (*Tetranychus urticae*), western tarnished plant bug (*Lygus hesperus*), western flower thrips (*Frankliniella occidentalis*), corn earworm (*Helicoverpa zea*), and white grubs (*Phyllophaga* ssp.) stand out (León-López *et al*., 2014); the main diseases in this crop are caused by *Botrytis* spp., *Colletotrichum* spp., and *Phytophthora* spp. (fruit rot); *Fusarium moniliforme*, *Rhizoctonia solani*, *Verticillium dahliae* and *Alternaria* spp. (strawberry blotch); *Fusarium oxysporum* (root and crown rot); strawberry mottle (SMoV) and strawberry sprouting (SCV) (Dávalos *et al*., 2011; Cordero *et al*., 2003; León López *et al*., 2014; Téliz-Ortíz *et al*., 1986). The "western flower thrips or Californian thrips" (*F. occidentalis*) is native to the southwestern United States and have a worldwide distribution. Its polyphagy, combined with its high biotic potential, allows it to generate large populations of individuals that infest the crops. The adults and larvae of *F. occidentalis* scrape and suck the fluid from the cells on the surface of stems, leaves, flowers and fruits; in flowers they cause veining, discoloration (silver-gray spots) and petal necrosis, which reduces yield and product quality (Albendin *et al*., 2012). In fruits, high populations (25 thrips/fruit) cause spots, scarring, tanning, deformations and softening that reduce commercial quality (especially for export) and shorten shelf life (Albendin *et al*., 2012; Coll *et al*., 2007). Additionally, the flower thrips is one of the most efficient species for the transmission of the tomato spotted wilt virus (TSWV) (Parrella *et al*., 2003). In Mexico and many areas of the world, thrips control is conventionally based on using synthetic translaminar contact and broad-spectrum chemical insecticides such as chloronicotinyls (imidacloprid), ketoenols (spiromesifen, spirotetramat), phenylpyrazoles (fipronil) and pyrethroids such as cypermethrin, deltamethrin and abamectin (Monteon-Ojeda *et al*., 2020). It is important to mention that, although effective, these types of products have other disadvantages such as the possible selection of resistance, environmental pollution, human intoxication during its application, in addition to placing food safety at risk (Monteon-Ojeda *et al*., 2020). On this basis, the objective of this research was to evaluate the insecticidal effectiveness of biorational and conventional products as alternatives for the control of flower thrips in strawberries, as well as their possible phytotoxic effects on the crop.

## MATERIALS AND METHODS

The study was carried out during June and July 2020 at the "ejido" Cacahuatlán, Tlayacapan, Morelos; 18° 55' 22'' N and 99° 00' 22'' W. The temperature and relative

humidity variables were recorded throughout the experiment using an electronic temperature and relative humidity meter, HOBO® model U12, and ranged between 23±2 °C and 65±10% respectively. The research took place in a commercial strawberry plantation (*Fragaria×ananassa*) cv. Camino Real during its flowering - fruiting stage and established in open ground. The plants were established in a double row furrow in an offset "tresbolillo" arrangement, at a 40 cm distance between plants and 1.25 meters between rows. In the furrow, gray embossed padding was used. For the irrigation system, drip tape was used, 8000 gauge with a dropper every 40 cm and a flow rate of 1.5 L $h^{-1}$. For nutrition, a Steiner nutrient solution adjusted to a pH of 5.5 was daily applied.

The treatment application was done in the mornings, from 7:30-8:30 am in cool conditions (15-18 °C) and low wind speed (0-3 km/h). Three applications were made for treatments T1, T2, T3 and T7 (control) and two for T4, T5 and T6 with a seven days interval between them. The spraying of insecticides was foliar, using a motorized gasoline sprayer backpack of 25 cc Honda® with a full cone nozzle at a constant pressure of 250-300 psi, calibrated at a flow rate of 400 L/ha. Treatments of both natural and synthetic origin were used, following: T1: neem oil extract (*Azadirachtin indica*) (80%) at a dose of 1.4 L/ha, T2: paraffinic oil (95%) at a dose of 2.0 L/ha, T3: garlic extract (*Allium sativum*) 25% + hot chili extract (*Capsicum frutescens*) 25% + cinnamon extract (*Cinnamomum zeylanicum*) 10% at a dose of 2.0 L/ha, T4: spinosad 12% at 60 mg $L^{-1}$ dose, T5: imidacloprid + lambda cyhalothrin (200 + 140 g/L SC) at 200 mL/ha dose, T6: bifenthrin (110 g/L EC) at 400 mL/ha dose and T7: control without insecticide application (water). The doses and active ingredients used were selected based on technical recommendations and previous studies.

For the experiment evaluation, systematic samplings (following a type Z pattern) of the thrips populations were carried out before and after the application of the insecticides. A previous evaluation and three subsequent ones were performed at 7, 14 and 21 days after the first spraying (dafs). From each experimental unit (EU), 10 plants were reviewed and from each plant two flowers (20 flowers per EU), the number of live thrips per flower was assessed. Also, samples of the found specimens were taken with a manual sprinkler and a water and fabric softener solution (9:1), the thrips were recovered in a plastic tray and with a sieve deposited in glass flasks with 70% methanol for laboratory identification. The identification was carried out with illustrated keys for the Thysanoptera genera and species of *Frankliniella* (Cavalleri and Mound, 2012; Moritz *et al.*, 2001; Soto and Retana, 2003) and corroborated by expert entomologist M.C. Jorge San Juan Lara. In addition to the aforementioned, an evaluation of the possible phytotoxic effect of the products applied on the crop was carried out three days after each application, EWRS damage scale was used (Champion, 2000). The efficacy of the treatments was evaluated using a completely randomized experimental design with four replications. Before the treatment application, the experimental units were randomized using the "design.ab" procedure in the R statistical software for Windows. This randomization determined which treatment was assigned to each EU. The efficacy percentage (%) of thrips control was calculated by counting the live individuals per flower in each EU after the application of the treatments, following the Abbott formula:

$$\%Effectiveness = \frac{IT - it}{IT} \times 100$$

Where: $IT$=absolute control infestation, $it$=treatment infestation.

A normality analysis of the distribution of the errors was performed on the obtained variable using the Shapiro-Wilk test, homogeneity of variances with the Bartlett test and graphically using residuals *vs*. predicted to verify assumptions. With the efficacy data obtained in the evaluations, an analysis of variance and a comparison test of means were performed with the Tukey method ($\alpha$=0.05) using the SAS V.9.4 statistical software for Windows®.

## RESULTS AND DISCUSSION

The present thrips specimens were counted, registering the presence of immature and adult forms. Most of the collected specimens were identified as *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae). This species has been reported as a common and high impact pest in this crop (González-Zamora and García-Marí, 2003; Matos and Obrycki, 2004; Solano-Rojas *et al*., 2018). The occurrence of immatures was occasional, the adults had the highest abundance. In multiple species of thrips, adults can be found in hundreds of plants, while larvae are hardly captured (Carrizo, 2002). The analysis of the thrips records during the evaluations showed that, at the beginning (previous evaluation), the population in the experimental units was homogeneous and had a normal behavior, so the corresponding statistical assumptions were met; the average number of thrips per inflorescence was of 9.5 individuals, an infestation high enough to cause substantial crop losses (Figure 1).

**Figure 1**. Infestation of thrips (*Frankliniella occidentalis*) in strawberry flowers (*Fragaria×ananassa*) cv. Camino Real previous evaluation of biorational and conventional control treatments at Tlayacapan, Morelos, 2020.

The analysis of variance and means comparison of the recorded field data identify significant differences in the populations of thrips during the first evaluation of insecticide treatments, carried out seven days after the first application; all insecticide treatments had a significant effect ($p \leq 0.05$) on the thrips population (Table 1). imidacloprid + lambda cyhalothrin and bifenthrin caused the highest mortality of thrips, spinosad also obtained good results, ranking as the second-best option; the neem extract showed a decrease of more than half of the population in its experimental units. Also, paraffinic oil and combined plant extract (T3) obtained the lowest mortality levels, although significant (Table 1). The treatment's behavior during the second and third evaluations was similar, highly significant differences ($p \leq 0.05$) were observed between them. Spinosad proved to be an exceptional control option, by causing mortality similar to that of conventional synthetic insecticide treatments (T6 and T7), which maintained the lowest populations throughout the experiment; although the other biorational treatments (T1, T2 and T3) did not reach the same mortality levels, they proved to be an excellent option, by reducing with just three applications, more than 60% the population affecting the strawberry flowers. Neem oil extract is highlighted as an excellent pest management alternative (Table 2). These results concur with that reported by Kharbade *et al.* (2018) who is a field experiment evaluated insecticidal treatments to reduce the infestation of thrips (*Thrips tabaci* L.) in onion crops. Their results revealed that the treatment with fipronil 5 SC was the most promising when registering the accumulated mean population lowest thrips of 4.38 thrips/plant and was on a par with clothianidin 50 WDG (4.69 trips/plant), the next best treatments in order of efficacy were carbosulfan + bifenthrin, lambda-cyhalothrin 5 EC, bifenthrin 10 EC, carbosulfan 25 EC which registered 6.44, 6.69, 6.91 and 7.15 trips/plant, respectively; followed by spinosad 45 SC, thiamethoxam 25 WG, rynaxypyr 18.5 SC and Metarhizium anisopliae in which 8.04, 8.22, 8.42, and 8.75 trips/plant were observed, respectively. The untreated control registered the highest population (23.70 trips/plant). Also coinciding with what was reported by Nderitu *et al.* (2010), who in a field experiment to evaluate and compare the effectiveness of synthetic insecticides (thiacloprid and chloropyrifos) and botanical insecticides (azadirachtin 0.15% and azadirachtin 0.06%) to control *Frankliniella occidentalis* populations and *Megalurothrips sjostedti* in beans (*Phaseolus vulgaris* L.) found significant differences ($p < 0.05$) between the four treatments, the lowest number of adult thrips and larvae were recorded in plots treated with 0.15% azadirachtin and Chlorpyrifos. In terms of yield, the plots treated with Chlorpyrifos, produced more marketable pods than those treated with botanical insecticides in both plantations, for which they recommended the use of chlorpyrifos at the beginning of the season to reduce thrips populations and applying 0.15% azadirachtin during the fruiting stage, to reduce the cost of production and allow the increase of natural enemies reducing the risk of resistance development. In Australia, Kay and Herron (2010) found that spinosad had the greatest impact on adults; in other studies, the effectiveness of spinosad to control immature and mature stages of *F. occidentalis* in cucumber greenhouses has been reported (Gholam and Sadeghi, 2016; Jones *et al.*, 2005). Alike to is reported in this bioassay, Thoeming *et al.* (2003) evaluated the systemic effects of neem (azadirachtin) applied to soil for the control of flower thrips (*F. occidentalis*) in beans (*P. vulgaris*) grown in multiple substrates. The applications of

**Table 1**. Results of the survival analysis of thrips (*Frankliniella occidentalis*) in strawberries (*fragaria×ananassa*) cv. Camino Real under biorational and conventional control treatments during the previous evaluation and first evaluation, at Tlayacapan, Morelos, Mexico (2020).

| Treatments | Pre- evaluation (0 dafs*) | First evaluation (7 dafs) |
|---|---|---|
| T1: Oil neem spray | 9.40 a** | 3.24 c |
| T2: Paraffinic oil | 9.30 a | 5.26 b |
| T3: Garlic extract + hot chili extract + cinnamon extract | 9.50 a | 5.28 b |
| T4: Spinosad | 9.55 a | 2.37 d |
| T5: Imidacloprid + lambda cyhalothrin | 9.35 a | 1.71 e |
| T6: Bifenthrin | 9.40 a | 1.75 e |
| T7: Control | 9.12 a | 9.67 a |

* Days after the first spraying.
** Means with different letters in the same column statistically differ according to the Tukey Test ($p<0.05$).

**Table 2**. Results of the survival analysis of thrips (*Frankliniella occidentalis*) in strawberries (*Fragaria×ananassa*) cv. Camino Real under biorational and conventional insecticide treatments for control during a second and third evaluation, Tlayacapan, Morelos, Mexico (2020).

| Tratamientos | Segunda evaluación (14 dafs*) | Tercera evaluación (21 dafs) |
|---|---|---|
| T1: Oil neem spray | 2.97 c | 2.15 c |
| T2: Paraffinic oil | 3.72 bc | 3.23 b |
| T3: Garlic extract + hot chili extract + cinnamon extract | 4.51 b | 3.62 b |
| T4: Spinosad | 1.09 d | 0.91 d |
| T5: Imidacloprid + lambda cyhalothrin | 0.74 d | 0.77 d |
| T6: Bifenthrin | 0.72 d | 0.65 d |
| T7: Control | 9.75 a | 9.17 a |

* Days after the first spraying.
** Means with different letters in the same column statistically differ according to the Tukey Test ($p<0.05$).

azadirachtin in the sand substrate recorded maximum mortality of 50.6% against immature when the substrate based on microcosm was used, 76% mortality was observed and in the mixture of substrates in a 1:1 ratio, 93% mortality was observed, the effects against thrips were kept up to 6 days after application. It should be taken into that the number of thrips in the crop after the application of neem-based products should not be a sole measure of the effectiveness of the product. This is because azadirachtin has a wide range of effects on pest insects, such as deterring feeding and oviposition, repellent effects, regulation of insect growth, sterilant, mating disruptor and toxicity (Nderitu *et al.*, 2010; Singh and Doharey, 2001).

The analysis of the efficacy percentages was able to identify a continuous increase and a similar behavior during the three evaluations carried out in all insecticide treatments (Figure 2). Although, for the biorational treatments (T1, T2 and T3), three consecutive weekly applications were necessary to reach acceptable levels of infestation control. These are justified considering its chemical origin, residuality and low cost. The neem oil extract

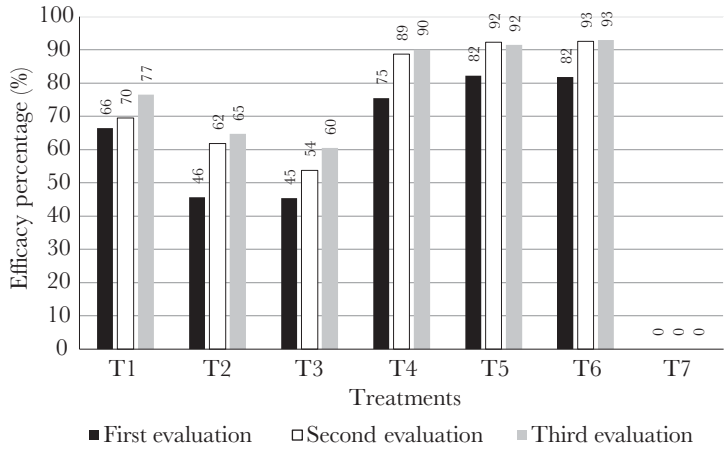


**Figure 2**. Control efficacy of biorational and conventional insecticides for the management of thrips (*Frankliniella occidentalis*) in strawberries (*Fragaria×ananassa*) cv. Camino Real, at Tlayacapan, Morelos, Mexico (2020). * T1: Neem (*Azadirachtin indica*) oil extract (80%) at a 1.4 L/ha dose, T2: paraffin oil (95%) at a 2.0 L/ha dose, T3: garlic (*Allium sativum*) 25% extract + hot chili (*Capsicum frutescens*) 25% extract + cinnamon (*Cinnamomum zeylanicum*) 10% extract at a dose of 2.0 L/ha, T4: Spinosad 12% at a60 mg $L^{-1}$ dose, T5: imidacloprid + lambda cyhalothrin (200 + 140 g/L SC) at a 200 mL/ha dose, T6: bifenthrin (110 g/L EC) at a 400 mL/ha dose and T7: control without insecticide application (water).

stands out because it reached 77% effectiveness during the third evaluation; spinosad was the best treatment with ecological characteristics, reaching 90% efficacy with only two applications, positioning it as the best non-synthetic option for the control of thrips in strawberries (Figure 2). The foregoing suggests that two or three consecutive applications every seven days of these biorational insecticides are sufficient to maintain the thrips populations at acceptable levels. The alternation of treatments is recommended, starting, for example, with an application of spinosad or bifenthrin, followed by two applications of neem extract, rotating the molecules in later production cycles. Coinciding with our results, Thoeming and Poehling (2006) evaluated using Neem-Azal-U (17% azadirachtin), a formulation developed for the absorption of roots against *F. occidentalis* in beans. There, the treatment caused mortalities from 70 to 98% after soil application, they also reported that a combination of azadirachtin with predatory mites resulted in efficiencies of up to 99%. Similarly, Kay and Herron (2010) evaluated insecticidal treatments for the control of *F. occidentalis* in peppers (*Capsicum frutescens*) and reported that spinosad achieved 98 to 100% control efficacy. It is important to mention that, throughout the trial, no treatment produced phytotoxic effects on the crop, the above contributes and justifies the use of biorational products.

## CONCLUSIONS

All insecticide treatments had a negative effect on the populations of *F. occidentalis* in strawberries; the highest efficacy percentages were recorded during the third evaluation, which occurred 21 days after the first application. These were obtained by spinosad, imidacloprid + lambda cyhalothrin and bifenthrin. However, three consecutive applications of neem extract are sufficient to reduce infestation of the pest by more than

75% in strawberries. This research shows that the incorporation of biorational products in the thrips management could reduce the high usage of synthetic pesticides and delay the development of resistance, as well as an ecological strategy to reduce the effect on natural enemies. Therefore, its incorporation into an integrated pest management system is recommended for this crop.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Characterization of cellulose and sugarcane (*Saccharum* spp.) straw from five cultivars grown in the humid tropic of Mexico

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## ABSTRACT

**Objective**: The aim of this study was to determine the cellulose content and characteristics of sugarcane straw from the cultivars MEX 69-290, MEX 68-P-23, CO-997, SP 70-1284 and CP 72-2086.

**Design/methodology/approach**: A completely random experimental design with six replicates was conducted; the study factor was the sugarcane straw from the five evaluated cultivars. For cellulose extraction, the sodium hydroxide (soda) method was used on dried sugarcane straw of 2 mm. The crystallinity and crystal size were determined with x-ray diffraction (XRD); the fiber length had achieved a measurement with a DMRE optical microscope.

**Results**: Among the results, it can be noted that the cultivars MEX 69-290 and SP 70-1284, which presented less cellulose content (8.4 g and 8.5 g) and lower yields (42.1% and 42.6%), while the cultivar CO-997, presented higher cellulose content and yield which ranged from 9.8 g to 49.8%. The crystallinity of cellulose was higher in the sugarcane straw from the cultivars SP 70-1284 and MEX 68-P23. The crystal size of cellulose was 2.3 nm. The length of cellulose fibers was small ($<945.7\ \mu m$).

**Study limitations/implications**: The collection of samples in the field, as well as selected materials for digestion.

**Findings/conclusions**: The sugarcane straw is a potential source of cellulose for the paper industry.

**Key words**: Agroindustrial sector, fiber length, pulp and paper industry, pulp mill effluents, sugarcane byproducts, XRD.

## INTRODUCTION

The sugarcane straw is a residue that is generated during the sugarcane harvest and remains in the field (in the form of green leaves, dry leaves, tips and pieces of stem). These residues can be harnessed to improve the soil organic matter contributing to the

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sustainability of the system (Salgado *et al.*, 2014). However, in the most of cases, the harvest residues are burned to prevent their re-integration into the crop system, affecting the recycling of soil nutrients. In some countries, the sugarcane straw from the mechanical harvesting is burned in coal-fired boilers to produce energy, feeding cattle, to produce cellulosic ethanol or to obtain paper (Ortiz *et al.*, 2012).

In Mexico, there are 57 sugarcane mills being the Presidente Benito Juarez (PBJ) mill, the most important located in the tropical humid region of Tabasco, Mexico. This sugarcane mill has 20923 hectares under sugarcane cultivation in the Cárdenas and Huimanguillo municipalities. Their 4500 producers of sugar cane belong to two cane associations: CNC and CNPR (NUC, 2014). The sugarcane field has 29 cultivars, being the cultivars MEX 57-473, MEX- 68P-23, CO-997, MEX-69-290 and SP-701284, which occupy 98% of the cultivated area. In recent years, in light of the lack of cutters, and to improve the sustainability of the system, a green harvest system, which generates around 18 t $ha^{-1}$ of sugarcane straw per harvest, was presented. The residue, considered waste in the Article 69 of the Sugar Cane Sustainable Development Law, has caused difficulties in cultivation work, generating accidental fires, and as a result, some farmers tend to incorporate them into the soil to take advantage of their positive effects on soil nutrient recycling and crop yield (Salgado *et al.*, 2014).

Sugarcane straw is mainly composed of cellulose (32-44%), hemicellulose (24-30%), lignin (12-36%) and ashes (2-7%). This chemical composition may vary depending on the site of cultivation, climatic conditions, state of development of the cane, and the cane variety (Costa *et al.*, 2015). On the other hand, cellulose has a wide use in the textile industry and in the paper manufacturing industry (Zhang *et al.*, 2010). The lignocellulosic compounds from the sugarcane straw present a wide potential for use as textile fibers, raw material for paper manufacturing, or generation of biofuels or energy (Costa *et al.*, 2015). Recently, several methods have been generated to modify the lignocellulosic components of the sugarcane straw, among them, are acetosolv (Saad *et al.*, 2008), ethanol/water (Moriya *et al.*, 2007), sodium hydroxide-soda/anthraquinone (AQ) (Costa *et al.*, 2013), and the biological usage of the fungus *Ceriporiopsis subvermispora* (Costa *et al.*, 2015).

It is important to note that in Mexico, have been harvested 790481 hectares of sugarcane in the 2013/2014 cycle, from which 91.0% was burned for its harvest and only 9% was harvested as green and incorporated into the crop system (NUC, 2014). It is evident that the sugarcane straw waste is not being used properly and it becomes urgent to show a potential use that would utilize lignocellulosic components of sugarcane straw, such as paper manufacture. Given these concerns, the aim of the present study is to evaluate the cellulose content and characteristics generated from sugarcane straw from five different sugarcane cultivars grown in the tropical humid region of Mexico.

## MATERIAL AND METHODS

**Straw samples**. Samples of sugarcane straw from the cultivars MEX-69-290, MEX-68-P23, SP 70-1284, CO-997 and CP-72 2086 were harvested from November to February 2014 in sugarcane plantations of 12 months of age prior to harvest. These plantations were located in the towns C-31 and C-34 belonging to the supply area of the

President Benito Juarez sugarcane mill in the Cárdenas municipality, Tabasco. These are located at 17° 58' 50" LN and 93° 27' 28" LO, at an altitude of 20 m.a.s.l. The average annual temperature is 26 °C; average rainfall is 2100 mm, with a relative humidity of 80%. Subsequently, thirty (30) stems of each sugarcane cultivar were randomly selected; the tips and dried leaves were collected and stored in large plastic bags. The sugarcane straw from each cultivar was milled through a 4 mm mesh in a forage chopper. Therefore, sub-samples of 3.0 kg have dried in a forced air oven at 65 °C for 48 h. additionally; the subsamples were ground through a 2 mm mesh in a Wiley mill. The material was stored in plastic bags. The cellulose extraction was carried out using acid hydrolysis method according to Bolio *et al.* (2011). The method consisted of two phases as follows: Pretreatment with NaOH and Pulpeo through acid hydrolysis with $H_2SO_4$, finally, with cellulose whitened based on NaClO.

**Pretreatment**. 800 mL of 10% NaOH was mixed with 40 g of dried sugarcane straw. The mixture was heated to 70 °C on a Thermo Scientific model CIMAREC heating plate until the first bubble was observed, then it was left boiling for 10 min. After that time, the mixture was left for 20 min to have allowed a temperature decreasing, therefore, washed with tap water five times, and then with purified water until reaching a pH 7. The mixture was passed through a sieve to remove the liquid, and the residue was crumbled and placed into an aluminum tray for drying process in a forced air oven at 65 °C for 12 h. Finally, the dry weight of the cellulose fiber was recorded.

**The pulping process**

**Hydrolysis**. 230 mL of 0.4% $H_2SO_4$ was added to 20 g of dried cellulose fiber and homogenized with a glass rod. The mixture was heated to 70 °C on the heating plate. Once the mixture reached its boiling point, the vessel was capped for one hour. The mixture was left for 10 min to cool down and therefore, washed eight times with purified water with the aim to retain the residue.

**Chlorination for lignin removal**. The previously washed residue was placed into a beaker, and 285 mL of 3.5 % sodium hypochlorite (105 mL of Chloralex® and 180 mL of purified water) was added. The mixture was placed in a boiling bath and have left to be heated for 10 min while being constantly stirred. Eight washes were then performed with purified water until had achieved a neutral pH. The product was returned into the beaker.

**Alkaline extraction**. 200 mL of 20% NaOH was added to the residue and stirred manually with the aid of a glass rod for 60 min. Additionally, 12 washes with purified water were performed until had achieved a neutral pH.

**Bleaching**. The cellulosic residue was placed into a 1000 mL beaker and 240 mL of 0.5% NaClO (15 mL of Cloralex® with 270 mL of purified water) was added. This was manually homogenized with a glass rod for one hour. Thereafter, eight washes with purified water were performed. The white residue that appeared, which was the cellulose, was placed in flat-bottomed aluminum trays and left for 24 h at room temperature. Therefore, have allowed drying in a forced air oven at 65 °C for 24 h. Finally, the dry weight of the cellulose obtained was recorded and stored in plastic bags.

**Studied variables**

**Cellulose fiber and cellulose yield**. The amount of cellulose fiber and cellulose extracted from the studied sample is 80 g of sugarcane straw, which was calculated using the Equation 1.

$$\textit{Yield of cellulose fiber or cellulose } (\%) = (C * 100)/80 \textit{ g of dry straw} \quad \text{Equation 1}$$

Where: $C$ is the weight of cellulose fiber or cellulose extracted in each sample of sugarcane straw (g).

**Properties of cellulose**. Length of the fiber ($\mu$m). For the fiber length, had achieved a measurement of the cellulose samples, TAPPI 271 standard pulp and paper fiber of om-12, which was used while employing an automated optical microscope with polarized light in the range of 0.1 mm to 7.2 mm. The Leica optical microscope used was the Model DMRE™, equipped with a DFC295 digital camera with lens at 2.5x and LAS Suite v4 software®. The studies were carried out in the Department of Wood, Cellulose and Paper (DMCP) at the Universidad de Guadalajara, Jalisco, Mexico.

**Crystallinity and crystal size**. Sugarcane straw and cellulose samples were extracted from the straw of five cultivars of sugar cane. Subsequently, samples were passed through a size 100 mesh screen.

To determine crystallinity and crystal size, 1g of straw and cellulose extracted from the sugarcane straw was used. Readings were taken according to the X-ray Diffraction, Powders method (PXRD) technique; using a "Bruker D8 Advance Vantec" Diffractometer with CuK$\alpha$ spectrum ($\alpha = 1.5418$ Å and 35 of the 20 mA applied current), at the Academic Division of Basic Sciences Research Department, Universidad Autonoma de Juarez, Tabasco-Mexico. The percentage of crystallinity (Xc%), was calculated using the Equation 2.

$$Xc\% = 100\left[1 - \left(I1 / I2\right)\right] \quad \text{Equation 2}$$

Where: $I1$ is the minimum peak intensity; $I2$ is the maximum intensity of the crystalline peak.

The crystal size was calculated using the Scherrer Equation (Equation 3).

$$t = 0.9\lambda / B\cos\theta\alpha \quad \text{Equation 3}$$

Where: $t$ is the crystal size;$\alpha$ is the wavelength of the utilized radiation ($\lambda$Cu); $B$ is the width at the medium height of the sample at the diffraction peak;$\theta$ is the position of the diffraction peak; 0.9 is the crystal form factor.

**Statistical analysis**. For the studied variables: cellulose, cellulose yield, cellulose fiber and cellulose fiber yield, analysis of variance was performed with a completely randomized

design, where the treatments were the sugarcane straw generated from five cultivars of sugarcane, which were analyzed with six replicates. To identify significant difference among treatments and statistical significance for all comparisons was made at $p<0.05$. Tukey's multiple range test was used to compare the mean values of treatments using the SAS statistical software version 9.0®. For the crystallinity and crystal size variables, only two readings of each sample were performed, therefore, the mean and the standard deviation were calculated as a measure of the error.

## RESULTS AND DISCUSSION

**Cellulose Fiber**. According to the analysis of variance results, there are highly significant differences in the cellulose fiber after the extraction of the evaluated cultivars in terms of the sugarcane straw (Table 1). The CV was 3.9%, which indicates a higher precision in the measurement of this parameter. According to the Tukey test, two groups of cultivars are observed in terms of the amount of the obtained cellulose fiber, with the cultivar MEX 69-290 being the smallest in comparison with the other cultivars, which is attributed to the fact that this cultivar lost more lignin and parenchymal material than the other evaluated sugarcane cultivars.

The other sugarcane cultivars were statistically the same, with values, which ranged from 31.4 to 32.8 g of cellulose fiber. In undertaking a sugarcane straw harvesting program to extract food-grade cellulose or cellulose fiber to create kraft paper, these sugarcane cultivars must be considered. Given these concerns, the yield of cellulose fiber also showed significant differences among the evaluated sugarcane cultivars (Table 1). The cultivar MEX 69-290 showed a significantly lower yield (35.1%). The rest of the evaluated sugarcane cultivars did not differ significantly in the yield of cellulose fiber variable. The values ranged from 39.3% to 41.0%, and coincided in the 39.5% yield of cellulose fiber obtained from the cultivar Mex 79-431 (García *et al.*, 2017).

**Table 1**. Cellulose fiber, yield of cellulose fiber, cellulose and cellulose yield of the sugarcane straw generated from five different sugarcane cultivars.

| Sugar cane cultivars | Cellulose fiber (g) | Fiber yield of Cellulose (%) | Cellulose (g) | Cellulose yield (%) |
|---|---|---|---|---|
| MEX 69-290 | 28.1b† | 35.1b | 8.4b | 42.1b |
| CP-72-2086 | 31.4a | 39.3a | 9.1a | 45.5a |
| MEX 68-P-23 | 32.2a | 40.3ab | 8.7ab | 43.5ab |
| SP 70-1284 | 32.8a | 41.0b | 8.5b | 42.6b |
| CO-997 | 31.5a | 39.3a | 9.9a | 49.8a |
| Mean (g) | 31.2 | 39 | 8.9 | 44.7 |
| CV (%) | 3.9 | 3.9 | 8.6 | 8.6 |
| Prob. F of T. | 0.01** | 0.01** | 0.01** | 0.01** |
| DMS | 2.1 | 2.6 | 1.3 | 6.6 |

† Averages with the same literal within the column are statistically equal to Tukey ($P\leq0.05$).
** Highly significant

**Cellulose (g)**. The analysis of variance showed significant differences in the cellulose content of the sugarcane straw from the evaluated cultivars (Table 2). The CV was 8.6%, which indicates a greater variation in the measurement of this parameter.

The Tukey test shows the cultivars MEX 69-290 and SP 70-1284, present the lowest cellulose content compared to the other studied sugar cane cultivars. The cultivars MEX 68-P23, CP 72-2086, and especially, the cultivar CO-997 contain more cellulose. With reference to the cellulose content, the yield of cellulose (%), showed significant differences among the evaluated sugarcane cultivars (Tables 2, 3).

The cultivars with the lowest yield of cellulose were as follows: MEX 69-290 with 42.1%, and SP 70-1284 with 42.6%, respectively. The cellulose contents from the other evaluated cultivars were statistically equal with values that ranged from 43.5% to 49.8%, where the cultivar CO-997 presented the highest yield (Figures 1, 2).

These yields are higher than reported by Costa *et al.* (2013), in cellulose obtained from sugarcane straw after an alkaline treatment using soda/AQ where the yield was 30%.

**Cellulose content**. During cellulose extraction, it was observed that the cultivar MEX 68-P-23 consumed more sulfuric acid than the other samples and presented residues of precipitates in the alkaline extraction. This cultivar and the cultivar MEX 69-290 presented a viscous consistency, which made it difficult to separate the fibers.

The yield of cellulose generated from the cultivar CO-997 was similar to the 48% reported for sugarcane bagasse (López *et al.*, 2016: Sánchez-Muñoz *et al.*, 2021). The results indicate that sugarcane straw could be a promising source of cellulose.

**Crystallinity**. The crystallinity of the cellulose is an indicative of the arrangement of the polymer chains in the cellulose fibrils. According to Candanedo, Roman & Gray

**Table 2**. Crystallinity of cellulose from sugarcane straw and extracted cellulose from five different sugarcane cultivars.

| Cultivar | Cellulose crystallinity of sugar cane straw (%) | Crystallinity of extracted Cellulose (%) |
|---|---|---|
| CO-997 | 38±0.5 | 62±0.8 |
| CP 72-2086 | 36±0.7 | 61±0.5 |
| MEX 68-P23 | 37±0.6 | 69±0.9 |
| MEX 69-290 | 42±0.5 | 62±0.8 |
| SP 70-1284 | 38±0.7 | 77±0.8 |

**Table 3**. Size of cellulose crystal of untreated sugarcane straw and cellulose extracted from the straw of five different sugarcane cultivars.

| Cultivar | Size of straw crystal from untreated cane (nm) | Crystal size of extracted cellulose (nm) |
|---|---|---|
| CO-997 | 2.3±0.1 | 2.2±0.05 |
| CP 72-2086 | 2.7±0.05 | 2.2±0.1 |
| MEX 68-P23 | 2.6±0.1 | 2.3±0.05 |
| MEX 69-290 | 2.5±0.1 | 2.3±0.1 |
| SP 70-1284 | 2.6±0.5 | 2.3±0.1 |

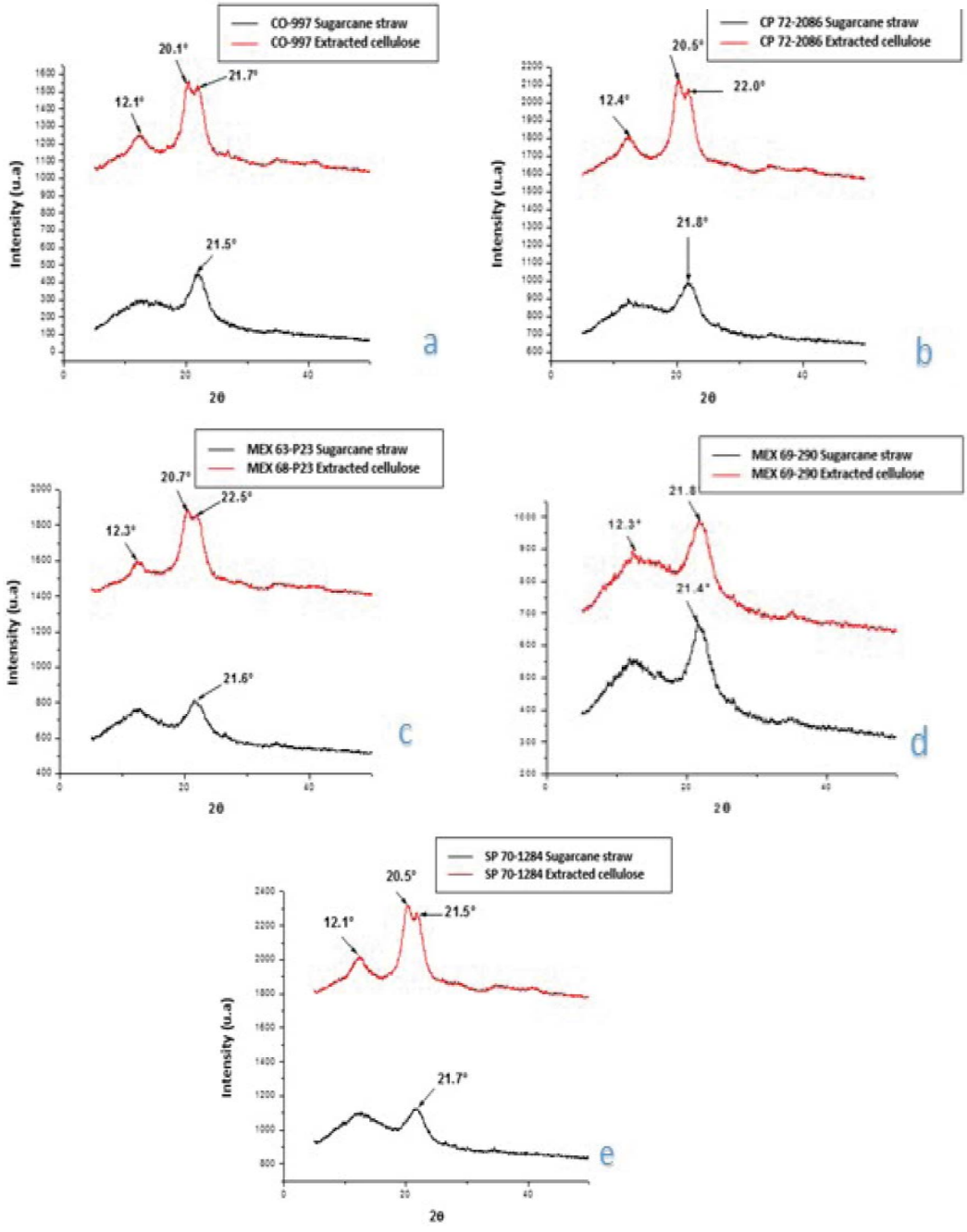


**Figure 1**. XRD patterns of untreated sugarcane straw and extracted cellulose from different sugarcane cultivars: a) CO-997, b) CP 72-2086, c) MEX 68-P23, d) SP 70-1284, and e) MEX 69-290.

