Advances in biology through agronomy, aquaculture, coastal and environmental sciences

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Prologue

Advances in biology through agronomy, aquaculture, coastal and environmental sciences is an electronic book, edited by Pantanal Editora, based on the compilation of research papers where the authors of the different chapters have used highly current scientific methodologies and research equipment.

The biological sciences as the main object of research in agriculture, aquaculture, coastal and environmental sciences generate every day an understanding of knowledge that allows raising the scientific level of society as part of universal access to knowledge.

This book mainly addresses issues related to the use of plants extracts as sustainable alternatives for biocontrol of pests and bacterial diseases. It also brings together information on viruses and other diseases in aquatic organisms. In addition, studies of mangroves structure and their contribution to carbon sinks in experimental sites in northwestern Mexico are presented. Finally, an analysis on educational strategies for environmental education based on plant biology is carried out.

Editors appreciate the participation of the authors who have come from higher education institutions and research centers of great scientific prestige in Mexico. The majority of them are members of the National Research System of CONACyT, Mexico.

The authors

Summary

Chapter 1

Phytotoxicity of hydroalcoholic extracts of *Parkinsonia aculeata* L. sp. Pl., in tomato plants. Polyphenol and flavonoid content

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ABSTRACT

This research aimed to determine the content of polyphenols and flavonoids in hydroalcoholic extracts obtained from the stems and leaves of *Parkinsonia aculeata* and their phytotoxicity in tomato seedlings applied at the early stages of growth. The extracts were applied at 15, 25 and 35 days after emergence (DAE). The highest content of polyphenols and flavonoids was obtained in the leaves, and the polyphenol concentration exceeded that of flavonoids. The hydroalcoholic extracts of both stems and leaves presented level 5 phytotoxicity in tomato plants at 15 DAE. However, from 25 DAE, there was no phytotoxicity. At 35 DAE, there was only phytotoxicity when the volume of both organs was 5 mL plant^{-1}. There was a significant interaction between organ and volume factors. The study shows that leaf and stem extracts can be used for biocontrol without causing phytotoxicity in tomato plants from 25 days, using volumes between 1 and 3 mL plant^{-1}.

Keywords: antioxidants, biocontrol, palo verde.

INTRODUCTION

Phytopathologists and producers, with an organic approach, employ various agrotechnical alternatives with the aim of reducing the contaminant load from the excessive application of broad-spectrum fungicides. The use of plant extracts to control pests and diseases is one of these agrotechnical alternatives (Báez et al., 2021). Among them, extracts of species such as creosote bush (*Larrea tridentate* (DC.) Coville) and oregano (*Origanum vulgare* L.) have been used to control phytopathogenic fungi of the

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genus Fusarium, which produces significant losses in the yield of crops of economic interest (Figueroa et al., 2019).

Plants sometimes produce a significant amount of primary and secondary metabolites as a regulatory action on a large number of pests and diseases; for this reason, the possibility of being used with a focus on environmental protection through integrated management is being studied (Zoppolo et al., 2008). Some of these metabolites are synthesized as defenses (repellents), and others intoxicate and directly eliminate microorganisms and pests. For example, saponins, amygdalins, and norhydroguaiaretic acid are used for the control of fungi and bacteria (Martínez-Olivo et al., 2020). Protocatechotic acid has a significant effect on the control of pathogens in general and in particular on preventing basal rot in tomatoes (El-Nagar et al., 2020). Among the metabolites that plants synthesize are polyphenols (PP), which are obtained from the shikimic acid biosynthetic pathway (Santos-Sánchez et al., 2019). PP is normally an antioxidant whose main function is to prevent damage to foliar organs. The damage can be due to biological oxidation induced by abiotic stress or caused by insects and microorganisms (Lee et al., 2020), including bacteria and fungi (Rivera-Solís et al., 2021).

Many species of the semidesert also show characteristics of tolerance to pests and diseases, subsisting in addition to prevailing adverse conditions such as salinity and drought (Gonzáles et al., 2021), as is the case for *Parkinsonia aculeata* L. Sp. Pl., commonly known as "palo verde or bacaporo" (Adhikari, White, 2014). This species has been studied in various regions of the world mainly for clinical purposes (Divya et al., 2011; Franco et al., 2022), and phytochemical studies have been developed to determine the presence of various metabolites that can be used for the biocontrol of pathogens, such as *Fusarium oxysporum* (Arvizu-Quintana et al., 2021). These studies are of great importance in the prevention of environmental contamination after having proven that they present minimal or no phytotoxicity in plants of economic interest. An important step is to evaluate whether these extracts obtained from model plants, such as "palo verde", affect the physiological, biochemical and/or agronomic performance of crops. Another important point is to substitute some chemical pesticides with these products. This substitution may reduce the amounts of pollutants in agricultural areas. Taking these elements into account, a study was carried out with the objective of determining the content of polyphenols and flavonoids in hydroalcoholic extracts obtained from stems and leaves of P. aculeata and evaluating the phytotoxicity in tomato seedlings applied in the initial stages of development.

MATERIALS AND METHODS

Study site

Location of the experimental area. The research was developed in the Biotechnology Laboratory of the National Institute of Technology of Mexico, Valle del Yaqui Campus, in the municipality of Bácum, Sonora, Mexico. Leaf and stem samples of *P. aculeata* plants were taken from the semidesert of

Sonora to obtain hydroalcoholic extracts by percolation according to the process described by Fecker et al. (2020).

Extract preparation

The samples were separated at a rate of 100 g and then remained in 1 L of 76% alcohol for 10 days (Figure. 1a-b, leaves and stems, respectively). After this period, the alcohol was separated using a rotary evaporator at 30 revolutions per minute and at a temperature of 65°C (BUCHI® R215, USA), with an extraction efficiency of 75%. The extracts were kept at 4°C until they were used (Figure 1c).

Figure 1. Sample maceration from (a) stem (b) and hydroalcoholic extracts from leaves (c).

Tomato variety used to evaluate phytotoxicity

As an experimental model, tomato seeds of the Río Grande® variety were used, with determined growth, cataloged as susceptible to fusarium wilt (Arellano-Aburto et al., 2021). The seeds were sown in 200-well polyfoam trays under semicontrolled conditions in a growth chamber. The conditions inside the chamber were adjusted to 10 hours of light, a temperature of 25°C and a relative humidity of 75%. Peat moss-type substrate (PROMIX®) was used for seed germination. At 15 days after emergence (DAE), the seedlings were selected and subjected to the established treatments.

Treatments and experimental design

The treatments consisted of the combination of two sources of variation: A) plant organs, with two levels (stems and leaves); and B) volumes of extracts applied, with four levels (0, 1, 3 and 5 mL plant^{-1}). The level of zero applications of the extract was taken as the control treatment. Each treatment of the eight conformed had a sample size of 30 plants. The application of the extracts was carried out on the roots and foliar route in unison three times after emergence: at 15, 25 and 35 DAE. This last factor was not included as a source of variation, and the results were compared separately in the respective statistical analyses.

The treatments were distributed under semicontrolled conditions, following a completely randomized experimental design with a bifactorial arrangement. In all treatments, the edge effect and neighboring variants were taken into account for phytotoxicity variable measurement. These plants were dispensed with to reduce the possible experimental error.

Evaluated variables

Polyphenol content was determined by the Folin-Ciocalteu method developed by Anesini et al. (2008). For the test, 125 µL of the gallic acid standard solution was prepared, and 0.5 mL of distilled H2O and 125 µL of Folin-Ciocalteu reagent were added. These reagents remained in the reaction for 6 min, and 1.25 mL of a 7% Na_2CO_3 solution was added. Finally, 1 mL of distilled H_2O was added and left to stand for 90 min at a temperature of 17°C and 65% relative humidity.

An absorbance reading was performed on the obtained solution in a UV Vis Genesys 10S spectrophotometer (Thermo Scientific®) at a wavelength (λ) of 760 nm. Then, both stem and leaf extracts were diluted at a 1:5 ratio with 50% methanolic solution, and the total polyphenol content was determined in the same way as gallic acid standards. Then, by interpolating the absorbance of the extracts in the gallic acid curve, the content of total polyphenols expressed in mg L^{-1} of extract was determined in triplicate samples.

Flavonoid content was determined by the method described by Muñoz et al. (2007). Samples of 250 µL of the extracts of *Parkinsonia aculeata* L. Sp. were dissolved in 1000 µL of deionized water. Then, 75 µL of $NaNO_2$ was added and allowed to react for 5 minutes. Subsequently, 75 µL of 10% $AlCl_3$ and 500 µL of 1 M NaOH were added. The mixture was centrifuged at 3500 r.p.m. for 5 minutes. Finally, the absorbance was measured at a wavelength of 510 nm. The final concentrations of total flavonoids were expressed in mg L^{-1} of stem and leaf extracts (Herrera et al., 2017).

Table 1. A phytotoxicity scale was established when evaluating extracts from leaves and stems of *P. aculeate* L. Sp.Pl.

Value	Description	Phytotoxycity
1	No foliar damage or death	Null
2	Foliar damage or death of at least 2 plants	mild
3	30% of leaf area damaged and 5 plants dead	half
4	More than 30% of the leaf area damaged and more than 10% dead plants	moderate
5	More than 50% of the leaf area damaged and more than 15% dead plants	high

Evaluation of the phytotoxicity of the extracts

The evaluation of the phytotoxicity of hydroalcoholic extracts from leaves and stems of *P. aculeata* was carried out in the initial stages of the Rio Grande tomato variety, following the scale described in Table 1, taking the scale proposed by Esparza-Díaz et al. (2010) as a reference. This evaluation was carried out twice (August-December 2020 and the same period of 2022).

Statistical analysis

To compare the concentrations of polyphenols and flavonoids, the theoretical distribution of student probabilities proposed by Gosset (1917) was used, establishing the differences between the organs where they were determined.

For the evaluation of phytotoxicity, a double classification analysis of variance was carried out based on a linear model of fixed effects (Fisher, 1937). The number of damaged or dead plants during each treatment (discrete quantitative variable) was taken as a variable response. When there were differences between the means of the treatments, Tukey's multiple comparison test was used for a significance level of 5% (Tukey, 1960). The statistical indicators of the coefficient of variation (CV), standard error of the mean of the treatments (ESx) and the coefficients of determination (R^2) were determined without adjusting for the isolated factors (organs and volume) and for the interaction between these two factors. STATISTICA professional software, version 14.1 for Windows (Statsoft, 2014), was used for statistical processing.

RESULTS

Polyphenol and flavonoid contents in the hydroalcoholic extracts of stems and leaves.

Polyphenol concentrations and total flavonoids showed highly significant differences between the organs from which the extracts were obtained. The concentration was in both parts, but it was higher in the leaves (Table 2).