(2005), if the fibrils are in disordered regions, they are amorphous, and if they are highly ordered, they are crystalline. In Figure 1, the diffractograms of the sugarcane straw sample and the extracted cellulose are presented.

The peak at 2=22.5° and 2=12.3°, 20.7° and 21.6° correspond to the cellulose structure (Wang *et al.*, 2007; Costa *et al.*, 2015). The crystallinity of the cellulose contained in the sugarcane straw was higher in the straw of the cultivar MEX 69-290. The rest of

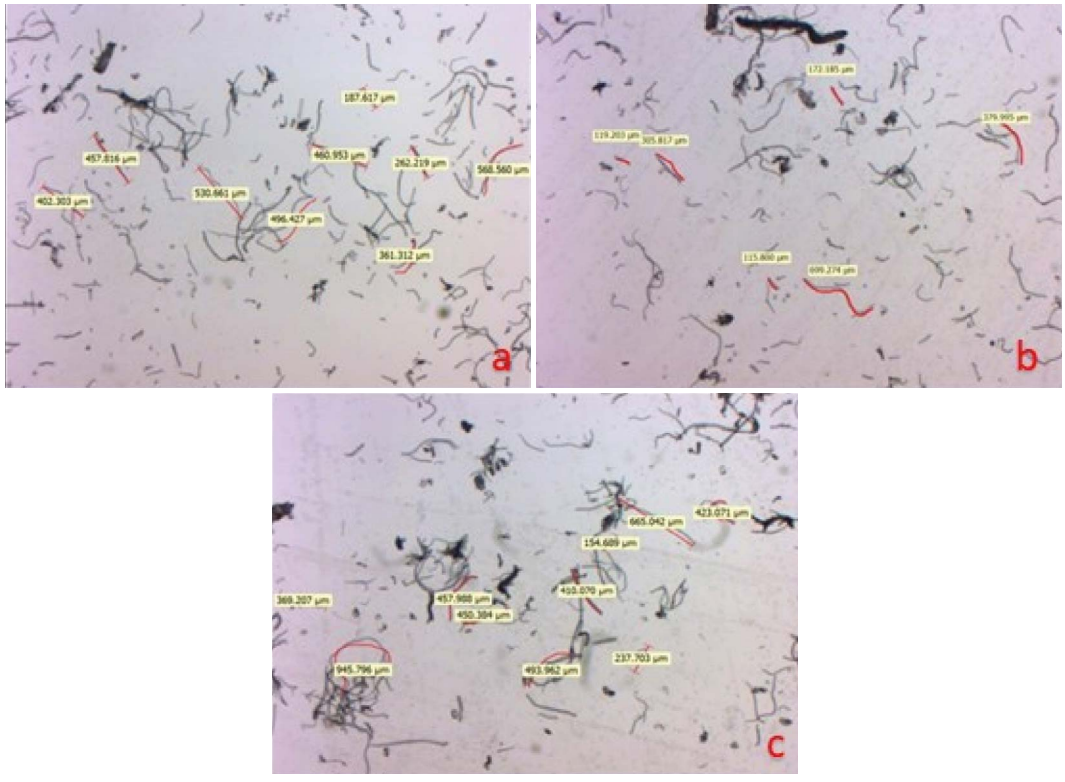


**Figure 2**. The fiber length of the studied sugarcane cultivars a) SP 70-1284, b) Mex 68-P23 and c) CP 72-2086, taken with an optical microscope, 25X amplification.

the studied sugarcane cultivars presented values similar to each other or below the average (Table 2). The average cellulose crystallinity of the sugarcane straw, 38.2%, which was lower than the 56.07% crystallinity of the cellulose of the sugarcane straw (García *et al.*, 2017). The crystallinity percentage of the extracted cellulose is higher in comparison to that observed in the cane straw cellulose (Table 2), which is due to the elimination of lignin and hemicellulose (Kumar *et al.*, 2015). It was also observed an increasing in the crystallinity of the cellulose when passing from untreated sweet sugarcane bagasse to treated sugarcane bagasse. The peaks of the interferogram correspond to the links O-H, H-C-H, C-H, and C-O-C of the cellulose molecules (Visakh & Thomas, 2010), which coincides with that reported by García *et al.* (2017), when analyzing the purity of the cellulose extracted from the sugarcane cultivar Mex 79-431, extracted with both methods.

The crystallinity of the cellulose extracted from the sugarcane straw was higher for the cultivar SP 70-1284 with 77%, followed by the cultivar MEX 68-P23 with 69%, and the other studied cultivars presented a cellulose crystallinity content ranged from 61 to 62% (Table 2).

The crystallinity of sugarcane straw cellulose is similar to the cellulose extracted from cotton, flax and ramie, which have a crystalline fraction ranged from 60 to 70% and higher than the 55% crystallinity determined for sugarcane bagasse cellulose (López *et al.*, 2016). The crystallinity of the cultivar SP-70-1284 was similar to the crystallinity of the cultivar Mex 79-431 (74%) reported by García *et al.* (2017).

The average size of the cellulose crystals of the untreated sugarcane straw in the studied cultivars was 2.6 nm (Table 3). This crystal size was similar to those reported for the *Asplenium fernandezianum* Kunze. (2.6 nm) (Newman, 1999), and for sugarcane bagasse (López *et al*., 2016), and lower than those reported for the rachis of the banana cluster (4.46 nm) (Bolio *et al*., 2011). The crystal size in the cellulose extracted from the sugarcane straw of the evaluated cultivars was similar with an average of 2.3 nm (Table 3); in comparison to the crystal size reported for the cellulose extracted from the sugarcane straw of the cultivar MEX 79-431 (García *et al*., 2017), which generated greater crystallinity by losing the amorphous area and better defining the arrangement of cellulose molecules.

**Fiber length**. The results obtained with the sugarcane cultivars SP 70-1284, MEX 68-P23, and CP 72-2086, showed that the size of the cellulose fiber extracted is small (Figure 2), which prevents the formation of kraft paper sheets. An increased order in the size of the fiber was reported in Figure 2 as follows: SP 70-1284 (568.5-187.6 $\mu$m) < MEX 68-P23 (699.2-115.8 $\mu$m) <CP 72-2086 (945.7-154.6 $\mu$m). The fiber length of the sugarcane cultivar CP 72-2086 is equal to the average length of the eucalyptus (*Eucalyptus occidentalis* Endl.) fiber with 940 $\mu$m, and less than maize (*Zea mays* L.) leaf (1860 $\mu$m), of sugarcane bagasse (1500 $\mu$m), and henequen "pineapple" (*Ananas comosus* (L.) Merr.) fibers (1700 $\mu$m) (Prado, Anzaldo, Becerra, Palacios, Vargas & Rentería, 2012). The small size of the cellulose fiber of the evaluated sugarcane cultivars is due to the initial sugarcane straw size used to extract the cellulose, which was 2 mm (the particle size after the straw milling process). This indicates that in a near future studies, the particle size would had achieved an increasing, which would range from 30 to 40 mm (Costa *et al*., 2013).

## CONCLUSIONS

The studied sugarcane cultivars showed significant differences in the cellulose content obtained from the straw. The highest content and yield of cellulose was obtained by treating the straw of the cultivar CO-997 and the lowest in the cultivars MEX 69-290 and SP-701284. The crystallinity of the cellulose extracted from treated sugarcane straw was higher in comparison to that observed in the cellulose of the untreated straw, which is due to the effect of treatment with acid hydrolysis. Among the different studied sugarcane cultivars, SP 70-1284 and MEX 68-P23 presented the highest crystallinity of the cellulose in treated straw. The crystal size of the cellulose in the untreated sugarcane straw presented an average of 2.6 nm, being larger, to the crystal size of the cellulose extracted from the treated sugarcane straw (2.3 nm). The length of the cellulose fibers extracted from the treated sugarcane straw was less than 1000 $\mu$m and presented wide ranges of variation within each cultivar. The sugarcane straw cellulose from the studied cultivars has similar characteristics and presents potential for use in various areas, in the paper and fabric industry, as well as in composite materials, biomaterials, etc.

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AGRO PRODUCTIVIDAD

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# Reproductive management in white-tailed deer (*Odocoileus virginianus* Zimmermann)

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## ABSTRACT

**Objective**: To review information related with the reproductive physiology and management of white-tailed deer for reproductive specialists and producers.

**Design/methodology/approach**: The information presented in this document relies on the review of scientific papers and on experience gained in white-tail deer production systems.

**Results**: White-tailed deer is a species with seasonal reproduction and one of the most important hunting species in Mexico. Currently, all reproductive biotechnologies applied to small ruminants can be used in white-tailed deer.

**Limitations of the study/implications**: Information regard the physiology and reproductive management of white-tailed deer is limited, probably due to conditions specific to its production system.

**Findings/conclusions**: Research and publication of information regard the physiology and reproductive management of white-tailed deer is needed.

**Key words**: hunting; physiology, biotechnologies, ruminant.

## INTRODUCTION

The white-tailed deer (Figure 1) naturally distributes throughout most of the Mexican territory and is perhaps the most important hunting species of interest in the country (SEMARNAT, 2014). According to the CONABIO report (2012), of the 365 species registered for wildlife management units for conservation and exploitation (UMA) in the wild, white-tailed deer was registered in 86% of them. This indicates the economic and social importance of this species in Mexico.

In the white-tail deer production units, it is necessary to perform reproductive management practices to increase the probability of births of fawns at the desired time and with adequate characteristics. Currently, most reproductive biotechnology used in sheep and goats can be adapted to deer, yet it is important to know the general aspects of the reproductive physiology of the species, as well as its management within the

**Figure 1**. Male white-tailed deer specimen.

production system. The objective of this document is to describe basic aspects of the physiology and reproductive management of white-tail deer.

**Seasonality**

The availability of forage for both wild and domestic ruminants vary throughout the year. Regarding this situation, white-tail deer have adaptive strategies for the efficient use of food availability, since the last third of gestation and lactation demand large amounts of nutrients (Osborn *et al.*, 2000). Therefore, the number of offspring per parturition will depend on the quantity and nutritional quality of the consumed diet by the females; if the diet is nutritionally poor, females will only procreate one offspring, but if the diet is adequate and abundant, she will have two or more fawns (Ramírez, 2004).

Female white-tail deer are seasonal polyestrous and in both temperate and warm climates, they begin their reproductive activity in autumn and give birth by summer (Harder and Moorhead, 1980), when food is most available (DelGiudice *et al.*, 2007; Asher, 2011). It is important to note that there is little or no information about the births of this species in its native warm environment, although there are studies that indicate that this species shows strictly seasonal birth patterns (Yamauchi & Matsuura, 2009; Asher, 2011). Changes in the photoperiod in temperate climate are neuroendocrine signals to initiate or stop reproductive activity (Jacobson, 2007). In contrast, in the warm climate, deer are not exposed to a well-defined photoperiod, so their reproductive patterns evolved as a response to factors such as seasonal fluctuations in food availability, local competition for forage resources with other wildlife species, predation pressure, and variations in climate (Asher, 2011).

Changes in photoperiod or day length are sensed by the retina and suprachiasmatic nucleus of the deer, and are measured through the neurochemical secretion of melatonin, which is secreted during the night. It is a neuroendocrine signal that activates the reproductive axis in the short-day season, but inhibits it in the long days (Ebling, 2010). The greater exposure to melatonin secretion on short days decrease the sensitivity of the hypothalamus to the negative feedback of estradiol (hormone responsible for seasonality reproduction), which allows an increase in the pulsatile secretion of gonadotropin-releasing

hormone (GnRH) to activate the reproductive axis (Yamauchi and Matsuura, 2009); contrary to the case when deer are exposed to long days (Malpaux *et al.*, 1996).

During the reproductive season, females exhibit estrous behavior, luteal progesterone secretion ($\geq 1$ ng $mL^{-1}$ a level considered indicative of ovulation), conception rate through natural mating higher than 85% and reduced early embryonic mortality. In contrast, during anestrus, deer have low plasma progesterone concentrations (on average $<0.06$ ng $mL^{-1}$), this is indicative of a complete cessation of ovarian activity and can last from 4 to 6 months, from spring to early fall (Harder and Moorhead, 1980).

**Puberty**

White-tail deer undergo pubertal changes prior to puberty onset. These changes are observed in secondary sexual characteristics and reproductive anatomy. In males, there is an increase in body size, neck bulging, development of calcified and hardened antlers during their first winter and appearance of velvet on them due to the production of testosterone; the rubbing of the antlers is the signal to start reproduction. The anatomical changes in the male's reproductive system are characterized by an increase in the secondary sex glands, the testicles and the epididymis. In females, changes in reproductive characteristics are less obvious than in males. Puberty may begin as early as six months of age, which depends on the food availability and quality. It is believed that up to 80% of fawns can conceive during their first year of life. But if food availability is limited or of poor nutritional quality, puberty is delayed by about one year. As in other cervid species, nutrition, especially energy intake and protein, plays an important role in the onset of puberty in females, since for this physiological process to begin, they must have an adequate body size to start reproducing (Jacobson, 2007).

**Estrous cycle**

The estrous cycle of the female white-tail deer (Figure 2) has an average duration of 26 days, and varies from 21 to 30 days (Knox *et al.*, 1988). Estrus occurs on day zero of the cycle, a stage in which females accepts male's mating and last 39 h on average (Verme & Ozoga, 1981). The female's receptivity at estrus is the result of the increase in estradiol concentrations coming from the preovulatory follicle(s); concentrations increase five days before estrus and reach their highest peak on the estrus day, which coincides with the preovulatory peak of luteinizing hormone (LH; Plotka *et al.*, 1980). During this period, the females may copulate with more than one sire, resulting in offspring from different parents (DeYoung *et al.*, 2002).

Ovulation occurs between 12 and 14 h after the end of estrus (Verme & Ozoga, 1981). Females ovulate between 1.22 to 2.18 oocytes, with higher ovulatory rate in adult females than in young females (Ransom, 1967), with 1.88 offspring on average and mating services that can be between 25-96 h after estrus (Verme & Ozoga, 1981). Following ovulation, the hormonal environment previously dominated by estradiol disappears with the rupture of a preovulatory follicle, giving way to the formation of the corpus luteum (CL) and its respective production of progesterone. Luteal progesterone is responsible for gestation maintenance, at least until day 156 (Plotka *et al.*, 1982). The production of this hormone progressively increases after ovulation. However, if the female does not become pregnant

**Figure 2**. White-tail deer female specimen.

after inseminated or mated, these steroid concentrations begin to decrease seven days before the next estrus (Plotka *et al.*, 1980).

**Manipulation of the estrous cycle**

The main objective of the estrous cycle manipulation is to control the onset of estrus at the most convenient time. For cattle, sheep and goats, the control of estrus onset as well as the use of artificial insemination (AI) have allowed controlling the productive development of herds and its genetic improvement. However, reports on the reproductive physiology, estrus synchronization and AI in white-tail deer females are scarce, despite their importance for hunting.

The basis for the estrus manipulation onset is to create a luteal phase, followed by a follicular phase, accompanied by a decrease in progesterone concentrations and an increase in estradiol concentrations from a developing follicle. The luteal phase can be simulated by insertion of a progesterone-releasing intravaginal device (PIDR), progesterone implants or sponges. Progesterone-releasing intravaginal device insertion has the advantage that it can be used at any time of the year, but their cost is a limitation. The intravaginal device can be applied for a period of seven (Gentry *et al.*, 2007) or 14 days (Mellado *et al.*, 2013).

In sheep and goats, it is common to administer equine chorionic gonadotropin (eCG) to females before or at the time of removal of the intravaginal device or progestogen, to increase the ovulation rate, as well as the synchrony of the onset of estrus after the device is removed. However, the application of 200 IU of eCG in white-tail deer females has not yielded satisfactory results (Gentry *et al.*, 2012; Haslag *et al.*, 2016), suggesting the need for studies to evaluate the optimal dose of this hormone. Using the progesterone implant (Norgestomet) is carried out by placing only half of the implant in the animal's ear for 14 days (Willard *et al.*, 2002). According to Randel *et al.* (1998), between 75 and 80% of deer synchronized with the intravaginal device or implant will show estrus at approximately 58 h after progestogen withdrawal.

Also, prostaglandin injection is used for estrus induction; although it is a cheaper method, it is only effective during the reproductive season, when regression of the existing CL is achieved to induce the onset of a new estrus or terminate an unwanted gestation. The

used prostaglandin dose should cautiously be chosen, since the doses that cause regression of the CL and termination of gestation in cattle and goats are not effective in white-tail deer females (Becker & Katz, 1994). In terms of estrus induction, satisfactory results have been obtained with the application of prostaglandin on day 11 of the estrous cycle (Magyar *et al.*, 1989). According to Magyar *et al.* (1992), the effectiveness of prostaglandin injection in estrous induction is of 60%.

Oestrus detection is commonly performed by repeated introduction of vasectomized males. During estrus, females remain immobile, with their ears folded back accepting males mounting, some females even adopt this position when handlers place their hand on the animal's rump. When performing the estrus check, care should be taken, as some males can be violent with females not in estrus or the staff (Warren *et al.*, 1978). Females that are not in estrus, quickly move away from males and urinate more frequently, being more tolerant to the presence of the male 24 h before estrus (Gasset *et al.*, 1998).

Once estrus presence of is guaranteed, on one side, groups of females are placed to be mounted by a stallion in a specific time; a strategy to obtain a harvest of fawns at the most desirable time of the year. Randel *et al.* (1998), with a ratio of 10 synchronized females per sire, obtaining a conception rate of 70% at the first service, higher than the 55% obtained in non-synchronized females. On the other hand, the AI of females, performed either by transcervical insemination with frozen semen in a range of 0 to 30 h after estrus detection (with 67 to 73% of gestations; 1989; Magyar *et al.*, 1989) or by fixed- insemination time (60 h after removal of the intravaginal device) by transcervical route (with 24 and 56% of gestations, Gentry *et al.*, 2007; Lambe *et al.*, 2009). Willard *et al.* (2002) reported that semen deposition can be performed in the uterus body via trans-vaginal route, using an insemination applicator or the Gourley endoscope in 92 and 100% of cases.

**Gestation**

High fertility tends to be a common characteristic of several cervids and specifically for female white-tail deer, fecundity is strongly influenced by habitat and the nutritional quality of the consumed food. Few studies have been conducted on this physiological stage, but it has been reported that the average duration of gestation is 200 days, with a 187 to 222 days variation (Ramírez, 2004). After fertilization, the embryo reaches the uterus 5 to 6.5 days after ovulation. Embryo implantation occurs shortly before day 27 post-ovulation. It has been established that the presence of a normal half-life CL for progesterone production is a major requirement for successful gestation (Harder & Moorhead, 1980).

To prevent lysis of the CL, the embryo secretes a molecule called interferon tau, which suppresses oxytocin and prostaglandins release, resulting in maternal recognition of gestation (Asher *et al.*, 2007). Fawn weight appears to be the main factor that triggers birth; however, the timing of birth slightly varies from year to year, depending on the conception timing and maternal nutrition (Heffelfinger, 2006). The number of offspring as well as their sex depends on the age and nutrition of the mother; usually one-year-old females have only one fawn per parturition, two-year-old females average 1.3 to 1.6 fawns, and females over three years of age have 1.5 to 1.8 fawns per parturition (full reproductive age; Jacobson, 2007; Gentry *et al.*, 2012; Figure 3).

**Figure 3**. Female white-tail deer specimens with their fawns.

Gestation may be altered by nutritional factors; females with poor nutrition, slightly increase gestation length, are more likely to have low birth weight offspring, abortion or simply abandon their young after birth, compared to well-fed females (Ramirez, 2004). As parturition approaches, the females separate from the rest of the group and looks for a suitable place to give birth. After birth, the mother leaves the fawns in a safe place, returning to see them only two or three times a day. When calving twins, the fawns are usually separated at different resting sites to reduce the likelihood of predator attack on both (Heffelfinger, 2006). Deer milk contains twice as many nutrients as cow's milk, which provides the young with the necessary nutrients for a rapid development. After a few weeks, fawns begin to consume green forage, and in the first two months of life begin to move around with their mother. Weaning occurs at between two and four months of age (Jacobson, 2007).

## CONCLUSIONS

The economic and hunting importance of white-tail deer (*Odocoileus virginianus*) in Mexico and the lack of information related to aspects of the physiology and reproductive management of this species, highlights the need to investigate, generate knowledge and disseminate the experiences that may arise from studies and field work with white-tail deer, in order to facilitate the application of reproductive biotechnologies.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Reproductive aspects of the male jaguar (*Panthera onca*): A review

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**ABSTRACT**

**Objective**: To describe the anatomy, morphology and physiology of the reproductive system of male jaguars, as well as assisted reproduction techniques.

**Methodology**: A literature review on the anatomy and morphology of the jaguar's reproductive system, its physiological characteristics and assisted reproduction techniques were carried out to document relevant information on the topic.

**Results**: With this review, basic aspects of the morphology of the reproductive system of the jaguars are disclosed, although scarce knowledge is available on their reproduction. The advances in the collection, evaluation and cryopreservation of semen of this feline are shown, in addition to assisted reproduction techniques such as artificial insemination and *in vitro* fertilization, which have a great potential to safeguard the species.

**Study limitations**: The jaguar, an emblematic species of Latinamerica, is an endangered species, like other wild felids species as ocelot (*Leopardus pardalis*) and margay (*Leopardus wiedii*), which makes it necessary to have a national assisted reproduction program. However, for this to be possible, information about their reproductive physiology is necessary, which is complicated in wild animals and even more so because the reproductive mechanisms greatly differ between felids species. There is scarce information in this regard from its free-living or Mexican zoos, it is for this reason necessary to generate such information.

**Conclusions**: It is necessary to continue working on designing protocols for artificial insemination and other assisted reproduction techniques such as *in-vitro* fertilization specifically for male *Panthera onca*.

**Keywords**: semen; free-living felids; physiology; reproduction.

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## INTRODUCTION

Within an ecosystem, felines are of great importance because they are predators and help to keep it in balance; however, many of them are in danger of extinction due to habitat loss and poaching. Six of the 36 reported species of the Felidae family that existing worldwide distribute in Mexico (Ceballos and Oliva, 2005); among them, the jaguar, an emblematic species for Latin America. According to PROFEPA (2016), among the members of the Felidae family, the jaguar (*Panthera onca*), the ocelot (*Leopardus pardalis*) and the margay (*Leopardus wiedii*) are in endangered status, while the jaguarundi (*Herpailurus yagouaroundi*) is in the threatened category. This unfavorable situation raises the importance of a national assisted feline reproduction program. For this to be possible, information about their reproductive characteristics is necessary, which is complicated in the case of free-living animals and even more so because the reproductive mechanisms greatly differ between species. Therefore, information must be generated for each of them (Roldan, 2010). In addition, it would be prudent to use assisted reproduction techniques generated for other mammals. According to the Yaguareté network in Argentina (http://www.redyaguarete.org.ar/el-yaguarete/celo-y-reproduccion/), there is no specific reproductive season for jaguars, although it is noted that they give birth during spring in extreme climates, which could be due to greater prey availability; and at any time in tropical areas, since light and humidity remain constant throughout the year. In the case of Mexico, Ceballos *et al.* (2011) mention that the jaguar's reproductive season occurs between December and January. Knowledge regards the jaguar's reproduction will allow a better understanding of the mechanisms involved in its occurrence. Therefore, the objective of this review was to describe the anatomy, morphology and physiology of the jaguar's reproductive system, as well as assisted reproduction techniques in this species.

## REPRODUCTION

### Anatomy of the reproductive system

The reproductive apparatus of the jaguar consists of a penis and testes. The penis is conical and located within a prepuce; when animals are not sexually active, it is caudally oriented, however, during coitus the position is reversed (Figure 1).


**Figure 1**. Reproductive apparatus of the jaguar, testicles (A) and penis (B).

The penis consists of a root, a body and a glans penis, the latter covered by numerous androgen-dependent cornified papillae that appear after puberty. The testes (Figure 2) are in the perineal region with a craniocaudal orientation and formed by testicular tissue, head, body and tail of the epididymis, efferent and deferent ducts, spermatic cord, testicular artery and veins. The head of the epididymis is situated craniolaterally, the body is dorsal and the tail is caudal to the testicles. The vas deferens surround the testis in a cranial direction penetrating the spermatic cord. Attached to the testes are two bulbourethral glands that contribute to the formation of seminal plasma and the prostate, which is formed by a compact and a disseminated portion (Mayor and Lopez, 2010).

## Reproductive process

The jaguar's reproductive process is poorly understood, but it is believed that more than one factor is involved in its regulation, including geographic space, photoperiod, temperature, food availability and psycho-social environment (Figure 3; Feldman and Nelson, 1996).

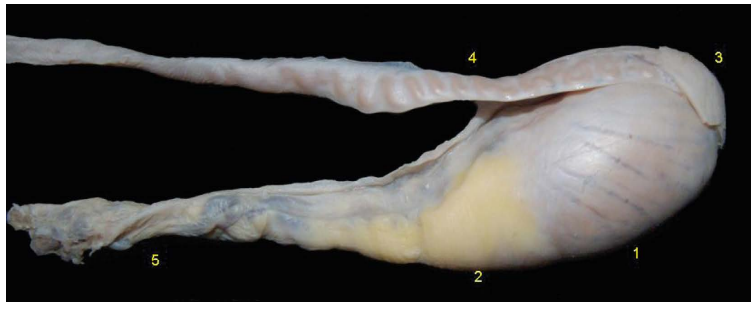


**Figure 2**. Male gonad showing its parts 1) testicle, 2) head of the epididymis, 3) tail of the epididymis, 4) vas deferens, 5) testicular vessels and nerves. With permission from Mayor and López (2010). Atlas de Anatomía de especies silvestres de la Amazonia peruana.

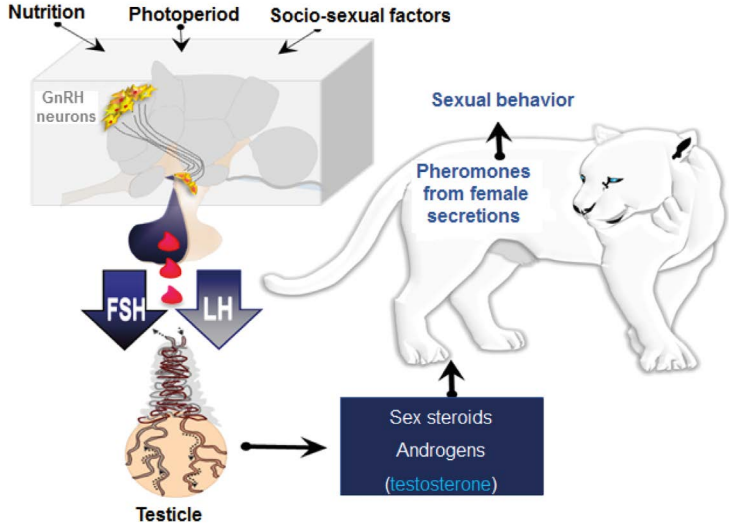


**Figure 3**. Influence of environmental factors on jaguar reproductive behavior. Pheromones help them to mark territory and avoid the entry of other males, androgens stimulate pheromone production, and secretion is constant or regulated by photoperiod.

**Reproductive hormones**. Neuroendocrine interactions between the hypothalamus and pituitary gland control testicular function. GnRH (gonadotropin-releasing hormone), a neurohormone secreted by the preoptic-hypothalamic area (APO-H), stimulates the gonadotropes in the pituitary to secrete LH (luteinizing hormone) and FSH (follicle-stimulating hormone). These gonadotropins enter and travel through the bloodstream to reach the testes to directly influence spermatogenesis through their effect on Leydig and Sertoli cells (Figure 4). For example, if the secreted testosterone concentration by Leydig cells is increased in the blood, negative feedback is exerted at the APO-H and pituitary level, inhibiting pulsatile GnRH/LH secretion, which decreases the testosterone secretion by Leydig cells. On other hand, FSH directly acts on sperm production, Sertoli cells, in turn, secrete inhibin and activin (Johnston *et al*., 2001).

**Photoperiod effect**. Photoperiod also influences males, who show a seasonal variation in androgen levels during the year. An increase in these hormones level seems to be linked both to the increase in prey and to the season in domestic cats (*Felis catus*), Pallas's cat (*Otocolobus manul*), snow leopard (*Uncia uncia*), among others. Swanson and Brown (1996) mentioned that changes in the spermatogenesis relate to the photoperiod; in addition, they indicated that there were variations in the quantity, sperm quality and hormone levels in the blood at different times of the year. Regard the free-living felids, particularly jaguars. Morato *et al.* (2001) conducted a study on reproductive aspects of this felid in captivity and found an average ejaculate volume of 8.6±1.3 mL (n=28), with a concentration of


**Figure 4**. Procedures to be performed before obtaining semen samples in jaguar. A) Anesthetized jaguar. B) Physiological constants check. C) Palpation of the testicles. D) Penile exposure.

$3.9\pm0.7\times10^{6}$ spermatozoa per mL. Although there were variations in ejaculate volume during the seasons (autumn 6.0±0.9 mL, 10.9±4.3 mL in winter, 9.0±3.7 mL in spring and summer 7.9±0.8 mL), there were no seasonal differences. For testosterone, these researchers indicated that there were also no differences due to the sampling season, although there were some numerical variations. For example, during fall, testosterone production was 170±20.7 ng $dL^{-1}$, while in winter and spring it was 324±60.4 and 287.9±52.5 ng $dL^{-1}$, respectively. Regard sperm quality, Morato *et al.* (1999) mentioned having found a high percentage of sperm malformations (51%), a large amount of sperm malformations is common in captive felids (Table 1). In the case of the domestic cats and the black-footed cats (*Felis nigripes*), Herrick *et al.* (2010) reported higher than 80% sperm abnormalities.

**Spermatogenesis**. Spermatogenesis is a sequence of cytological events of male germ cells (multiplication and differentiation) that result in spermatozoa formation. Spermatogenesis takes place cyclically in the epithelium of the seminiferous tubules of the testis, starting at the onset of puberty. At the end of the spermatogenic process, spermatozoa are released into the lumen of the seminiferous tubules and taken to the epididymis, where they complete the maturation process and acquire fertilizing capacity (Gilbert, 2005). The sperm membrane is capable of absorbing different substances produced in the seminiferous tubules, epididymis, vas deferens and accessory sex glands (semen). Another interesting aspect is that, unlike other mammals, feline sperm capacitation is simpler and takes less time (Holstein *et al.*, 2003).

## Assisted reproduction techniques

In Mexico, as in other countries, the jaguar is an endangered species due to its habitat destruction, illegal hunting and prey scarcity (Ceballos, 2010). Therefore, captive reproduction of this feline is an important option for its preservation by properly managing assisted reproduction techniques such as semen freezing, artificial insemination, in vitro fertilization and embryo transfer (Morrell *et al.*, 1998). These techniques can help genetic exchange between populations (Morato *et al.*, 2001), improve reproductive success, reduce aggressive behavior, female-male incompatibility and physical problems, and reduce the transmission of infectious diseases during mating. It is also possible to transfer semen from captive males to free-living females, or between geographically separated wild populations, which contributes to increasing genetic variability (Swanson, 2006; Morato *et al.*, 2001). This can be done with fresh or frozen semen. Therefore, there is a need for genetically characterized specimens, which is not easy to obtain (Morato *et al.*, 2001). According to Paz (2000), in Brazil only 4% of captive jaguars has reproduced in recent years, possibly due to the difficulty of determining behavior during the estrous cycle, lack of knowledge of the hierarchical structure when kept in the same cage, photoperiod (induced ovulation *vs.* spontaneous ovulation; Wildt *et al.*, 1995) and perhaps also to nutritional status (undernutrition).

In some countries, assisted reproduction techniques are applied in wild animals based on the pharmacological protocols used in domestic animals, although sometimes it is not

possible to apply the same protocols from one species to another, due to the differences in their reproductive mechanisms and their behavior.

**Semen collection**

**Animal handling**. The characteristics of wild animals are special, therefore, obtaining semen samples is performed in anesthetized animal (Herrera *et al.*, 2017) with an electro-ejaculator, whereas in any other medical procedures using anesthetics, it requires to withdrawal water and food from the specimens at least 12 and 24 h, respectively before its application. According to the AZA (2016), the following products can be used 1) Telazol® [4-8 mg $kg^{-1}$ IM (intramuscular), Kreeger and Armstrong, 2010], 2) Xylazine® (2 mg $kg^{-1}$) via IM combined with Ketamine®. It is recommended to give Rohimbine® (0.125 mg $kg^{-1}$) after anesthesia to reverse the Xylazine® effects. Atropine sulfate® (0.04 mg $kg^{-1}$) or Glycopyrrolate® (0.01-0.02 mg $kg^{-1}$) can also be administered as a single dose IM or subcutaneously in case, the animal presents excessive salivation. In some cases, anesthesia can cause contamination of the seminal sample with urine, as the bladder relaxes. Therefore, in jaguars, it is recommended to use Zoletil®, 6-8 mg $kg^{-1}$ and even supplement it with ketamine. Once the animal is anesthetized (Figure 4), it is advisable to determine their body condition, palpate the testicles and evaluate their consistency (flaccid, normal or turgid) and measure their length and width (Morato *et al.*, 2001) to determine the volume. The penis is extruded from the sheath and examined to visualize the presence of cornified papillae (1-3 scale, 3=most prominent papillae) as indicated by Swanson *et al.* (1995).