Table 2. Total polyphenol and flavonoid contents in extracts of *P. aculeata* L. Sp.Pl. [A1.3: absorbance, Ax: average absorbance; F: dilution factor].

Organ	Absorbance for polyphenols					Dilution factor	
	A_1	A_2	A_3	A_x	Without Factor	F=5	F=20
Stem	0.75	0.76	0.76	0.76	74.88	374.44	
Leaves	0.89	0.90	0.92	0.91	91.52		1830.34**
							Y = 0.0089x + 0.0925
	Absorbance for flavonoids						
Stem	0.09	0.09	0.089	0.09	1.53	7.63	
Leaves	0.11	0.11	0.10	0.10	1.75		35.04**
							y = 0.0576x + 0.0038

The results demonstrate the plant's ability to store these compounds to be protected from pests and diseases. The storage is more in the leaves than in the stem. It was verified that hydroalcoholic extracts of leaves and stems of *P. aculeata* at concentrations of 10% were effective in promoting a low severity of the disease (3.7 and 3.3, respectively). These results may explain the low abundance of reports

showing the presence of pests and diseases in this species. This was attributed to the synthesis and accumulation of these compounds as main biocontrol agents.

Phytotoxicity of extracts in tomato seedlings

The application at 15 DAE of treatments T2 to T8 showed highly significant phytotoxicity (p=0.0017) in the Rio Grande tomato variety, causing damage to 77% of the plants of each mentioned treatment (value of 5). For this reason, the hydroalcoholic extract application of *P. aculeata* at 15 DAE is not recommended (Table 3). In the statistical analysis, at this moment of application (15 DAE), although there was a significant interaction between the organ*volume factors (p=0.01796), it was observed that the effect of the applied volume explained 98% of the total variability obtained (R^2 (volumes)=0.98). These findings show that any volume used close to emergence can generate phytotoxicity and that this increases significantly as the volume of extract applied increases (Table 3).

Table 3. Evaluation of the phytotoxicity of the hydroalcoholic extracts of *Parkinsonia aculeata* L. Sp. Pl. in tomato seedlings, Rio Grande variety.

Treatments	Number of dead plants (%)			Phytotoxicity (1-5)		
	15 DAE[1]	25 DAE	35 DAE	15 DAE	25 DAE	35 DAE
T1	0.33a	0.3a	0.2a	1	1	1
T2	24.3b	2.3b	1.7ab	5	2	1
T3	25.6b	3.0 cb	2.3ab	5	2	2
T4	28.6c	3.3b	3b	5	2	1
T5	0.35a	2.3b	0.3a	5	2	1
T6	25.6b	3.6b	3.3b	5	2	2
T7	28c	3.3b	1.6ab	5	2	1
T8	29c	3.3b	1.6b	5	2	1
$R^2_{(organs)}$	0.03	0.14	0.01			
$R^2_{(volume)}$	0.98	0.64	0.79			
$R^2_{(interaction)}$	0.02	0.15	0.12			
ES	0.11	0.33	0.21			
CV	26.2	6.04	5.1			

[1]DAE: days after emergence; R^2: unadjusted coefficients of determination for the isolated factors and their interaction. ES: standard error of the mean of the treatments; CV: coefficient of variation of the treatments. Means with equal superscripts in the columns of number of dead plants do not differ statistically by Tukey's test, p<0.005.

When the treatments were applied at 25 DAE, there were also significant differences between the organs used to obtain the extracts (p=0.00274), as well as between the volumes (p=0.0003), with a significant interaction (p=0.0179). When the extracts were applied during 25 DAE, a total variability found in phytotoxicity was explained by 64%, and the average phytotoxicity between treatments was 2. A similar result was found at 35 DAE, where the source of volume variation contributed 79% to the total variability found in phytotoxicity, although the average value of phytotoxicity was 1 (Table 3). The results obtained indicate that high volumes of the extract can cause phytotoxicity in plants. Because of this, these

studies must be carried out to recommend its use. This can be useful to producers to control diseases in seedlings without causing damage to the initial morphological and physiological characteristics of plants.

DISCUSSION

Various investigations on the use of crude plant extracts have revealed their inhibitory activities on microorganisms. For example, the antimicrobial activity of *Pinus wallichiana* A.B. Jack leaf extracts against *Fusarium oxysporum* f. sp. *cubense* (Foc), attributed to the significant presence of flavonoids and polyphenols (Ain et al., 2022). These results confirm that the palo verde extract has important potential as a biocontrol agent because it comes from a plant. This product easily mitigates into the plant (Stefanovic; Comic, 2012), so it would generate little or no phytotoxicity. This characteristic confirms the importance of this study.

Many of these extracts contain terpenoids, alkaloids, tannins, saponins, phenylpropanoids, and flavonoids, which are used to manufacture fungicides and pesticides at high concentrations (Nxumalo et al., 2021).

In Mexico, various plant species extracts have also been obtained. They have a significant concentration of flavonoids and polyphenols used as biocontrol agents of insects. A major control of 48-hour-old larvae with seven concentrations was obtained when the insecticidal activity of mistletoe dust (*Phoradendron densum* Torr. ex Trel.) on *Spodoptera frugiperda* was evaluated by Hernández et al. (2018).

In general, flavonoids play an important role in protection against biological oxidation induced by biotic and abiotic stresses (Sun et al., 2022). The content of polyphenols in plants and fruits varies depending on the genotype, species, environmental conditions, degree of maturity, soil composition, geographic location, and storage conditions (Shen et al., 2022). Flavonoids are also a frequent object of research due to their diverse functions, such as nutrient assimilation, protein synthesis, enzymatic activity, photosynthesis, formation of structural components, and defense against adverse environmental factors such as aggression of pathogens and insects (Figueirinha et al., 2008; Vélez-Terranova et al., 2014).

Multiple extracts have been obtained from semidesert plants for agronomic purposes, with an organic and integrated management approach for pest and disease control (Heikal et al., 2021). The governor species (*Larrea tridentata* (DC.) Coville) has been used for the biocontrol of Fusarium sp., with reductions in radial growth of 10% (Martínez-Olivo et al., 2020). Neem extract (*Azadirachta indica* A. Juss) has been used to evaluate antifungal activity against tomato vascular wilt and showed high control efficiency and minimal phytotoxicity in seedlings (Ayvar-Serna et al., 2021).

For fungal diseases such as vascular wilt, the hydroalcoholic extract of *Acacia farnesiana* was tested for a decade (Rodríguez et al., 2012) under in vitro culture conditions, with significant decreases in the mycelial growth of the fungus and minimal phytotoxicity in plants. Rivera-Solis et al. (2021) also tested extracts of *Sargassum* spp. as inducers of tolerance to *Fusarium oxysporum* in tomato seedlings without finding significant phytotoxicity. These results demonstrate the practical value of plant extracts in

controlling diseases that affect agricultural crops and their contribution to caring for the environment by reducing the application of chemical products.

Plant extracts in pest and disease management are currently recognized as environmentally safe, less hazardous and cheaper. In their most natural form, many plant species have insecticidal characteristics (Tavares et al., 2021). Its use constitutes an alternative to mitigate contaminant loads due to concentrated chemical products that sometimes generate resistance in organisms.

The production of plant extracts is still important in the discovery of innovative and environmentally safe antimicrobials to overcome problems with resistance to multiple pesticides. The use of extracts to minimize or eliminate the damage caused by pests and diseases can contribute to the national and international scientific community. This could have great economic and ecological significance.

CONCLUSIONS

The hydroalcoholic extracts of *Parkinsonia aculeata* L. Sp.Pl. present a higher concentration of flavonoids and polyphenols in the leaves than in the stems. The Río Grande tomato variety presents high phytotoxicity (f=5) at 15 DAE. Therefore, the application of hydroalcoholic extracts of *P. aculeata* at this time is not recommended. The safe application of the hydroalcoholic extract of *Parkinsonia aculeata* L. Sp.Pl. without symptoms of phytotoxicity appearing, must be carried out from 25 to 35 DAE of the seedlings.

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Chapter 2

Evaluation of the antioxidant and antimicrobial activity of hydroalcoholic extracts of *Larrea tridentata* leaves

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ABSTRACT

Approximately 80% of the world's traditional medicines use plant species to meet primary health care needs. In recent years, it has been discovered that the essential oil compounds of *larrea tridentata* have beneficial activities: antioxidant, antitumor, neuroprotective, regenerative, antibacterial, antiviral, antifungal, anthelmintic, antiprotozoal and insecticidal activities. However, few investigations have evaluated the secondary metabolites of their ethanolic extracts. The objective of present study is evaluation of the antioxidant and antimicrobial activity of hydroalcoholic extracts of *larrea tridentata* leaves. The ethanolic extracts evaluated contained 25.08±2.47 mg of anthocyanins 100 g^{-1} of plant and 228.88±3.45 mg gae polyphenols 100 g^{-1} of plant. The antioxidant activity evaluated by dpph is 47.12±2.43%. The ethanolic extracts had a high efficiency in inhibiting the growth of *staphylococcus aureus, escherichia coli, salmonella* and *shigella,* with inhibition halos of 13.41±0.51 to 19.67±0.41 mm. The determination of the minimum inhibitory concentration (mic) was 0.5 μg ml^{-1} against *escherichia coli*, 7.74 μg ml^{-1} against *salmonella,* 9.14 μg ml^{-1} against *shigella* and 12.25 μg ml^{-1} against *staphylococcus aureus*. These results showed that the tested ethanolic extracts possess antibacterial activities against bacteria that cause foodborne illness.

Keywords: foodborne illness; inhibition halos; anthocyanins; polyphenols; gae polyphenols; minimum inhibitory concentration (mic).

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INTRODUCTION

Human beings have used medicinal plants since ancient times to meet primary health care needs. In general, these plants are one of the main sources of phytochemicals (Martins et al., 2013). Approximately 80% of the world's traditional medicines are made up of bioactive compounds found in plants (Oliveira et al., 2005). Due to the biodiversity present on our planet, science has shown that many plants can be used in medicine (Corell-Doménech, 2019). *L. tridentata,* commonly known as gobernadora, is a plant that grows in the northern part of Mexico and the southwestern United States (Ross, 2005).

Mexico and especially the state of Sonora have great biodiversity, with more than 26,500 species of flowering plants, 1,600 ferns, 976 species of mosses and 1,700 species of lichens recorded. Several cultures have used diverse plant species, including *L. tridentatta*, for medicinal purposes (Andrango-Quisaguano, 2022). Today, scientific reports on these species are very limited, with less than 10% of the world's angiosperm species evaluated for their chemical composition and pharmacological properties, which motivates research on medicinal plants, as they possess valuable phytochemical and pharmacological information, becoming important in modern medicine. Other species can be a direct source of therapeutic agents as raw material for the manufacture of semisynthetic drugs. The chemical structure of their active principles can serve as a model for the development of synthetic drugs, and these principles can be used as taxonomic markers in the search for new drugs (Marcano; Hasegawa, 2018).

The increase in diseases caused by pathogenic microorganisms is a generalized concern and constitutes a risk factor for public health, which is why compounds from natural sources that inhibit bacterial growth are being sought (Corzo-Barragán, 2012). *L. tridentata* is an exceptional source of polyphenols, and its main exponent in this plant is nordihydroguaiaretic lignan (NDGA), which has been noted as being responsible for the biological activity of *L. tridentata* (Lambert et al., 2004; Martins et al., 2013).

However, the secondary metabolites found in *L. tridentata* leaves include lignans, flavonoids, saponins, triterpenes and triterpenoids, among others. Most of these compounds are studied for their antimicrobial, antiviral (Reyes-Melo et al., 2021), antifungal (Tequida-Meneses et al., 2002; Tucuch-Pérez et al., 2020) and antibacterial (Núñez-Mojica et al., 2021) properties. Nevertheless, only a few studies have reported the antibacterial and antioxidant activity of ethanolic extracts of *L. tridentata* leaves. These types of plants are an important source of antioxidants, which have an impact on food quality and consumer health.

MATERIALS AND METHODS

Experimental area

The equivalent of 2 kg of governor's weed was used. The grass was obtained in the proximity of Ciudad Obregón, Sonora. With coordinates 27°28'55.9 "N 110°00'02.0 "W. Once the plant was collected, it was transported to the Tecnológico Nacional de México campus Valle del Yaqui, located at Avenida

Tecnológico, Block 611, Bácum, Sonora, México. C.P. 85276, to the ultrasonic pulse laboratory and the aquaculture laboratory. These laboratories have the appropriate equipment for this research.

Extraction of phytochemicals from L. tridentata

For the extraction of phytochemicals, an ethanol-acetic acid-water solution was prepared in a 50:10:40 v/v ratio for both corns in a 1:30 w/v ratio, and the pH was adjusted to 1 by adding 2 molar acetic acid and placed in constant agitation for 15 days. Finally, the sample was centrifuged at 4000 g, and the supernatant was collected. The extract obtained was concentrated to dryness in a Rotavapor (DLAB model RE100-S) at 50 °C and stored in the dark at -20 °C until use. The total phenol content was expressed as mg 100 g^{-1} (Abdel-Aal; Hucl, 1999; Ju; Howard, 2003).

Determination of total phenols

Based on the determination of Singleton and Rossi (1965). For the reaction, 90 µL of a 50 mg mL^{-1} solution of extract (per genotype) was added, 1.91 mL of deionized water and 200 µL of Folin-Ciocalteu's reagent were mixed; after 2 minutes, 0.8 mL of 15.9% sodium carbonate was added and then incubated at a temperature of 50 °C for five minutes, and the absorbance was measured at 765 nm. The total phenol content was expressed as mg gallic acid equivalent (GAE). 100 g^{-1} dry extract (Pérez-Pérez et al., 2014; Singleton; Rossi, 1965).

Determination of total flavonoids

For this variable, 0.5 mg mL^{-1} of the extract in solution was mixed with 2 mL of distilled water and 150 µL of sodium nitrite, left to stand for five minutes by adding 150 µL of a 2.5% aluminum chloride solution, and then left to stand for six minutes by adding 1 mL of 0.5 N sodium hydroxide and 5 mL of distilled water, the absorbance was measured at 510 nm. To determine the flavonoid content, a calibration curve with quercetin was generated, and the total flavonoid content was expressed as mg of kersetin 100 g^{-1} of dry extract (Reyes et al., 2013; Zhishen et al., 1999).

Antioxidative capacity by DPPH

The capacity to scavenge the DPPH radical (2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazin-1-il) is based on the reduction of the absorbance at 517 nm by the action of antioxidants. The performance of this experiment consisted of mixing 3.9 mL of 100 µM DPPH radical dissolved in 80% methanol with 0.1 mL of the sample or standard and kept in the dark for 30 minutes. The reading was made at 517 nm after an incubation period of 30 minutes, and the results were expressed in % inhibition (Molyneux, 2004).

Antimicrobial activity by disk diffusion

The qualitative analysis of the antimicrobial activity was performed using the disk diffusion method, and the bacteria used were the following*: Staphylococcus aureus* (ATCC 8532), *Escherichia coli* (ATCC 12210), *Salmonella* (ATCC 8230) and *Shigella* (ATCC 4837). Bacteria were grown in 2% (w/v) trypticase soy broth (TSB). Purity control assays were used to verify the status of the bacteria. The strains were preserved in TSB broth with 20% sterile glycerol (v/v) at -20 °C (Ma et al., 2019).

The procedure was performed by impregnating sterile 6 mm diameter Whatman No. 1 filter paper discs with a concentration of 10 mg/disc of extract. Gentamicin was used as a positive control (30 µg/disc), and sterile distilled water was used as a negative control. The inhibition halo was measured after 24 hours of incubation at 37 °C. The criterion for interpretation was gentamicin (450 µg $disc^{-1}$) as a reference. Subsequently, inhibition halos were measured starting from the center of the discs to the end of the inhibition halo (Riverón-Rodríguez et al., 2012).

Statistical analysis

Biochemical analyses were performed in triplicate using Statgraphics Software (Version 18) to calculate the mean and standard deviation. The significant difference between two mean values was determined by analysis of variance (ANOVA) at the 95% confidence level ($p \leq 0.05$), and the significant difference between groups was determined by Tukey's comparison of means ($p<0.05$).

RESULTS AND DISCUSSION

Table 1 shows the concentrations of polyphenols extracted from *L. tridentata*, evidencing the presence of 228.88±3.45 mg of GAE 100 g^{-1} of plant. One possible justification for this high value is the environmental and surface conditions. This is because the environment directly affects a plant's ability to produce phytochemicals (Björkman et al., 2011). Studies on medicinal plants have found that factors such as water stress and temperature influence the phytochemical composition of medicinal plants (Shin et al., 2021). They also found that secondary metabolites play an important role in plant adaptation to the environment and plant recovery under stress conditions (Oh et al., 2009). Among the different types of stresses, water shortage, temperature and salt water are considered negative factors, as they lead to reduced yields of various crops. However, plants grown under dry conditions often produce higher concentrations of active ingredients that protect against free radicals and reactive oxygen species and help prevent photosynthetic damage (Albergaria et al., 2020; Oh et al., 2009).

The total amount of anthocyanins obtained from ethanol was higher than that reported for L. tridentatta elsewhere in Mexico (Saldívar-Lira, 2003). With respect to food technology and the use of medicinal herbs, it has been found that adding herbs to juice reduces the anthocyanin content due to the soaking effect of the extract on the phytochemicals in dried herbs (Teneva et al., 2022). Therefore, it is difficult to retain sufficient anthocyanins in the juice. However, although it is difficult to link the increase

in anthocyanins with their stability, it is certain that plant extracts can protect juice components from decomposition during processing. However, the effectiveness of phenolic compounds in food preparations was not related to the polyphenol content or the resulting antioxidant properties but to their quality, suggesting that the phenolic composition is qualitative. was more important than the total polyphenol content of the extract (Klisurova et al., 2019).

Table 1. Anthocyanin composition, polyphenols and antioxidant activity of gobernadora.

	Anthocyanins mg 100 g^{-1} de plant	**Polyphenols mg of GAE 100 g^{-1} de plant**	**DPPH (% of inhibition)**
L. tridentatta	25.08±2.47	228.88±3.45	47.12±2.43

[1] Gallic Acid Equivalents (GAE); 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The total amount of anthocyanins obtained from the extract was superior to that reported for L. tridentatta in other places of Mexico (Saldívar-Lira, 2003; Urias-Lugo et al., 2015). With respect to food technology and the use of medicinal herbs, it has been found that adding herbs to juice reduces the anthocyanin content due to the soaking effect of the extract on the phytochemicals in dried herbs (Teneva et al., 2022). Therefore, it is difficult to retain sufficient anthocyanins in the juice. Nevertheless, the effectiveness of phenolic compounds in food preparations was not related to the polyphenol content or the resulting antioxidant properties but to their quality, suggesting that the phenolic composition is qualitative. was more important than the total polyphenol content of the extract (Klisurova et al., 2019).

Antimicrobial activity

The bioassays were evaluated with strains of *Staphylococcus aureus* (ATCC 8532), *Escherichia coli* (ATCC 12210), *Salmonella* (ATCC 8230) and *Shigella* (ATCC 4837). The inhibition range shown is between 13.41 and 19.67 mm. This was compared to the gentamicin positive control, where an inhibitory halo of 16.01±1.17 to 22.47±0.41 mm was observed, and no inhibition was observed in the water negative control. However, it is important to note that the dose of gentamicin is 22.5 times the amount of plant extract used. A recent study demonstrated the in vitro growth inhibitory effect of 14 strains of *Xanthomonas axonopodis* using methanolic extracts of leaves and stems of *Bixa orellana, Gliricidia sepium, Ocimum basilicum* and *Petiveria alliacea*. The study concluded that these four plants have fungicidal effects and may be promising for controlling plant diseases (Nalimova et al., 2005). The inhibition halos reported were 17 to 21 mm in diameter for the four extracts used, very similar to the results obtained in this study (Table 2). Martinez-Valverde and Colmenares (1999) studied the effects of several extracts, including eucalyptus and *L. tridentata,* and found that the growth of pathogen colonies was inhibited by 90-100%. Similarly, results were obtained for 7 monocots, 46 dicots, 1 gymnosperm and 2 ferns. However, they reported that only nine showed in vitro growth inhibition of common pathogens. These inhibitory halos

were similar to eucalyptus extract (12.4 mm) and palo santo extract (11.3 mm) (Martínez-Valverde et al., 2000; Stauffer et al., 2000).

Table 2. Inhibition of extracts against pathogens.