**Sperm samples obtention**. It consists in following protocols for collecting and storing by cooling or freezing the sperm samples obtained by electro-ejaculation, epididymal lavage or testicular tissue (Garde *et al.*, 1998), once the sampling is done, the semen is evaluated to determine its macroscopic and microscopic characteristics, deposited in straws and stored in liquid nitrogen tanks for later study or use.

**Electroejaculation**. It consists of electrical stimulation through a transrectal probe coupled to a voltage unit (Figure 5).

On jaguars, Morato *et al.* (2001) used a 2.6 cm diameter probe with a 29 cm length and a 60 Hz battery-powered electrostimulation (AC, 60 Hz), applying 80 electrical stimuli divided into three series; 30, 30 and 20 stimuli with 10-minute intervals between series.

**Sperm recovery from the epididymis**. This technique can be used when a genetically valuable animal dies. With this technique, spermatozoa are obtained directly from the tail of the epididymis (Chatdarong *et al.*, 2010). Two techniques can be used for this purpose 1) flotation, which consists of cutting the epididymis into small pieces in a diluent solution for the sperm extraction (Morton *et al.*, 2010); and 2) retrograde lavage which consists of injecting a buffered solution into the vas deferens and then retrieving.

**Testicular tissues preservation**. Another technique is the preservation of testicular tissue, which represents a challenge for cryobiology; there are predictions that if the testicular tissue retained active spermatogenesis, elongated spermatids could be obtained for oocyte fertilization by using intracytoplasmic sperm injections (Oliveira *et al.*, 2015). On other hand, Abrishami *et al.* (2010) proposed that testicular freezing can be used in

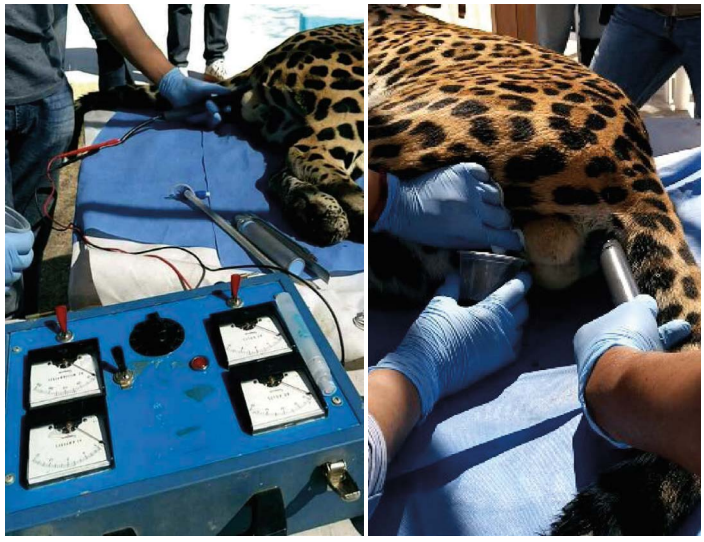

**Figure 5**. Electro-ejaculation technique applied to a 3-year-old jaguar in captivity.

cases where animals suddenly die. Regard the above, in recent years Campos-Junior *et al.* (2014) published a successful study in collared peccary.

**Sperm evaluation**. Once the semen is collected, their evaluated parameters are their total volume (mL), pH, sperm count ($\times 10^6$), motility (0-100%), advancement on a scale of 0 to 5 (0=no movement and 5=rapid forward movement; Wildt *et al.*, 1983), sperm concentration, normal or abnormal classification (Josthon *et al.*, 1994; Morato *et al.*, 2001) and acrosomal integrity (Yanagimachi, 1994). Some data on sperm evaluation are shown in Table 1, where some differences in reproductive parameters measured in captive and free-living jaguars can be observed, as well as the high percentage of abnormal sperm cells found.

**Sperm conservation**. Techniques and protocols in semen preservation should focus on the specific characteristics of each species, given the differences in the physiology during sperm production and the inherent changes in the preservation process to which the spermatozoa are subjected. How the semen is evaluated, its type of packaging (straws), the composition of the extender (diluters), duration of equilibration time, freezing curve, storage and thawing speed are determining factors for success in the conservation of male gametes from wild felines (Roldan, 2010).

**Table 1**. Testicular, seminal and hormonal characteristics in jaguar males.

| Species | Weight (kg) | TV ($cm^3$) | Vol (mL) | C ($\times 10^6/mL^{-1}$) | A (%) | M (I) (%) | ST ($ng\ mL^{-1}$) | Author |
|---|---|---|---|---|---|---|---|---|
| Captive Jaguar | | 45.3±3.9 | 8.6±1.3 | 3.9±0.7** | 51.0 | 50.6±5.8 | 114-445*** | Morato *et al.* (1999) |
| Free Jaguar | 96.0±7.7 | 52.4±3.4 | 4.1±0.7 | 35.0±21.3 | 26.5 | 73.0±6.1 | 2.1±0.8 | Morato *et al.* (2001) |
| Captive Jaguar | 72.0±11.0 | 41.6±0.6 | 8.3±0.7 | 8.0±1.7 | 50.0 | 64.0±2.4 | 3.1±0.7 | Morato *et al.* (2001) |
| Captive Jaguar | 83.1±20.3 | ND | ND | ND | ND | 32±24.3* | ND | Gaviria-Sciolle y Arias-Bernal (2011) |

VT=Testicular volume, C=Concentration, A=abnormalities, M=motility, I=index. *Progressive motility after semen thawing at 37 °C, **$\times 10^6$/mL, ST=Serum testosterone, *** (ng/dL). ND=Not determined in the works cited.

### Artificial insemination

It is a technique used to deposit spermatozoa in the female reproductive tract at the "right" time to achieve oocyte fertilization. Despite its popularity in animals of zootechnical interest, it is rarely used in reproductive programs of free-living animals due to the scarce knowledge of the female's reproductive physiology (Roldan, 2010). Despite this, they have been able to obtain offspring in ocelot females by laparoscopy (Swanson *et al.*, 1995) and in puma, tiger, cheetah, clouded panther, snow leopard, ocelot and margay females with artificial insemination using fresh semen (Roldan, 2010).

### *In vitro* fertilization and sperm microinjection

These techniques can be used when the quantity or quality of spermatozoa is reduced or in cases where the sperm do not survive the freezing process. In the case of *in vitro* fertilization, it is recommended that the conditions are like those in the genital tract, although further research is required (Roldán, 2010).

## CONCLUSIONS

Advances in assisted reproductive techniques involving male jaguars were presented. However, the necessity of specific protocols for seminal conservation and its possible use in in vitro fertilization and artificial insemination is made evident.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Combined effects of cover crops and herbicide rotation as proactive weed management in pineapple (*Ananas comosus* L. Merr)

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**ABSTRACT**

**Objective**: to evaluate the effect of two proactive strategies for weed management in pineapple (*Ananas comosus*), including (1) cover crop rotation and reduced rate of herbicide (RRH) and (2) cover crop association and RRH.

**Design/Methodology/Approach**: We conducted pineapple field experiments in Huimanguillo, Tabasco Mexico, using a complete randomized block design for both rotation and association experiments. Weed occurrence were registered and classified. The weed management effect of cover crops such as cowpea (*Vigna unguiculata*), sunnhemp (*Crotalaria juncea*), stylo (*Stylozanthes guanensis*) and velvet bean (*Mucuna pruriens*) were evaluated alone and combined with three herbicides. Data of soil ground cover and weed suppression levels were analyzed by one-way ANOVA and the means were separated by Least Significant Differences (LSD) at P=0.05.

**Results**: Synergistic interaction was detected for weed suppression in all cover crops and herbicide treatments. Combined effects of metribuzin and pendimethalin herbicides with cover crops varied from 80% - 90% of weed suppression until 90 days after treatment (DAT); however, when cover crops were combined with haloxifop plus diuron, 100% of weed control was achieved until 90 DAT.

**Study limitations/implications**: Irrigation, weather conditions may affect observations.

**Findings/Conclusions**: Our results showed that all cover crops, specially *Vigna unguiculata* and *Mucuna pruriens* in a rotation system, along with reduced rate of herbicides is novel approach strategy for weed management in pineapple plantation. Cover crops such as cowpea might improve crop performance, productivity and feasibility for farmers. The reduced rates of preemergence herbicides and cover crops will be very helpful for the farmers and for protection of environment.

**Keywords**: Cover crops, *Anananas comosus*, herbicide, weed suppression, integrated weed management.

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## INTRODUCCIÓN

Weeds causes substantial declining of crop productivity and quality, which are directly related to food security and safety. Approximately one-third yield losses occur worldwide due to weeds, in which contribution of introduced, invasive or noxious weeds is exhaustive. In 2020, 1,209,000 tons of pineapples were produced which leads Mexico to be positioned

Image by Matthias Böckel at Pixabay

as the world's ninth biggest pineapple producer (SIAP, 2020). However, weeds are one of the major constrains on pineapple production and cause elevated cost of production. Identification of the major weed species, biology and ecology is imperative to develop integrated weed management. On the other hand, herbicide are main tool for managing weeds in many crops including pineapple around the world. However, most registered herbicides in pineapple are older molecules also used in different crops. They are applied up to five times during the plant crop cycle and twice in the ratoon crop period. For more than 15 yr before its banning, farmers typically applied bromacil twice during this period, at the onset of either the plant or ratoon crop (Valverde and Chaves, 2020). However, bromacil has been banned in Costa Rica in 2017, and glyphosate in Mexico is expected to be banned by 2024.

Cover crops are largely known to provide several eco-biological services in agroecosystems (Hunter *et al.*, 2017; Kladivko *et al.*, 2014). In addition, cover crops have increasingly being studied recently for their approach as weed suppression, intentionally to promote a better management of proactive herbicide-resistant weeds strategy, especially in the overuses of glyphosate (Valverde and Chaves, 2020; Norsworthy *et al.*, 2012; Price *et al.*, 2011; Wallace *et al.*, 2019). Wiggins *et al.* (2016) pointed out that some cover crops suppress weeds that are already herbicide resistant, thereby reducing the intensity of selection for future resistance. Furthermore, weed cover abundance and biomass in the soil ground cover crop are reduced due to suppression of seedling emergence from the seedbank (Wallace *et al.*, 2019). Also, mixing a grass or legume cover crop in intercropping cash crops system results in greater productivity, low impact of plant diseases and stability, and weed suppression compared with cash crop monoculture (Brainard *et al.*, 2011; Garcia-De la Cruz *et al.*, 2002). For instance, the velvet bean (*Mucuna pruriens* (L.) is cultivated in sustainable and organic cropping systems and to increase the productivity crops such as corn and pineapple (Sasamoto *et al.*, 2013; Ortiz-Ceballos *et al.*, 2012; Garcia-De la Cruz *et al.*, 2006). Research on *Crotalaria* sp. has focused on nematode suppression. However, its vigorous growing provide good ground coverage for weed control. Phophy *et al.* (2017) pointed out that that cowpea and lablab are effective for weed suppression in conservation agricultural systems.

We wanted to answer the following questions. Is a intercropping of cover crops more effective at suppressing weeds compared with crop rotation grown in monoculture or combined with minimum rate application of herbicide? In addition, if cover crop performance is expected to be feasible and less variable from year to year in terms of the weed-suppressive effects? To address these questions, we conducted 2-yr cicle experiments, each involving a different suite of cover crop species grown as monocultures and three herbicides at different time of application in which we quantified weed ground cover, weed abundance, weed suppression levels and soil ground cover crops.

## MATERIALS AND METHODS

### Site description

Two-field experiment were conducted at the Ejido La Esperanza municipality of Huimanguillo, Tabasco, México NM (431955 N y 1980750 W, 24 m). The soil type is

cutanic umbric acrisol (Salgado *et al*., 2017). According to Murillo-Hernandez *et al.* (2019) the pH is strong acid, no salinity, high organic matter content and nitrogen, intermediate levels of phosphorus content and very low in P, Ca and Mg. High content of Fe, Zn and Co.

## Experimental design set up and cultural practices

*Crop rotation experiment*

The cover crop plots (2×2 m each) were established in a randomized complete block design with eighteen treatments with four replications. The cover crop treatments included cover crops *Mucuna pruriens*, *Crotalaria juncea*, *Sylosanthes guanensis, Vigna unguiculata*, weedy check (control without cover crop) and weedy check (without herbicide). In the rotation experiment, after cover crop fallow finalization, three herbicides (pendimethalin, metribuzin and mixture of haloxyfop-r-methyl and diuron were evaluated in each of the six treatments cover crops. Cover crops and herbicides were applied according to Table 1. Cover crops were established in May 2016 and repeated in 2018 (Figure 1). Herbicides

**Table 1**. Detail of cover crops and herbicide rate treatments in the field experiment.

| Trade name | Common name | Application time | MOA | Dose g. a. i. $ha^{-1}$ |
|---|---|---|---|---|
| Velvet bean | *Mucuna pruriens* | PRE, POST | Allelopathy | 10 kg |
| Stlylo | *Stylosanthes guanensis* | PRE, POST | Cover crop | 5 Kg |
| Sunnhemp | *Crotalaria juncea* | PRE, POST | Allelopathy | 47 Kg |
| Cowpea | *Vigna unguiculata* | PRE, POST | Allelopathy | 10 kg |
| Prowl | Pendimethalin | PSI, PRE | Mitosis inhibitor | 700 |
| Sencor | Metribuzin | PRE | Photosynthesis inhibitor | 560 |
| Galant | Haloxyfop r- methyl | POST | ACCase inhibitor | 200 |
| Karmex | Diuron | PRE, POST | Photosynthesis inhibitor | 800 |
| Weedy check | a) no cover crop<br>b) no herbicide | | | -CC<br>-H |

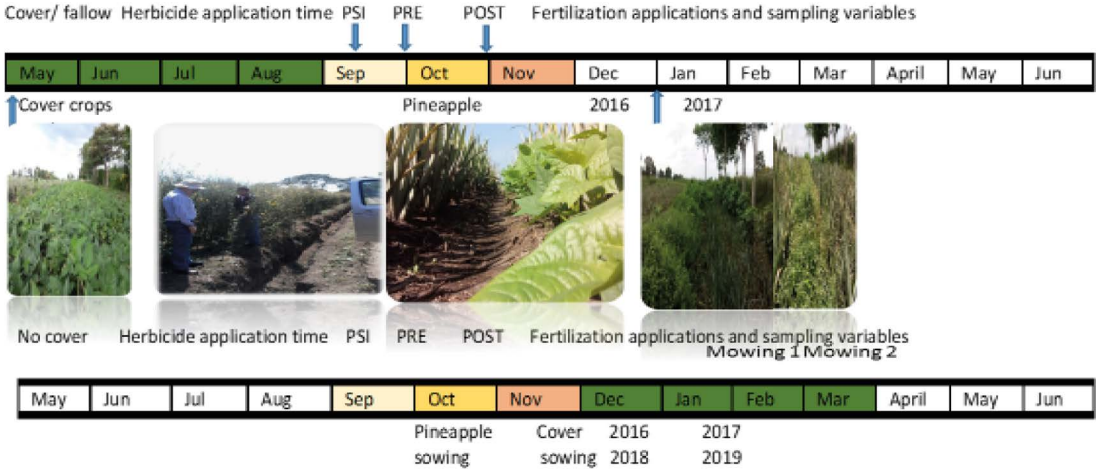


**Figure 1**. Schematic timeline showing the field work under crop rotation (up) and association (dawn) experiments in Huimanguillo, Tabasco, Mexico.

were applied at different times according their mode of action and time of application (Figure 1). Suckers of pineapple were sown in October 2016 and repeated sown in 2018. Both weed soil ground cover and cover crop ground cover were evaluated four five times during the growing season at 15, 30, 60, and 90 days after treatments (DAT) and expressed in percentage (%) using a scale from 0 to 100% (0% meaning no cover, 100% meaning plot completely covered).

*Crop association (Intercropping) experiment.*

The experiment started with the planting of pineapple and then cover crops were sown in a randomized complete block design with 18 treatments as in the previous rotation experiment. Pineapple was intercropped with: (i) *M. pruriens*, (ii) *C. juncea*, (iii) *S. guanensis*, (iv) *V. unguiculata* (v) weedy check without cover crops and (vi) weedy check without herbicide. All cover crops were seeded as shown in Table 1. The experimental design was a randomized complete block with four replications. Each experimental plots were one-intercrop beds and two pineapple beds. Each bed was 2 m×4 m. Prior to pineapple planting, plots were amended with lime (200 kg/ha). Cover crops were sown at the same time as pineapple between two pairs of pineapple rows (Table 1). Pineapples were fertilized according to standard plantation practice (400 kg/ha/year for N and K, and 5 kg/ha/year for Fe). At 90 days after cover crops plantation, each experimental cover crop plots were mowed (Figure 2). Visual rating of weed infestation and cover crop ground cover was based on 1 to 10 where 1 represents complete weed free situation while 10 represents complete weed cover. Weed samples were collected using quadrants of 1 $m^2$, placed randomly in each plot. The weed samples were separated into broad leaves, sedges and grasses.

## RESULTS AND DISCUSSION

This study revealed 14 weed species belonging to 13 genera and 7 families. The weeds were predominantly grasses and all of them have an invasive status in Mexico (Table 2). The family order of abundance in their occurrence were Poaceae, Asteraceae, Euphorbiaceae, Cucurbitaceae, Cyperaceae, Rubiaceae and Phylantaceae. Also, some of these weeds were introduced and have an invasive status in Mexico (Table 2). Thus, they might have been threatened and displacing the original flora in the savanna of Huimanguillo, Tabasco, Mexico. The intermediate level of abundance of the broadleaved weed species could be attributed to the frequently disturbed conventional tillage practices being carried out in the experimental site, coupled with the high use of nitrogen fertilizer. According to Streit *et al*., (2003) tillage practices and nitrogen fertilizer application increases the abundance of broadleaved weeds. We observed high abundance of *Momordica charantia*, present in the field site. The weed community present in the field site experiment and surrounding areas was made up primarily of grasses species (making up to 90% of the total weed composition). High abundance of large crabgrass [*Digitaria sanguinalis* (L.) Scop.] being the dominant species followed by *Eleusine indica*. This high abundance of grasses might be attributed to agricultural practices such as intensive tillage and herbicide resistance, due the fact that high doses and repeated application of herbicides such as glyphosate, bromacil and diuron have been used during many years in this region. The success of *D. sanguinalis* as

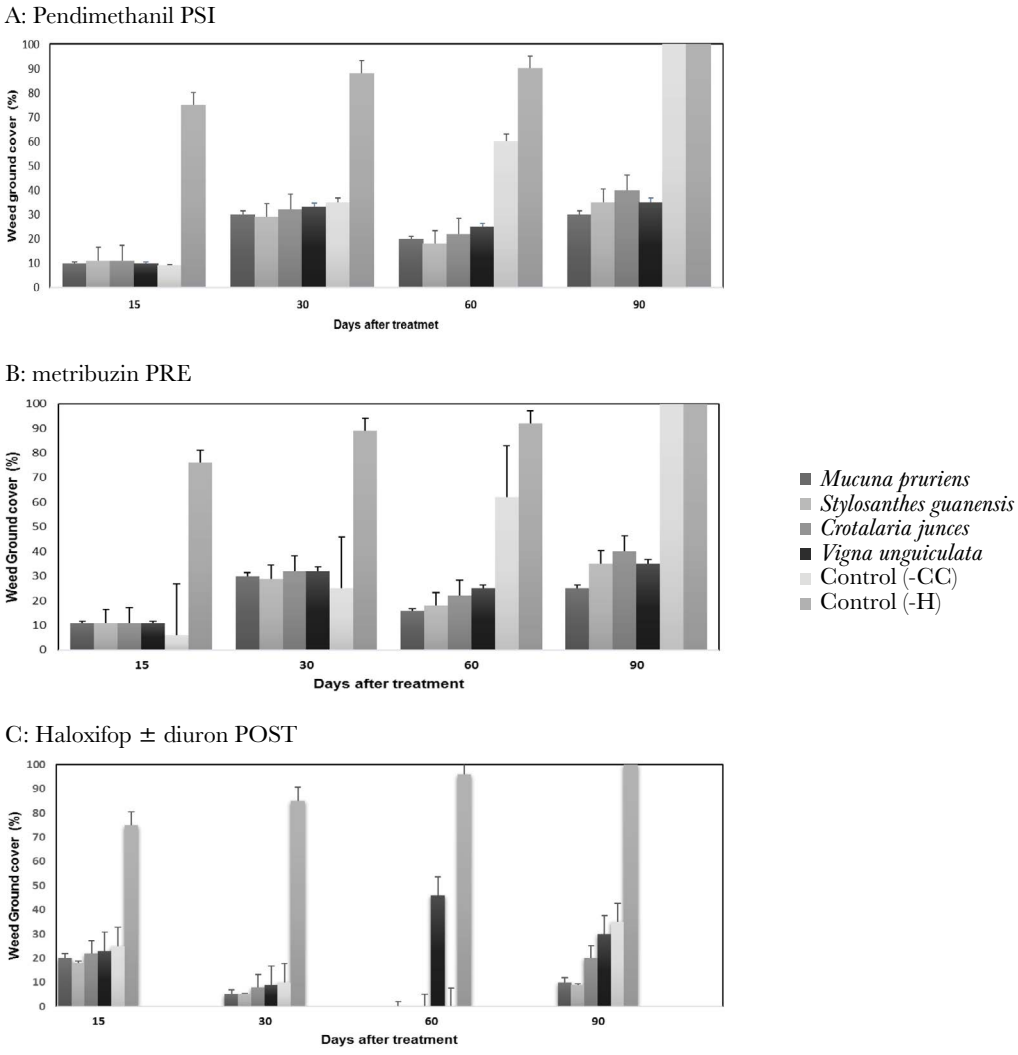


**Figure 2**. Synergistic effects of herbicide and cover crop in the association experiment (Intercropping). -CC= weedy check without cover crop; -H=weedy check without herbicide. Vertical bars represent ±1 standard error of the mean.

a weed in extensive crops is mainly due to its high seed production and the long period of seedling emergence in the field, distributed from spring to mid-summer (Gallart *et al.*, 2010; Cardina *et al.*, 2011). *E. indica*, was found to be the most prevalent grass (82.2%) in immature oil palm plantations throughout Malaysia (Maizatul-Suriza and Idris, 2017). The presence of *E. indica* biotypes with resistance to some groups of herbicides is likely to become a concern. Thus, ecological approaches for weedy grass management is an unexploded strategy to overcome herbicide resistance.

In the field, all the cover crops had good establishment *M. pruriens* produced the highest ground cover, which was significantly higher than the cover produced by other fabaces, at both 30, 60 and 90 days after sowing (DAS) (Table 3). The treatments with the lowest weed coverage were the combination of both *M. pruriens*, and *S. guanensis* with both pendimethalin and metribuzin herbicide in a preemergence time application at 30 and 60 DAT. In the

**Table 2**. Common weed flora, level of occurrence and invasiveness status.

| Family | Weed species | Level of infestation | Invasiveness status/origin |
|---|---|---|---|
| **a) Broadleaves** | | | |
| Euphorbiaceae | *Croton hirtus* | + | Native/America |
| Euphorbiaceae | *Croton lobatus* | + | Native/America |
| Astereaceae | *Vernonia cinerea* | + | Exotic /Africa |
| Astereaceae | *Eupatorium pycnocephalum* | + | Native/Mexico |
| Cucurbitaceae | *Momordica charantia* | +++ | Invasive/africa |
| Euphorbiaceae | *Euphorbia heterophylla* | ++ | Native/America |
| Rubiaceae | *Borreria leavis* | + | Native/America |
| Asteraceae | *Emilia sonchifolia* | + | Native/America |
| Phyllanthaceae | *Phylanthus* spp. | + | Exotic/Asia |
| **b) Sedge** | | | |
| Cyperaceae | *Cyperus rotundus* | ++ | Exotic/Asia |
| **c) Grasses** | | | |
| Poacea | *Eleusine indica* | +++ | Invasive/Asia |
| Poacea | *Digitaria sanguinalis* | +++ | Invasive/Europe |
| Poacea | *Sorghum halepense* | ++ | Invasive/Asia |
| Poacea | *Bothriochloa pertusa* | ++ | Invasive/África |

+++ High infestation (60 - 90% occurrence)
++ moderate infestation (30 - 59% occurrence)
+ low infestation (1 - 29% occurrence)

rotation experiment, after the end on cover crops monocultures, 60% of weed ground cover was observed (Figure 2 A, B). Both pendimethalin, a preplant or pre-sown incorporate (PSI), and metribuzin, a pre-emergence, herbicides have broad-spectrum weed control, affecting seed bank, and seed emergence. The synergism effect observed between cover crops and herbicide might have delayed weed emergence in the intercropping system. However, in the rotation experiment grass weed density was not positively affected by the legume cover crops in both years (2016-2017, 2018-2019). However, *S. guianensis* at 15 and 30 DAS produced significantly low grass weed density while broadleaved weed density was generally low in all the plots. Weed suppression levels, ground cover crop, and weed cover were similar for *S. guanenesis* and *C. juncea* both intercropping and monocultures experiments across the time evaluated. However better weed control was resulted when combined with pre and postemergence herbicide. Herbicide effect alone has positive effect on weed suppression ranging from 85-100% depending on the time of application (Table 3). Performance of herbicide and crop rotation resulted in delayed weed emergence, which was observed at 60 DAT (Figure 3), however weed reemergence was observed at 90 DAT. Haloxyfop plus diuron treatments along with cover crops have the best weed control achievement in both experiment with 100% efficacy until 90 DAT (Table 3). While time and rotation of herbicides provided a proactive management of resistance, overuse of herbicides such as bromacil, glyphosate and the evolution of glyphosate-resistant weeds poses one the greatest threats to conservation tillage as it has forced some farmers to revert

**Table 3**. Weed suppression (WS) and cover crop ground cover (GC) levels (%) at 30 and 60 days after herbicide treatment (DAT).

| Cover crop (CC) | Herbicide (H) | Intercrop- cc- pineapple | | | | Rotation cc-pineapple | |
|---|---|---|---|---|---|---|---|
| | | 30 DAT | | 60 DAT | | 30 DAT | 60 DAT |
| | | WS % | GC % | WS % | GC % | WS % | WS % |
| *Mucuna pruriens* | pendimethalin | 70 | 50 | 85 | 78 | 92 | 80 |
| | metribuzin | 70 | 51 | 85 | 78 | 89 | 80 |
| | haloxyfop + diuron | 95 | 50 | 100 | 80 | 100 | 100 |
| | - H | 50 | 55 | 75 | 81 | 30 | 20 |
| *Crotalaria juncea* | pendimethalin | 65 | 40 | 80 | 60 | 85 | 78 |
| | metribuzin | 65 | 40 | 80 | 61 | 85 | 78 |
| | haloxyfop + diuron | 94 | 45 | 100 | 61 | 100 | 100 |
| | -H | 45 | 44 | 70 | 62 | 29 | 18 |
| *Stylozanthes guanensis* | pendimethalin | 72 | 52 | 86 | 81 | 86 | 75 |
| | metribuzin | 72 | 52 | 86 | 81 | 85 | 78 |
| | haloxyfop + diuron | 95 | 50 | 100 | 80 | 100 | 100 |
| | -H | 51 | 52 | 76 | 80 | 28 | 15 |
| *Vingna unguiculata* | pendimethalin | 69 | 46 | 82 | 74 | 84 | 70 |
| | metribuzin | 69 | 46 | 82 | 73 | 84 | 75 |
| | haloxyfop + diuron | 94 | 47 | 100 | 74 | 100 | 100 |
| | -H | 45 | 46 | 69 | 75 | 30 | 16 |
| - CC | pendimethalin | 65 | 0 | 55 | 0 | 89 | 75 |
| | metribuzin | 65 | 0 | 50 | 0 | 90 | 75 |
| | haloxyfop + diuron | 88 | 0 | 100 | 0 | 100 | 90 |
| | -H | 0 | 0 | 0 | 0 | 0 | 0 |

to conventional tillage for effective weed control. Cover crops have the potential to delay weed emergence, decrease weed size, and decrease weed number. In addition to the weed suppression and ecological benefits from the all cover crops used. Our results supports previous research indicating that utilizing soil-residual herbicides along with cover crops improves control of palmer amaranth and/or waterhemp (Perkins *et al.*, 2021). The use of cowpea as monoculture before pineapple plantation but also as intercrop is highly important as food source. After harvesting, the living mulch leads to a considerable reduction in weed coverage (about 65% at 90 DAS a density of 20 plants/$m^2$). However, this weed control level was more efficient combined with low dose rate rotation with pre-plant or pre-emergence herbicides. Previous research by Soti and Raceli (2020) demonstrated that methanol and ethyl acetate extracts of cowpea contained allelopathic compounds and that might has phytotoxicity properties. Thus, identification and isolation of the allelochemicals from all cover crops used in these experiments will be useful.

In general, the highest levels of weed suppression were associated with the *M. pruriens* when combined with all three herbicides, ranging from 95% to 100%. The high level of weed suppression (100%) were observed when *M. pruriens* where combined with treatments with post emergence herbicides (diuron and haloxyfop) (Table 3). The treatments with the

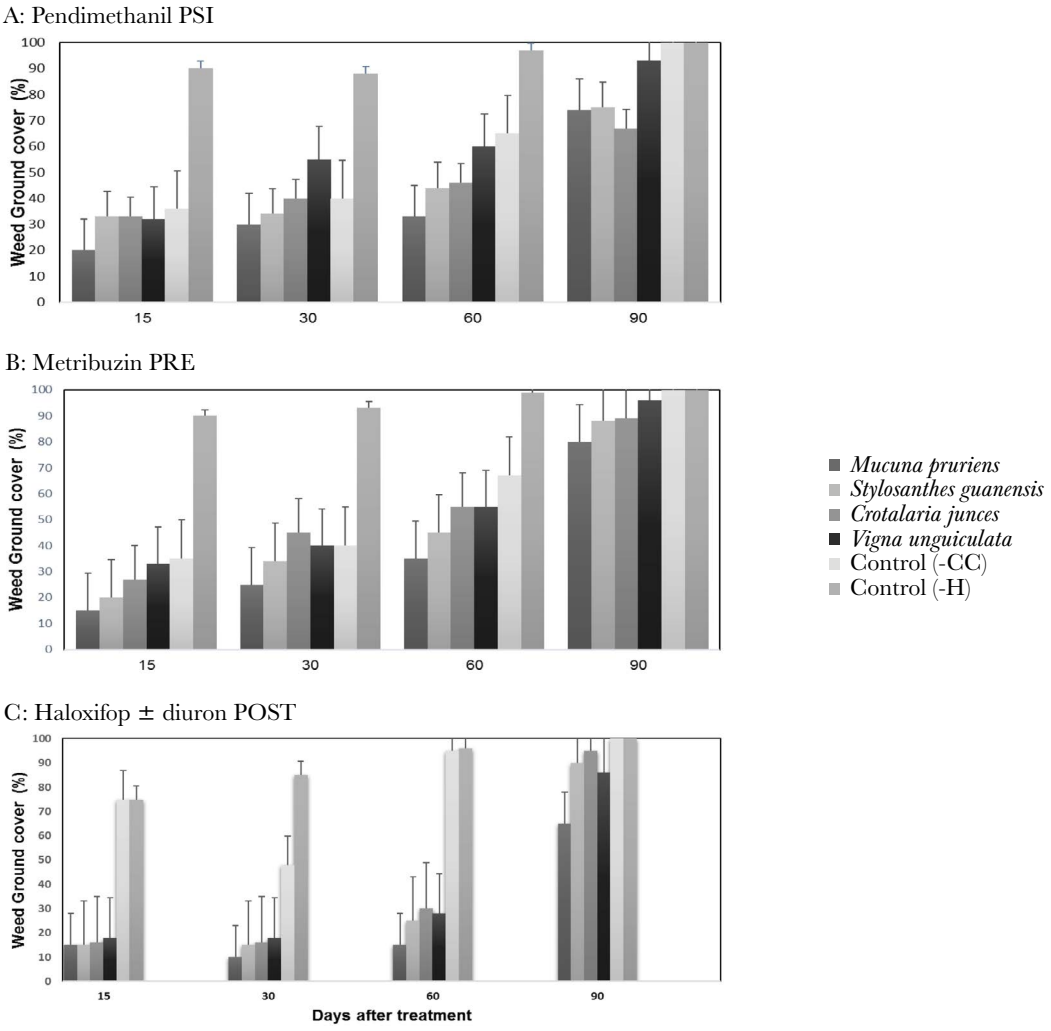


**Figure 3**. Combined effects of herbicide and cover crops in the rotation experiment. -CC=without cover crop; -H=weedy check without herbicide. Vertical bars represent ±1 standard error of the mean.

highest levels of weed suppression were also those that produced the highest ground cover crop (Table 3). These results support the hypothesis that dual approach with cover crop along with low dose herbicide rate strategy provide greater weed-suppression benefits than the most-suppressive cover crop grown as a monoculture. Our results are congruent with previous research showing cover crop have weed suppressive abilities (Osipitan *et al.*, 2019). However, we are aware that there might be other mechanisms of action to suppress weeds, rather that ground cover crop canopy alone. For instance, the action of biological control agents that inhabits the cover crop as well as allelopathy, which has unexploited potential in integrated weed management and ought to be further studied in our research laboratory and facilities. Prediction changes in seedbank level, timing of seedling emergence in the field are strictly related to the dormancy state of the seedbank (Batlla & Benech-Arnold, 2010) and could be useful to decide the crop sowing date or the timing of herbicides applications. A large body of research in literature indicate that weed population density and biomass

production may be markedly reduced using crop rotation and intercropping strategies. In a meta-analysis study, Osipitan *et al.*, (2019) pointed out that crop rotation resulted in emerged weed densities in test crops that were lower in 21 cases, higher in 1 case, and equivalent in five cases in comparison to monoculture systems. In 12 cases where weed seed density was reported, seed density in crop rotation was lower in 9 cases and equivalent in three cases when compared to monocultures of the component crops. In addition, weed biomass in the intercrop was lower in 47 cases and higher in four cases than in the main crop grown alone. For the previous studies, it seems that success of cover crop rotation for weed suppression appears to be based on the use of crop sequences that create varying patterns of resource competition, allelopathic interference, soil disturbance, and mechanical damage to provide an unstable and frequently inhospitable environment that prevents the proliferation of a particular weed species (Osipitan *et al.*, 2019). Alternatively, intercropping with fabaces such as cowpea, as food source may provide yield advantages without suppressing weed growth below levels observed in component sole crops if intercrops use resources that are not exploitable by weeds or convert resources to harvestable material more efficiently than sole crops. Parameters such as weed seed longevity, weed seedling emergence, weed seed production and dormancy, endophytes biological control agents of weed mortality, and allelopathic interactions needs to be investigated. Compatibility of these strategies with current technologies and farming practices might become more accessible and feasible to farmers.