	Inhibition zone size (mm) Bacteria			
	Staphylococcus aureus	***Escherichia coli***	***Salmonella***	***Shigella***
L. tridentata extract	12.25[b]	0.5[c]	7.74[a]	9.14[a]
Gentamicin	21.35[a]	19.88[b]	14.21[c]	17.44[b]
Water	NA	NA	NA	NA

[1]NA, no activity; Values are expressed in μg mL^{-1}. Different letters in the columns indicate significant differences ($p \leq 0.05$)

The antimicrobial efficacy of *L. tridentata* extracts is shown in Table 3. The lowest concentration was observed for Escherichia coli at 0.5 mg mL^{-1}, which agrees with the sensitivity and inhibition rate reported in Table 2. The other bacteria were affected by the extract of *L. tridentata*. However, the highest minimum concentration was for Staphylococcus aureus (ATCC 8532). Previous studies found that the antibacterial activity of the plant was equal for gram-positive and gram-negative bacteria (Mendez et al., 2012). The range of action of antibacterial activity in crude methanol extracts ranges from 62.5 to 250 μg mL^{-1} (Snowden et al., 2014). However, other authors have found inhibition ranges from 0.35 to 15 μg mL^{-1} (Gerstel et al., 2018), similar to the concentrations reported in our study (0.5-12.5 g mL^{-1}). In addition, the evaluation of *L. tridentata* flower extracts against Staphylococcus aureus yielded an MIC of 60 μg mL^{-1} (Gerstel et al., 2018).

Although the concentrations between studies differ from one another, the antibacterial activity is still present. Different factors can influence the nature and quantity of the secondary metabolites extracted. among the most important of which are solvent, pH, temperature, extraction time and sample composition, which could explain the different concentrations determined among the different studies, including the present investigation (Dirar et al., 2019; Do et al., 2014). Among the wide variety of bacteria to which *L. tridentatta* has shown inhibitory activity are *Clavibacter michiganensis* subspecies *michiganensis* and *P. syringae* at a concentration of 6.25 mg mL^{-1}. However, the concentration reported for these species is higher than the range of action of the antibacterial activity. This difference may be related to the extraction solvent (Morales-Ubaldo et al., 2021). The low concentrations to inhibit bacteria may be associated with the polarity of the compounds extracted with different solvents; for example, compounds extracted with ethyl acetate can easily penetrate bacterial cell walls and, therefore, can disrupt the barrier function of the cell membrane or cause membrane fusion, a process that results in the leakage of intramembranous materials. This mechanism of action decreases the necessary concentration of the compound to inhibit a specific bacterium (Renzetti et al., 2020); therefore, compounds of medium and

low polarity may have a higher activity against microorganisms (Gómez-Guiñán et al., 2003; Lobiuc et al., 2023; Martínez-Aguilar et al., 2012).

Table 3. Inhibitory minimum concentration.

	Concentration (µg mL^{-1})	Inhibition zone size (mm)			
		Staphylococcus aureus	***Escherichia coli***	***Salmonella***	***Shigella***
L. tridentata extract	20±0.45	13.41±0.51[a]	17.33±1.54[a]	14.67±0.90[a]	19.67±0.41[a]
Gentamicin	450 ±1.53	21.11±1.21[b]	21.04±0.58[b]	16.01±1.17[a]	22.47±0.41[b]
Water	0	0	0	0	0

[1]Different letters in the columns indicate significant differences ($p \leq 0.05$)

The use of natural extracts is a promising alternative in the control of bacterial infections (Esposito; Turku, 2023). There is considerable variation among the results in the study of secondary metabolites of *L. tridentata* because of the variety of phytochemicals contained in the plant (Qaderi et al., 2023; Reyes-Melo et al., 2021). Some secondary metabolites reported are terpenes, saponins, tannins, quercetin, kaempferol, ellagic acid, gallic acid, methyl gallate, resorcinol, cinnamic acid, catechins and lignans. However, these vary according to the environmental conditions of the area. In conclusion, phytochemicals exist in nature as nutraceuticals that are beneficial to human health, especially for their antioxidant and antimicrobial effects. The great variety of different compounds present in plants provide a rich source of potential pharmaceuticals to improve human health. Moreover, these compounds are very diverse in their mode of action, making them unlikely to cause bacterial resistance.

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Chapter 3

Morphological characterization of creole populations of ancho pepper of San Luis de la Paz, Guanajuato, Mexico

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ABSTRACT

The objective of this research was to carry out a morphological characterization of four creole populations of ancho pepper (*Capsicum annuum* L.) from the municipality of San Luis de la Paz, Guanajuato. The research was conducted under greenhouse conditions at Inifap-Cebaj in 2015 and 2016. A completely randomized design with 4 populations and two replications was used, considering 18 plants per replication as indicated in the National System for Seed Inspection and Certification (SNICS) descriptors manual. Plant height (PA), color by anthocyanins at the node level (CAN), leaf length (LH), leaf width (AH), leaf pubescence (PH), fruit diameter (DF), fruit length (LF), fruit length-to-width ratio (RLAF), fruit peduncle cavity (CPD), fruit peduncle cavity depth (PCPF), fruit peduncle thickness (GPF), fruit peduncle length (LPF), fruit pericarp thickness (GPF), flowering initiation time (TIF) and ripening time (TM) were recorded. The joint principal component analysis across years showed that the first two components explained 74,79% of the total morphological variability. On the other hand, the variables with the greatest explanatory capacity for CP1 were AH, LH and LF, and in CP2, GPF, LPF and CAN stood out. These results indicate that leaf and fruit traits are the most important components for the characterization of ancho pepper populations.

Keywords: *Capsicum annuum*, creole populations, morphological characterization.

INTRODUCTION

Mesoamerica and Mexico, as an important part of this region, are recognized as centers of origin and/or domestication of agricultural crops of worldwide importance. In Mexico, pepper (*Capsicum*

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annuum L.) is one of the most important horticultural species due to the value of its production and the high labor demand it generates. It is cultivated in almost all the states of the republic, from sea level altitudes up to 2500 m, and because it is its center of origin, a great diversity of types and varieties have been generated, which constitutes a valuable resource for its genetic improvement (Laborde; Pozo, 1984; Hernández-Pérez et al., 2011). In this regard, MacNeish (1964) and Hernández-Verdugo et al. (2012) state that *Capsicum* spp. was one of the first plants domesticated in the Americas.

Aguilar-Rincón et al. (2010) report that ancho pepper is grown in the states of Zacatecas, Durango, San Luis Potosí, Guanajuato and to a lesser extent in the state of Puebla. In green, it is used in the preparation of stuffed pepper or rajas. However, most of it is consumed dry as a condiment in the preparation of marinades or moles. Despite the great genetic and phenotypic diversity of *C. annuum* in Mexico, the regional variants of great economic and social importance are little recognized at the national level.

Knowledge of morphological variation and their geographic distribution patterns is of considerable interest for understanding the evolution of plant species and working on their conservation (Solís-Neffa, 2010). Among the geographic factors that influence the differentiation of populations are climate, latitude and altitude. Climate is considered one of the main factors affecting the distribution and variation of plant species because it can act directly on the fisiological processes of growth and reproduction or indirectly through ecological interactions, such as competition for resources. Several studies have shown that precipitation and temperature influence geographic patterns of morphological variation (Hernández-Verdugo et al., 2012).

Morphological or phenotypic markers have traditionally been used to differentiate varieties (Tapia et al., 2005; Adugna et al., 2006; Piña-Escutia et al., 2010). In this regard, Hernández-Villarreal (2013) proposed that morphological characterization of plant genetic resources is the determination of a set of characters through the use of defined descriptors that allow taxonomic differentiation of plants and concluded that characterization is the first step in crop improvement and conservation programs.

In 2002, the International Union for the Protection of New Varieties of Plants (UPOV) recommended that to measure variability, it is necessary to use discriminatory descriptors and to establish the experiment with a minimum of five plants per accession in homogeneous lots in two replications, thus obtaining better and greater information in the statistical analysis. When performing the characterization, reliable morphological variables should be used to discriminate, thus allowing differentiation between groups. These variables are already established in the so-called "technical guides for varietal description". In this regard, Villota-Cerón et al. (2012) conducted a morphological characterization study of 68 *Capsicum* introductions and selected promising introductions to increase the varietal supply of this genus. On the other hand, Santiago-Luna et al. (2016) mention that with respect to phenotypic diversity patterns, differences were determined between the populations of Santa María Tonameca and Santo Domingo de Morelos. The latter were highly variable in the characters evaluated.

Three groups of phenotypic diversity were determined, in plant, fruit and those associated with yield per plant.

On the other hand, Elizondo-Cabalceta and Monge-Pérez (2017) conducted a morphological characterization of 15 bell pepper genotypes with square- or rectangular-shaped fruits grown under a greenhouse; they found 5 variables at the qualitative and 8 quantitative levels. The data showed wide variability in plant height, leaf area, stem diameter, stem length, fruit width, fruit length, fruit length, fruit length/fruit width ratio and fruit wall thickness. They also stated that this information is useful for producers in the process of genotype selection in their production system, according to the market niche of interest, and that the morphological characterization of genotypes is an activity that allows the selection of the most promising varieties of a crop for subsequent use in breeding programs.

In the Macro Vegetable Network in 2016, it is mentioned that a total of 1,226 accessions have been characterized, where 50% refers to morphological characters and 27% corresponds to agronomic evaluation. Therefore, the main results of the chile crop characterization projects stand out, with 715 accessions identified for fresh and dry yields, earliness, fruit quality and size, resistance to pathogens, pigments, colorants and capsaicin. Based on the above, the objective of the present research was to carry out the phenotypic description of four creole populations of ancho pepper from San Luis de la Paz, Guanajuato, under controlled greenhouse conditions.

MATERIALS AND METHODS

The research was carried out in a glass chapel greenhouse belonging to the National Institute of Forestry, Agricultural and Livestock Research, Bajío Experimental Field (INIFAP-CEBAJ), located at km 6 of the federal highway Celaya-San Miguel de Allende, in Celaya, Guanajuato, located at 20° 34' North Latitude and 100° 50' West Longitude and an altitude of 1765 m (Google maps). The climate is semiwarm with an average annual rainfall of 400 to 700 mm.

The biological material (Table 1) was donated by Ing. José Antonio Morín Prado of the Agrisan company, located at Avenida Juárez No. 306, and the seed hoarder Juan amigo of the Estación de Lourdes community in the municipality of San Luis de la Paz, Guanajuato, Mexico. Both companies are located at 21° 29' North Latitude and 100° 70' West Longitude and an altitude of 1990 m (Google maps).

Sanitary management consisted of seed disinfection for fungal prevention using the fungicide Captan 80 WG at a dose of 150 g/100 kg of seed, which was mixed in a container with 500 ml of water along with the seed. Fifty-cavity plastic planting trays were used, which were disinfected in a 10% chlorine solution. Sterile Peat Moss No. 3 was used for seedling production, depositing one seed per cavity at a depth of 0,5 cm. The trays were placed in a germination chamber at a temperature of 25°C, light irrigation was applied to promote seed germination, and the moist substrate was covered with black plastic to prevent evaporation.

Table 1. Creole populations of ancho pepper. San Luis de Paz, Guanajuato, Mexico.

Name of the population	Type of pepper	Origin of the population	Origin
San Luis 1	Ancho	Creole	Acaparadora Juan amigo
San Luis 2	Ancho	Creole	Acaparadora Juan amigo
Especial	Ancho	Creole	Empresa Agrisan
Esmeralda	Ancho	F_2 Population	Empresa Agrisan

The experiment was carried out in two cycles; in the first cycle, sowing was performed on 30/03/2015, and in the second cycle, sowing was performed on 18/03/2016. A total of 144 pots of 12 inches were used, which were disinfected in a 10% chlorine solution. The substrate preparation was made with 10% Tezontle, 10% leaf and 80% lama soil and was sterilized with Busan 30 W (2-thiocyanomethylthio benzothiazole) for the prevention of Phytophthora capsici. Transplanting was performed when the plants were an average of 11 cm tall and had 6 true leaves.

A completely randomized design with four populations and two replicates was used; each replicate consisted of 18 plants per population.

The fertilization dosage recommended by INIFAP was applied based on the Technical Guide for Pepper Wilt (INIFAP, 2011). This fertilization program is dosed by phenological stages to be applied in irrigation during crop development and up to the first 115 days after transplanting; at transplanting, ammonium sulfate (110 kg ha^{-1}) was applied in a period of 10 days; in development, 10 applications were made in a period of 15 days using potassium nitrate (35 kg ha^{-1}), ammonium sulfate (130 kg ha^{-1}) and technical MAP (30 kg ha^{-1}); for growth, there were 10 applications in a period of 20 days using potassium chloride (70 kg ha^{-1}), calcium nitrate (40 kg ha^{-1}), phosphonitrate (110 kg ha^{-1}) and potassium nitrate (75 kg ha^{-1}); and for fruit set and fruit development, there were 15 applications in a period of 30 days with the fertilizers magnesium nitrate (75 kg ha^{-1}) and calcium nitrate (75 kg ha^{-1}). At transplanting, base fertilization with 40 g YaraMila Complex fertilizer (15-15-15) per pot was applied.

On the other hand, imidacloprid and methylcarbamoyl were applied at doses of 15 and 12 ml, respectively, in the irrigation system to control whitefly and red spider mites. In addition, a preventive application of the fungicide Busan 30 W (2-thiocyanomethylthio benzothiazole) was made to control P. capsici at a concentration of 50 ppm of commercial product, applied every 20 days after transplanting. Forum (dimatomorph) was also applied simultaneously at a dose of 22,45 ml of the commercial product and Cercobin (thiaphanate methyl) at a dose of 20 ml of the commercial product against *Fusarium* spp. and R. *solani*, applied 18 and 39 days after transplanting.

Harvesting was performed manually for both cycles. The fruits were harvested individually per plant from each experimental unit and were placed in different bags for labeling. On the other hand, imidacloprid and methylcarbamoyl were applied at doses of 15 and 12 ml, respectively, in the irrigation system to control whitefly and red spider mites. In addition, a preventive application of the fungicide

Busan 30 W (2-thiocyanomethylthio benzothiazole) was made to control P. *capsici* at a concentration of 50 ppm of commercial product, applied every 20 days after transplanting. Forum (dimatomorph) was also applied simultaneously at a dose of 22.45 ml of the commercial product and Cercobin (thiaphanate methyl) at a dose of 20 ml of the commercial product against *Fusarium* spp. and *R. solani*, applied 18 and 39 days after transplanting. Harvesting was performed manually for both cycles. The fruits were harvested individually per plant from each experimental unit and were placed in different bags for labeling.

Forty-seven morphological characteristics were recorded; these were organized by character type and averaged to obtain descriptive statistical values using the technical guide for varietal description of chili developed by the National System for Seed Inspection and Certification (SNICS, 2014).

Of the 47 characters recorded, a mode and mean analysis was carried out to discriminate variables from noninformative characters, obtaining 15 variables with the greatest explanatory capacity for the variability observed among the Creole populations of broad chili (data not shown).

A principal component analysis (PCA) was performed, and the scatter plot of the components, the percentage of variance, the eigenvalues of the variables and the two-dimensional plot of the dispersion of the variables within the components were obtained using the program past (paleontological statistics) ver. 3.15.

RESULTS AND DISCUSSION

In the principal component analysis (PCA) for the pooled data of the 2015-2016 cycles, seven principal components were formed and explained 64.939, 9.857, 8.015, 7.691, 5.633, 3.640 and 0.223%, respectively, of the total morphological variability observed among the studied populations (Table 2).

Table 2. Grouping of components and percentage of variability in ancho pepper materials. Cycles 2015 and 2016.

Principal Component (PC)	Eigenvalues	Percentage of variance
1	9.740	64.939
2	1.478	9.857
3	1.202	8.015
4	1.153	7.691
5	0.844	5.633
6	0.546	3.640
7	0.033	0.223

According to the interpretation and decision making of the data presented in the sedimentation graph (Figure 1), the first two components should be selected since together they explain 74.796% of the total variability of the populations in the two study cycles.

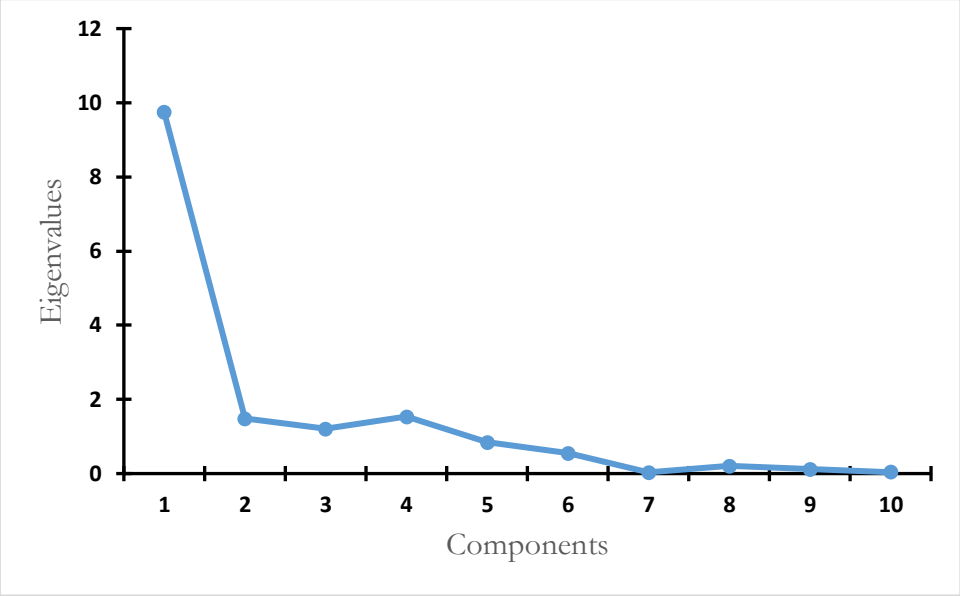

Figure 1. Graph of sedimentation in ancho pepper populations. Cycles 2015 and 2016.

Table 3 shows the variables with the greatest influence for each component; in this sense, the first principal component explained 64.939% of the total variability among the populations and was explained by the width (0.316) and length of leaf (0.313), length (-0.312) and diameter of fruit (0.295), fruit length-width ratio (-0.297), thickness of fruit stalk (-0.296) and time of initiation to flowering (0.293). The second principal component explained 9.857% of the total variability and was related to the variables anthocyanin color at the node level (0.444), fruit stalk length (0.462) and fruit pericarp thickness (0.613). These results agree with those of Ramírez-Novoa et al. (2018), who studied morphological diversity in chili piquín populations from Queretaro and Guanajuato and stated that the characteristics that had the greatest proportion of influence in explaining the total variation among populations were those related to fruits and leaves.

They are also similar to the results obtained by Toledo-Aguilar (2010), who studied morphological diversity in native varieties of poblano chile and concluded that fruit weight and color are important characteristics in phenotypic diversity. On this topic, Narez-Jiménez et al. (2014), in their study of the in situ morphological diversity of wild chiles in the State of Tabasco, found that CP1 explained 43.61% of the total variation and was explained by fruit variables. Another study reported by Pardey et al. (2006) affirms that variability among chili bell pepper populations is explained by fruit characteristics, plant architecture, flower structure and number of flowers per axil.

Likewise, Medina et al. (2006) found morphological differences, mainly in fruit and foliage, in different populations of the genus *Capsicum*. Similarly, it has been reported that fruit width in chili can vary between 5.9 and 10.2 cm (Dasgan and Abak, 2003; Hutton and Handley, 2007; Moreno-Pérez, et al., 2011). Similarly, Castañón-Nájera et al. (2008) mention that wild morphotypes of chile have fruits of

small length and width and affirm that this difference between morphotypes is probably due to changes produced by domestication.

Table 3. Eigenvalues of the variables in the PCA in the characterization of ancho pepper populations. Cycles 2015 and 2016.

Characteristic	ABV	CP1	CP2
Plant height	AP	0.224	0.179
Anthocyanin color at the knot level	CANN	0.202	0.444
Leaf length	LH	0.313	0.052
Leaf width	AH	0.316	0.010
Leaf pubescence	PH	0.253	-0.266
Fruit length	LF	-0.312	0.013
Fruit diameter	DF	0.295	-0.023
Fruit length/breadth ratio	RLAF	-0.297	-0.025
Peduncular cavity of fruit	CPF	-0.099	0.222
Depth of fruit peduncular cavity	PCPF	0.274	-0.060
Fruit stalk thickness	GPF	-0.296	0.188
Fruit stalk length	LPF	0.202	0.462
Thickness of fruit pericarp	GPF	-0.147	0.613
Time to flowering	TIF	0.293	-0.043
Maturation time	TM	0.228	0.115