## CONCLUSION

Intermediate to high weed suppression levels with cover crops was resulted within the population at the time of herbicide application. Integration of cover crops as a complementary tactic in herbicide based production systems seems to be feasible. This study shows that *M. pruriens*, *S. guanensis*, *C. juncea* and *V. unguiculata* achieve effective weed suppression alone, however synergism effects were observed when combined with herbicides in both experiments. Intercropped cover crops and herbicides did not affect pineapple growth visually; however, weed control improved with herbicide application in

**Figure 4**. Cover crop with *Vingna unguiculata* in intercropped with pineapple at 30 DAS. March 16, 2018.

crop rotation or fallow. Integrating cover crops into the agricultural systems as an effective strategy to enhance crop production sustainability and resiliency is a friendly and feasible new approach for farmers.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Optimization of culture media to produce *Bacillus subtilis* strain QST 713 in a handcrafted bioreactor

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**ABSTRACT**

**Objective**. To optimize a nutrient medium based on fertilizers for the cultivation of *Bacillus subtilis* in an Airlift-type handcrafted bioreactor.

**Design/Methodology/Approach**. Twenty-seven nutrient media, fixed by combining five factors with three levels, including sucrose, ammonium sulfate, triple superphosphate, UltraK® formula, and *B. subtilis* inoculum (Serenade® Max) were tested in a 50L handcrafted by the authors. The variables monitored in the media were absorbance, dissolved oxygen, pH, and temperature. The first was the one that was considered for optimization as it is the indirect indicator of bacterial growth. On the statistical analysis, the option "*Larger is better*" was chosen for Signal/Noise for the ANOVA of the main effects according to the Taguchi method.

**Results**. The highest level of sucrose, together with the lowest level of triple superphosphate were determinants for maximum growth of *Bacillus* in the time studied. On the other hand, the components such as ammonium sulfate, UltraK® formula, or the amount of inoculum were not significant, which means that they can be added from the mid to low levels.

**Study limitations/Implications**. This new information can be scaled to bioreactors of 2500 L for *B. subtilis* that we have previously developed.

**Finding/Conclusions**. Maximum bacterial growth depends on a good supply of sucrose, limiting triple superphosphate. Additionally, it is prudent to decrease additions of ammonium sulfate because it reduces dissolved oxygen in the nutrient medium.

**Keywords**: nutrient broth, optimization, airlift bioreactor, *Bacillus subtilis.*

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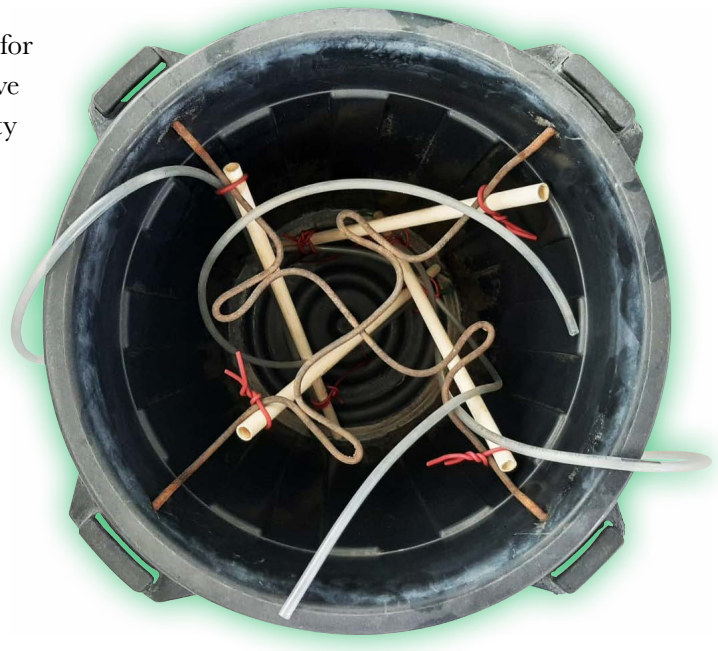
## INTRODUCTION

Bioreactors used for experimentation tend to be expensive equipment, even those with a capacity of 10 or fewer liters since their cost usually exceeds € 20,000; and the possibility of its acquisition is usually remote for laboratories in general, for which researchers implement their designs, but they are usually handcrafted and low-capacity models (Lamping, 2004; Serrat-Díaz & Méndez-Hernández, 2015).



There are examples of the performance of small factory-built bioreactors, with tests using *E. coli* and media prepared from basic reagents that are not economical for application outside of medicine (Schirmer *et al.*, 2019). Others, who are the vast majority, prefer simplicity when using bioreactors that work by shaking, with even smaller culture volumes (Carvalho *et al.*, 2010). In these cases, researchers carry out 90% of the culture experiments in these bioreactors and less than 2% of the publications mention the engineering aspects of the equipment, with some exceptions (Bourne *et al.*, 1992; Büchs, 2001).

However, massive, or commercial production of beneficial microorganisms for agriculture from the practical point of view occurs in fermenters or bioreactors, and to achieve this, it is necessary to previously make a good choice of the microorganism, its culture medium or its optimization and a good start-up of the fermentation process (Seletzky *et al.*, 2007). And if the production, of beneficial microorganisms, is approached from the economic point of view, then its application to crops becomes exclusive to a small sector of farmers who have enough money to do it. These high prices for both the reactor, its operation, and its culture media, have led some to consider the need to lower costs based on the concept "*Do it yourself*". Under this condition, control focuses on simple monitoring of optical density, fluorescence, pH, and dissolved oxygen; all of this at affordable prices. These alternatives have a high degree of flexibility for different applications and requirements in the design and construction of simple bioreactors (Pilizota & Yang, 2018; Theodore *et al.*, 2019). At the same time, this type of reactor can also enhance what has been called "synthetic biology", as it includes genetically modified microorganisms that can be used in the development of new products, with a regulated expression of metabolic routes or with the introduction of new routes, becoming in factories of biological products (Pleiss, 2006; Scognamiglio *et al.*, 2015). Then, the task of bioengineers is to grow microorganisms of interest, at higher speeds than those that occur in nature in containers that contain the nutrients and the optimal aeration and temperature conditions (Galindo *et al.*, 2007).

Therefore, the objective of this work was to optimize a nutrient medium based on fertilizers for the cultivation of *Bacillus subtilis* in an Airlift-type bioreactor. This handcrafted Airlift-type bioreactor is of our own design with a volume of 50 liters of capacity, in which performance tests were carried out with different media considering the basic variables previously mentioned. Although there are typical batch Airlift bioreactors (Katoh & Yoshida, 2009) described as internal lift (IL) and external lift (EL) of turbulent motion, it is important to note that the bioreactor presented in this work is a variant of the first case and that promotes excellent aeration and movement of the culture medium.

## MATERIALS Y METHODS

Our group has experience in the construction of a fully operating 2500-liter Airlift-type bioreactor to produce *Bacillus subtilis* for direct application in the irrigation of chili pepper cv. 'Sequoia' for the control of *Phytophthora capsici* in an agricultural company in the municipality of Pabellón de Arteaga, Aguascalientes (Paulino-Martínez, 2017). In this study, the goal was to optimize the materials used for the preparation of the culture medium in a small-scale artisanal bioreactor.

**Bioreactor characteristics**

The present study was intended to optimize the use of material inputs to reduce broth costs; and to carry out experiments, a 50-liter reactor was used under laboratory conditions. This was devised in a 60-liter container with metal supports to fix four PVC pipes of ½" diameter by 50 cm long submerged and in an inclined position. The pipes were fed separately in the lower part using fish tank tubing so that each of the four pipes would bubble freely. This feeding was carried out using two double-outlet air pumps for fish tanks (Elite 799®, Hagen HA799).

**Raw materials for the nutritive broth**

To make the estimates of the basic nutrient requirements, several studies were consulted that report the maximum biomass and the dry matter obtained in bioreactors, as well as the elemental composition of the bacteria (Matar *et al.*, 2009; Bratbak, & Dundas, 1984; Glazyrina *et al.*, 2010; Novoselov *et al.*, 2013). In addition, the appropriate materials for the preparation of the broth were reviewed (Hernández-Bustos, 2003). Based on the above, it was determined to use commercial sucrose, ammonium sulfate, triple superphosphate powder (ground), and UltraK®. All of these, except for sucrose, are fertilizers or agrochemicals that are easily available and cheaper than laboratory reagents. These materials proved to be suitable for the rapid growth of *B. subtilis*.

**Bacterial strain**

Previously, in the use of our artisanal Airlift reactors in the field (Paulino-Martínez, 2017), *Bacillus subtilis* strain QST 713 (Serenade® Max, Bayer; AgraQuest, 2001) was used since according to farmers it provides adequate protection against *P. capsici*. For this reason, this strain was kept being part of this study to optimize the factors of its production.

**Data recorded**

Variables measured were turbidity (Absorbance at $\lambda$=600 nm) using a visible light spectrophotometer (Spectronic® 20D, Milton Roy Co.), dissolved oxygen using a sensor (LAQUA act, DO120, Horiba), pH (pH meter WT-40, AMPROBE) and temperature using a glass thermometer. Each variable was measured every hour during 12 h for the 27 runs.

**Treatment design**

The treatment design was done under the Taguchi method with five factors and three levels per factor. Table 1 shows the factors and levels, defined in grams/50 L. The data analyzed were those of the last measurement.

The resulting data were subjected to the analysis of variance of means and signal-to-noise using Minitab® software (version 16) to determine the importance or contribution of the studied factors in the measured variables. The analysis was performed with the Signal/Noise option "*Larger is better*" $\left(S/N\right)_L = -10 * \log_{10}\left\{\sum\left(1/y^2\right) * 1/n\right\}$ (Cruz-Trejos

**Table 1**. Levels for raw materials used for the preparation of the nutritive broth of the 50 L bioreactor.

| Material (Factor) | Low (g) | Medium (g) | High (g) |
|---|---|---|---|
| Sucrose (commercial type) | 154.5 | 463.5 | 772.5 |
| Ammonium Sulfate | 60.5 | 181.5 | 302.5 |
| Triple Superphosphate | 6.3 | 18.9 | 31.5 |
| Ultra K® | 1.05 | 3.15 | 5.25 |
| Inoculum (Serenade® Max ) | 1.1 | 3.3 | 5.5 |

*et al.*, 2012). The 27 combinations or runs generated by Minitab16® according to the Taguchi design in g/50 L appear in Table 2.

**Table 2**. Runs or combinations of the nutrient broth for the bioreactor (g/50 L).

| Run | Sucrose (g) | Ammonium sulfate (g) | Triple Superphosphate (g) | Ultra K® (g) | Serenade® (g) |
|---|---|---|---|---|---|
| $L_1$ | 154.5 | 60.5 | 6.3 | 1.05 | 1.1 |
| $L_2$ | 154.5 | 60.5 | 6.3 | 1.05 | 3.3 |
| $L_3$ | 154.5 | 60.5 | 6.3 | 1.05 | 5.5 |
| $L_4$ | 154.5 | 181.5 | 18.9 | 3.315 | 1.1 |
| $L_5$ | 154.5 | 181.5 | 18.9 | 3.315 | 3.3 |
| $L_6$ | 154.5 | 181.5 | 18.9 | 3.315 | 5.5 |
| $L_7$ | 154.5 | 302.5 | 31.5 | 5.25 | 1.1 |
| $L_8$ | 154.5 | 302.5 | 31.5 | 5.25 | 3.3 |
| $L_9$ | 154.5 | 302.5 | 31.5 | 5.25 | 5.5 |
| $L_{10}$ | 463.5 | 60.5 | 18.9 | 5.25 | 1.1 |
| $L_{11}$ | 463.5 | 60.5 | 18.9 | 5.25 | 3.3 |
| $L_{12}$ | 463.5 | 60.5 | 18.9 | 5.25 | 5.5 |
| $L_{13}$ | 463.5 | 181.5 | 31.5 | 1.05 | 1.1 |
| $L_{14}$ | 463.5 | 181.5 | 31.5 | 1.05 | 3.3 |
| $L_{15}$ | 463.5 | 181.5 | 31.5 | 1.05 | 5.5 |
| $L_{16}$ | 463.5 | 302.5 | 6.3 | 3.315 | 1.1 |
| $L_{17}$ | 463.5 | 302.5 | 6.3 | 3.315 | 3.3 |
| $L_{18}$ | 463.5 | 302.5 | 6.3 | 3.315 | 5.5 |
| $L_{19}$ | 772.5 | 60.5 | 31.5 | 3.315 | 1.1 |
| $L_{20}$ | 772.5 | 60.5 | 31.5 | 3.315 | 3.3 |
| $L_{21}$ | 772.5 | 60.5 | 31.5 | 3.315 | 5.5 |
| $L_{22}$ | 772.5 | 181.5 | 6.3 | 5.25 | 1.1 |
| $L_{23}$ | 772.5 | 181.5 | 6.3 | 5.25 | 3.3 |
| $L_{24}$ | 772.5 | 181.5 | 6.3 | 5.25 | 5.5 |
| $L_{25}$ | 772.5 | 302.5 | 18.9 | 1.05 | 1.1 |
| $L_{26}$ | 772.5 | 302.5 | 18.9 | 1.05 | 3.3 |
| $L_{27}$ | 772.5 | 302.5 | 18.9 | 1.05 | 5.5 |

## RESULTS AND DISCUSSION

The bioreactor worked perfectly for the 27, 12 h runs causing the culture medium to move in a spiral-upward way, creating a clockwise movement bubbling through the pipes. In none of the cases was the accumulation of sediment or precipitate observed inside the bioreactor (Figure 1).

### Turbidity

All the media studied were translucent at the time of preparation and subsequently, their maximum turbidity increased at the end of 12 h in which each of the broth was being monitored (Figure 2). This means that there was sustained bacterial growth in all cases, but not in all cases the same growth was obtained. The treatments that obtained the highest turbidity at the end of 12 h were: $L_{22}$, $L_{26}$, $L_{23}$, and $L_{24}$ (Table 2, Figure 2). In a subsequent analysis of the turbidity, taking into account the last measurement, significance was found $p=0.001$ for the commercial sugar factor (sucrose) and $p=0.054$ for the triple

**Figure 1**. Arrangement of the artisanal Airlift bioreactor.

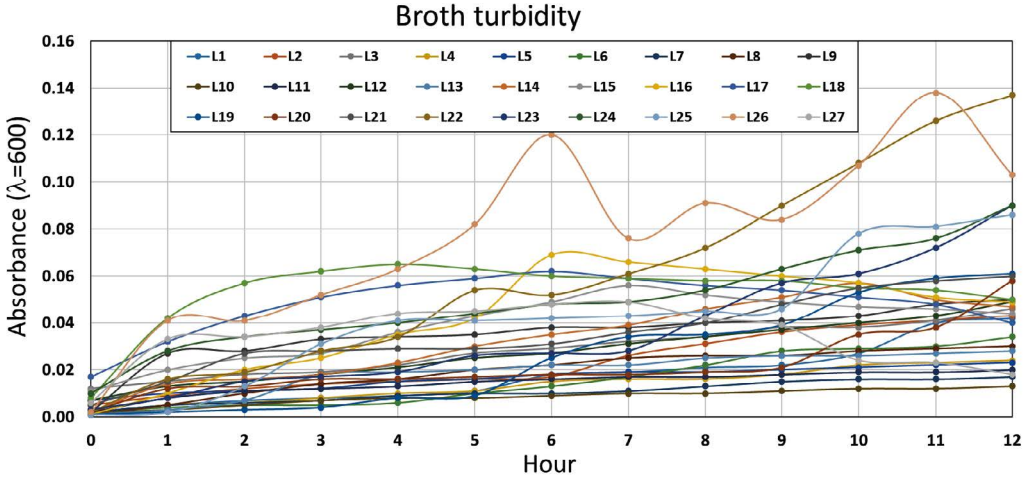



**Figure 2**. Evolution of the turbidity of the 27 media in 12 h of culture.

superphosphate factor (Figure 3). The rest of the factors seemed not to have significance. Taking the matching levels of these treatments, all have the maximum amount of sucrose, and triple superphosphate mainly at the lowest or medium values. The addition of UltraK® or the dosage did not result in notable differences.

Checking the graphs of the main effects of each of the factors and taking into account the analysis of variance of the turbidity means, it was observed that the maximum value was achieved when the sugar level was the highest (772.5 g/50L). On the other hand, triple superphosphate had a negative influence at medium and high levels, since the highest bacterial growth was observed when 6.3 g/50L were added (Figure 4).

For non-significant main effects (Figure 3), the lowest or medium levels can be taken without influencing the results. In this case, the criterion for selecting the best level turns out to be economic, that is, it is better to take lower levels for ammonium sulfate, UltraK®, and the inoculum dose without diminishing bacterial growth.

**ANOVA FOR TURBIDITY**

| SOURCE | GL | SS | Adj. SS | Adj. MS | F | P |
|---|---|---|---|---|---|---|
| Sucrose (g) | 2 | 0.010665 | 0.010665 | 0.005333 | 11.95 | 0.001 |
| Ammonium sulfate (g) | 2 | 0.000878 | 0.000878 | 0.000439 | 0.98 | 0.396 |
| Triple superphosphate (g) | 2 | 0.003133 | 0.003133 | 0.001566 | 3.51 | 0.054 |
| Ultra K (g) | 2 | 0.000529 | 0.000529 | 0.000265 | 0.59 | 0.564 |
| Inoculum (g) | 2 | 0.000016 | 0.000016 | 0.000008 | 0.02 | 0.982 |
| Error | 16 | 0.007141 | 0.007141 | 0.000446 | | |
| Total | 26 | 0.022362 | | | | |

**Figure 3**. Analysis of variance of means of the absorbance variable ($\lambda$=600 nm).

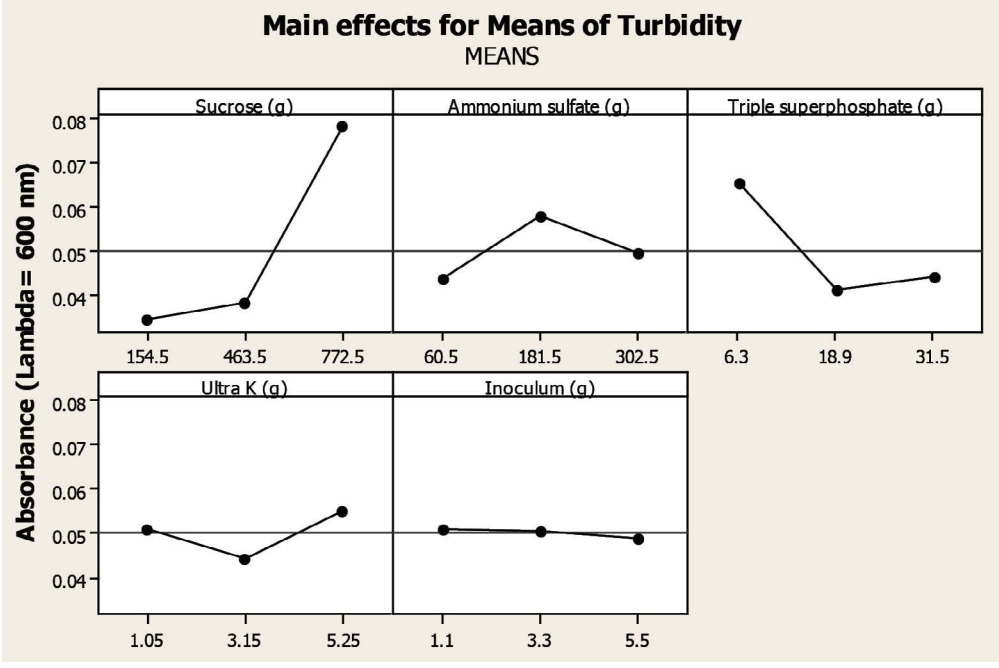



**Figure 4**. Graphical analysis of the turbidity means (absorbance $\lambda$=600 nm) from nine replications.

Confirmation of the above results is also observed in the analysis of variance of the relationships $\left(S/N\right)_L$, as mentioned by Cruz-Trejos *et al.* (2012). Again, the significant factors were only for sugar (sucrose) p=0.006 and triple superphosphate p=0.047; meanwhile, the rest of the factors were not significant (Figure 5). Coincidentally with the previous graph of the main effects of the means, in the analysis for the S/N values, the highest level of sucrose induced the highest turbidity, and the lowest level of triple superphosphate was the one that allowed growth bacterial and, therefore, the highest turbidity. In the rest of the factors, there are no major changes in the main effects.

By selecting the media that showed the highest turbidity 12 h after starting the culture, it was found that they contained the highest level of sucrose and most contained the lowest level of triple calcium superphosphate, matching with the graphic analysis of the main effects both for means and for Signal/Noise (Table 3).

## Dissolved oxygen

Dissolved oxygen dropped close to zero in most of the treatments, to the point that a proper analysis using the final values cannot be made through the Taguchi method. However, taking the graph as a reference, the evolution of the phenomenon can be observed (Figure 7). The treatments that initially had little dissolved oxygen and that fell more quickly to values close to zero, were those that had the highest content of ammonium sulfate, such as $L_{16}$, $L_{17}$, $L_{18}$, $L_{26}$, and $L_{27}$. On the other hand, those with the highest sucrose content were the next to drop their values close to zero. And finally, those treatments that remained with slightly higher values at the end of the run were mainly those that contained the lowest values of ammonium sulfate, such as $L_{10}$, $L_{11}$, and $L_{12}$.

ANOVA for S/N relations

| SOURCE | GL | SS | Adj. SS | Adj. MS | F | P |
|---|---|---|---|---|---|---|
| Sucrose (g) | 2 | 241.398 | 241.398 | 120.699 | 7.03 | 0.006 |
| Ammonium sulfate (g) | 2 | 12.234 | 12.234 | 6.117 | 0.36 | 0.706 |
| Triple superphosphate (g) | 2 | 127.515 | 127.515 | 63.758 | 3.72 | 0.047 |
| Ultra K (g) | 2 | 2.884 | 2.884 | 1.442 | 0.08 | 0.920 |
| Inoculum (g) | 2 | 7.934 | 7.934 | 3.967 | 0.23 | 0.796 |
| Error | 16 | 274.543 | 274.543 | 17.159 | | |
| Total | 26 | 666.509 | | | | |

**Figure 5**. Analysis of variance of S/N for Absorbance ($\lambda$=600 nm).

**Table 3**. Runs of four broths with the highest absorbance values at 12 h.

| Run | Sucrose (g) | Ammonium sulfate (g) | Triple Superphosphate (g) | Ultra K® (g) | Serenade® (g) | Absorbance ($\lambda$=600 nm) |
|---|---|---|---|---|---|---|
| $L_{22}$ | 772.5 | 181.5 | 6.3 | 5.25 | 1.1 | 0.137 |
| $L_{26}$ | 772.5 | 302.5 | 18.9 | 1.05 | 3.3 | 0.103 |
| $L_{23}$ | 772.5 | 181.5 | 6.3 | 5.25 | 3.3 | 0.09 |
| $L_{24}$ | 772.5 | 181.5 | 6.3 | 5.25 | 5.5 | 0.09 |
| Significance | ** | | * | | | |

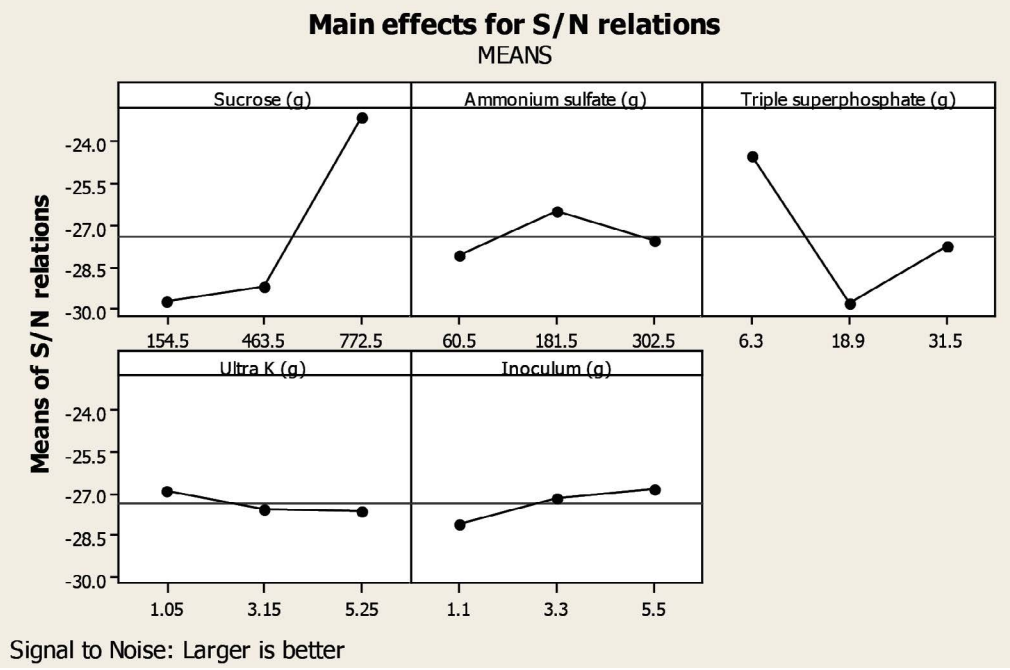



**Figure 6**. Analysis of the main effects of S/N means on the absorbance variable ($\lambda$=600 nm) from nine replications.

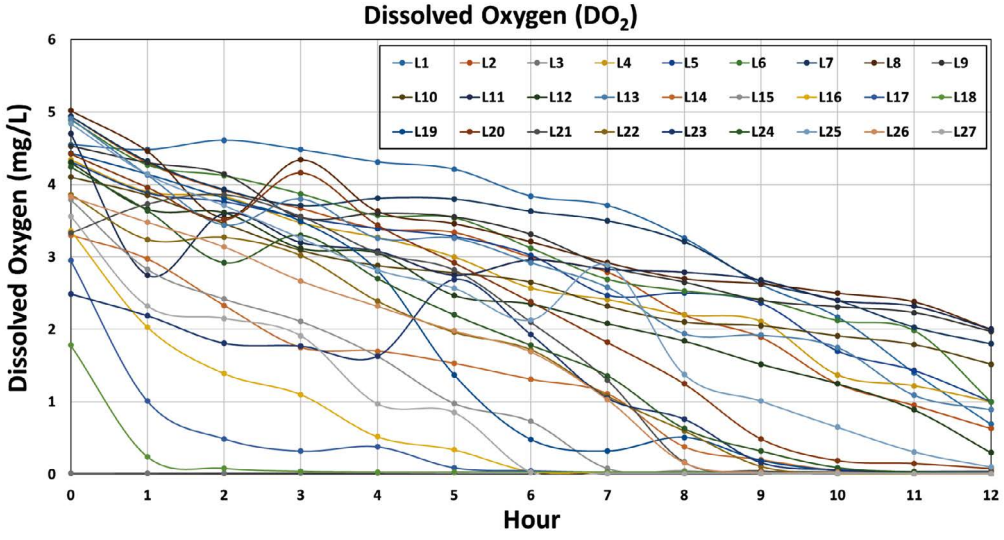



**Figure 7**. Evolution of dissolved oxygen in the media prepared for the bioreactor.

## Temperature and pH

These two variables did not show appreciable changes throughout the runs. Most of the media were in the 7.0 to 7.5 pH range, with two exceptions that were 6.8 ($L_{17}$ and $L_{18}$). Regarding the temperature of the media, it ranged between 17 and 19 °C in each run without differences among all the cases. This was, perhaps, due to the little variation of temperature within the laboratory.

## CONCLUSIONS

The media prepared in the selected combinations allowed good discrimination of those that produced greater turbidity derived from cell growth inside the bioreactor, finding that the combinations having the high level of sucrose combined with the low level of triple superphosphate generated the best response. On the other hand, the other factors such as ammonium sulfate, a formulation with potassium (UltraK®) and the amount of inoculum can be added at their mid or lowest levels because there was no significance for these components of the medium. The presence of high levels of ammonium sulfate caused the content of dissolved oxygen in the medium to drop precipitously, which is why supplying this type of excess should be avoided for a bioreactor of this type. Also, the high amount of sucrose led to a reduction in dissolved oxygen at approximately nine h after the run, but this may be due to the high bacterial growth as we detected in previous works (De la Cruz-De la Cruz *et al*., 2016). Sensitivity to oxygenation of *B. subtilis* under fermentation conditions is decisive for generating adequate bacterial populations (Bourne *et al*., 1992), and this is important for its application in the field for fertigation, as it is currently performed in a local farm in Pabellón de Arteaga, Aguascalientes (Paulino-Martínez, 2017).

Finally, the 50 L bioreactor was as efficient as the field bioreactors of which we have experienced since the accelerated growth of *B. subtilis* was achieved, ensuring that the components of the nutrient medium remained in constant agitation, without "dead zones" that usually affect bacterial growth (Galindo *et al*., 2007).

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Morphological and biochemical characteristics in fruits of *Mangifera indica* L. var. Ataulfo with and without conventional management

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**ABSTRACT**

**Objective**: To identify the morphometric and biochemical variation in mango fruits var. Ataulfo (*Mangifera indica* L.) in two contrasting environments with and without conventional management.

**Design/methodology/approximation**: Morphological and biochemical variables were studied in mango fruits var. Ataulfo in two environments, one of them (La Norteña) with Leptosol soil and Aw climate with conventional agrochemical-based management and the other (Santa Cecilia) on Acrisol soil and Am climate with agroecological management. There were 30 fruits used, all from five trees (n=150) per study garden in a state of commercial maturity. Each fruit was considered as an experimental unit, and morphological and biochemical variables were evaluated for each fruit.

**Results**: Increase in fruit weight, higher pH and increase in total soluble solids, but decrease in pulp weight on site with conventional handling. Increase in pulp content and firmness in fruits from the site without handling.

**Study Limitations/implications**: Changes in the amount and distribution of rainfall in both environments each year.

**Findings/conclusions**: Morphological and biochemical modifications are presented. Greater size and weight, pH and TSS content in the conventional production system, but increased pulp and greater firmness, as well as higher citric acid content in the agroecological system. The results suggest differential effects in mango fruits according to the management and environment where they develop.

**Key words**: Fruit morphometry, color, pH, Total soluble solids (TSS).

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## INTRODUCTION

The mango (*Mangifera indica* L.) var. Ataulfo has a Mexican origin with a designation of origin from Soconusco, Chiapas, Mexico (NOM-188-SCFI-2012) and preference in national and international markets for its firmness, flavor, shelf life (Palacio and Avendaño, 2019), color, smell and consistency (De Gyves *et al.*, 2009). It has been distributed to various tropical regions of Mexico and some of Central and South America (Infante *et al.*, 2011).

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The mango fruit is a source of carbohydrates, amino acids, vitamins, and minerals (Maldonado-Celis *et al*., 2019) with nutraceutical properties due to the antioxidant content of phenols and carotenoids (Maldonado-Astudillo *et al*., 2014) or the inhibition of pro-inflammatory cytokines formation (Márquez *et al*, 2012). At present, campaigns have been promoted to disseminate the nutritional value of mango to encourage its consumption and add value to the production chain (Sumaya-Martínez *et al*., 2012) with an emphasis on production without agrochemicals.

The increase of the mango yield is traditionally addressed through conventional agrochemical-based practices, such as synthetic chemical fertilization and chemical control of weeds, pests and diseases (Prieto Martínez *et al*., 2005; Palacio *et al*., 2011), however, the increase in organic products consumption derives on the strategy of using other sources of nutrition (Márquez *et al*., 2013; Berdeja-Arbeu *et al*., 2018), due in part, to the increase in the costs of synthetic chemical fertilizers, in addition to their polluting effects and alteration of the microbiota in the rhizosphere (Caballero-Mellado and Martínez-Romero, 1999), which are fundamental elements in the sustainability and productivity of agrosystems.

By applying organic sources of nutrients, the physical and chemical properties of soils are improved and concomitantly it is expressed in the crop development without detriment of the yield or quality of the fruit (Aguirre *et al*., 2009). In various crops, the application of chemical and or organic nutrition presents a different response in some morphometric and biochemical characteristics of the fruits. In general, the morphometric characteristics of the fruits vary according to the soil and management (Maldonado-Astudillo *et al*., 2016) and the quality of the fruits in physiological maturity and consumption is influenced by the supply of nutrients in the different stages of growth (García *et al*., 2015), in addition, its scarcity induces biochemical and physiological changes that generate softening of the fruits.

In mandarin (*Citrus reticulata*), weight, fruit diameter, vitamin C content, concentration of total soluble solids, citric acid percentage and maturity index are not affected by conventional and organic handling, but they did increase the mineral content of Ca, Mg, K, Na, Fe, Cu, Mn, and Zn in fruits with organic management (Pérez-López *et al*., 2007). The tomato production *Solanum lycopersicum* Saladette type, Sahel variety, produced in a mixture (v/v 80:20) of sand plus vermicompost in the greenhouse, it was statistically similar when using Steiner solution, also without variation, in the lycopene content (González *et al*., 2016). In eggplant (*Solanum melogena* L.) the number and width of fruits increased when growing only in worm humus compared to the fertilized treatment and other combinations of worm humus plus fertilization (Montaño-Mata *et al*., 2009). Therefore, the objective of this work was to identify the morphometric and biochemical variation of the mango var Ataulfo in two contrasting environments with and without conventional management.

## MATERIALS AND METHODS

The research was developed in two mango orchards in the region of the Soconusco, Chiapas, Mexico, in contrasting soil and climate environments and differences in agronomic management. One of them, "La Norteña", belonging to the National Institute of Forestry, Agricultural and Livestock Research (INIFAP) (14° 45' 36.8" N and 92° 23' 4.61" O) at 22 m

altitude, at the Km 20.5 of the Tapachula-Puerto Chiapas highway section, municipality of Tapachula, Chiapas. The trees are 18 years old and have a distance between plants of 15×15 m. The soil presents the following physicochemical characteristics; clay-loam texture, pH 6.53 (1: 2 water), Organic Matter (OM) 1.9%, $NO_3^-$ 12.0 (mg $kg^{-1}$), $NH_4^+$ 14 (mg $kg^{-1}$), P 13 (mg $kg^{-1}$ Olsen), $Fe_2^+$ 21.9 (mg $kg^{-1}$), $Mn_2^+$ 19.8 (mg $kg^{-1}$), $Zn_2^+$ 9.1 (mg $kg^{-1}$), B 6.1 (mg $kg^{-1}$), $K^+$ int. 289 (mg $kg^{-1}$), $Ca_2^+$ 2863 (mg $kg^{-1}$) y $Mg_2^+$ 185 (mg $kg^{-1}$), CE 0.07 ($Dsm^{-1}$ a 25 °C), 2% slope, medium superficial drainage of slow infiltration and apparent density of 1.53. The climate corresponds to the $Aw_2$ (w") ig type, with 1200 to 1500 mm of annual rainfall, distributed between the months from June to November and an average annual temperature of 28 °C (García 1973).

The general management of the plantation is based on practices of cleaning with tractor and edge bander and pruning after harvest. The nutrition through chemical fertilization with the application of 150 kg of urea $ha^{-1}$ (46-0-0), and 90 kg of potassium sulfate $ha^{-1}$ (20.5-0-0). As a flowering inducer, from the beginning to mid-October, 7 kg of potassium nitrate $ha^{-1}$ (22.5-0-52) are applied. In addition, at the beginning of flowering they were sprayed with mL $ha^{-1}$ of fungicide (Trifloxystrobin) in a preventive way. During the months of february and march, auxiliary irrigation by sub foliar sprinkling is applied every two weeks for two hours.