CP1=principal component 1, CP2=principal component 2

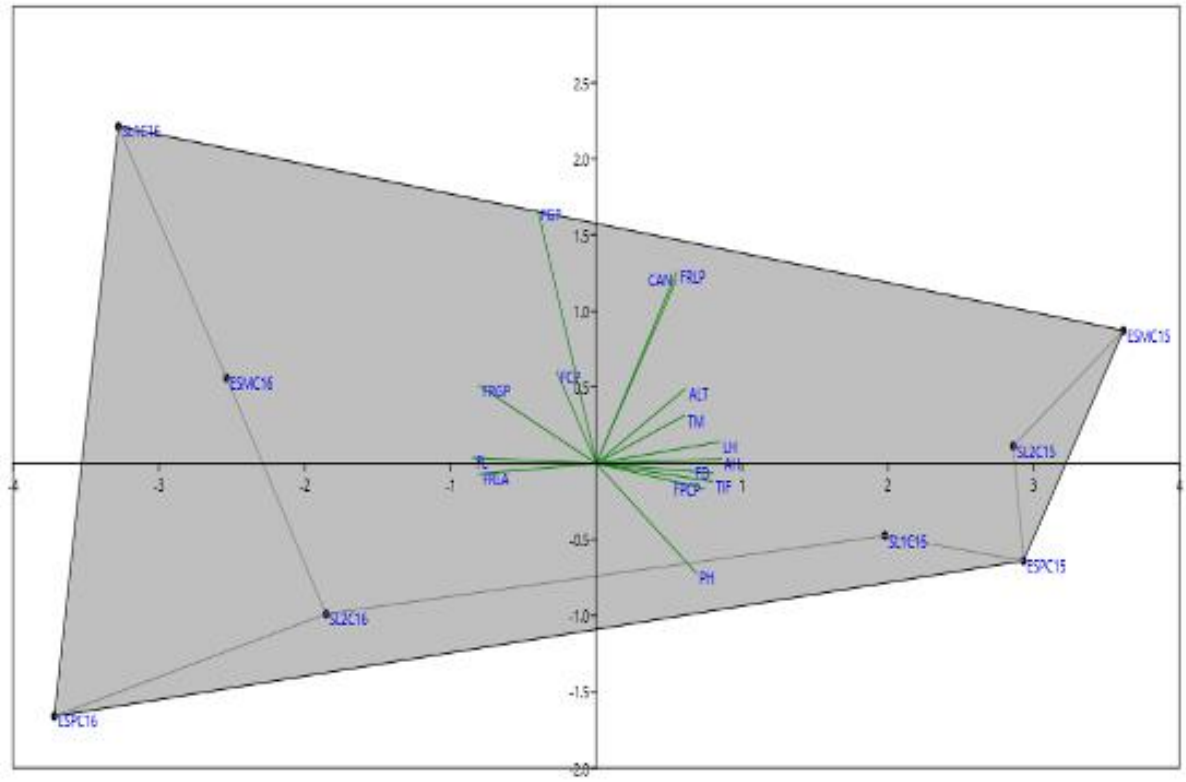

Figure 2. Two-dimensional plot of CP1 and CP2 in ancho pepper populations. Cycles 2015-2016.

Figure 2 shows the formation of two groups on different axes of the two principal components. The first group was formed by the Esmeralda (ESMC15) and San Luis 2 (SL2C15) populations of the

2015 cycle, and the variables positively related to the first axis were anthocyanin color at the node level (CAN), fruit stalk length (FRLP), plant height (ALT), ripening time (TM), leaf length (LH) and leaf width (AH). The second axis shows the Special (ESPC15) and San Luis 1 (SL1C15) populations of the 2015 cycle, explained by the variables TIF, FD, FPCP and PH. The second group is found in the third axis of the two principal components, where the San Luis 1 (SL1C16) and Esmeralda (ESMC16) populations of the 2016 cycle stand out with the variables FGP, FCP, FRGP and FL. The fourth axis is negatively linked, and the Special (ESPC16) and San Luis 2 (SL2C16) populations of the 2016 cycle are found with the variable FRLA. According to López et al. (2016), the predominant shape of the longitudinal section is ongular and angular with a strong transverse undulation, as well as the predominant characteristic of the presence of the peduncular cavity of medium depth in ancho poblano pepper hybrids.

CONCLUSIONS

The CP analysis for the pooled data from the 2015 and 2016 evaluation cycles highlights two principal components that explain 74.79% of the total variability.

The characteristics that contributed most to the total variation among the populations studied were mainly those related to leaves and fruits.

In the 2015 and 2016 cycles, with respect to the two-dimensional plot of CP1 and CP2, two groups were obtained, distributed in 4 axes. On the first axis were the Esmeralda (ESMC15) and San Luis 2 (SL2C15) populations, explained by the fruit, leaf and plant height variables. On the second axis, the Special (ESPC15) and San Luis 1 (SL1C15) populations were found with the flowering variables. The second group, on the third axis, included the populations San Luis 1 (SL1C16) and Esmeralda (ESMC16) explained by the fruit depth variables, and on the fourth axis, the populations Especial (ESPC16) and San Luis 2 (SL2C16) and the fruit length/width ratio variable.

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Chapter 4

Extraction of carotenoids present in the byproducts of bell pepper (*Capsicum annuum L.*) using the solvent method assisted with ultrasonic pulses

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ABSTRACT

In this project, the bell pepper byproduct was proposed to obtain carotenoids using the solvent-assisted method with ultrasonic pulses to evaluate the best extraction method and the effect of the pulses on the antioxidant capacity of the carotenoids. The extraction of carotenoids from the byproducts of bell pepper (*Capsicum annuum* L.) was carried by solvent extraction. The extractions consisted of using different solvents (hexane, ethanol, and acetone) assisted by ultrasonic pulses for 20 and 40 minutes with an amplitude of 40 kHz. After obtaining the oleoresin extracts, the antioxidant activity was determined using spectrophotometry techniques and physical-chemical analysis, determining pH, humidity, ashes, lipids, and proteins. The results of the extraction of carotenoids show that a better performance of the antioxidant activity was obtained for TEAC (trolox equivalent antioxidant capacity) as for DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) when the assistance of ultrasonic pulses was applied, with the solvents ethanol and acetone. In both methods, the treatments with hexane did not have the expected performance, taking into account the results of the other two solvents that were used during the extractions. Regarding the determination of flavonoids and phenols, a similar behavior was presented in both studies, presenting a minimum yield in the methods where the hexane solvent was used.

Keywords: ultrasound, antioxidants, phenols, flavonoids

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INTRODUCTION

The chili (*Capsicum annuum* L.) is among the main species of domesticated plants in Mexico; within its varieties, the bell pepper is one of the best, and approximately 5,800 hectares are planted throughout the country. Field yield can reach up to 50 tons per hectare per year (Reséndiz-Melgar et al., 2010). Of this enormous production, approximately 3 tons per hectare are discarded, given the demands made during chili packing.

The main component of chili peppers is water, followed by proteins and carbohydrates, as explained by the Spanish Nutrition Foundation (FEN); it is a good source of fiber, minerals, and vitamins as well as a good source of carotenes, among which capsaicin and pigments with antioxidant properties are found (Castillo-Olivera, 2021).

Carotenoids are compounds responsible for coloring a large number of plant and animal foods; it is known that some of these compounds, such as α and β carotene, are provitamin A (Meléndez-Martínez et al., 2004). Recent studies have revealed the antioxidant properties of these pigments, as well as their effectiveness in certain human diseases, increasing interest in these pigments (Acacio et al., 2013).

The traditional method for extracting these pigments is solid–liquid extraction, where high residence times and large amounts of solvents are needed, using heat and agitation. The use of these solvents always generates residues in the oil, and their toxicity causes scientific interest.

Many advanced techniques for the extraction of bioactive compounds have been investigated to improve extraction efficiency and overcome the disadvantages of conventional extractions. Ultrasound is a crucial technology to achieve the goal of sustainable "green" extraction. Recent studies show that ultrasound significantly affects the speed of various processes in the chemical and food industry (Chemat et al., 2017). Ultrasonic-assisted extraction improves the mass transfer of the extraction process by generating cavitation within the material. When cavitation bubbles are produced and collapse, the cell walls of the material are destroyed, and solute release is promoted.

Different studies report a significant increase in the yield of extracts with carotenoids with antioxidant capacity when using ultrasound-assisted extractions, even more than with microwave-assisted extractions (Chutia et al., 2021; Chuyen et al., 2018).

Therefore, this project aims to extract the carotenoids present in bell pepper coproducts through a solvent-assisted method with ultrasonic pulses and to evaluate their antioxidant capacity. The results will allow for determining the best method for extracting carotenoids and establishing the most appropriate temperature conditions, pepper pulp/solvent volume ratio, extraction time, and number of stages to obtain pepper oleoresin.

MATERIALS AND METHODS

Raw material

The peppers (*Capsicum annuum*) used in this study come from "Agrícola Badilla Flores S. A de C. V," in the Yaqui Valley in southern Sonora, grown in shade mesh. The samples were obtained in their highest state of maturation (red) after having been discarded for failing the quality of product packaging. The solvents used to extract carotenoids from bell pepper (*Capsicum annuum*) were hexane, acetone, and commercial-grade ethanol.

Sample pretreatment

The fresh peppers were subjected to a pretreatment with heat to eliminate the excess moisture, the peppers were cut between 1 to 2 cm thick (using only the skin and pulp of the fruit), and the seeds were discarded. They were dried in a food dehydrator for 24 h at below 60 °C to avoid carotenoid denaturation. The dry material was ground in a blade mill to obtain a particle size between 0.5 and 1.7 mm, following the methodology of (AOAC, 2005). This adaptation facilitated the extraction process of the carotenoids in the fruits. Likewise, it allowed better handling of the sample and a better application of the extraction method. A general diagram of the methodology followed is shown in Figure 1.

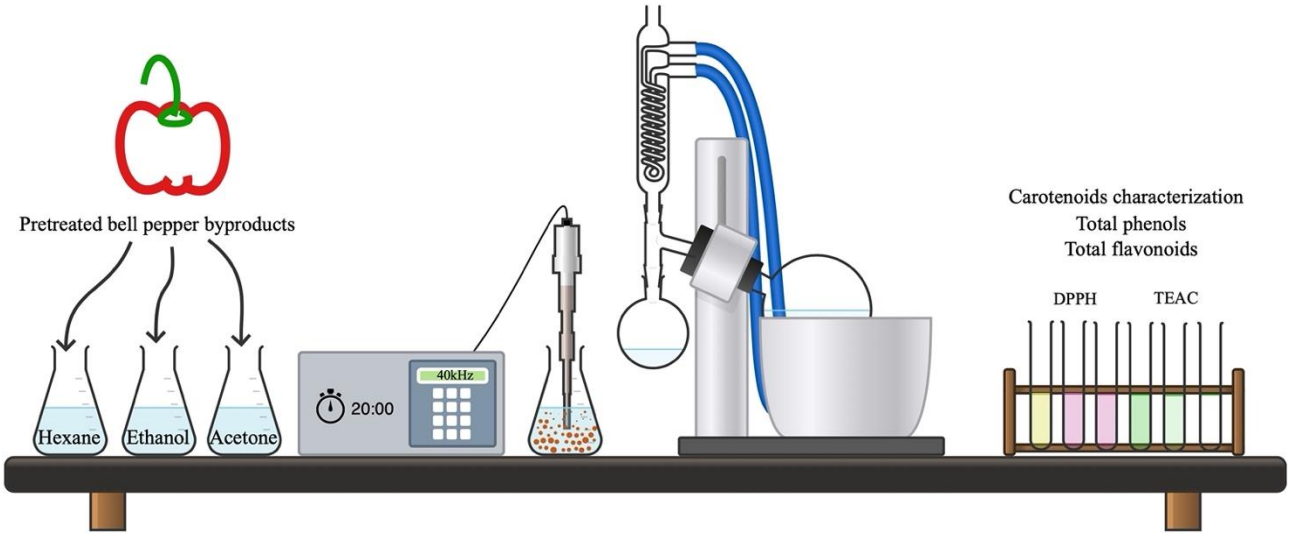

Figure 1. Diagram of bell pepper by-products processing.

Obtaining extracts

Solvent extraction

Fifty grams of processed bell pepper was used for every 100 mL of solvent in a 1:2 W/V ratio. The extraction was carried out in one stage for six h at 50 °C on a hot plate with constant stirring. The extract was separated from the solid material by settling/filtration and then stored in amber glass bottles in a dark place following the methodology proposed by Cardona et al. (2006).

Solvent extraction assisted by ultrasonic pulses

The same methodology was followed, but pulse assistance was given during the warm-up time: pulses with an amplitude of 40 kHz at two different times of 20 and 40 min. The extracts were then placed on a hot plate with constant stirring. The extract was separated from the solid material by settling/filtration and then stored in amber glass bottles in a dark place following the methodology proposed by Cardona et al. (2006) with some modifications.

Obtaining oleoresin

The extracts obtained by the methods above were subjected to a solvent reduction process using a Büchi brand R-215 rotary evaporator coupled with a vacuum pump at a temperature below 40 °C to avoid the loss of carotenoids due to oxidation. The oleoresin obtained was stored in a cool place and in an amber container to protect it from light and other factors that may cause denaturation and loss of antioxidant capacity.

Physical-chemical analysis of the raw material

The physicochemical characteristics were determined following the methodology of the AOAC, 2005, to analyze the pH, moisture, ashes, lipids, and proteins.

pH

The bell pepper (*Capsicum annuum*) was liquefied with distilled water 1:1 P/V to obtain an aqueous solution. The HANNA Instruments brand potentiometer model HI 2211 pH/ORP Meter was calibrated to carry out the analysis (AOAC, 2005).

Moisture content

A Memmert atmospheric pressure oven was used. The crucibles were washed and weighed and then placed in the oven at a temperature of 120°C for two h; the crucibles were weighed and recorded. With the help of an analytical balance, 3-4 g of sample was weighed and placed in the crucibles. Subsequently, they were placed in the oven at 110°C for one h. After this time, the crucibles were removed to room temperature. The dried sample was left to reach a constant weight, and the final weight was registered (925.10 AOAC, 2005).

Ash content

*Dried samples (*1-2 g) were placed in the crucibles and into the muffle. The temperature in the muffle was set to 550 °C for two hours. The weight of the samples was registered, and the inorganic matter was reported as the ash content (940.26 AOAC, 2005).

Lipid content

For the determination of lipids, Soxhlet extraction equipment was used. This method is based on continuously extracting all solvent-soluble substances from the dried bell pepper sample. Approximately 3 to 4 g of dry sample was accurately weighed and transferred to an extraction cartridge, covered with cotton, and placed in the extractor of the apparatus whose flask had already been brought to constant weight. The solvent was added over the cartridge until it siphoned two times. The extraction time was between 4 and 8 hours. After the extraction, it is removed from the heat to recover the solvent; the solvent is taken to the rotary evaporator to eliminate as much of the solvent as possible. While the sample is cooled down at room temperature for 15 minutes, it is placed in an oven with an air current at 80 °C for 30 min, cooled in the desiccator, and weighed to determine the percentage of lipids (963.15 AOAC, 2005).

Protein content

The protein determination of bell pepper (*Capsicum annuum*) was obtained by the Microkjeldahl (1883) method. The method consists of 3 stages, digestion, distillation, and titration; this methodology determines the amount of nitrogen related to the protein content (955.04D AOAC, 2005).

Determination of antioxidant activity

The oleoresins were evaporated in a vacuum rotary evaporator and resuspended in 80% methanol. The new methanolic extracts were diluted 1:20 and used to carry out the reactions of total phenols and flavonoids.

Determination of antioxidant capacity by TEAC

The ABTS radical scavenging capacity of the extracts was assessed by the method of Re [20]. A stock solution of the ABTS radical was prepared and kept in the dark at room temperature for 18 h. The stock solution was diluted with phosphate buffer to prepare the working solution of the ABTS radical with an absorbance of 0.70 ± 0.02 at 734 nm. For this analysis, an aliquot of the sample was mixed with 280 µL of the ABTS radical working solution, which was incubated for 30 min at room temperature in the dark, and then the absorbance was measured at 734 nm by using a microplate reader (Thermo Scientific Multiskan Sky; USA). A standard curve of Trolox was used to express the results as Trolox equivalents/mg sample (TE/g). Six replicates were carried out for each sample.

Determination of antioxidant capacity by DPPH

A 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was performed as reported by Dewanto et al. (2002). The vials of each of the samples were weighed and diluted in 1 mL of DMSO to obtain stock solutions, from which 100 µL was taken and diluted in 900 µL to obtain the first dilution. From this

dilution, 100 µL was taken and diluted in 900 µL to obtain the second dilution. To prepare the DPPH solution, 1.23 mg of reagent was weighed in a previously tared volumetric flask and dissolved in 25 mL of DMSO to obtain a 125 µM solution, which was stirred in a sonicator.

Samples and standards were prepared in triplicate following the methodology (Dewanto et al., 2002). The antioxidant capacity was expressed as µmol equivalents of Trolox/g of lyophilized extract (µmol of TE/g of LE).

Determination of Total Phenols

All extracts' total phenolic content (TPC) was determined spectrophotometrically using the Folin-Ciocalteu method (Singleton et al., 1999). The phenolic content was expressed as mg of gallic acid equivalents per 100 g of lyophilized extract (mg GAE/100 g LE).

Determination of total flavonoids

The total flavonoid content (TFC) was determined using a colorimetric method (Zhishen et al., 1999); (Dewanto et al., 2002). The results were expressed as mg of (+) – quercetin equivalents per 100 g of lyophilized extract (mg EC/100 gLE).

RESULTS AND DISCUSSION

Physicochemical analysis

The results of the characterization of red bell pepper (*Caspsicum annuum*) pulp are shown in Table 1. The pH values are similar to those Carranza (2009) described, with a pH of 5.3. There was no significant difference in the values of the proximal analyses (moisture, ash, lipids, and proteins) carried out in the present study compared to previous studies carried out by Rincón (2017) and Carranza (2009).

Table 1. Proximal composition of the bell pepper pulp.

Proximal composition	Present study	Bell pepper (Red, yellow, orange)*	Bell pepper (Red)**
pH	5.3 ± 0.34	5.2	5.3
Humidity	92.42 ± 0.61	92.1	92.3
Ash	8.19 ± 1.33	9.01	8.1
Lipids	0.74 ± 0.61	0.19	1.3
Proteins	1.04 ± 0.04	0.89	1.2

The results are presented in %,
Results are mean of 3 replicates ± standard deviation
* (Rincón-Alvarez, 2017)
** (Carranza-Rodriguez, 2009)

Antioxidant activity

Table 2 shows the results obtained from the determination of antioxidant activity equivalent to Trolox (TEAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), total phenols (TPC), and total flavonoids (TFC). The values are presented as the average of 3 replicates ± standard deviations and have been expressed in μM equivalents to Trolox for TEAC and DPPH and in Mg/ml for TPC and TFC.

The highest values for the TEAC test were obtained by the treatments where the solvents acetone and ethanol were used without the assistance of ultrasonic pulses, and the lowest value was obtained by hexane.

Table 2. Antioxidant activity.

Treatment	TEAC*	DPPH*	TPC**	TFC***
Acetone	6864.1 ± 11.6	3340.56 ± 10.48	1.29 ± 0.11	2.36 ± 0.51
Acetone/Pulses 20 min	6223.7 ± 3.16	2812.39 ± 6.64	1.73 ± 0.19	2.57 ± 0.07
Acetone/Pulses 40 min	6711.2 ± 1.59	3810.96 ± 19.29	1.45 ± 0.02	2.8 ± 0.05
Ethanol	14169.3 ± 7.45	5131.39 ± 7.50	2.34 ± 0.18	3.26 ± 0.34
Ethanol/Pulses 20 min	18875 ± 3.87	7475.15 ± 3.27	3.59 ± 0.18	4.31 ± 0.03
Ethanol/Pulses 40 min	17343.2 ± 1.98	6171.23 ± 13.80	2.84 ± 0.14	3.27 ± 0.03
Hexane	1093.2 ± 0.98	83.17 ± 64.54	0.69 ± 0.04	1.43 ± 0.08
Hexane/Pulses 20 min	903.3 ± 1.36	-	0.65 ± 0.09	1.26 ± 0.04
Hexane/Pulses 40 min	938.9 ± 6.54	-	0.44 ± 0.04	1.56 ± 0.02

Results are presented in μM TROLOX equivalents/g for TEAC and DPPH; for Total Phenols (TPC) and Total Flavonoids (TFC) were expressed in Mg/ml. * Abs 734 nm, ** Abs 765 nm, *** Abs 415 nm. Results are mean of 3 replicates ± Standard Deviation

When subjected to the assistance of ultrasonic pulses, the lowest times are those obtained by the solvents acetone and hexane at 20 min of treatment, compared to treatments without pulse assistance. Likewise, the highest values obtained when the treatments are submitted to the assistance with ultrasonic pulses are those obtained by acetone and hexane at 40 min of treatment, the lowest being the sample with ethanol at 40 min.

The results corresponding to the DPPH test showed that the highest value was obtained by treatment with the ethanol solvent with 20 min of pulse assistance, followed by acetone with 40 min of pulse assistance.

When not subjected to the assistance of ultrasonic pulses, the values obtained below the treatments with assistance were obtained by acetone followed by ethanol and hexane.

The results obtained for total phenols show a similar behavior in the treatments with solvents assisted by ultrasonic pulses in the DPPH test. The ethanol solvent with 20-min pulses had the highest value, followed by acetone with 20-min pulse assistance. under hexane with pulse assistance.

The values obtained by the solvents without the assistance of pulses were lower in the solvents acetone and ethanol, and only the hexane without treatment did not show a significant difference from the hexane assisted with pulses for 20 min.

Regarding the total flavonoids, they show the highest values for the treatments assisted with ultrasonic pulses, being the solvents with the highest values, ethanol with 20 min of assistance followed by acetone with 40 min of assistance and the lowest value obtained by hexane. with 40 min of assistance.