The "Santa Cecilia" orchard is located in the municipality of Huehuetán, Chiapas (15° 03' 12.4" N and 92° 20' 60" O) and 311 m altitude. The trees are 21 years old and the distance between plants is 20×20 m and one in the center at "five of golds". The vegetative soil cover is dominated by bijahua (*Calathea lutea* (Aubl.) E.Mey. ex Schult.) and kudzu (*Pueraria phaseoloides* (Roxb.) Benth.), also has intercropping of *Theobroma cacao* L., *Ananas comosus* (L.) Merr. and *Cedrela odorata* L. The dominant soil group is Acrisol, with a clay-loam texture, pH 6.12 (1:2 water), MO 3.4 %, $NO_3^-$ 3.16 (mg $kg^{-1}$), P 1.72 (mg $kg^{-1}$ Bray), $Fe_2^+$ 22.9 (mg $kg^{-1}$), $Mn_2^+$ 13.4 (mg $kg^{-1}$), $Zn_2^+$ 0.1 (mg $kg^{-1}$), B 0.1 (mg $kg^{-1}$), $K^+$ int. 58.7 (mg $kg^{-1}$), $Ca_2^+$ + 397 (mg $kg^{-1}$) y $Mg_2^+$ 98.3 (mg $kg^{-1}$), CE 0.07 ($Dsm^{-1}$ a 25 °C) and slope ≥18%, a 63% saturation point, field capacity of 33.8%, permanent wilting point of 20.1%, hydraulic conductivity of 0.90 $cm.h^{-1}$ and an apparent density of 1.03. The climate belongs to the Am (w") ig type with 3000 to 3500 mm of precipitation and temperatures of 19, 27 and 35 °C (minimum, average and maximum) (García, 1973).

Agricultural practices carried out by the producer have been cleaning with a machete and pruning after harvest.

The physical and biochemical analysis of the fruits were carried out in the Postharvest Physiology Laboratory and the Integral Laboratory of the Faculty of Agricultural Sciences of the Autonomous University of Chiapas, located in Huehuetán, Chiapas, Mexico.

**Variables**

There were 30 fruits used in a state of commercial maturity as experimental units from five trees per study orchard (n=150), physical and biochemical variables were evaluated for each fruit. The fruits were weighed individually with a digital scale (Ohaus® USA) with a 0.1 g sensitivity.

The CIE (International Commission of the Illumination) system was used to evaluate the color of the epicarp of the fruits in the L*C*h° (L* luminosity, C* chromaticity and h° hue or tone) space, which were measured with an X-Rite® brand colorimeter. In the end, the colors obtained from the fruits of both orchards were compared, expressed as the color difference:

$$(\Delta) \text{ leaving } \Delta L^*, \Delta C^* \text{ and } \Delta h^\circ$$

where: $\Delta L^*$=difference in the brightness value; +=lighter, −=darker; $\Delta C^*$=difference in the chroma; +=saturated, −=opaque; $\Delta h^\circ$=difference in the hue.

For the fruits shape, polar or longitudinal diameter (DL) and equatorial diameter (DE) (mm) were measured with SureBilt® (USA) digital vernier.

The firmness of the epicarp in the fruits was determined following the Official Mexican Standard NMX-FF-058-SCFI-2006, which establishes the minimum quality specifications that *Mangifera indica* L. must meet, with the help of a texturometer (Chatillon, FDV-30 model, USA) measuring the necessary force to penetrate the fruit peel expressed in Newtons (N).

Destructive sampling was carried out to quantify the morphological and biochemical components of the fruit. The weight of the peel, pulp and seed was obtained. With these values the pulp/seed ratio was determined.

Additionally, the total soluble solids (TSS) expressed as °Brix. For this purpose, 10 g of fresh pulp (mesocarp) were homogenized and adjusted to a final volume of 50 mL with distilled water. The °Brix were determined with a refractometer (ATAGO Model Pallete PR-32α USA: 0-32%) following the AOAC (1990) methodology. The pH was determined with a potentiometer (Thermo Orion, Model 230A USA).

The titratable acidity quantification (expressed as citric acid %) was carried out by the AOAC (2000) volumetric method. 10 g of liquefied pulp were used in 50 mL of distilled water. An aliquot of 20 mL was taken from the mixture and titrated with NaOH (0.1 N). The result was expressed as citric acid percentage. With the previous data, the °Brix/acidity ratio was calculated, using the quotient of the variables °Brix and the percentage of acidity that is expressed as % °Brix / acidity and represents one of the fruit maturity indices. The data obtained were processed with the help of the SAS software ver. 9.0 (SAS Institute, 2009), to establish the statistical difference between the mango orchards, an analysis of variance (ANOVA) was performed, and the differences were compared with the Tukey's mean test ($r \leq 0.05$).

## RESULTS AND DISCUSSION

The fruits weight from La Norteña site ranged from 256 to 320 g and they are classified as size 16 or large, and the fruits from the Santa Cecilia site weighed from 227 to 295 g within the 18 or medium caliber classification, according to the range standardized by the Official Mexican Standard (NOM-188-SCFI-2012). The differences in the weight of fruits produced with conventional management in the Aw climate and clay soils of La

Norteña Site represented 9.5% more, compared to the Santa Cecilia site, with Am climate, acrisol soils and with agroecological management. The difference is influenced by the weight of the peel and seed, morphological components that weighed 31.4 and 10% more respectively compared to the fruits produced at the Santa Cecilia site. However, the fruits of the Santa Cecilia Site registered an average increase of 9.3 g in the pulp weight, and it was statistically different ($P \leq 0.05$) compared to La Norteña site. This value increased its pulp/seed ratio (Table 1).

In other environments, such as in the agroclimatic conditions of Irapuato, Guanajuato, mango fruits var. Ataulfo weighed 223.9 g (Almanza *et al.* 2016), a lower weight than those found in the two sites on the Chiapas Coast, however, Maldonado-Astudillo *et al.* (2016) reported fruits of mango var. Ataulfo with an average weight of 387.8 g in the agroclimatic conditions of the Guerrero Coast and conventional management.

In relation to the morphological variables of the fruits such as the polar diameter of the mangoes that grown with conventional management was on average 4.8 mm higher compared to mangoes grown in the agroecological system. The equatorial diameter does not show changes in the fruits produced in both production systems. In this regard, Almanza *et al.* (2016) cite lower longitudinal diameter of mango fruits var Ataulfo with

**Table 1**. Morphological and biochemical variables of the fruits of *Mangifera indica* L. var. Ataulfo in two production systems at the Soconusco Chiapas, Mexico.

| Variable | La Norteña (Conventional Managementl) | Santa Cecilia (Agroecological Management) | CV % |
|---|---|---|---|
| Fruit Biomass | | | |
| Total Weight (g) | 276.62±8.08 a* | 251.43±6.56 b | 15.27 |
| Peel (g) | 43.89±1.08 a | 30.10±0.96 b | 15.22 |
| Pulp (g) | 206.51±7.08 b | 227.85±6.17 a | 16.76 |
| Seed (g) | 26.19±0.94 a | 23.56±0.85 b | 19.77 |
| Pulp/seed Ratio | 8.11±0.31 b | 9.96±0.37 a | 21.01 |
| | Fruit Morphology | | |
| Polar diameter (mm) | 122.51±1.25 a* | 117.68±0.95 b | 5.09 |
| Equatorial diameter (mm) | 71.09±0.70 a | 71.87±0.47 a | 4.58 |
| Fruit indices (Polar/equatorial) | 1.72±0.013 a | 1.63±0.014 b | 4.53 |
| Firmness (N) | 18.97±0.66 b | 23.71±0.97 a | 21.4 |
| | Fruit Color | | |
| Luminosity (L*) | 66.40±0.51 a | 64.91±0.44 b* | 4.00 |
| Chrome (C*) | 53.93±0.71 a | 53.06±0.73 a | 7.42 |
| Hue (h°) | 69.02±0.52 a | 69.38±1.27 a | 7.74 |
| ΔL* | −1.49 | | |
| ΔC* | −0.87 | | |
| Δh° | +0.36 | | |

* Values with the same letter within each factor and column are equal according to Tukey's test at $P \leq 0.05$.
** CV: Coefficient of Variation.

conventional management. In contrast, Maldonado-Astudillo et al. (2016) describe larger fruits of mango var Ataulfo obtained from the municipality of Atoyac de Álvarez, Guerrero. In this regard, it is suggested that the differences in weights and sizes in the fruits of the same mango crops are influenced by environmental conditions and management (Santos-Villalobos *et al.*, 2011).

The firmness of the mango fruits produced in Santa Cecilia was 19.9% higher and statistically different ($P \leq 0.05$) compared with the mangoes produced in the La Norteña Site. On the other hand, in the Nayarit Coast, with the same mango variety, var Ataulfo, there were no statistical differences in the firmness of the fruits subjected to fertilized and unfertilized treatment (Nolasco-González *et al.*, 2016). Cancino-Vázquez *et al.* (2020) cite an inverse relationship between firmness and fibrousness in mango fruits var. Ataulfo, when treated with low dose of gamma irradiation on the Chiapas coast. During ripening there are physiological and biochemical activities, such as respiration, that affect the firmness of the fruits (Martínez-González *et al.*, 2017).

The luminosity (L*) or color assessment in the epicarp (peel) of the var. Ataulfo mango fruits at La Norteña site presents a statistically different increase ($P \leq 0.05$) than the Santa Cecilia fruits. In the values of chroma (C*) and hue (h°) in the fruits, they did not present statistical differences between both sites (Table 1). If postharvest physical treatments are applied to the fruits, such as thermal and cold with coatings, both the luminosity as it is the hue and chroma, significant changes are obtained in that color space (L*C*h°) (Bello-Lara *et al.*, 2016; Ariza-Flores *et al.*, 2018).

When comparing the color of the fruits applying the difference $\Delta$ of the values for L*, C* and h°, it gives a negative result in luminosity $\Delta$L*. The above indicates that the fruits from La Norteña Site are lighter compared to the fruits from Santa Cecilia. For the $\Delta$C* chroma or saturation, the Santa Cecilia fruits are less saturated than La Norteña fruits and regarding the $\Delta$h° hue, the positive value indicates a greener color tone in Santa Cecilia compared with La Norteña fruits. In this regard, it has been identified that the modification of the color of the fruits during maturity is influenced by changes in the content of chlorophyll, carotenoids, accumulation of flavonoids (Martínez-González *et al.*, 2017) and anthocyanins (Brownleader *et al.*, 1999), derived from environmental conditions, plantation management, especially fertilization, which influence its coloration (Bouzayen *et al.*, 2010).

In addition, during the ripening stage of the fruit, sugars, volatile compounds, and organic acids that affect the flavor, aroma, and nutritional quality, are modified (Martínez-González *et al.*, 2017). Among the components of the fruits flavor, the acidity, which is formed by organic acids such as malic, citric, oxalic, and tartaric, forms an important element for the palate of the consumers. Additionally, of being inversely related to pH and total soluble solids (TSS). The fruits pH of La Norteña site was higher compared to the Santa Cecilia fruits and statistically different ($p \leq 0.05$). Likewise, the citric acid content was 3.5% higher compared to the Santa Cecilia mangoes, but without statistical difference. The pH and the percentage of citric acid are related to organic acids, since they are used during the respiration process (Tsouvaltziz *et al.*, 2007), thus reducing acidity, and while acidity decreases pH, total soluble solids increase (Ortiz-Franco *et al.*, 2016).

On the other hand, under conditions of Galeana, Guerrero and with thermal and chemical treatment, the pH and citric acid values are 4.5 and 0.6%, respectively (Ariza-Flores *et al.*, 2018), and citric acid tends to change significantly if the mango fruits var Ataulfo are treated with gamma radiation (Cancino-Velázquez *et al.*, 2020).

The TSS found at La Norteña site are 31% higher compared to the Santa Cecilia site. This is very significant, since the flavor in the fruits is the TSS content, they are also an estimate of the total sugar content, and in mango var. Ataulfo this characteristic is very attractive due to its high sugar content (Cancino-Vázquez *et al.*, 2020).

During the ripening of the fruits the acidity content tends to decrease, therefore, the total soluble solids tend to increase. This relationship is expressed as the quotient of the soluble solids content (°Brix) between the titratable acidity values (TSS/Acidity ratio). In this work, a higher TSS/Acidity ratio was found in La Norteña site, and it is 30% higher compared to the Santa Cecilia fruits. This difference in between the values of the TSS/Acidity ratio is due to the citric acid content, the lower the acidity value, the greater the relationship with the TSS.

## CONCLUSIONS

Morphological and biochemical modifications are presented in mangoes produced in the two production systems and contrasting environments. Greater size and weight, pH and TSS content in the conventional production system, but increased pulp and greater firmness, as well as higher citric acid content in the system with agroecological management. The results suggest differential effects in mango fruits according to the management and environment where they develop.

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AGRO PRODUCTIVIDAD

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# Agroecological coverings for the sustainable production of Rambutan (*Nephelium lappaceum* L.)

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**ABSTRACT**

**Objective**: To evaluate the influence of living and dead covers on the yield and quality of rambutan fruits (*Nephelium lappaceum* L.) in Soconusco, Chiapas, Mexico.

**Design/methodology/approach**: Five treatments were evaluated, two live covers, two dead covers and an always clean control without covers. The following were evaluated: plant height, crown volume, fruit quality, fruit yield (t $ha^{-1}$). The data were analyzed under a randomized block experimental design.

**Results**: All the agroecological modalities of hedging evaluated produced fruits with the quality required for national and international commercialization.

**Study limitations/implications**: The morphological and physiological response of the crop can change with the age of the tree.

**Findings/conclusions**: An agroecological management strategy is presented to develop rambutan cultivation in Soconusco region.

**Keywords**: Covers, Rambutan, Agroecology, quality of fruits.

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## INTRODUCTION

Rambutan (*Nephelium lappaceum* L.) is a tropical fruit native to the Malay Archipelago that belongs to the Sapindaceae family (Tindall, 1994). It was introduced around 1960 into Soconusco, Chiapas, Mexico, where it adapted to the warm-humid environment, a condition which has favored its expansion in the last years. The fruit is consumed fresh due to its sweet flavor, juicy pulp, vitamin C and riboflavin content (Pérez & Jürgen, 2004). Additionally, it has been found that it contains polyphenolic compound bioactives (Hernández *et al*., 2017; Ling *et al*., 2010; Thitilertdecha *et al*., 2010), antimicrobial activity (Mohamed *et al*., 1994; Solanki, 2010), and antihyperglycemic activity (Palanisamy *et al*.,

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2011; Manaharan *et al.*, 2012), which are considered natural compounds with both high added value and high antioxidant activity.

At present, there are more than 2000 ha of rambutan crops in Chiapas, ranging from 100 to 700 m of altitude, which have allowed the region to diversify with regards to production and economy (Caballero *et al.*, 2011). This crop has also been established in other regions of Mexico, like Veracruz, Tabasco, and Nayarit (García-Gurría *et al.*, 2013). The production of rambutan as a traditional monoculture produces an average yield of 10 t $ha^{-1}$ year (SIAP, 2020) with the use of high amounts of agrochemicals (SAGARPA, 2015). Moreover, these technologies include non-quantified energy expenditure, which diminishes crop profitability while it leads to phreatic surface contamination with collateral damage to wild flora, whose function is to maintain ecological balance of agroecosystems (Ruiz *et al.*, 2013). In view of this situation, some agroecological practices, such as the use of live and dead covers, have proven their efficiency in crop management and weed management (Cairo *et al.*, 2017). These practices allow the conservation of soil moisture (Leyva, 2002; Vanderlinden *et al.*, 2004; Gómez *et al.*, 2017), and boost the number and diversity of macro-organisms (Toledo, 2008). As for rambutan, there is no research done with such purposes. Therefore, the objective of the research hereby presented was to evaluate the influence of live and dead covers on the yield and quality of rambutan fruits.

## METHODS AND MATERIALS

The research was carried out in the Cantón Barrio Nuevo, Villa Comaltitlán municipality (15° 08' 7,14" N and 92° 37' 32,11" O) at 23 m of altitude. The climate corresponds to type Aw (w") IG, with 2500 mm of annual rainfall, distributed between June to November, and an annual average temperature of 23 °C (García, 1973). The experimental area is characterized by having moderately fertile soil and classified as a cambic, deep Feozem, with a sandy crumb texture, granular structure, slightly acidic pH (6.0), low organic matter content (2.6%), 0.07% N, 26.6 mg $kg^{-1}$ P, 230.0 mg $kg^{-1}$ K, 804 mg $kg^{-1}$ Ca, 457.5 mg $kg^{-1}$ Mg, and 168.0 mg $kg^{-1}$ Na (USS, Working Group WRB, 2015).

Uniformly growing 18-month-old rambutan plants of the Adelita variety were acquired. The cultivation was done on November 8, 2009, through a 7×7 m spatial arrangement (Fraire, 2001).

We set live and dead covers and a core with no cover 15 days after the cultivation, under an experimental design of random blocks with 5 treatments and four replications. The covers were established from the base of the rambutan plant and over the soil surface up to 2 m away at the cardinal points. A total of 16 $m^2$ were covered.

The treatments were as follows:

1. *Arachis pintoi* Krapov. & W. C. Greg cover (ApC). *A. pintoi* stolons were set at a rate of 12 stolons 15-20 cm long and with 5 to 6 knots per $m^2$. After sowing, the cultivated area was irrigated with 32 liters of water, in two applications with 30-minute intervals.
2. Weeds cover (WeC). The population of weeds was kept and was pruned with a machete and by hand to 0,20 m high.

3. Rambutan crop residues cover (RCR). The rambutan pruning residues were cut and spread in 0,10 m pieces over the soil surface at an average height of 0,20 m.
4. Cover including short-cycle crop residues (corn straws, beans, and sesame) and weeds (CRW). The area was covered with the crop residues and the rest was maintained with weeds at 0,20 m high.
5. Uncovered core with herbicides (UCC). Herbicide paraquat was used, six times a year, four during rainy period and two during dry period, at the rate of 2.0 L $ha^{-1}$. When the effect of paraquat concluded, the same dose of glyphosate was used covering 16 $m^2$ from the base of the rambutan plant. The surviving species were extracted manually.

All five treatments were set in an experimental design of randomized blocks with four replications. A tree was considered as one experimental unit.

Plant Height variables were recorded bimonthly from 2009. They were obtained using a graduated ruler 4 m long (Error ±0,01 m). The crown volume was measured bimonthly from 2011 to 2014. Crown volume was estimated from the equatorial diameter, using a measuring tape (Error ±0,01 m) at a medium height of the crown in two directions (N-S and E-W), and crown height was measured with a graduated ruler 4 m long (Error ±0,01 m). The total number of fruits was counted per treatment and replication. The Weight of the fruits (g) was achieved by weighing them individually on a digital scale (Ohaus® USA) with a sensitivity of 0.01 g. Degrees Brix (°Brix) were obtained in 30 fruits per plant and taken at the four cardinal points at two different heights of the plant with a refractometer (ATAGO Model Pallete PR-32α USA: 0-32%) following the AOAC methodology (1990). pH was obtained with a potentiometer (Thermo Orion, Modelo 230A USA). Titratable acidity quantification (expressed as % citric acid) was measured by means of the volumetric method of AOAC (2000).

**Statistical analysis**

The data obtained were processed with the help of Statgraphics Centurion XVI.I software. A variance analysis (ANOVA) was conducted to establish the statistical difference. When differences were found, the comparison of Duncan's means was carried out ($P \leq 0,05$).

**Economic Analysis**

The economic evaluation was calculated considering the SAGARPA proposal (2014), which contemplates: (i) the direct costs of the work and inputs used for treatments, (ii) the indirect costs during the experimental phase, and (iii) the economic results of the three rambutan harvests. To determine the total benefits per treatment (in Mexican pesos), the total investment (benefit-cost-ratio) was subtracted from the gross income.

## RESULTS AND DISCUSSION

**Plant height and Crown volume**

The results obtained show that the covers did not influence both variables. There were no significant differences between the treatments during the experimental period (Table 1).

**Table 1**. Height and crown volume in *Nephelium lappaceum* L., with different coverage treatments in Soconusco, Chiapas, Mexico, area for five years. The values are averages of four repetitions and without statistical difference (P≤0.05).

| Year | Date | Plant height (m) | | | | | Crown volume ($m^3$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ApC | WeC | RCR | CRW | UCC | ApC | WeC | RCR | CRW | UCC |
| 2009 | Nov | 1.11 | 1.11 | 1.11 | 1.11 | 1.11 | | | | | |
| 2010 | Jan | 1.16 | 1.13 | 1.13 | 1.13 | 1.13 | | | | | |
| | Mar | 1.22 | 1.21 | 1.21 | 1.22 | 1.22 | | | | | |
| | May | 1.29 | 1.28 | 1.29 | 1.30 | 1.31 | | | | | |
| | Jul | 1.40 | 1.38 | 1.36 | 1.41 | 1.42 | | | | | |
| | Sep | 1.52 | 1.51 | 1.44 | 1.51 | 1.51 | | | | | |
| | Nov | 1.64 | 1.60 | 1.54 | 1.64 | 1.65 | | | | | |
| 2011 | Jan | 1.76 | 1.72 | 1.62 | 1.71 | 1.75 | 3.03 | 2.41 | 1.77 | 2.68 | 3.12 |
| | Mar | 1.88 | 1.77 | 1.70 | 1.76 | 1.82 | 4.11 | 2.93 | 2.24 | 3.05 | 3.70 |
| | May | 1.94 | 1.82 | 1.75 | 1.81 | 1.87 | 4.70 | 3.29 | 2.59 | 3.46 | 4.16 |
| | Jul | 2.13 | 2.11 | 1.87 | 1.90 | 1.95 | 6.86 | 5.96 | 3.52 | 4.33 | 5.03 |
| | Sep | 2.25 | 2.29 | 2.03 | 2.02 | 2.14 | 8.56 | 8.04 | 5.07 | 5.66 | 7.31 |
| | Nov | 2.37 | 2.41 | 2.21 | 2.17 | 2.27 | 11.04 | 10.39 | 7.55 | 7.87 | 9.20 |
| 2012 | Jan | 2.43 | 2.49 | 2.28 | 2.27 | 2.35 | 12.29 | 11.68 | 8.63 | 9.44 | 10.58 |
| | Mar | 2.56 | 2.54 | 2.38 | 2.34 | 2.42 | 14.76 | 13.13 | 10.08 | 10.48 | 11.81 |
| | May | 2.64 | 2.59 | 2.44 | 2.36 | 2.45 | 15.00 | 13.33 | 11.14 | 11.88 | 11.84 |
| | Jul | 2.72 | 2.64 | 2.49 | 2.46 | 2.51 | 16.32 | 14.38 | 12.33 | 12.53 | 12.41 |
| | Sep | 2.75 | 2.67 | 2.52 | 2.51 | 2.54 | 17.70 | 15.26 | 13.00 | 13.14 | 13.27 |
| | Nov | 2.79 | 2.69 | 2.55 | 2.56 | 2.57 | 18.53 | 16.82 | 14.38 | 14.68 | 14.70 |
| 2013 | Jan | 2.82 | 2.75 | 2.67 | 2.71 | 2.69 | 19.37 | 17.99 | 15.74 | 18.24 | 16.06 |
| | Mar | 2.84 | 2.81 | 2.75 | 2.82 | 2.76 | 20.33 | 19.13 | 17.63 | 18.72 | 17.41 |
| | May | 2.87 | 2.83 | 2.79 | 2.89 | 2.80 | 21.12 | 19.80 | 17.79 | 18.87 | 18.02 |
| | Jul | 3.00 | 2.88 | 2.78 | 2.95 | 2.83 | 21.46 | 19.97 | 18.89 | 20.76 | 19.23 |
| | Sep | 3.06 | 2.91 | 2.77 | 2.99 | 2.85 | 24.91 | 23.97 | 22.52 | 23.66 | 22.18 |
| | Nov | 3.13 | 2.94 | 2.77 | 3.02 | 2.87 | 27.60 | 25.98 | 25.05 | 26.38 | 25.01 |
| 2014 | Jan | 3.26 | 3.13 | 2.96 | 3.23 | 3.03 | 30.46 | 28.21 | 27.15 | 29.54 | 27.13 |
| | Mar | 3.32 | 3.29 | 3.18 | 3.29 | 3.17 | 35.05 | 30.08 | 30.16 | 34.06 | 30.38 |
| | May | 3.37 | 3.32 | 3.25 | 3.30 | 3.18 | 35.20 | 30.17 | 30.29 | 34.19 | 30.55 |
| | Jul | 3.41 | 3.34 | 3.30 | 3.37 | 3.20 | 35.29 | 30.25 | 30.43 | 34.60 | 30.64 |
| | Sep | 3.42 | 3.35 | 3.32 | 3.40 | 3.22 | 37.91 | 33.59 | 33.36 | 37.07 | 32.24 |
| | Nov | 3.64 | 3.56 | 3.55 | 3.64 | 3.43 | 39.79 | 37.09 | 36.29 | 39.25 | 36.00 |

ApC (*Arachis pintoi* Krapov. & W. C. Greg cover), WeC (Weeds cover), RCR (Rambutan crop residues cover), RCR (Rambutan crop residues cover), UCC (Uncovered core with herbicides).

The plants reached an average height of 3,3 m by the end of 2014. Plant height of fruit trees is a growth variable closely linked to crop yield, and, from the practical point of view, it is of great importance as harvesting fruits more easily or vice versa depends on that. The architecture of the rambutan plant shows that the side branches define productivity.

The initial growth of the crown volume in the ApC treatment increased from the second year, a trend maintained until the last sampling. In contrast, the CRW treatment produced the smallest crown size during evaluation.

At the end of the third harvest, the core, WeC and RCR had the smallest crown size while ApC and CRW reached a significantly higher development. In this regard, Barreto *et al.* (2015) explain that plants produced by grafting the same variety grow in a similar way if set in similar edaphoclimatic conditions. This suggests covers contribute to it, mainly with *Arachis pintoi* (ApC) by improving soil fertility through the activity of nitrogen-fixing bacteria and moisture conservation with CRW.

In general, throughout the March-July period there is a greater increase in the plant growth, which coincides with the induction of the flowering-fruiting stage. This effect in the region was pointed out by Fraire (2001). The treatments with the greatest increase in crown volumes (ApC and CRW), close to 40 $m^3$ and compared to the rest of the treatments, show a difference of about 4 $m^3$. The increase in crown volume may be related to fruit production and the volume of branches that must be pruned on each tree, after harvests. This morphological expression may influence the results of subsequent harvests (Doruska and Burkhart, 1994 and Brunner, 1998).

**Influence of the covers on the quality of the fruits**

The results show significant statistical differences ($p \leq 0.05$) in the weight of the fruit and the °Brix. In the case of fruit acidity and pH, there are no significant differences over the years (Table 2). However, the quality of rambutan fruits for export is considered optimal when they reach a weight greater than 30 g and a total soluble solids content of 16 to 18% (Codex Alimentarius, 2008), in addition to presenting a uniform red color, free of lesions and pest damage. In our case, fruit mass was greater than 30 g, including the core.

A less efficient result was expected with the WeC treatment, that is, smaller fruits, when considering the weeds competition for nutrients and water with rambutan; however, up to now, only in the second year of harvest, lower weight was obtained in comparison with the other treatments. On the other hand, the treatment without weeds (UCC), produced fruits of greater size in the years 2013 and 2014 which was statistically superior to the rest of the treatments ($p \leq 0.05$).

In general, ApC treatment was considered to produce better results. This was partially because it was easier to set *Arachis pintoi* under the shade of the rambutan and because of its promiscuous condition of association with rhizobia, which is capable of fixing nitrogen and improving the nitrogen supply to the plant.

Rambutan residues covers (RCR) and annual crop residues (CRW) had intermediate values during the three years of production. In 2013 all treatments decreased their fruit weight, which increased in the following year. This could have been due to environmental conditions. °Brix content in the fruits differed between years and treatments, with no apparent relationship. They increased with WeC and RCR in the first year, and only RCR was again statistically different from other treatments in 2014. WeC was inserted into the first statistical group only the first year and ApC in the last. Rambutan is a crop that

can reach productions that fluctuate between 12 and 16 t $ha^{-1}$ in Chiapas (Fraire, 2001). These productions can be achieved after crop yield stabilization, which occurs after the sixth harvest (Arias and Calvo, 2014).

**Table 2**. Fruit weight Evaluation of the quality of *Nephelium lappaceum* L., fruits three years after being set with different covers in Soconusco, Chiapas, Mexico.

| Treatments | Fruit weight (g) | | |
|---|---|---|---|
| | 2012 | 2013 | 2014 |
| ApC | 35,0 b | 33,4 b | 32,3 bc |
| WeC | 31,8 d | 27,3 d | 32,9 bc |
| RCR | 37,4 a | 31,9 c | 31,1 c |
| CRW | 33,2 c | 30,8 b | 33,2 b |
| UCC | 31,9 d | 37,2 a | 34,0 a |
| CV % | 6,51 | 10,60 | 4,93 |
| SE | 0,281 | 0,494 | 0,438 |
| Acidity | | | |
| ApC | 1,67 | 1,64 | 1,69 |
| WeC | 1,55 | 1,59 | 1,44 |
| RCR | 1,54 | 1,56 | 1,52 |
| CRW | 1,66 | 1,64 | 1,69 |
| UCC | 1,64 | 1,62 | 1,64 |
| CV % | 7,43 | 3,79 | 14,36 |
| SE | 0,047 | 0,021 | 0,09 |
| pH | | | |
| ApC | 4,66 | 4,68 | 4,65 |
| WeC | 4,67 | 4,59 | 4,74 |
| RCR | 4,68 | 4,63 | 4,68 |
| CRW | 4,68 | 4,68 | 4,68 |
| UCC | 4,62 | 4,66 | 4,60 |
| CV % | 1,54 | 1,16 | 4,22 |
| SE | 0,023 | 0,019 | 0,063 |
| $^{o}$Brix | | | |
| ApC | 19,0 ab | 20,1 b | 20,6 a |
| WeC | 20,1 a | 18,9 c | 19,0 bc |
| RCR | 19,0 a | 20,2 b | 20,7 a |
| CRW | 17,9 b | 21,4 a | 19,9 ba |
| UCC | 19,0 ab | 20,9 ab | 19,2 cb |
| CV % | 7,22 | 5,87 | 6,85 |
| SE | 0,509 | 0,318 | 0,467 |

ApC (*Arachis pintoi* Krapov. & W. C. Greg cover), WeC (Weeds cover), RCR (Rambutan crop residues cover), RCR (Rambutan crop residues cover), UCC (Uncovered core with herbicides). Means with different letters in each column, indicate significant statistical differences according to Duncan ($P \leq 0.05$).
CV: Coefficient of Variation. SE: Estandard Error.

**Influence of covers on rambutan crop yield**

The analysis of the first three harvests (Table 3) showed that for the first two harvests the influence of covers was extremely low and highly variable between treatments, which usually occurs with all perennial fruit trees.

The highest fruit yield was achieved with the treatment where protection with CRW was used and was statistically different in the three harvest years compared to the rest of the treatments. ApC also achieved the highest fruit production during 2014 and is positioned as the second-best option in yield. In this case, it is considered as an alternative of great validity as it favors the presence of beneficial entomofauna for the pollination of the crop flowers, and, therefore, for greater fruit production (Marroquín *et al*., 2015).

WeC treatment produced the lowest yields of all the treatments in the three harvests. This suggests interspecific competition crop-weeds for light, water, and nutrients, during the growth and development of crops under adequate spatial arrangements (Franke, 1995).

However, the results obtained confirm the benefits of covers and the disadvantages of keeping the soil uncovered, corroborating what Febles *et al*. (2010) proposed regarding the effectiveness of the cover to protect the soil against erosion, solar radiation, rains, winds, weed and extreme temperatures.

The results obtained shed light on the importance of the agroecological view of considering any living or dead cover for the benefit of the crop compared to bare soil.

**Economic evaluation of rambutan cultivation with a cover**

The profitability of the two best treatments (CRW and ApC) amounted to 163 000 Mexican pesos. Harvest with ApC contributed the most with more than 80% of the profits (Table 4).

**Table 3**. Rambutan crop (*Nephelium lappaceum* L.) yield with different cover treatments in the first years of harvest in Soconusco, Chiapas, Mexico.

| Treatments | Performance (t ha$^{-1}$) | | |
|---|---|---|---|
| | 2012 | 2013 | 2014 |
| ApC | 0,604 b | 3,055 b | 11,694 a |
| WeC | 0,459 cd | 2,383 e | 8,640 c |
| RCR | 0,497 c | 2,811 c | 10,133 b |
| CRW | 0,716 a | 3,269 a | 11,555 a |
| UCC | 0,417 d | 2,516 d | 10,409 b |
| CV % | 23,95 | 13,02 | 13,33 |
| SE | 0,023 | 0,044 | 0,336 |

ApC (*Arachis pintoi* Krapov. & W. C. Greg cover), WeC (Weeds cover), RCR (Rambutan crop residues cover), RCR (Rambutan crop residues cover), UCC (Uncovered core with herbicides). Means with different letters in each column, indicate significant statistical differences according to Duncan (P±0,05).
CV: Coefficient of Variation. SE: Estandard Error.

**Table 4**. Economic evaluation of *Nephelium lappaceum* L. fruit production with different covers in the Soconusco, Chiapas, Mexico.

| | ApC | WeC | RCR | CRW | UCC |
|---|---|---|---|---|---|
| Establishment Costs ($/ha$^{-1}$) | 50 337,00 | 50 237,00 | 50 137,00 | 50 137,00 | 76 037,00 |
| Maintenance costs ($/ha$^{-1}$) | 14 650,00 | 15 470,00 | 15 670,00 | 15 470,00 | 18 730,00 |
| Income | 227 595,00 | 173 085,00 | 204 105,00 | 229 485,00 | 200 055,00 |
| Net Profit ($/ha$^{-1}$) | 162 068,00 | 107 378,00 | 138 298,00 | 163 878,00 | 105 288,00 |

ApC (*Arachis pintoi* Krapov. & W. C. Greg cover), WeC (Weeds cover), RCR (Rambutan crop residues cover), RCR (Rambutan crop residues cover), UCC (Uncovered core with herbicides).

Both treatments can be successful from an agroecological point of view. While dead covers with crop residues offer protection from the moment they are applied, live covers take time to offer such protection. One advantage of the latter is that they can attract beneficial insects, although they retain less moisture compared to dead covers. Therefore, each alternative must be adjusted to the main interest of farmers, considering that both are as efficient economically speaking.

The least successful treatment was UCC with a utility similar to the conservation of regulated natural vegetation (WeC). Both treatments were surpassed by CRW with a difference that fluctuated between $56,000 and $58,000 Mexican pesos (US$ 2,800-2,900). The treatment with the use of herbicides (UCC) was less efficient in terms of profitability, as it increases the main crop production costs, facilitates erosion and loss of soil moisture, apart from reducing biodiversity within the agroecosystem, which is counterproductive for the conservation of agroecosystems considering the problems caused by climate change.

## CONCLUSIONS

*A. pintoi* live covers and crop residues covers used in rambutan harvesting do not limit the growth, yield, and quality of rambutan crop fruits regarding national and international commerce. This research project generated a strategic proposal aimed at enhancing sustainable rambutan harvesting for all producers with increased opportunities for national and international trade.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Processes in meat oxidation and usage of rosemary (*Salvia rosmarinus* (L.) Schleid.,) as a natural antioxidant

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**ABSTRACT**

**Objective**: Lipid oxidation of meat is one of the most important factors affecting shelf life and is one of the decisive factors in the consumer's purchase decision of the product. Therefore, information related to lipid oxidation using rosemary (*Salvia rosmarinus* (L.) Schleid.) as a natural antioxidant is described and analyzed.

**Design/methodology/approach**: Mechanisms of oxidation were explored and described, as well as the alternatives to stop this process and different methodologies to measure antioxidant activity and innovative alternatives that are currently being investigated.

**Results**: Appling antioxidants is one of the most widely used methods to counteract the oxidation process in meat. Currently, using herbs and spices has gained great acceptance, as in the case of rosemary. Its usage obtained satisfactory results for inhibiting and delaying lipid oxidation.

**Limitations of the study/implications**: Using rosemary may have some drawbacks such as incorporating a strong flavor to the meat and the effects that its active compounds may have when exposed to oxygen, heat and humidity. Therefore, it is necessary to research for alternatives that will allow better preservation and availability of its compounds.

**Findings/conclusions**: Nanoencapsulation of rosemary may be an alternative to the drawbacks of its use, working as a protective barrier for improved performance and improving food safety. However, this innovation is just being explored and is therefore not possible to have a certainty of success when using these new technological alternatives.

**Keywords**: oxidation, lipids, meat, rosemary, nanoencapsulation.

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## INTRODUCTION

Meat and meat products are rich in nutrients such as proteins, fats and minerals; they are perishable foods with a short shelf life (Heinz and Hautzinger, 2007; Rashidaie *et al.*, 2019). One of the factors that affect the most the shelf life is lipid oxidation (LO) in the unsaturated fatty acid (UFA) fraction of the membrane, forming peroxides and hydroperoxides, which are susceptible of decomposition into secondary oxidation products, such as hydrocarbons, aldehydes, ketones, alcohols, among others (Chen *et al.*, 2017; Garcia *et al.*, 2017).



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These compounds produce unpleasant aromas and flavors in foods, typical of rancidity which negatively alter sensory attributes such as color, texture, odor and flavor (Gallego *et al.*, 2015). It is, therefore, necessary to use alternatives that allow meat preservation, but at the same time seek for consumer acceptance, like using natural antioxidants (Cheng *et al.*, 2017). The food industry commonly uses synthetic antioxidants, which may be unsuitable for consumers, due to their potential toxicity and carcinogenic risks (Aminzare *et al.*, 2019). Some plants are a source of bioactive substances containing phenolic compounds with antioxidant properties; among these plants is rosemary (*Salvia rosmarinus*) which contains potent antioxidants such as phenolics, diterpenes, carnosic acid, carnosol and rosmarinic acid (Chao *et al.*, 2020). However, these phenolic compounds may lose their beneficial effects when exposed to oxygen, heat, humidity and light. Therefore, it is necessary to use specific methods to protect them to achieve a higher antioxidant activity, one way is nanoencapsulation (Duarte and Larroza, 2019; Rashidaie *et al.*, 2019). The usage of rosemary oil is limited due to its hydrophobicity, as it is difficult to dissolve in the aqueous phase of food. One possible way to overcome this barrier is through nanotechnology (Boskovic *et al.*, 2019), achieving increased stability, protection, controlled release and reducing the possible adverse impact on the organoleptic properties in meat and meat products (Duarte and Larroza, 2019). On this basis, a review of the information related to lipid oxidation and the application of rosemary (*Salvia rosmarinus* (L.) Schleid.) as a natural antioxidant has been developed.

**Shelf life of meat and meat products**

Meat and meat products are an excellent source of essential nutrients containing high-quality proteins, fats and minerals, which make them highly perishable foods, therefore their shelf life is short (Aminzare *et al.*, 2019; Tsironi *et al.*, 2019). The shelf life of meat and meat products is defined as the maximum recommended time where products can be stored, in specific temperature and humidity conditions, without losing an acceptable quality (Donohue, 2016). These products are susceptible to changes that lead to LO, whether in a fresh or cooked state. The fresh form is affected by its storage and packaging, as well as other factors related to the animal species they come from and their fat content. When cooking, oxidative stability and shelf life are again affected by the type of meat (solid, ground, or mechanically deboned) and the employed thermal processing method (boiling, frying, grilling, or curing) (Shahidi, 2016). Spoilage results in changes in the sensory characteristics of those products such as off-flavors and colorings, which make the product undesirable or unacceptable for the consumers (Donohue, 2016). Some strategies have been applied to maintain food quality and extend shelf life, these include the addition of antioxidants, which slow oxidation and extend the shelf life of packaged foods (Chao *et al.*, 2020).

**Meat oxidation and ways to prevent or retard it**

Oxidation is a complex and irreversible process in oils and fats. So, in meat, the membrane fraction of UFAs oxidizes resulting in the formation of hydroperoxides (Kumar *et al.*, 2015). The oxidation process comprises three stages: initiation, propagation and

termination (Figure 1). In the initiation stage, the alkyl radical is formed from UFAs. Factors such as heat, light, or metal ions generate instability in fatty acid unsaturation; this instability breaks the instauration, causing labile hydrogen adjacent to the double bond to be lost and forms an alkyl radical. The propagation phase is associated with increased oxygen consumption, which reacts with the alkyl radical to form peroxides and reacts with new UFAs to form hydroperoxides that are primary compounds.

These compounds are labile molecules that decompose to produce alkoxyl radicals, and in turn, originate a complex mixture of secondary oxidation products, known as low molecular weight volatile and non-volatile compounds such as hydrocarbons, aldehydes, ketones, alcohols, among others. These compounds generate unpleasant aromas and flavors in foods, typical of rancidity (Wasowicz *et al.*, 2004; Venegas and Perez, 2009; Kumar *et al.*, 2015). In the termination stage, free radicals and hydroperoxides react in various combinations, forming non-radical or stable products of low molecular weight (Wasowicz *et al.*, 2004; Venegas and Pérez, 2009; Kumar *et al.*, 2015).

Lipid oxidation should be prevented and regulated to preserve food quality, given the impact that it has on the physical and nutritional characteristics of food. Lipid oxidation

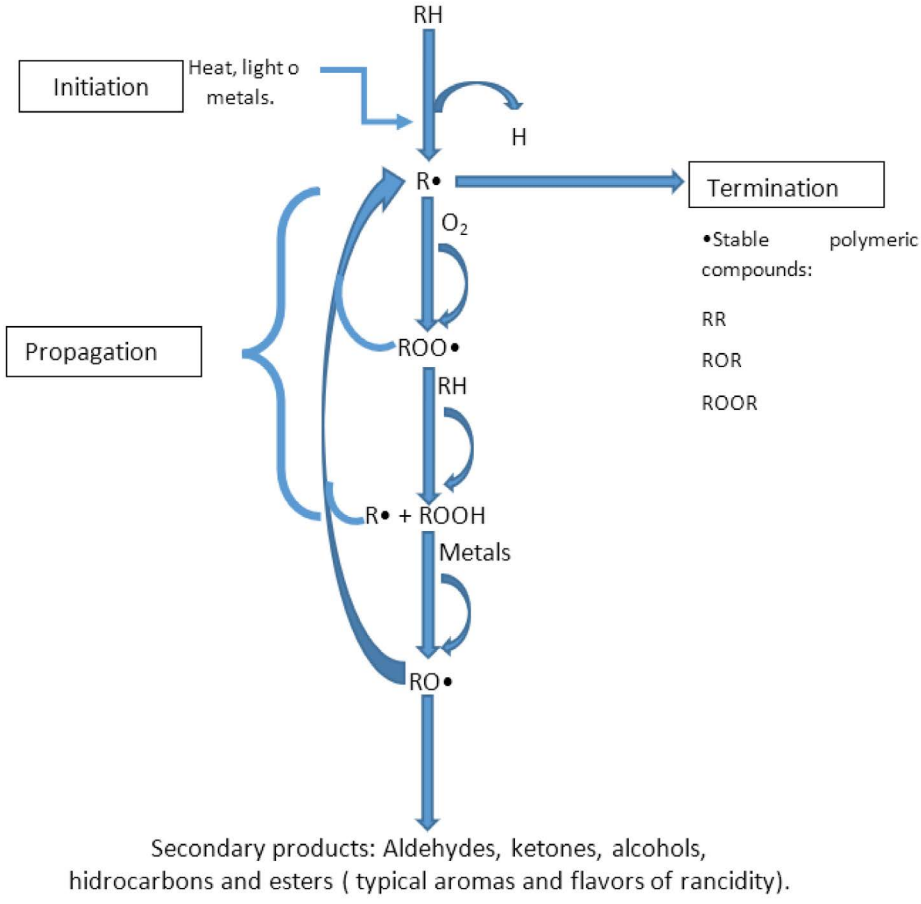


**Figure 1**. Unsaturated fatty acids oxidation process. RH=unsaturated fatty acid; R•= alkyl radical; ROO•=peroxide radical; ROOH=hydroperoxide; RO•=alkoxyl radical.

can negatively alter sensory attributes such as color, texture, odor and flavor (Gallego *et al.*, 2015; Cheng *et al.*, 2017). Generally, by rancid odors, discoloration, loss of nutritional values and off-flavors (Xiong *et al.*, 2020). Lipid oxidation is one of the main factors that reduce the shelf life of meat and meat products (Aminzare *et al.*, 2019; Xiong *et al.*, 2020) since these foods have high lipid content and can generate oxidized compounds such as ketones, alcohols, aldehydes, alkanes and alkenes of low molecular weight and high volatility (Rojano *et al.*, 2008; García *et al.*, 2017). Also, mincing, cooking and other processes that meat undergoes before storage and refrigeration, alter the membranes of muscle cells, thus facilitating the interaction of UFAs with prooxidant substances such as non-heme iron; LO accelerates and rapid deterioration of quality and development rancidity (Gallego *et al.*, 2015).

Heating meat decreases the UFAs content, increases hydroxyl radicals and non-heme iron and decreases the activity of endogenous antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase (Aminzare *et al.*, 2019; Xiong *et al.*, 2020). Cooking meat involves the formation of hydroperoxides that can easily decompose into various already mentioned volatile organic compounds and are responsible for reducing sensory quality due to oxidative flavors (Aminzare *et al.*, 2019).

## Prevention of lipid oxidation

One of the strategies to reduce meat spoilage due to LO and improve its shelf life is applying antioxidants (Cheng *et al.*, 2017; Aminzara *et al.*, 2019). Antioxidant activity refers to the capacity of a substance to inhibit oxidative degradation caused by the reaction of free radicals (Figure 2).

Antioxidants retard the oxidation of easily oxidizable biomolecules, such as lipids in meat and meat products. These compounds can donate hydrogen radicals to free radicals, preventing oxidative damage (Londoño, 2012; Amaral *et al.*, 2018). The key mechanism in the reaction with free radicals is to form stable inactive products. The action occurs in the initiation and propagation stage when the radicals formed in these phases are removed or during the degradation of hydroperoxides (Kumar *et al.*, 2015).

## Methods to assess antioxidant activity and oxidative status in meat

Antioxidants can stabilize free radicals *via* two mechanisms, the first is called hydrogen atom transfer which comprises 2,2-diphenyl-1-picryl hydrazyl (DPPH), Oxygen Radical

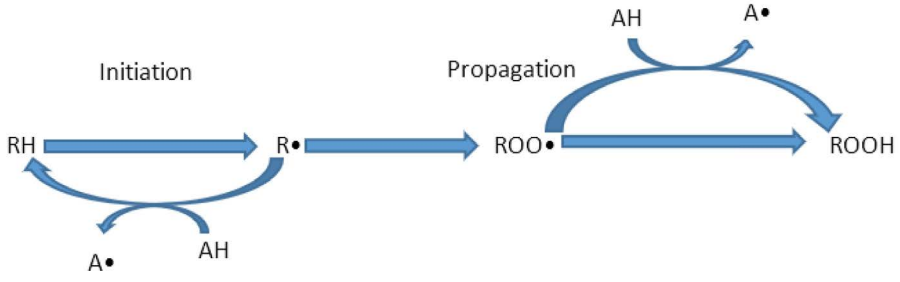


**Figure 2**. Action of antioxidants on lipid oxidation. RH=unsaturated fatty acid; R•=alkyl radical; AH=antioxidant; A•=antioxidant or scavenger radical; ROO•=peroxide radical; ROOH=hydroperoxide.

Absorbance Capacity (ORAC) and N1, N1-di-methyl-1,4-phenylenediamine (DMPD). The first mechanism measures the capacity of an antioxidant by stabilizing the free radicals through transferring hydrogen atoms. The second is called electron transfer involving azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP), both of which stabilize free radicals (Londoño, 2012; Ácsová *et al.*, 2019). One of the most applied strategies to determine the antioxidant capacity of a compound, mixture, or food is to measure the activity of the antioxidant against free radical substances or solutions. Various chromogenic compounds are used to determine the capacity of phenolic compounds contained in fruits, vegetables or species extracts to trap the free radicals generated (Kuskoski *et al.*, 2005; Sotelo *et al.*, 2015). Some of them are described below.

**DPPH (2,2-diphenyl-1-picryl hydrazyl)**

The test is based on determining the capacity of an antioxidant to stabilize the DPPHα radical, this measurement can be made by spectrophotometry with a maximum absorbance peak at 515 nm. It is one of the few stable organic radicals, it presents a strong violet coloration, whose absorbance decreases when reduced by an antioxidant. Its reduction depends on the ABTS method and the ability of the antioxidant compounds to transfer electrons or donate protons (Santacruz, 2011). It is a commercially available free radical that can be directly obtained and does not have to be generated in situ like $ABTS^{\bullet+}$, but it can only be dissolved in organic media. The technique has been developed to measure antioxidant capacity mainly in plants and food extracts. The method is simple and requires little instrumental material; however, on the downside, the technique makes it difficult to interpret the results when you have substances whose absorption spectrum is opposite to that of the radical (Kuskoski *et al.*, 2005; Santacruz, 2011; Londoño, 2012).

**ORAC (Oxygen Radical Absorbance Capacity)**

This test has advantages such as high adaptability to antioxidants, biological samples, foods and the ability to analyze the antioxidant potential of non-protein samples using a wide range of extraction agents. The reaction is conceptually simple but difficult in practice. The reactions begin with the heating of azide compounds to release nitrogen gas and generate two radicals ($R^{\bullet}$). During the radical generation, it is very important to maintain the optimum heating temperature to ensure complete decomposition of the azides. If the required temperature is not maintained, unclear and incomparable results are obtained (Ácsová *et al.*, 2019).

**DMPD (N1, N1-Di-methyl-1,4-phenylenediamine)**

Oxidants in samples are reduced and color changes are evaluated spectrophotometrically. First, the $DMPD^{\bullet+}$ radical is formed by mixing a solution of DMPD in an acetate buffer and ferric chloride. The prepared red-colored solution of the DMPD cation is let to rest at laboratory temperature for 12 h before being used to evaluate the antioxidant activity of the samples. The oxidative state of the $DMPD^{\bullet+}$ substance is readable at 515 nm.

Choosing methanol as a solvent for DMPD is not suitable (Ácsová *et al.*, 2019). The results from this method are low, poorly reproducible and inconsistent (Kuskoski *et al.*, 2005).

**ABTS (azinobis 3-ethylbenzothiazoline-6-sulfonic acid).**

It is based on the uptake capacity of an antioxidant to stabilize the $ABTS^{\bullet+}$ cation radical. It is a free radical obtained after a chemical (manganese dioxide or potassium persulfate), enzymatic (peroxidase, myoglobulin), or electrochemical reaction. Azinobis 3-ethylbenzothiazoline-6-sulfonic acid can measure the activity of compounds of lipophilic and hydrophilic nature including carotenoids and flavonoids. This method is applied along with DPPH, both of which have good stability in certain conditions. It is a highly sensitive, practical and fast method. It has the advantage that its spectrum presents maximum absorbance at 414, 654, 754 and 815 nm in an alcoholic medium (Kuskoski *et al.*, 2005; Santacruz, 2011; Londoño, 2012).

**FRAP (Ferric Reducing Antioxidant Power)**

This method is based on the evaluation of the ability of an antioxidant to reduce ferric iron ($Fe^{+3}$) present in a complex with 2,4,6-tri(2-pyridyl)-s-triazine to the ferrous form ($Fe^{+2}$), causing the formation of a ferrous tripyridyltriazine complex. The reduction is indicated by an intense blue color with maximum absorption at 593 nm; however, the solution is yellow if no reduction of ferric ions occurs (Silva *et al.*, 2017; Ácsová *et al.*, 2019).

**TBARS (thiobarbituric acid reactive species)**

The TBARS technique assesses the oxidation state in muscle components such as meat, and is one of the most widely used techniques (Isaza *et al.*, 2013). The calculation is based on the determination of the content of the substance reactive to 2-thiobarbituric acid. A spectrophotometer at $1_{max}$=532 nm is used to monitor the formation of a pink-colored product, resulting from the addition of 2-thiobarbituric acid with malonaldehyde (product of IFA oxidation) (Wenjiao *et al.*, 2014; Silva *et al.*, 2017). Values are expressed in mg malonaldehyde $kg^{-1}$. This technique has limitations, but TBA is useful to compare the oxidation of a meat sample or byproduct at different stages. The TBARS value correlates with the results of sensory analysis in a product, as an LO indicator (Venegas and Perez, 2009).

**Rosemary as an antioxidant and nano encapsulation**

Synthetic and natural antioxidants have been used in the food industry to delay or prevent LO in meat (Cheng *et al.*, 2017). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butyl hydroquinone (TBHQ), propyl gallate (PG) and nitrite have been used in the meat industry (Gallego *et al.*, 2015; Aminzare *et al.*, 2019). However, they are unsafe for consumers, due to their toxicological and carcinogenic effects, therefore, their use has nowadays been limited.

Currently, there is a growing trend to consume natural products. Plants are a source of bioactive substances with phenolic compounds, which are the main molecules responsible for the antioxidant properties which result in an effective alternative to synthetic

antioxidants (Kumar *et al.*, 2015; Aminzare *et al.*, 2019; García *et al.*, 2020). Phenolic compounds, the main antioxidant in plants, may be classified and include phenolic diterpenes (carnosic acid and carnosol), flavonoids (quercetin and catechin), phenolic acids (gallic acid, rosmarinic acid, and caffeic acid), and volatile oils (carvacrol, eugenol, thymol, and menthol) (Aminzare *et al.*, 2019).

Among the plants that have antimicrobial and antioxidant properties is rosemary (*Salvia rosmarinus* L. (Lamiaceae), a perennial woody herb native to the Mediterranean region, worldwide cultivated as an ornamental and aromatic plant. Rosemary leaves are commonly used to flavor foods as a condiment (Rašković *et al.*, 2014; Rashidaie *et al.*, 2019). The antioxidant properties of rosemary are attributed to its major phenolic diterpenes, such as carnosic acid, carnosol and rosmarinic (caffeic acid and 3,4-dihydroxyphenylacetate ester). Rosemary has the potential effect to inhibit the LO of food, by eliminating free radicals and the chain reaction of metal ions such as $Fe^{2+}$ is terminated, reducing the activated oxygen molecules rate of formation (Afonso *et al.*, 2013; Rašković *et al.*, 2014; Aminzare *et al.*, 2019; Rashidaie *et al.*, 2019). Rosemary is a natural antioxidant widely used for food conservation (Feng *et al.*, 2016), due to its antioxidant activity in meat and meat products, described in Table 1.

Plant extracts such as that from rosemary have highly active compounds but can lose their beneficial effects by oxygen exposure, heat, moisture, light, or during processing. Therefore, it is necessary to use specific methods to protect them and achieve the highest antioxidant activity, one possible way is by encapsulation that reaches incorporation through nanometric delivery systems (Rashidaie *et al.*, 2019; Duarte and Larroza, 2019). Nanoencapsulation is a technology focused on coating the active agent onto another

**Table 1**. Rosemary usage (*Salvia rosmarinus*) as a natural antioxidant in meat and meat products.

| Study components | Objective | Effect | Reference |
|---|---|---|---|
| Rosemary extract (RE) | Effect on the quality and stability of ground chicken meat. | The TBARS values of RE (350 ppm) were significantly ($P<0.05$) lower than the control at day 7 of storage time. | Hijazeen and Rawashdeh, 2019. |
| Lyophilized Rosemay Extract (LRE) | Evaluate the effect of the use of LRE on the oxidative stability of pork sausages stored at −12 °C. | The lipid oxidation of sausage was significantly inhibited at 49 days of frozen storage with LRE compared to the control (47.28%). | Bianchin *et al.*, 2017. |
| Rosemary extract | Its quality in chicken breast was evaluated during 10 days of refrigerated storage. | The lipid oxidation of chicken breast was strongly inhibited by rosemary extract. TBARS values were significantly lower than control samples from 6 to 10 days ($P<0.05$). | Feng *et al.*, 2016. |
| Rosemary essential oil (REO) and modified-atmosphere packaging (MAP) | Effect on meat quality of pultry fillets during 7 days of refrigerated storage. | The addition of REO in combination with MAP reduced the level of lipid oxidation. | Kahraman *et al.*, 2015. |
| Rosemary essential oil (REO) | Determine the increase in shelf life of fresh Barbarine lamb's meat. | TBARS values significantly increased for both treatments (control and REO) with storage time without significant effect. | Smeti *et al.*, 2013. |
| Rosemary essential oil (REO) | Improves the lipid stability and sensory characteristics of chicken meat. | Addition of essential oils of rosemary at level of 200 mg/kg ($P<0.05$) reduced the TBARS and ($P<0.05$) increased the sensory scores of beef patties during frozen storage period. | Mohamed and Mansour, 2012. |

material at a nano-scale of 1 to 100 nm sizes. Nanocarriers have been made from safe materials including biodegradable polymers, lipids and polysaccharides (Mohamed *et al.*, 2020; Boskovic *et al.*, 2019). Although using rosemary essential oil is limited due to its hydrophobicity, which prevents it from dissolving in aqueous phases of food. One possible way to incorporate essential oils is by nanoencapsulation, with this technique stability, protection and a controlled release increase. The bioavailability of essential oils reduces adverse impacts on the organoleptic properties of meat and meat products by preventing undesirable interactions with food components (Aminzare *et al.*, 2019; Boskovic *et al.*, 2019; Duarte and Larroza 2019). Food acceptability mainly depends on its sensory attributes, in the quality of meat and meat products is influenced by taste perception (Cao *et al.*, 2018). Rosemary extract encapsulation or rosemary oil increases the antioxidant properties and shelf life of meat during storage. Encapsulation of rosemary extract at 1600 ppm has shown better results for antioxidant properties and increases the shelf life of beef up to day 21 of storage (Rashidaie *et al.*, 2019). The use of rosemary extract nanocapsules as a dietary supplement in broilers has also shown beneficial effects on lipid profile and antioxidant status (Mohamed *et al.*, 2020).

## CONCLUSIONS

The process of lipid oxidation (LO) in meat and meat products decreases the consumer's health. To prevent or delay this process, it is important to know how the physiological activity between the formation of oxidants and the effect of antioxidants takes place. Rosemary is one of the most widely used natural antioxidants currently under research that have satisfactory results on LO inhibition. This plant is a safe and efficient alternative for meat preservation, its incorporation can be through new technological tools such as nanoencapsulation. However, further studies on the incorporation of rosemary extract or essential oil in nanocapsules are still necessary to establish its antioxidant potential and to understand its application.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Reproductive management of the ram (*Ovis orientalis aries*)

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**ABSTRACT**

**Objective**: To describe briefly the physiology, the anatomy and the reproductive management of the ram.

**Design/Methodology/Approach**: Review of the anatomy, physiology and general considerations for an adequate reproductive management of the ram.

**Results**: The ram presents a less seasonal reproductive activity than the female and its reproductive behavior is easier to observe. However, it is in the fall, the reproductive season of seasonal sheep, when their behavior is much more complex and complete. It begins with smelling the external genitals of the female, goes through the flehmen response and ends with mating, intromission and ejaculation. It is necessary to consider the anatomical and physiological aspects of the ram and its meticulous management to avoid reproductive failures in the flock. Frequently, the rams are less important for the flock's handler during the season of reproductive rest. Monitoring their diet and parasite and disease control is necessary to keep them apt for reproduction. The evaluation of the reproductive aptitude of the ram before mating is convenient and can be planned as part of its management; and once mating begins, considering the adequate proportion of rams/females, in addition to monitoring their performance during this event.

**Study Limitations/Implications**: To consider that the ram shows a good capacity for mating, capacity for service, libido, quality of semen, since it is responsible for a proportion of lamb production.

**Findings/Conclusions**: Reproduction in sheep is regulated by the photoperiod, genetic potential, nutritional status, health status and other factors, which are important both in the ram and in the female. Some sheep breeds have potential of prolificacy; a good reproductive management of the ram can influence these factors and increase the reproduction rate in the flocks.

**Keywords**: stud, fertility, sheep, reproduction.

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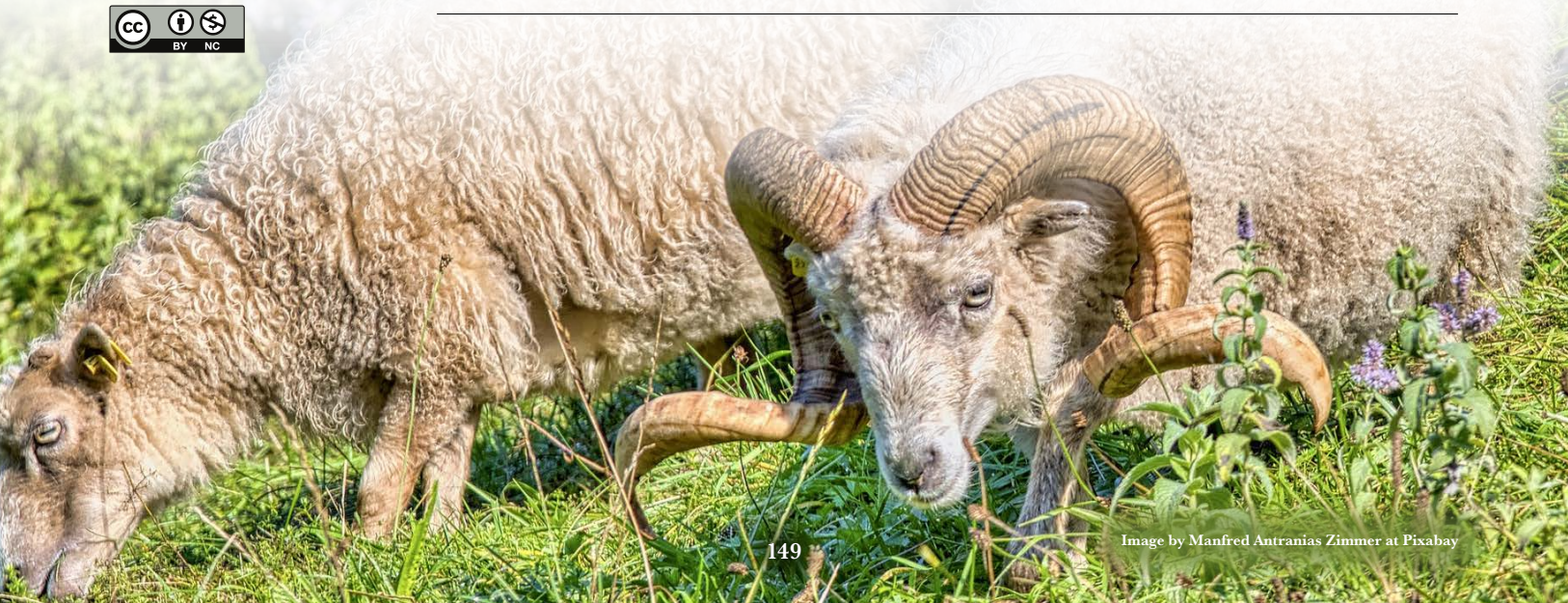

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## INTRODUCTION

The aspects that refer to the reproductive management of the ram have attracted more interest, reason why studies that review this process have increased, since in sheep production systems the contribution of genetic characteristics depends 50% on the reproductive capacity of the ram.

The ram is defined as a seasonal reproducer, since the photoperiod is one of the main factors that regulates its reproductive activity and provokes physiological changes due to the seasonal fluctuations of light hours. This exerts important effects on its endocrine and gonad activity, which must be in sync with the seasonal activity of the sheep. There are other factors that also affect the reproductive efficiency of sheep, such as nutrition (Martin *et al.*, 1994; Martin and Walkden-Brown, 1995) and socio-sexual factors (Blache *et al.*, 2000). Another important physiological characteristic that defines the ram are the different stages that make up its productive cycle, for example, the onset of puberty and with it, the start of spermatogenesis; this is slightly related with the chronological age, since the onset of puberty depends to a great extent on the body development of the male, which is in function of the daily weight gain. In the productive aspect, the ram can be selected because of its ability to transmit its productive and reproductive characteristics, reason why the producer ought to have well-defined objectives for the genetic improvement program, and based on them, perform the selection of reproducers. Recent studies are focused on the implementation and development of techniques on semen evaluation, sperm cryopreservation, artificial insemination, libido tests, estrus induction and synchronization, among others. These techniques are used increasingly more and with very good results in sheep production units, with the objective of improving their reproductive efficiency. It is important to mention that the incorporation of these technologies should be adapted to the different production systems and geographic conditions of the country, since each of them have their own particularities. Finally, a deficient management can cause health problems in the rams, and those not apt for reproduction must be sacrificed or they will die much before the end of their productive life (Ridler *et al.*, 2012).

### Anatomy and physiology of the reproductive system of the ram

The reproductive system of the ram is made up of the testicles (masculine gonads) that perform two main functions: hormone production, process known as steroidogenesis, and production of mature sperm cells through spermatogenesis; both functions are closely linked since sperm production depends on hormone synthesis.

The ram's testicles weigh from 200 to 300 g each, although this varies with the season of the year and the breed, reaching their maximum weight at the middle of the reproduction season (short days; October-November, latitude North). The testicles are found in a scrotal sac that provides support and protection, in addition to regulating the temperature, since sperm production happens at 4 to 7 °C below body temperature (Durán Ramírez *et al.*, 2008). The testicles have a system of conducts such as the epididymis, in charge of the storage, maturation and transport of sperm cells, in addition to the vas deferens that are situated on the end of the epididymis and open towards the urethra, and these have the main function of transporting sperm cells until their ejaculation. Next to the

urethra and the union with the vas deferens there is a group of accessory sexual glands (vesicular glands, prostate, and two bulbourethral glands), which produce liquids that pour into the reproductive tract and mix with the sperm cells forming the semen and the penis (Figure 1). The penis has the function of depositing semen in the female's vagina, as well as the expulsion of urine, and both (semen and urine) pass through the urethra (Durán Ramírez *et al.*, 2008).

**Spermatogenesis, hormonal regulation and semen production**

Spermatogenesis happens in the seminiferous tubules, where diverse processes of division and cellular differentiation intervene leading to the formation of sperm cells. The first liberation of mobile and fertile sperm cells takes place after the sexual maturation of the male. In the maturation process, the testicles are regulated by gonadotropins: the luteinizing hormone (LH) and the follicle stimulating hormone (FSH), secreted towards the circulatory system from the anterior pituitary. However, the secretion of LH and FSH is regulated by the gonadotropin-releasing hormone (GnRH), secreted by the localized neurons in the preoptic-hypothalamic area. LH acts by binding itself to the receptors in the plasma membrane of the Leydig cells (located in the interstitial space of the testicle) and stimulates the production of androgens (testosterone). Androgens are secreted by the Leydig cells by diffusion and pass to the lymph, another part goes to the liquid that is found in the light of the seminiferous tubules, and most of it reaches the blood stream. The androgens travel linked to proteins (approximately 98%), such as albumin and sex hormone-binding globulin (SHBG), some of it (2%) circulates freely and it is the biologically active fraction. FSH exerts its action on the Sertoli cells that are part of the seminiferous tubules and are interspersed between the germinal cells. The Sertoli cells are large, cytoplasmic prolongations, and extend from

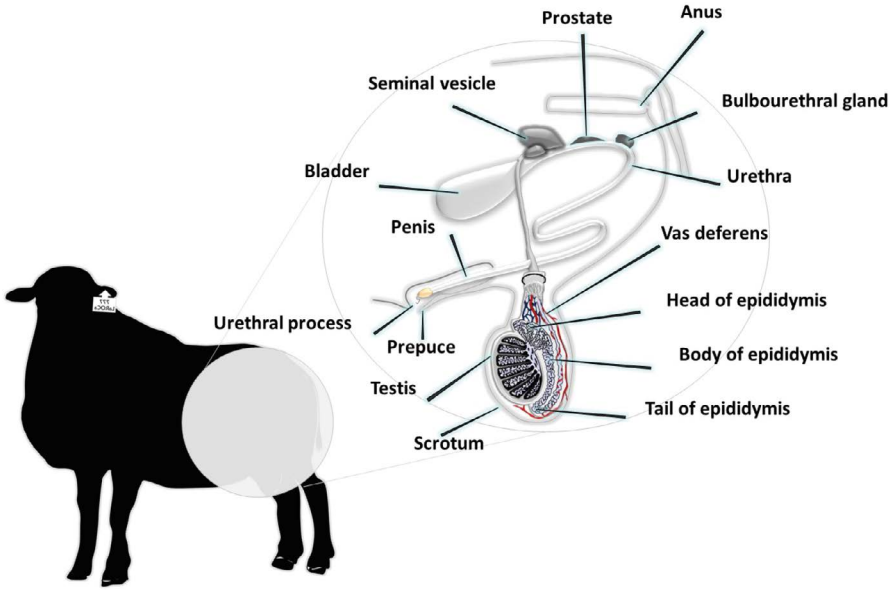


**Figure 1.** Anatomy of the ram's reproductive system.

the basal lamina to the light of the seminiferous tubule; they present receptors for FSH, stimulate the production of the aromatase responsible of converting the androgens (secreted by the Leyding cells) to estrogens, synthesize and secrete inhibin, and the androgen-binding protein, which has a high affinity for testosterone and guarantees an adequate concentration of this important hormone for the maturation of the sperm cell. Sertoli cells also provide support and nutrition to the germinal cells, and participate in their maturation. The close links present between adjacent Sertoli cells are part of the hematotesticular barrier, which separates the compartments in which germinal cells are found: a basal one (which includes spermatogonia) and an adluminal one (where the spermatocytes, spermatids, and sperm cells are found). The function of this barrier consists in protecting the germinal cells from pathogens present in the blood, by preventing the passage of the large molecules from the basal region towards the adluminal region and the space of the seminiferous tubules (Griswold, 1995). In rams, spermatogenesis is defined as the transformation of sperm cells (masculine sex cells) from germinal cells or spermatogonia, and takes place in the seminiferous tubules, begins approximately between 10 and 15 weeks of age, without considering the season (Olster and Foster, 1986). The product of spermatogenesis is the spermatozoon (sperm cell), the anterior part of its head is surrounded by the acrosome, carrier of the enzymes that will be used during the fertilization process, and the tail (flagellum) responsible for the locomotion and propulsion of the spermatozoon is connected to the head by a short neck (implantation region) that contains the mitochondria, responsible for contributing energy for locomotion (Durán Ramírez *et al.*, 2008).

**Seminal quality tests**

The development of sperm in the ram takes approximately 50 days. Therefore, a test is recommended to evaluate the reproductive variables in males, at least between 6 and 8 weeks before the beginning of mating, sufficient time to solve possible reproductive problems that rams can present (Ridler *et al.*, 2012). After undergoing tests such as capacity for mating and semen evaluation, it is known that up to 36% of the rams can be non-apt for reproduction and are the possible cause of low fertility in the flock (Aké-Villanueva *et al.*, 2019).

The aspects to monitor routinely should include a general health exam, bodily condition and genital inspection (evaluation of the scrotal circumference, testicular tone and anomalies). A semen evaluation, libido tests and health tests are recommended. For semen evaluation, there are two semen collection methods that are the most used: artificial vagina and electro-ejaculation; with the artificial vagina, the results obtained in the semen evaluation are very similar to those obtained from natural mating. The semen obtained with an electro-ejaculator presents higher value, although lower concentration of sperm cells, so its use is recommended when the studs are not trained (Haféz, 1987). With the semen evaluation, the fertility is estimated indirectly and is recommendable for all the rams before the mating. The evaluation includes the volume of semen, individual mass motility, sperm cell concentration and abnormalities (Haféz, 1987). In the ram, the volume per ejaculate varies from 1 to 1.5 mL with a concentration of 2 to $6\times10^{-9}$ sperm

cells per mL. The seminal parameters can be altered by disease, frequency of ejaculation, nutrition problems, management factors, season, age, sexual preparation (first-timers *vs.* experienced), collection method, management of the ejaculate during and after collection, and analysis techniques, among others (McDonald and Pineda, 1991).

## General considerations: puberty, seasonality and nutrition

### *Puberty*

Puberty is defined as the moment when the male achieves its first mounts with introduction of the penis into the female's vagina and also presents sperm cells in the ejaculate. The quality of the nutrition and the speed of growth are factors that modify the moment of the onset of puberty; it is known, for example, that when males have a normal diet, puberty can start at between 100 and 150 days of age; however, it will depend on the photoperiod (Haresing, 1989). Similar to other species, it is difficult to determine the age of puberty in rams, since this event is scarcely related with the chronological age, since body development is determinant. This relationship includes growth index, compensatory growth and maturity with regards to the body size (Martín and Walkden-Brown, 1995). Concerning their reproductive physiology, it has been proven that the sexual maturation in males takes place in response to internal factors (growth) and external factors (photoperiod), the frequency of LH/FSH secretion in response to increasing GnRH secretion, to stimulate the start of the process of spermatogenesis (Olster and Foster, 1986); the levels of testosterone continues to increase for at least the first 21 months of life, and changes begin to take place in the morphology of the testicle, sperm cells appear in the seminiferous tubules, in the epididymis or in the ejaculate (Haresing, 1989). Most of the rams begin puberty at the age of 4 to 6 months with 60% of the adult body weight (Jainudeen *et al.*, 2000); in seasonal breeds, it is common to wait until the following reproductive season, when rams are 17 to 19 months of age.

### *Reproductive seasonality*

The ram is considered to be a seasonal reproducer, and there are variations between breeds, some that are markedly seasonal (Blackface, Suffolk and Texel) and other less so (Pelibuey, Dorset, Merino). However, in all the sheep breeds the reproductive activity reaches its maximum in the fall (latitude North) or spring (latitude South) and coincides with the reproductive cyclicity of the females (Robinson and Karsch, 1984). In sheep, reproductive seasonality is regulated by melatonin, which translates the changes in the photoperiod and coordinates the changes in reproduction according to the seasons of the year. Melatonin is secreted only during the night, becoming an endocrine signal that acts in the brain together with other hormones, to regulate the pulsating secretion of GnRH (Lincoln and Clarke, 1997). In sheep, the changes from long days to short days causes an increase in the secretion of LH and FSH, as well as the activation of the reproductive axis, with the highest concentrations of testosterone during the fall, and the lowest in the spring. The levels of gonadotropins increase as the reproduction season begins coinciding with the reduction of light hours (Lincoln and Clarke, 1997). It is important to mention that most of the rams from different sheep breeds produce semen throughout the year;

however, a decrease in semen production and sexual activity takes place, in the spring (non-reproductive season, latitude North; Haresing, 1989).

*Nutrition*

In the ram, nutrition is one of the factors that affect testicular size and sperm production. It has been proven that in nearly all the mechanisms where nutrition participates to regulate reproduction, changes are provoked in the secretion of LH/FSH, main hormones that regulate the physiology of the testicles, reason why the frequency of secretion of the GnRH pulses is an important factor in the control of testicular growth (Martin *et al.*, 1994). Testicular growth and the frequency of secretion of the LH pulses increase after improving the nutritional status, and can decrease after a loss of weight or body condition; in rams, with a diet high in energy and protein, the secretion frequency of the GnRh pulses increases, but the effect disappears after three weeks. Despite this, the testicular mass and sperm production continues to increase for several months (Oldham *et al.*, 1978; Martin *et al.*, 1994). This is why a restriction in the diet also reduces testicular growth (Martín *et al.*, 1994).

**Reproductive management of the ram in the production systems**

*Programming of mating*

Mating consists in pairing ewes with one or several rams, to guarantee that most of the ewes become pregnant; it is one of the activities of greatest importance in the production units, and the total production depends on this activity. Regardless of the type of mating (short or long, continuous mating) that is used, the producer must always consider the stud as one of the main assets of the ranch and one of the most important. The ram should be monitored regularly in the production unit with the purpose of it always being in optimal physical, sanitary conditions and apt for reproduction. Although it is not common to find sterile males in the flocks, 10 to 15% of the rams in the flocks can show some decrease in the reproductive capacity (Schoennian, 2021). Therefore, the flock's productivity could be compromised if one of the rams with low reproductive capacity is used as a reproducer. Thus, the rams that are used in short mating periods should have gestation rates between 85 and 95% in periods of 30 to 35 days with a group of up to 50 ewes, which means that the ram should be in optimal bodily and health conditions, in order to withstand the work load. Its capacity to mount and copulate should be considered, as well as libido, semen evaluation and in addition they should be free of transmissible diseases (Gordon, 1997; Ridler *et al.*, 2012), since the ram is responsible for the gestation rate and a proportion of lamb production (Ridler *et al.*, 2012). For example, if one ewe from a lot of 50 females is not pregnant, lambing will be reduced in 2%, while if a ram fails, from two that were introduced into a flock of 50 ewes, lamb production can be reduced in up to 50%. This is why a reproductive management program of the ram ought to have a constant and detailed examination of its reproductive capacity, since the benefits that will be obtained in the short and long term will depend on it (McDonald and Pineda, 1991).

*Ram selection for studs*

The selection of a stud should be conducted with all the care possible. With the experience of the producer or managing the flock, studs can be selected at first sight; the males selected ought to be apt for reproduction. The studs should be of thick, symmetrical, broad and rectangular bones and extremities and with a large amount of muscle mass. The limbs represent the support for the male, and they should be strong and well-implanted, since they are determinant for the stud to be able to mount the proportion of females assigned; on the contrary, it will not be able to move long distances, which will limit the search for females in estrus. The lower back should be long, broad, strong, muscular and straight. The testicles should have good development, they ought to be well-located and hanging, without reaching down below the hocks. The skin on the scrotum should be thick and loose, in order to allow the testicles to retract and relax, which allows the stud a better production of sperm cells. The head should be of moderate size, with convex and thickset profile, and with good insertion of neck and shoulders; the neck should be thick and muscular, with or without mane, under and over, for hair sheep breeds (Gordon, 1997). The stud ought to be selected due to its ability to transmit its phenotype and conformation, and its ability to transmit its productive characteristics. Studs that are proven to have genetic capacity to produce, for example, meat or milk, or both, should be selected (McDonald and Pineda, 1991). Reproductive diseases can be an important cause of subfertility or infertility in males. The males affected can be identified early by serological exams and reproductive solidity exams. The early diagnosis will maximize the success of the treatment or accelerate the selection decisions (Stewart and Shipley, 2021).

*Preparation of the rams for mating*

Consider the following points:

- The duration of the production of a spermatozoon demands at least 45 days, and its passage through the epididymis lasts from 12 to 15 days, which is why the rams should be prepared at least 8 weeks before the beginning of mating.
- During continuous mating, there should be one male for 30 to 50 females available.
- Inspect periodically the reproductive system of the male to prevent lesions.
- Palpate externally and examine the different parts of the reproductive system, and ensure that the ram does not present any lesion on the prepuce, the testicles or the epididymis.
- Improve the dietary level of the ram; flushing can also be used in studs, when they are outside the reproduction period, feeding them with a maintenance ration, and two months before mating, changing the ration for one that would cover the requirements for maintenance and reproduction.
- Stimulate the libido, particularly for spring mating, which is achieved by introducing the ram with females in estrus.

## CONCLUSIONS

A careful management of the ram ensures the success of the flock's reproduction and maximizes its reproductive life. Frequently rams are not monitored outside the reproduction

season, although it is convenient to monitor nutrition, control parasites and diseases, and with this keep them apt for reproduction. Mating outside the reproductive season requires the stud to always be in optimal conditions. Therefore, in order to understand the reproductive capacity of the ram, semen evaluations can be performed every six months, as well as monitoring the effectiveness of the stud during breeding.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Suckling, male effect and kisspeptin in the reproductive management of ewes in postpartum anestrus

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**ABSTRACT**

**Objective**: To describe the management strategies of controlled suckling and the male effect to reduce postpartum anestrus, and to show the participation of kisspeptin to regulate the effect of both factors.

**Design/Methodology/Approach**: A review of scientific publications was conducted, in order to show the importance of suckling and the male effect as strategies to reduce postpartum anestrus in the ewe, as well as the relation of kisspeptin with both factors.

**Results**: Seasonal anestrus can be avoided with the use of breeds adapted to the local environment, such as Pelibuey. Postpartum anestrus occurs mainly as a result of suckling, since the latter inhibits the pulsating secretion of the gonadotropin-releasing hormone (GnRH) and the luteinizing hormone (LH). The exact path of this inhibition is unknown, although it seems that endogenous opioid peptides and kisspeptin are intermediaries. Controlled suckling and the male effect are management strategies that improve the reproductive behavior of postpartum ewes. Kisspeptin regulates the influence of the male effect through the secretion of GnRH/LH.

**Study Limitations/Implications**: To understand the impacts of suckling and the male effect on the duration of postpartum anestrus, as well as the participation of kisspeptin in the regulation of both effects, will allow designing management strategies to improve the reproductive efficiency of the ewes.

**Findings/Conclusions**: Controlled suckling and the male effect reduce postpartum anestrus and improve the reproductive behavior of the ewes; advancing knowledge of the kisspeptin effect could improve the effectiveness of both techniques.

**Keywords**: lamb breeding, postpartum, LH, Pelibuey sheep, first lambing ovulation.

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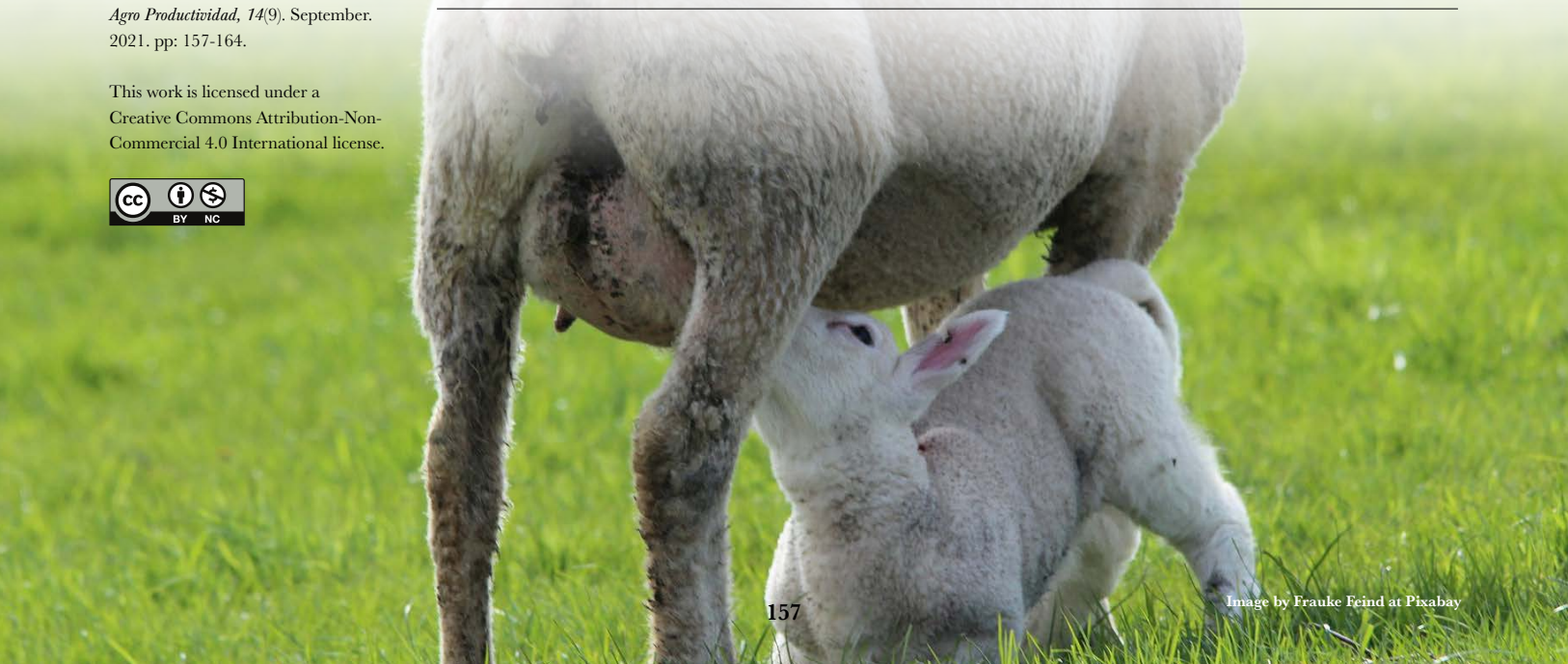

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## INTRODUCTION

Sheep reproduction is key for the profitability of the flocks because the lamb is the main product, which is why the reproductive management needs to be the most efficient possible. In an intensive production system there are two births per year, and the number of lambs born depends on the breed.

The season of the year (photoperiod) is one of the factors that inhibit the reproduction of the ewe (Clarke *et al.*, 1984), because this species evolved and adapted to the climate conditions, to reproduce in the days with less light hours in the year (fall-winter) and give birth during the spring and the summer, when the climate conditions and availability of food are the best.

The difference of light and darkness hours is lower the closer to the Equator, which is why in Mexico the hair breeds introduced adapted and are less susceptible to changes in the photoperiod. Thus, studies about seasonality performed in Mexico with the Pelibuey breed show that it is capable of reproducing throughout the year, while wool-producing ewes enter anestrus in spring (Arroyo *et al.*, 2007). Therefore, using hair breeds in cross-breeding with wool breeds is a strategy to decrease the effect of seasonality; and to improve the daily weight gain of the lambs and the quality of the carcass, Dorper or meat-producing breeds can be used.

When seasonality is not the problem, the ewe has a reproductive cycle approximately every 17 days; however, when lambing takes place, suckling by the lamb takes on importance to inhibit the reproductive activity. The filial relationship established between the mother and the lamb plus the suction of the mammary gland (suckling) begins during the first minutes after the birth, while the mother "cleans" the lamb of the placenta residues and stimulates it to consume colostrum (Viker *et al.*, 1993). Suckling inhibits the pulsating secretion of the gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH); that is, it inhibits the capacity of the ewe to enter estrus and to ovulate (Camacho *et al.*, 2008); thus, lamb weaning restarts the estrus and the ewe's ovulation (Salloum and Claus, 2005).

The effect of suckling begins with the suction of the mammary gland, the sight and detection of the lamb's aroma (Griffith and Williams, 1996). This stimulus reaches the hypothalamus via the spinal cord, where it inhibits the pulsating secretion of GnRH (Yamada *et al.*, 2007) and as consequence of the pulsating secretion of LH, which is essential for the final maturation of the follicles and the ovulation (Mandiki *et al.*, 1990). In recent years the discovery of kisspeptin (Kp) has helped to explain several of the unknowns in the reproductive physiology of the ewe and it seems to also be related to suckling (Yamada *et al.*, 2007).

The effect of suckling in postpartum anestrus in the ewe can be reduced through controlled suckling and the male effect, and Kp seems to have a relation in both effects (Fabre-Nys *et al.*, 2015). Therefore, the objective of this literature review is to describe the management strategies of controlled suckling and the male effect in order to reduce postpartum anestrus, and to show the relationship there is between suckling, the male effect and the Kp during this period.

**Reduction of the postpartum anestrus with controlled suckling**

Controlled suckling is a reproductive management strategy that reduces the negative effect of suckling on sheep reproduction after lambing, and it is conducted as follows: 1) Immediately after the birth, making sure that the lamb is consuming colostrum and suckling continually from the mother during the first week of life; 2) Starting on day 7 postpartum, separating the mother from the lamb in pens to avoid direct contact, mutual viewing and calling; 3) The lambs have free-access water in the pen, concentrated food according to age, and shade; 4) Since day 7 of age and until weaning, uniting the mothers for 30 min with their lambs to suckle twice a day (morning and afternoon). Starting on day 35 postpartum, introducing a male to induce heat and mounting for females in estrus, or performing the synchronization of estruses and artificially inseminating the ewes.

Controlled suckling, twice per day for 30 min, improves the postpartum reproductive behavior of the ewes. With this modality of suckling, the ovulation rate increases from 70 to 88.8% and the interval from birth to first ovulation is reduced from 60.5 to 52.6 days in comparison to ewes with continuous suckling (Morales-Terán *et al.*, 2004); the ovulation rate increases, although the days until first ovulation were not different (51.1 *vs.* 56.7; Morales-Terán *et al.*, 2011); and the reproductive behavior of ewes synchronized with progesterone improves (Camacho *et al.*, 2008). This happens because controlled suckling reduces the suction stimulus, visual and olfactory contact of the lambs with the ewes, which stimulates the pulsating secretion of GnRH/LH gradually and provokes the ovulation and manifestation of estrus (Pérez-Hernández *et al.*, 2009).

Controlled suckling does not affect the weight gain of the lambs compared to continuous suckling when creep-feeding and basic cares of the lambs are offered (Morales-Terán *et al.*, 2011). In addition, this management of ewes and lambs decreases the levels of stress that happen at the moment of weaning in mothers and lambs observed in continuous suckling, and the weight gain from birth to 41 days of age of the lambs is similar in controlled suckling and continuous suckling (Castillo-Maldonado *et al.*, 2013), when their diet depends most on the milk consumed from their mothers (Pérez-Hernández *et al.*, 2009).

**Male effect**

The separation and reintroduction of rams to ewes in seasonal anestrus stimulates their reproductive activity (Hawken *et al.*, 2007), and this is known as the "male effect". The management consists in the following: 1) The females and the males must be separated before lambing to avoid visual, auditory contact and the aroma or pheromones; 2) Starting on day 7 postpartum and until weaning or mating, males selected previously with high libido are introduced to the group of females; 3) To avoid copulation, the male is protected with an apron and introduced into the pens with ewes in anestrus during 30 min, twice per day (morning and afternoon); 4) To avoid familiarization, maintain novelty and the potency of the stimulus, it is recommended to rotate males every day.

Exposure of ewes to the male in postpartum anestrus decreases the interval from birth to first ovulation to 27.7 days and manages for 100% of the ewes to ovulate in the first 60 days postpartum compared to 53.6 d and 83.3%, respectively, in ewes with continuous suckling (Cruz-Espinoza, 2011, unpublished data), and improves the percentage of gestating ewes

(90%) in comparison to those without exposure to the male (43%; Hernández-Hernández, 2018; unpublished data).

There are factors that influence the response of ewes to the male effect, such as the prior isolation of females and males, the breed and the previous experience of ewes with the male. Initially, it was believed that a period of separation of males and females previous to the introduction of the males was necessary for this technique to be effective (Martin *et al.*, 1986); however, new studies show contradictory results (Delgadillo *et al.*, 2009). Hawken *et al.* (2009) found that the continuous presence of the male does not increase the pulsating secretion of LH ($0.26 \pm 0.04$ pulses in 6 h); however, introducing new males to the group of ewes (without the need for prior isolation) increases the secretion of LH ($0.87 \pm 0.06$ pulses in 6 h). In turn, in ewes in seasonal anestrus that did not present ovulation after 65 d of contact with males, 90% ovulated after 4 d of contact with new males (Pearce and Oldham, 1988).

In sheep breeds with marked seasonality, they only respond to the male effect in the last days of the anestrus period, and the less seasonal ones can still respond in the middle of the anestrus season (Martin *et al.*, 1986). In the Merino breed, the male effect is always effective; however, in the Suffolk breed it functions at the end of the reproductive season and at the beginning of it (Martin *et al.*, 2004). On the other hand, the response to the male effect among ewes with and without previous experience with males is different, so the experience could be affecting the meaning that ewes assign to the aroma of the males (Gelez and Fabre-Nys, 2004). The main olfactory system projects towards areas responsible for the cognitive analysis of the stimuli that the animal perceives or from past experience, before reaching the area that controls the secretion of LH and it is possible that this is how the experience of the male effect has an impact (Cohen-Tannoudji *et al.*, 1989). Therefore, it is important to consider the factors previously mentioned at the time of introducing the male effect in the flocks.

The male effect happens through the pheromones produced by the stud, so impregnating the sebaceous glands with wax or the exposure of ewes in seasonal anestrus to wool from the studs induces ovulation, but this does not happen when urine is used (Knight and Lynch, 1980). The substances that exert the male effect are secreted on the skin and impregnated on the wool, at least in sheep (Knight *et al.*, 1983). Although mixed fatty acid compounds have been identified, the pheromones or exact substances responsible for the male effect have not yet been isolated (Rosa and Bryant, 2002) and it has been proposed that such a substance could be derived from bacterial fermentation from skin exudates that are impregnated on the wool (Hawken and Martin, 2012). The pheromones stimulate the pulsating secretion of GnRH/LH and with this, the final maturation of the follicles that will potentially be ovulatory.

**Kisspeptin and its relationship with the male effect and suckling**

Kp is derived from the Kiss1 gene and gives origin to a hormone of 54 amino acids (kisspeptin 54 or metastine) which can be divided enzymatically to form peptides of 10, 13, 14 amino acids (Kotani *et al.*, 2001). The last 10 amino acids of Kp contain the biological power (Caraty *et al.*, 2012). In ewes, the existence of large populations of neuronal Kp

bodies in the preoptic area (POA) and the arcuate nucleus (ARC; Smith *et al.*, 2007) of the hypothalamus has been proven (Figure 1).

Relaciones neuroendócrinas de la kisspeptina (Kp), efecto macho, amamantamiento y fotoperiodo en la secreción de GnRH y LH de ovejas. A15: núcleo dopaminérgico; ARC: núcleo arcuato; $D_2$: receptor de dopamina; Dyn: dinorfina; EM: eminencia media; $E_2$: estradiol; ER$\alpha$: receptor alfa de $E_2$; Kiss1r: receptor de Kp; KOR: receptor de Dyn; NKB: neuroquinina; NK3R: receptor de NKB; $P_4$: progesterona; POA: área preóptica; POE: péptidos opioides endógenos.

There are a large number of neuronal projections of Kp towards the internal and external region of the middle eminence, and in apparent contact with small blood capillaries (Franceschini *et al.*, 2006), where projections of the GnRH neurons end in the portal hypothalamus hypophysis system, and they secrete their content (Figure 1). In addition to this, it has been shown that the presence of the Kp receptor (Kiss1r) in GnRH neurons (Messager *et al.*, 2005). Also, in ewes, 93% of Kp neurons in the ARC express the alfa estradiol receptor (ER$\alpha$), while 50% of the Kp neurons express it in the POA (Franceschini *et al.*, 2006).

Kp regulates the secretion of GnRH and LH in sheep and other species like humans, bovines, monkeys and many other. In ewes, exogenous Kp stimulates the secretion of

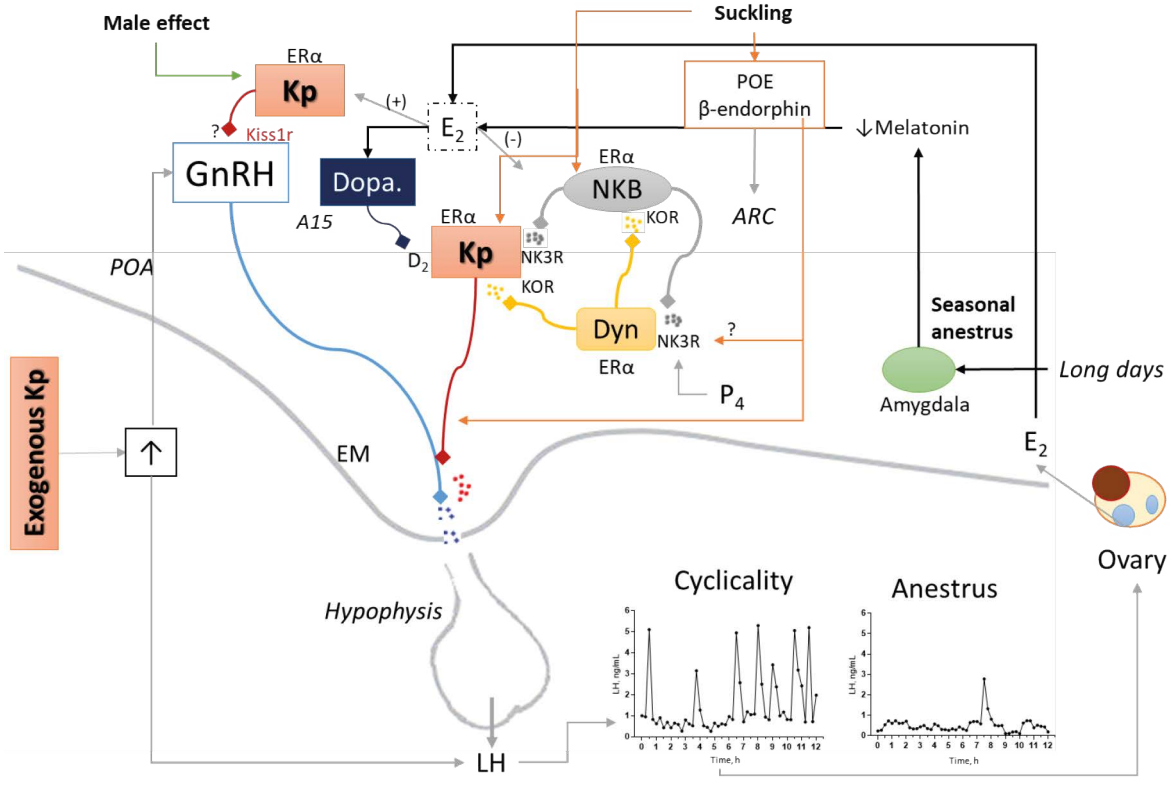


**Figure 1**. Neuroendocrine relationships of kisspeptin (Kp), male effect, suckling and photoperiod in the secretion of GnRH and LH of sheep. A15: dopaminergic nucleus; ARC: arcuate nucleus; $D_2$: dopamine receptor; Dyn: dynorphin; EM: median eminence; $E_2$: estradiol; ER$\alpha$: $E_2$ receptor alpha; Kiss1r: Kp receptor; KOR: Dyn receptor; NKB: neurokinin; NK3R: NKB receptor; $P_4$: progesterone; POA: preoptic area; POE: endogenous opioid peptides.

GnRH, LH, FSH and estradiol ($E_2$) during the seasonal anestrus, the reproductive season (Caraty *et al.*, 2007) and the ewe's puberty (Redmond *et al.*, 2011). It also induces the pre-ovulatory peak of LH in ewes during seasonal anestrus (Sébert *et al.*, 2010). Just as Kp participates in these reproductive processes, its participation in the regulation of postpartum anestrus cannot be dismissed. In rats, suckling inhibits the expression of Kiss1 and the application of exogenous Kp increases the pulsating secretion and concentration of LH (Yamada *et al.*, 2007). The application of Kp in ewes in postpartum anestrus increases the pulsating secretion of LH (Hernández-Hernández, 2018; unpublished data).

Kp regulates the action of the pheromones of the male effect on the secretion of GnRH/LH (Figure 1). The specific form by which the stimulus of the male effect reaches the GnRH neurons is still unknown, although there is the possibility of Kp being involved, because its secretion is activated at the moment of the male effect (Fabre-Nys *et al.*, 2015). In goats there are neuronal projections from the amygdala to the ARC; in sheep a residue derived from Fos genetic activation has been detected in the nucleus which indicates neuronal activity due to the male effect (Jouhanneau *et al.*, 2013). The ewes subjected to the male effect increase ARNm of Kiss1 in the ARC, while the administration of an antagonist (P-271) to Kp completely blocks the response to the male effect (Bond *et al.*, 2013).

## CONCLUSIONS

Suckling prolongs the duration of postpartum anestrus of ewes by inhibiting the pulsating secretion of GnRH/LH necessary for final follicle maturation and ovulation. Kp participates in the regulation of the secretion of GnRH/LH in different reproductive stages and possibly also participates in postpartum anestrus. Controlled suckling and the male effect are management strategies that the producer can implement to improve the reproductive behavior of the ewes in the postpartum period and seasonal anestrus. Kp also regulates the influence of the male effect on the secretion of GnRH/LH; however, the mechanism is still not clear. The exact mechanism of the regulation of suckling on the secretion of GnRH/LH and how the Kp influences in it is also unknown.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Reproductive management of the goat

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**ABSTRACT**

**Objective**: To share technical aspects with specialists in animal reproduction and producers that could help to improve the reproductive capacity of caprine livestock.

**Design/Methodology/Approach**: Scientific evidence and experience in the reproductive management of goats are the basis that sustains the information presented in this article.

**Results**: The goat is widely distributed in Mexico; it is a species with seasonal reproductive activity, but of easy manipulation with hormonal and natural means. Presently there is a large variety of biotechnologies that can be applied in the production units, to potentiate the reproductive activity of the goat.

**Study Limitations/Implications**: The lack of knowledge and the lack of consulting and technical training limit the productive and reproductive potential of goat breeding in Mexico.

**Findings/Conclusions**: Knowledge of the reproductive physiology of the goat and understanding of the means available to manipulate it guarantees its reproduction at the time and in the conditions desired by the producer and the market.

**Keywords**: biotechnology; reproductive strategy; fertility; gestation.

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## INTRODUCTION

The goat is a seasonal polyestrous species; that is, it presents many estruses, at specific intervals of time but only during one season of the year, when the days are shorter than the nights (reproductive season). During these days, the male is also sexually active and the intensity of its smell is increased, stimulating the reproductive activity of the doe. During seasonal anestrus (season of the year when the days are longer than the night), even when there is follicular development in the females' ovaries, external signs of estrus, ovulations or formation of corpora lutea are not observed. In general, in Mexico the reproductive season of goats happens from September to February, and in the males from May to December. In particular, in the central part of the country it has been observed that mounting begins at the end of May. The differences in the beginning and length of the reproductive season is an effect of the latitude: as it approaches the equator, the effect of the photoperiod is lower and other factors such as the rainy season and the availability of food are of greater importance in the establishment of the reproductive season. The objective of this study is to present technical aspects that help to improve the reproductive ability of caprine livestock.

### Estrus cycle

The estrus cycle (time interval between one estrus and the next) of the doe has an average duration of 21 days (Fatet *et al.*, 2011) but it can vary from 16 to 28 (Chemineau, 1983). The duration of the estrus (period in which the doe accepts mounting) varies from 14 to 48 h (Fatet *et al.*, 2011). The time at which the pre-ovulatory peak of the luteinizing hormone (LH) and the ovulation take place varies between 8-13 and 28-37 h, with regard to the beginning of the estrus (Menchaca *et al.*, 2007). After ovulation, the teak and granulose cells will originate the one or many corpora lutea (CL), in charge of producing progesterone ($P_4$). In the case of does that have not been inseminated or when the maternal recognition of gestation does not happen, a regression process of the CL will begin, induced by prostaglandin $F_{2\alpha}$(PG$F_{2\alpha}$), between days 16-18 of the estrus cycle and there will be a new estrus (Figure 1; Fatet *et al.*, 2011; Balaro *et al.*, 2017). On the contrary, if the doe is pregnant, the CL will remain during the gestation period (148-154 days).

### Estrus synchronization

Once females have reached puberty, they can be inseminated in the next estrus. If the producer requires for the females to be inseminated in a short period of time or outside the reproductive season, then synchronization or induction of the estrus can be done. This allows the homogeneous presentation of the estruses in a specific moment. One of the main advantages of the synchronization and induction of estrus is that it allows programming births, obtaining the kid harvest and the milk production when the price and the market conditions are the most favorable for the producer. The synchronization of estruses is achieved through the exogenous application of some of the hormones that control the estrus cycle of the doe. They are GnRH, $P_4$, and PG$F_{2\alpha}$. The first induces the liberation of the follicle-stimulating hormone (FSH) and LH. The FSH stimulates follicular growth and the LH unchains the events that cause the ovulation of pre-ovulatory follicle or follicles (Figure 1).

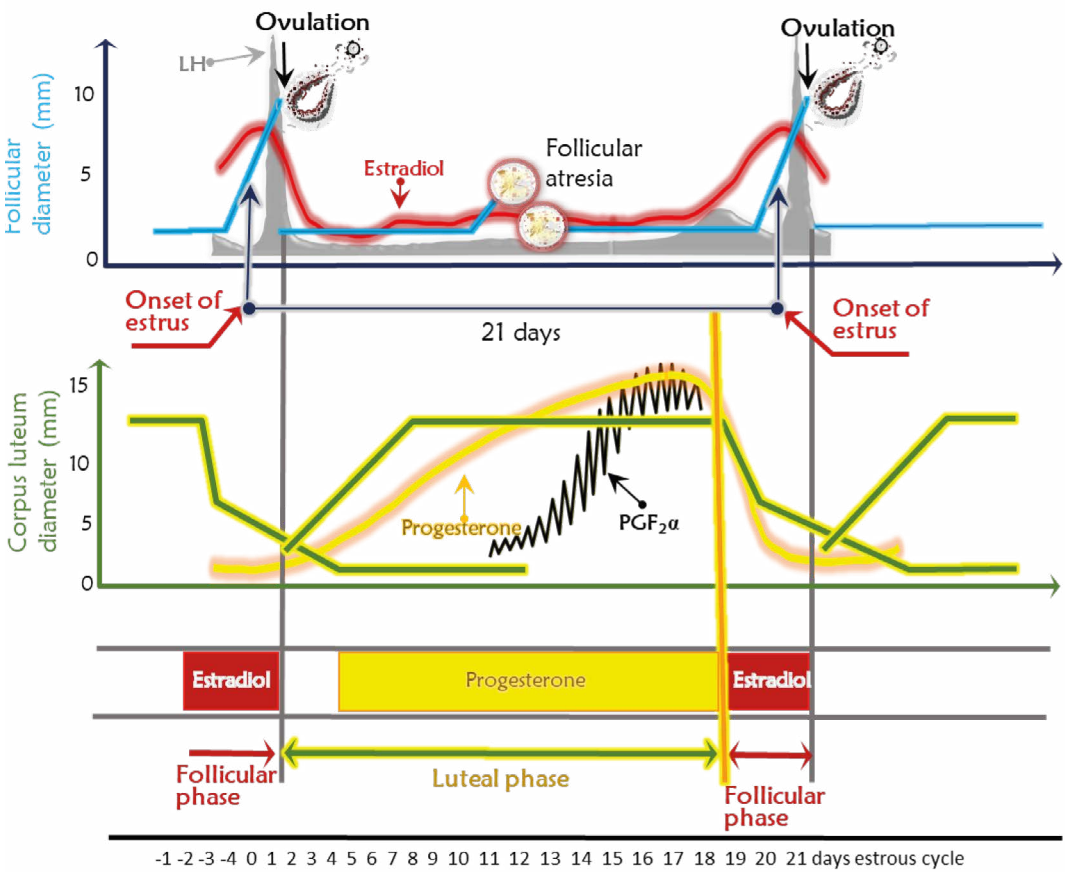


**Figure 1**. Picture depicting the estrous cycle in goats. The red, yellow, green, blue and black lines indicate the variations in blood estradiol and progesterone concentrations, the diameter of the corpus luteum and follicle, and the prostaglandin ($PGF_{2\alpha}$) secretion pattern throughout the estrous cycle, respectively. The grey chart depicts the LH pattern secretion.

The synchronization protocols can be divided into two: those that use exogenous progesterone and those that do not use it. However, when $P_4$ is not used, the other hormones must be applied during the luteum phase. $P_4$ is applied for periods that generally vary from five to 14 days, since it is known that $P_4$ participates in the presentation of estrus so its presence in the organism, whether supplemented or natural, is mandatory. In the Mexican market there are two mediums to supplement it, by sponges or by controlled release devices (CIDR; Figure 2). Both are applied intra-vaginally. Apart from the material that they are made of, the main difference between both is that the sponges contain progesterone analogues (fluorogestone acetate or medroxyprogesterone), while the CIDR contain the natural source of this hormone. In addition, the CIDR can be reused while the sponges are used only once.

$PGF_{2\alpha}$ is generally applied 48 h before or at the moment of withdrawing the sponge or the CIDR, or at the time of applying the equine chorionic gonadotropin (eCG). The regression of CL by the action of $PGF_{2\alpha}$, in combination with the withdrawal of the sponge or CIDR, causes a sudden decrease of the concentrations of $P_4$, provoking the appearance of the external signs of estrus, as a result of the increase of $E_2$ produced by the one or

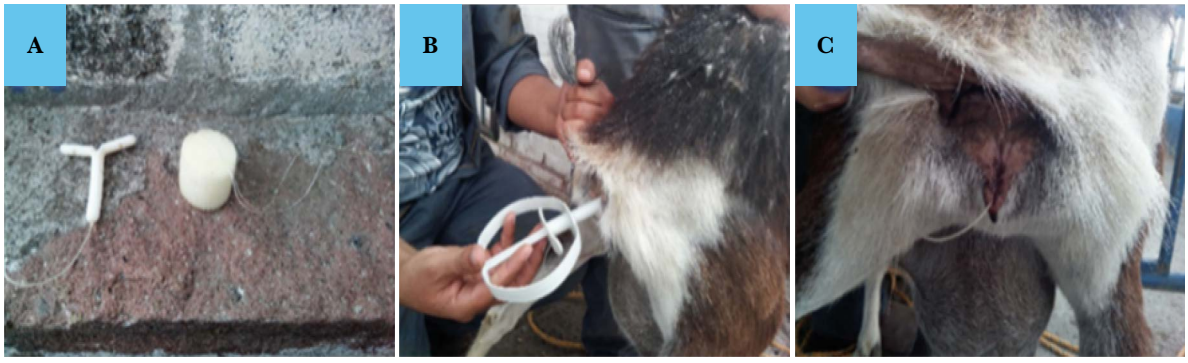


**Figure 2**. (A) Picture depicting a CIDR and sponge used in goats to estrus synchronization. (B) The CIDR and sponge are intravaginally inserted into the animal. These vaginal inserts have a tail that normally remains exteriorized after their application. (C) The removal of the CIDR and sponge is carried out by pulling out their protruding tail; if a goat shows not protruding tail of the vaginal inserts, a vaginal examination by speculum is recommended to ensure that they are not located deep into the vagina.

many pre-ovulatory follicles. The eCG has a similar action to the FSH; the objectives of its application, according to the injected dose, are to increase the number of pre-ovulatory follicles and the homogenization of the appearance of estruses. The estrus in does takes place between 26 and 47 h after the withdrawal of the sponge or CIDR (Navanukraw *et al.*, 2014) and it shortens even more the time with the application of the eCG.

The protocols that do not use exogenous $P_4$ are based mainly on the intramuscular application of $PGF_{2\alpha}$, alone or in combination with the GnRH. The main disadvantage of these protocols is that they can only be used during the reproductive season (short days) and in presence of a CL. This happens because it depends on the natural contribution of $P_4$ by the CL and since this ovarian structure does not exist, the exogenous supplementation of $P_4$ during anestrus is essential for the implementation of any induction protocol. The effectiveness of the induction of estruses, through the use of $PGF_{2\alpha}$, depends on the presence of a CL at the time of its application.

**Synchronization scheme**

The detection of estruses is done with the help of a buck with askew penis, vasectomized, or a "whole" one with apron (Figure 3), to avoid early or unwanted mating. The bucks are introduced to the pen of the does treated at intervals of 6 h, after having ended the hormonal treatment. The does that accept mounting are considered to be in estrus. It is advisable to record the time at which estrus was detected, to program the insemination (natural mounting, NM; artificial insemination, AI). In the case that it is not possible or when detecting the estrus is not meant to be done, there is the option of the use of induction or synchronization protocols with insemination at a fixed time. The insemination can be performed between 43 and 54 h after withdrawing the sponge or CIDR (Holtz *et al.*, 2008; Vilariño *et al.*, 2011; Sen and Onder, 2016). An insemination protocol at a set time is ovsynch, which is adopted from bovines. It consists in the injection of GnRH on day zero, followed by another of $PGF_{2\alpha}$ and GnRH seven and nine days later. The insemination is performed 16 h after the second injection of GnRH (Holtz *et al.*, 2008).

**Figure 3**. A Sannen buck wearing and apron and harness to avoid copulation and to identified ewes in estrus.

**Mating and artificial insemination (AI)**

Mating can be carried out naturally or controlled. In the first case, the buck is incorporated into a pen or a group of does at the beginning of the reproductive season. Mounting is carried out as the does enter estrus naturally. In controlled mating the buck is allowed to mount the doe 12 to 18 h after the estrus is detected, and mounting can be repeated at intervals of 12 h until the end of the estrus. This is generally done with does with synchronized estrus. In the case that there is not enough time to expose the females in estrus to the male, it can be introduced into the pen of synchronized does.

The proportion females:males to be used will depend on whether it is natural mating or with synchronized does. In the latter, the number of females per male should be lower, because the estruses will present in a short period of time. Fonseca *et al.* (2008) suggested that the proportion should not exceed 8:1 with synchronized estruses, with intervals between synchronizations of 3 to 4 days. This is adequate, considering that Mellado *et al.* (2000) reported that the males perform nine mounts per day. In natural mating, the number of females can be increased up to 75 for each male (Mellado *et al.*, 1996). However, this will depend on the libido and experience of the buck to be used. It is advisable to place a harness (Figure 3) on the male or simply paint its chest continually, to be able to identify the females the have been mounted. Therefore, the birth date and the mating ability of the buck could be estimated. In the case that no marked does are observed, the buck should be replaced, since this indicates the inability to mount.

The goat breeder has the option of introducing new genetic material to his flock through AI; the insemination can be conducted with fresh, refrigerated or frozen semen. The first two are generally obtained from males within or close to the production unit, since their average life is limited. Contrary to these, the frozen semen can be conserved in liquid nitrogen indefinitely and transported to any part of the world. The types of insemination, available to goats, can be classified according to the site where semen is deposited in the reproductive system of the goat. These are intra-vaginal, peri-cervical, intra-cervical, and intra-uterine via trans-cervical and laparoscopy.

The AI techniques can be carried out by the producer. However, laparoscopy implies the use of specialized equipment (laparoscope, sources of light and trocars), which can limit its routine use. In addition, it requires for animals to be deprived of food and water for a minimum period of 12 h before the insemination but uses a lower number of sperm cells per dose, since the semen is deposited in the uterine horns. Ritar *et al.* (1990) obtained between 50 and 66% pregnancies using semen doses with 5 to 60 million sperm cells through laparoscopy, while using the uterine intra- and trans-cervical insemination they obtained between 34 and 42%, inseminated with doses of 80 to 160 million sperm cells. In general, the further in the reproductive system of the doe the semen is deposited, the gestation percentages will be higher. In this regard, Salvador *et al.* (2005) obtained 82% of pregnancies when the semen was deposited past the cervix and 37% when it was deposited into the vagina. The insemination can be carried out between 12 and 24 h after the estrus is detected.

**Male effect**

The male effect is an alternative to the conventional protocols of synchronization, which consists in the stimulation of the reproductive activity of the doe, induced by the effect (presence, smell, vocalization and mating) of the sudden introduction of the buck. The stimulation consists in the increase of the frequency of LH secretion, presentation of estrus and ovulation. According to Martínez-Alfaro *et al.* (2014) the pre-ovulatory peak of LH and the ovulation is presented at 41 and 65 h after the introduction of the male. However, the period between the introduction of the male and the beginning of the estrus can vary from 1.8 to 8 days (Fernández *et al.*, 2011; Zarazaga *et al.*, 2018). Chemineau *et al.* (2006) indicated that after the first ovulation induced by the male effect, if the does are not inseminated, some of them will present an estrus cycle of normal duration, while the rest will suffer a premature regression of the corpus luteum, starting a new cycle at between six and nine days after the introduction of the male.

The male effect is used during anestrus and its effectiveness in the induction of estrus depends on the male's libido. Therefore, the males should be treated in a way that they are active during seasonal anestrus. Flores *et al.* (2000) indicated that the males that are not stimulated prior to their incorporation with the does are incapable of inducing the reproductive activity in them. A way of stimulating the reproductive activity in the males during anestrus is through testosterone, administered at a dose of 50 mg every third day for three weeks, which is effective in inducing the sexual behavior of the male during anestrus (Luna-Orozco *et al.*, 2012).

The response to the male effect will depend on the physical state of the does at the time of the stimulus; the response to the male effect is lower in does with ≤33 kg of live weight and higher in does with ≥34 kg (Véliz *et al.*, 2006). This is normal, since the heaviest does are generally dominant in the flock and will be the first to have access to the male. In addition, the proportion male:females should be taken into account. For example, it has been found that the fertility is reduced in 22% when a proportion of 1:30 is used in comparison to 1:20 (Zarazaga *et al.*, 2018).

**Reproductive biotechnologies in goats**

Reproductive biotechnologies are tools that can be used by the technician or producer to increase the reproductive potential of the flock and to promote the dissemination of outstanding animals. Some of the most used biotechnologies in goats include estrus synchronization, AI, semen extraction and freezing, super-stimulation, production, division and freezing of embryos, follicular aspiration and production of *in vitro* embryos. Other less common ones such as cloning, intra-cytoplasmic sperm injection, freezing of oocytes, cloning and sexing of embryos can be reviewed in Nasar *et al.* (2008).

Estrus synchronization and artificial insemination were reviewed in prior sections of this document. Semen extraction tends to be conducted with the help of an artificial vagina or the electro-ejaculator. The artificial vagina is the most frequently used method, since it is less stressful for the male. The volume and concentration of semen extracted varies from 0.4 to 1.27 mL and 1.4-3.6×$10^9$ sperm cells $mL^{-1}$ (Memon *et al.*, 1986; Karagiaannidis *et al.*, 2000). The collected semen is mixed with cryo-preservatives and stored in liquid nitrogen, for indefinite time, for its use through AI or in the production of *in vitro* embryos.

Super-stimulation consists in the use of gonadotropins to induce the development of pre-ovulatory follicles, and therefore ovulations, higher than the number that would naturally occur in each estrus cycle. The gonadotropins commonly used are purified sources of FSH and eCG. The first is applied several times at an interval of 12 h before, during and after withdrawal of the sponge or CIDR. The eCG is applied only once, due to its longer average life. However, there is the disadvantage of provoking the persistence of pre-ovulatory follicles after the estrus, which is unfavorable for embryo development. The collection of embryos is carried out six to seven days after the estrus. The collection methods are laparotomy or via the cervix. The embryos collected can be immediately transferred to receiving does or frozen for their posterior transference. Another alternative is to divide every embryo into two halves, each of them with potential to generate a kid. It is important to mention that the receiving does of embryos must be in the same estrus cycle than the donating, at the time of the transference, to guarantee a similar hormonal medium between donor and receptor.

Follicle aspiration is carried out with the aim of collecting oocytes *in situ* for the production of embryos *in vitro*. The visualization and fixation of the ovary, to aspirate the follicles present, is carried out through laparoscopy. Once collected, the oocytes are evaluated, and those of good quality are subjected to a process of maturation, fertilization and cultivation under laboratory conditions.

## CONCLUSIONS

The knowledge available about the reproductive physiology of the goat is abundant. However, it is still necessary to work on the implementation of reproductive biotechnologies within production units, so that their use becomes generalized and improvements can be made not just in reproductive parameters, but also genetics and the productivity of the flock in general.

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AGRO PRODUCTIVIDAD

Colegio de Postgraduados

# Exploitation of plantain (*Musa* spp.) plantations as an agrotourist element

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**ABSTRACT**

**Objective**: To analyze the processes and alternative uses that plantain cultivation may have, in addition to places with tourist potential in the town Monte Salas, municipality of Fortín de las Flores, Veracruz, Mexico.

**Design/Methodology/Approach**: A review of the space of the study area was performed, as well as an anthropological analysis, description of the process of the plantain leaf used to prepare local food, commercial analysis, development of a sustainable product, and a research stay in Colombia.

**Results**: In Monte Salas, Fortín de las Flores, Veracruz, Mexico, plantain cultivation is regarded as an outstanding element of the landscape, together with the process of leaf roast production as a value-adding strategy to generate biodegradable co-products. Through the dissemination of audiovisual capsules on social networks, places with tourist potential were made known and together with the implementation of the offer of biodegradable co-products, it was demonstrated that plantain leaf can be used alternately.

**Study Limitations/Implications**: Due to the SARS-CoV-2 (COVID-19) coronavirus pandemic, the dissemination of our findings and products were restricted.

**Conclusions**: Novel biodegradable products can be manufactured from plantain leaves, which created a special interest of local tourists to visit Monte Salas and enjoy the agrosystemic landscape.

**Keywords**: agrosystemic, landscape, co-product, biodegradable.

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## INTRODUCTION

Bananas and plantains belong to the *Musa* genus within the Musaceae family, and are native to South Asia. Within this family there are two species of current commercial importance: *Musa cavendish*, which includes bananas, and *Musa*×*paradisiaca*, which includes plantains (Díaz Arango *et al.*, 2015). In addition, there are numerous interspecific hybrids that are easily catalogued as *Musa* spp. From Asia, this genus reached the Canary Islands in the 15th century and was later introduced to the American continent. Currently, this crop has spread to many regions of Central and South America, constituting the basis of the diet and of the economy of many tropical regions (Secretaría de Agricultura y Desarrollo Rural, 2020).



Image by Se Kimseng at Pixabay

In addition to its nutritional importance, this crop is an important source of employment and income in developing countries. As an agrifood power, Mexico produces a variety of crops for both domestic consumption and export, including bananas and plantains (Villalobos-Arámbula, 2019). The main banana and plantain producing states are Chiapas with 35% of national production; Tabasco with 22%; Veracruz with 19%; and Michoacán and Colima with 7% each (Secretaría de Agricultura y Desarrollo Rural, 2020).

In addition to the fruit, this crop produces other products and by-products, such as pseudostems, leaves and veins. Furthermore, banana agroecosystems can be a tourist attraction for visitors. As a landscape element, banana plantations tend to enrich the value of sites by incorporating a diversity of species within the *Musa* genus, as well as other plant species, both cultivated and wild. This plant diversity also results in a greater flow of animals such as reptiles, birds and mammals, among others.

In order to have a regional and international view of the potential uses of plantain in agro-ecotourism perspectives, a study and liaison stay was conducted in the community of San Rafael, Antioquia, Colombia. This stay made it possible to record and to analyze various processes within the management and utilization of banana, and its potential inclusion as an attraction in agro-ecotourism projects, applicable to the reality of Monte Salas in Mexico.

## MATERIALS AND METHODS

The first part of the study consisted of analyzing banana cultivation from a visual perspective, taking agroecosystemic landscapes as a tourist attraction. This section was divided into four stages:

**Understanding the space through landscape ecology**. In order to understand the study of the landscape, the basic concepts that integrate it and the critical path of analysis were first defined. Subsequently, the way in which elements of the study area interact with each other was studied, that is, analyzing how agriculture is the predominant activity in the area and how banana plantations are the second most important crop. The relationship that exists among banana producers in Monte Salas, Fortín de las Flores, Veracruz, Mexico (Coordinates: 18° 54'ø16"øN, 96° 59'ø21"øW, 1400 m.a.s.l.) was established with this approach (CEIEG, 2020) (CEIEG, 2020).

The understanding stage was used to determine how widespread the crop is in the area. For this purpose, photographs were taken from a high point, according to the criteria of landscape ecology described by Forman and Godron (2009), whose structure of a landscape consists of matrices, patches and corridors (Vila *et al.*, 2019).

**Anthropological analysis**. Another important aspect for the analysis of this study was the anthropological explanation of how the crop emerged in this region. Based on documentary research, historical processes were compiled by searching for maps and written documents containing information about activities in local, state and national archives. This section described how banana cultivation was introduced in the High Mountains Region of Veracruz (Región de las Altas Montañas de Veracruz, RAMV) and how it eventually became more important than coffee cultivation (*Coffea arabica* and *C. canephora*), becoming the main economic activity in different localities of the region (Chávez-Hita and Florescano, 2013).

**Description of the production process**. In the third stage, the banana leaf production process was described, for which velillo cutters (local name for the plantain leaf) were identified, as well as the drivers who transport velillo to Mexico City. With the use of audiovisual tools, the process of roasting the leaf for use in the preparation of tamales and other regional foods was documented.

**Commercial analysis**. During field work, the profitability of banana leaf cutting for the main cutters in the area was investigated. In addition, a more integral and novel use of the plant's products was proposed, in addition to the production of fruits and velillo. This new approach could increase the flow of tourists to the area, and the use of audiovisual tools could enhance its diffusion.

**Alternative uses of the plantain crop**. Based on the field work with the cutters, it was determined that after the fruit, the leaf is the main usable and marketable product. Tests were carried out to find a different use for the leaf, and it was concluded that the optimal use would be in the manufacture of disposable utensils for serving food. In order to have a broader knowledge about plantain cultivation, an internship was carried out in San Rafael, Antioquia, Colombia, place where more practical knowledge was generated about the use of the banana leaf in a sustainable and applicable way for sustainable tourism. To conclude the research and evaluate whether these product alternatives were viable, trials were conducted to create a biodegradable product. For this purpose, a prototype of a plate was made with the leaf and starch of cassava (*Manihot esculenta*), making it resistant to liquids without leaks, achieving a product that can be industrialized. This prototype developed in Colombia was tested in the town of Monte Salas, Mexico.

## RESULTS AND DISCUSSION

Understanding the physical space constitutes the first step in formulating concrete questions about the landscape and its analysis (Thiébaut, 2017). To describe the landscape it is important to understand that the territory is understood as the space appropriated by a social group to ensure its reproduction and the satisfaction of its vital needs, which can be material and symbolic. It can be considered a refuge zone, a means of subsistence, a source of resources, and also a landscape, a privileged ecological environment, an object of emotional attachment, a homeland, and a geographic symbol. The result of the transformation of the environment by agricultural activity constitutes a key element of landscapes in the rural environment, and hence geography is considered as a science of the landscape, of the territory organized by human societies, and of the relationships of human beings with the environment (Ramírez and López, 2015).

To begin with the first part of the analysis process, concepts and methods were used framed in landscape ecology, a discipline that deals with planning of landscape and nature (Troll, 2003), and with which units that make up the landscape can be delimited. Thus, we looked for the observation point where there is a panoramic view and an overview of the landscape, taking into account land use, plots, vegetation forms, dominant function of an area, areas where the population is established (Hiernaux and Lindón, 2006), which in this case is the study zone and other neighboring localities. Within this framework, the various crops that represent the locality of Monte Salas were also recorded and analyzed.

The analysis of banana cultivation in the locality of Monte Salas showed two high altitude perspectives that serve as a contrast between one and the other. From a high point, different patches of the landscape are visible (Figure 1A). This photograph was taken from the Cerro de Santa Lucía Potrerillo, near Monte Blanco, and shows elements of the landscape ecology and its structure. Human settlements the patches and the tropical evergreen mountain forest is the dominant matrix. Both climate and soil in the region are conducive to the cultivation of sugarcane (*Saccharum* spp. hybrids) (Figure 1B) and banana-coffee polyculture (Figure 1C), which are the basis of the local people's economy.

In the second scale, the association of the banana crop with coffee was observed. This association is the most applied in the study area, due to its fast growth and production of edible fruits. Its main characteristic is the way it is planted, since in addition to coffee, vanilla (*Vanilla planifolia*), orange (*Citrus sinensis*), bamboo (*Bambusa* spp.), avocado (*Persea americana*), and lemon (*Citrus*×*limon*) can be grown, both for family consumption and for local sale.

In the management of banana plants, growers try to keep them at a low height in order to facilitate harvesting of the leaf and midrib. Depending on the stage of growth, the species cultivated and the time of year, a banana plant can measure from 2 to 6 m approximately (Licona, 2007).

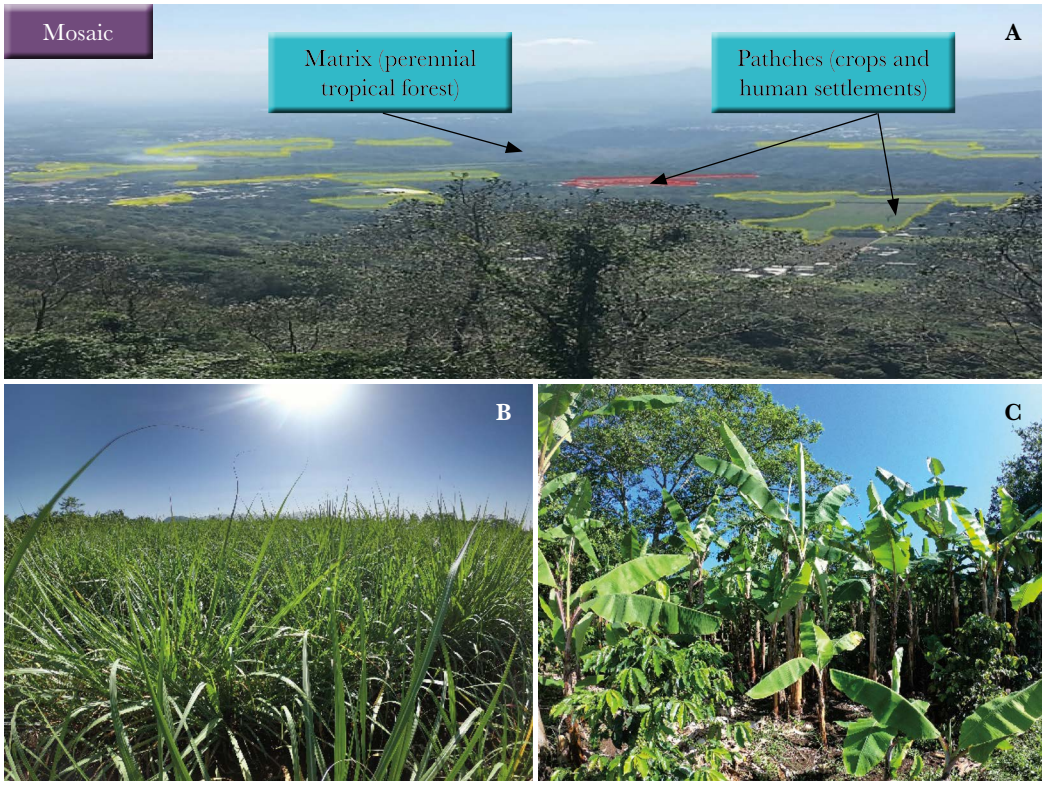


**Figure 1**. Panoramic picture of the landscape elements of Monte Salas, municipality of Fortín de las Flores, Veracruz, Mexico. The picture was taken from a high mountain in order to appreciate the community and the crops such as sugarcane (*Saccharum* spp. hybrids) and the polyculture plantain-coffee (*Coffea* spp.). A) Ecology of the landscape; B) Sugarcane plantations; C) Polyculture plantains-crops.

The introduction of the banana crop in the High Mountains region came about when landowners became interested in export crops such as sugarcane and coffee. According to Chávez-Hita and Florescano (2013), in 1898 coffee growers in the region were advised not to use banana as a shade crop because it stole moisture from the soil. However, one of the most important haciendas in the locality of Monte Blanco was a pioneer in introducing and adapting the cultivation of coffee in plains and ravines, as well as experimenting with different shades for the harvest. In the decade of 1950 in Monte Salas, a sector of producers opted to maintain their culture based on multiple systems, so since the first half of the decade they began to promote the marketing of banana leaves, locally known as *velillo*. In addition to the production of edible fruits, there are banana genotypes whose most significant use is the cutting of the leaf to make tamales and wrap other foods produced in the region. Given their Asian origin, according to Pilcher (2001), tamales wrapped in banana leaves began to be made from very early colonial times, mainly in the cultures of the coastal areas of the country. Before colonial times, tamales were made with leaves of species related to the plantain, such as *Heliconia*, which are native to the American continent.

The elaboration of tamales using banana leaf as a wrapper is an activity considered a driving force of the production system of banana-coffee associations (Susan *et al.*, 2017). Thanks to these systems, it is possible to obtain three agricultural products on the same land: coffee beans, banana fruits and banana leaves. Under this system, leaf cutters earn additional income every two weeks from the sale of leaves; weed growth in the production unit is delayed by the shade and the banana crop residues are composted.

**Description of the plantain crop production process**

Currently, banana cultivation represents the second most important commercial activity in the locality of Monte Salas (CEIEG, 2020). In order to intensify this crop, producers have opted to increase the population density within coffee plantations. Thus, more than 80% of the producers have population densities ranging from 289 to 625 banana vines per hectare with three or four pseudostems each, for a total of 867 and 2500 plants, respectively (Table 1).

This activity is profitable and yields two to three times higher than the specialized system for the production of fruits under the same market conditions of the products are reported. Leaf harvesting is carried out by specialized cutters hired by local leaf collectors.

The process of leaf harvesting and processing consists of four stages. The first stage is leaf cutting, which is carried out by specialized cutters hired by local collectors. The cutting of these leaves has a technique that each cutter must handle perfectly, as they must hold the leaves in the air to prevent them from falling to the ground, getting dirty and damaged (Figure 2A). As they cut, the workers must form rolls of 50 leaves. The second

**Table 1**. Average production of leaf rolls of plantain (*Musa* spp.) leaves per pseudostem, hectare and year in Monte Salas, Fortín de las Flores, Veracruz, Mexico.

| Pseudostems per hectare | Rolls per hectare | Rolls per hectare a year |
|---|---|---|
| 2,300 | 150 | 480 |

stage of the process consists of passing each leaf sheet directly over fire, an activity carried out by specialized persons in roasting leaves (Figure 2B). The third level of processing is leaf "deveining", which consists of removing the central rib and packaging it (Figure 2C). Finally, the processed leaves are packaged and sold in local markets (Figure 2D).

The commercialization of the leaf or *velillo* is today the main source of employment in the communities of Monte Salas and Monte Blanco in the municipality of Fortín de las Flores, Veracruz, Mexico. In the town of Monte Salas, Mr. Jacobo Gil Olmedo has formed a microenterprise and has become the main banana leaf collector and marketer. This microenterprise currently has two galleys, each with two wood-fired ovens where the leaves are roasted and then deveined and packaged. A space in his home is used for temporary storage of the *velillo* rolls.

The cutters are employed by the traders who, through a verbal contract, make agreements with the plantation owners for the cutters to enter their farms to harvest the leaves.


**Figure 2**. Process of production and manufacturing of plantain (*Musa* spp.) leaves to prepare tamales and other regional and local foods in Monte Salas, Fortín de las Flores, Veracruz, Mexico. A) Leaf cutting; B) Leaf roasting; C) Leaf deveining (removing the central vain of leaves); D) Packing.

**Table 2**. Average production of leaf rolls per cutter, price, and cost effectiveness according to the number of rolls estimated during the Summer 2020 (prices in Mexican pesos).

| Leaves per toll | Price per roll (MX$) | Rolls per cutter | Cost effectiveness (MX$) |
|---|---|---|---|
| 50 | $70 | 15 | $1,050 |

Growers know that the management of banana residues favors moisture conservation and also reduces the weed population in banana plantations. When decomposed, these agricultural residues become compost that improves soil quality and provides nutrients to plants in production. In the last five years, *velillo* has been the only product that has risen in price, making it possible to recover production costs and generate constant income throughout the year, since leaf cuts are made biweekly.

*Velillo* can be sold both raw and roasted. The package of raw *velillo* has a commercial value of $130 pesos per roll and has a shelf life of approximately 8 days after cutting, and the package of roasted *velillo* is sold for $150 pesos, with a shelf life of 6 days, considering average temperature conditions of no more than 24 °C. During periods of high demand that include from October to February, the price of the *velillo* may rise.

**Banana cultivation as an agrotourism attraction**

Promotional videos were made in order to promote the banana production and leaf processing activities, and thus attract tourism to the area under study. To this end, places with tourist potential such as the Tule Lagoon, which belongs to Monte Blanco (Figure 3A) were visited, a trail towards the plantation where there are different tree species, as well as the banana crop (Figure 3B), a trail of palms that make the landscape attractive (Figure 3B), and the river that runs through the entire Monte Salas ravine. Crops such as chayote (*Secchium edule*), coffee and, of course, banana can be seen along these routes (Figure 3D).


**Figure 3**. Places with touristic potential in Monte Salas, municipality of Fortín de las Flores, Veracruz, Mexico. 3A) Tule Lagoon. 3B) Road to Tule Lagoon. 3C) Road to Monte Salas river. 3D) Monte Salas river.

**Alternative and sustainable uses of the banana crop**

In addition to the sale of the fruit and leaf, this study raised the possibility of developing some other alternative and sustainable product that could be derived from the same plant. The approach was made taking into consideration that the people of the community themselves could carry out with the raw materials and the tools that were available in the area.

Based on the studies of Mazzeo *et al.* (2010) on the industrial utilization of banana harvest and post-harvest residues, it was determined that the leaf could be a good element and source of raw material for the elaboration of other useful products in the food service industry. This is because the leaf shows resistance to high temperatures and at the same time sufficient hardness and malleability for handling. Traditionally, the leaf is also used to wrap foods such as cheeses and meats in the region.

In support of this initiative and with the purpose of enriching knowledge on the cultivation and use of banana, a liaison stay in the Republic of Colombia was organized. This decision was made considering that Colombia is one of the main producers and exporters of bananas and plantains in the world (Atlasbig, 2021; FAO, 2021), and that, within that country, the Department of Antioquia concentrates the largest cultivated area with about 70,000 hectares planted with bananas and plantains (Minagricultura, 2020). In order to choose the place where to work, it was necessary to take into account the rural tourism approach, and the municipality of San Rafael in that department was chosen. The Local Tourism Network was contacted to carry out this trip. This network is an organization that, in addition to being the main face of San Rafael in terms of tourism, is also the organization responsible for activities such as seminars, tourism fairs, festivals and the region's festivals. Thanks to them, it was possible to successfully implement the research project within the framework of the research stay.

The activities were carried out in September 2019. First, together with the producers of San Rafael, it was decided to use banana leaves as raw material to produce an alternative product. Subsequently, to coat the leaf, tests were carried out with materials of 100% natural origin such as propolis from Melipona bees (Apidae family, Meliponini tribe) and cassava starch. Having done these tests, models of biodegradable plates and utensils useful in food service were developed. After roasting the leaves over medium heat, they were made malleable enough to be handled and the starch extracted from the cassava was applied according to the process proposed by Cobana and Antezana (2007) (Figure 4A). To shape the leaves, they are placed in an aluminum mold in the shape of a plate (Figure 4B). This mold is placed in the fire at a temperature of approximately 120 °C and a pressure of 60 kg. The final product obtained is a 100% biodegradable sheet made of banana leaf and covered with cassava starch, subjected to temperature and pressure (Figure 4C).

After showing the process of elaboration of the biodegradable dish, the second Tourism Fair of the Province of Water, Forests and Tourism was held in San Rafael. We participated as exhibitors on the first day of the fair, where products made from banana leaves were shown, as well as a brochure explaining how to make them and the benefits of creating ecological alternatives that help the environment. On the second day of the fair, we


**Figure 4**. Process of manufacturing a biodegradable dish made from plantain (*Musa* spp.) leaves and starch extracted from cassava or tapioca (*Manihot esculenta*). 4A) Leaf covered with starch extracted from cassava or tapioca (*Manihot esculenta*); 4B) Molded leaf; 4C) Final product taking the form of a dish, useful for serving local foods.

participated as speaker and presented the topic of the elaboration of biodegradable utensils made from banana leaves and cassava starch.

Once the presentation was over, people interested in the project approached to propose a class-workshop to discuss the benefits of creating biodegradable products that reduce the emission of polluting waste, with the intention of creating sustainable tourism in the community of San Rafael. The workshops were held on October 16 and 17, 2019. The first day the workshop was held in a rural school called "Bosqueschool Cariba" in the municipality of San Carlos, in the same Department of Antioquia (Figure 5A), and the second workshop was held in the municipality of San Rafael (Figure 5B).

Back in Mexico, the same initiative was implemented in Monte Salas. Biodegradable plates were made for a festival, and they served as replacement for disposable plastic or styrofoam materials (Figure 6A). In such biodegradable plates, food was served (Figure 6B) previously taking the sanitation measures, which included the perfect cleaning of the leaf


**Figure 5**. Workshops organized to demosntrate the techniques of elabotation of biodegradable plated from plantain (*Musa* spp.) leaves and cassava (*Manihot esculenta*) starch. A) Bosqueschool Cariba, San Carlos, Antioquia, Colombia; B) Association of the Local Tourism Network, San Rafael, Antioquia, Colombia.


**Figure 6**. Public demonstration of the biodegradable plates made from plantain (*Musa* spp.) leaves and cassava (*Manihot esculenta*) starch in Monte Salas, municipality of Fortín de las Flores, Veracruz, Mexico. A) Prototype of the biodegradable plate; B) Local foods served in the plates; C) People from Monte Salas, consuming local foods served in the biodegradable plates.

to later proceed to the elaboration of the biodegradable plates. The event was attended by people from the town of Monte Salas, who were served in these prototypes that tested their resistance to solid food.

This strategy is serving local dishes as a tourist attraction for people interested in banana cultivation, as well as in the production of biodegradable products based on the banana or plantain leaf.

## CONCLUSIONS

Through this project it was possible to record and analyze the banana production activity in the community of Monte Salas, Fortín de las Flores, Veracruz, Mexico, as well as to offer sustainable alternatives that diversify the income of the producers and in turn attract interested tourists. The alternative use of banana leaves in the production of biodegradable dishes offers the possibility of obtaining extraordinary income, is a sustainable strategy, and can function as a tourist attraction for the community that offers a natural and agro-ecological landscape of interest.

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