On the other hand, the lowest values are obtained by the treatments without the assistance of ultrasonic pulses, the lowest being the one obtained by the treatment with acetone as a solvent, the others showed us a significant difference with the minimum values for the assistance with pulses.

Under the conditions of the present study, it was possible to obtain carotenoids from red bell pepper (*Capsicum annuum L.*) byproducts by assisting with ultrasonic pulses.

It is possible to affirm that the use of this emerging technology, such as ultrasonic pulses as an assistant for the extraction of bioactive compounds such as carotenoids, has particular effectiveness depending on the solvent in enhancing their antioxidant properties since by generating membrane rupture, it helps to release almost in its entirety the compound of interest, thus generating a greater extraction.

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Chapter 5

Intracellular Holosporaceae pathogen intensifies the susceptibility of shrimp (*Litopenaeus vannamei)* to the white spot syndrome virus (WSSV): a preliminary approach

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ABSTRACT

The susceptibility to white spot syndrome virus (WSSV) in the white shrimp *Litopenaeus vannamei* previously infected with an intracellular Holosporaceae pathogen known as necrotizing hepatopancreatitis bacteria (NHPB) was evaluated. Coinfected shrimp mortality was recorded over 20 days and compared with those infected with WSSV or NHPB alone. NHPB-WSSV-co-infected shrimp reached 100% mortality after 15 days of challenge, whereas shrimp infected with WSSV alone reached the same mortality level after 19 days. Control shrimp and those infected only with NHPB did not show mortality during the trial. These results suggest that NHPB may not be an aggressive pathogen causing mortality but can trigger the susceptibility of shrimp to WSSV.

Keywords: Coinfection; White spot syndrome virus; Necrotizing hepatopancreatitis bacterium; Holosporaceae

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INTRODUCTION

Infectious diseases are still one of the major challenges for shrimp aquaculture in the 2020 decade (Gallaga-Maldonado et al., 2020; Patil et al., 2021). Stressful conditions make aquatic organisms prone to diseases; in this regard, various biotic and abiotic factors may favor opportunistic infections.

In shrimp, intracellular pathogens are considered opportunistic agents that may go unnoticed for long periods unless molecular detection tests are used (Vincent; Lotz, 2005). The necrotizing hepatopancreatitis bacterium (NHPB) is perhaps the most important intracellular pathogen for penaeid shrimp. This bacterium was formerly considered a rickettsia-like pathogen due to its intracellular nature (Gollas-Galván et al., 2014); however, recent evidence derived from multilocus sequence analysis demonstrated that the intracellular agent belonged to the Holosporaceae family, similar to some species considered pathogens of other arthropods (Leyva et al., 2018).

The NHPB resides and multiplies exclusively in the tubular epithelial cells of penaeids but commonly does not cause high mortality rates except in extreme cases involving highly stressful conditions, and the first dead organisms could be detected as long as three weeks after the initial infection (Ávila-Villa et al., 2012; Figueroa-Pizano et al., 2014). Moreover, the immune system is highly activated weeks postinfection; for instance, Figueroa-Pizano et al. (2014) reported the participation of the proPO system and the clotting reaction against NHPB, mostly on days ^{12} and 18 postinfection. The evidence suggests a prolonged latent phase of this pathogen that could go unnoticed in nonacute phases. However, this pathogen has physiological implications in shrimp, including immunodepression, leading to susceptibility to other pathogens or coinfections.

Coinfection involves the simultaneous infection of a host by multiple pathogen species; in this regard, the primary infection reduces the immune resistance, leading to second or more concurrent infections and aggravating the scenario for shrimp health. White spot syndrome virus, the most severe virus for white shrimp, is perhaps the most common in coinfections with other viruses or bacteria (Macías-Rodríguez et al., 2014; Pang et al., 2019; Rubio-Castro et al., 2016). In this sense, the problem with the late detection of NHPB due to its intracellular nature and its prolonged latent phase, as well as the greater susceptibility of shrimp to be coinfected with WSSV, is a latent risk; however, to date, there is no evidence of concurrent infections between these two pathogens, and one of the reasons is that NHPB is still an unculturable bacterium. Therefore, this work evaluated the survival of shrimp (*L. vannamei*) previously infected with necrotizing hepatopancreatitis bacteria (NHPB) to white spot syndrome virus (WSSV).

MATERIALS AND METHODS

Animals.

The experimental approach was carried out with healthy adult shrimp (*Litopenaeus vannamei*) weighing 25-30 g, acclimated to optimal conditions in artificial seawater with salinity at 30 PSU,

temperature 28 °C, constant aeration, photoperiod 12:12, and 10% water change per day. Shrimp were fed *ad libitum* twice a day with commercial food pellets (Camaronina, Purina™) containing 35% crude protein. Aquariums were cleaned daily to remove feces, food debris, and dead organisms.

Preparation of NHPB inoculum.

Because the Holosporaceae bacterium NHPB is unculturable, the inoculum consisted of a homogenate of hepatopancreatic tissue from shrimp positive for NHPB and negative for WSSV, IHHNV, TSV, and *Vibrio parahemolyticus* after employing molecular detection through PCR. The dissected hepatopancreas was homogenized and suspended in 0.9% saline solution with NaCl in pyrogen-free water.

Genomic DNA was extracted from the hepatopancreas using the Gene Clean commercial kit (BioQ® Inc., 1101-601) following the manufacturer's specifications. The presence of NHPB was determined by PCR through amplification of the ribosomal subunit 16S gene-specific for NHPB using the primers forward: 5´ - CGT TGG AGG TTC GTC CTT CAG T - 3´ and reverse: 5´- GCC ATG AGG ACC TGA CAT CAT C - 3´ (Nunan et al., 2008). Reactions were performed in 25 µL using a gradient Px2 Thermal Cycler (Thermo, USA) under the following conditions: Step 1: 95 °C/2 min; Step 2: 25 cycles of 95 °C/30 sec, 60 °C/30 sec and 70 °C/30 sec; Step 3: 60 °C/1 min and 72 °C in 2 min. The PCR products were visualized in agarose gel (1.2%) under UV light using the KODAK Imaging System 4.0. Samples positive for NHP-B were homogenized at 4 °C, and 300 µL of glycerol was added and stored at -20 °C until use as inoculum.

Preparation of viral inoculum

Virions of the white spot syndrome virus (WSSV) were obtained from the muscle of experimentally infected shrimp, according to Gracia-Valenzuela et al. (2009). One gram of tissue was diluted in 4 mL of 150 mM nuclease-free sterile saline solution, homogenized, centrifuged at 3000 x g, and filtered through 0.45 µm (MF-Millipore Membrane Filters, Millipore™), followed by 0.22 µm. The filtered solution was placed in a 100 kDa cutoff microfilter (Amicon Ultra15, MIllipore™) and centrifuged at 1000 × g for 30 min at 4 °C. The microfilter material retained and containing WSSV virions was recovered and placed in 1 mL of 150 mM saline, sterile, and nuclease-free solution. The filtered suspension was stored without additional buffer at -20 °C and used as viral inoculum. The presence of WSSV in the filtered suspension was confirmed by PCR based on Gracia-Valenzuela et al. (2009).

The quantification of WSSV virions was estimated by following the instructions of the IQREAL kit in a 15 µL volume reaction. The quantification was determined by calibration curve construction using 10^1, 10^2, 10^3, 10^4, and 10^5 WSSV copy/µL solutions included in the i-screen kit (GeneReach™ Biotechnol Corp., Taiwan) based on the method of Nunan et al. (2004).

Experimental infections

Eighty shrimp were infected with NHPB by oral supplementation with infective inoculum. Forty other shrimp were inoculated with NHPB-free hepatopancreas. NHPB was detected in the feces and hepatopancreas of randomly sampled shrimp to confirm the infection, while no detection was expected for the control shrimp. Thirty NHPB-positive shrimp were selected for the NHPB group, 30 for the NHPB-WSSV coinfection group, and 30 of the NHPB-negative shrimp for the NHPB control.

Infection with WSSV was performed via intramuscular injection to the 30 shrimp of the NHPB-WSSV coinfection group and an additional 30 of the WSSV group. A WSSV-free control group of shrimp was also injected with saline solution. Each group was stocked in a 60 L aquarium. Finally, the survival was evaluated for each group, and WSSV and NHPB were monitored in each dead and living shrimp at the end of the trial.

RESULTS AND DISCUSSION

Except for the control group, all shrimp were positive for their corresponding infection and coinfection (Figure 1). No mortalities were detected for any of the control groups (NHPB-free and WSSV-free) or the NHPB-infected group; however, mortalities were recorded in the WSSV-NHPB coinfection and WSSV infection groups.

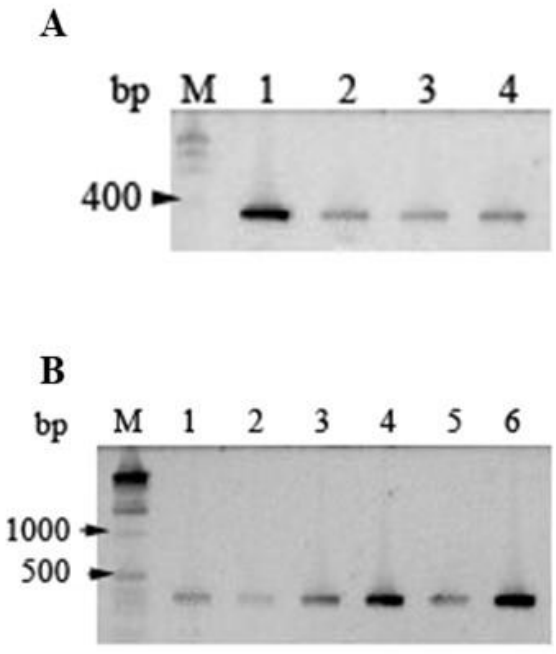

Figure 1. A. Electrophoresis gel with PCR products for the detection of NHP-B in shrimp. Lane M, molecular mass marker. Lane 1, control positive of NHPB. Lanes 2 to 4, hepatopancreas of shrimp samples fed with infective NHPB inoculum. **B.** Electrophoresis gel with PCR products to detect WSSV in shrimp. Lane M, molecular mass marker. Lanes 1 to 5, shrimp inoculated with WSSV. Lane 7, positive control of WSSV. bp= DNA base pairs.

Survival started to decline on the 9th day of the trial; however, mortality occurrence was more pronounced in coinfected shrimp, reaching 100% on the 15th day, whereas 100% mortality was detected on the 19th day in shrimp infected with WSSV alone (Figure 2).

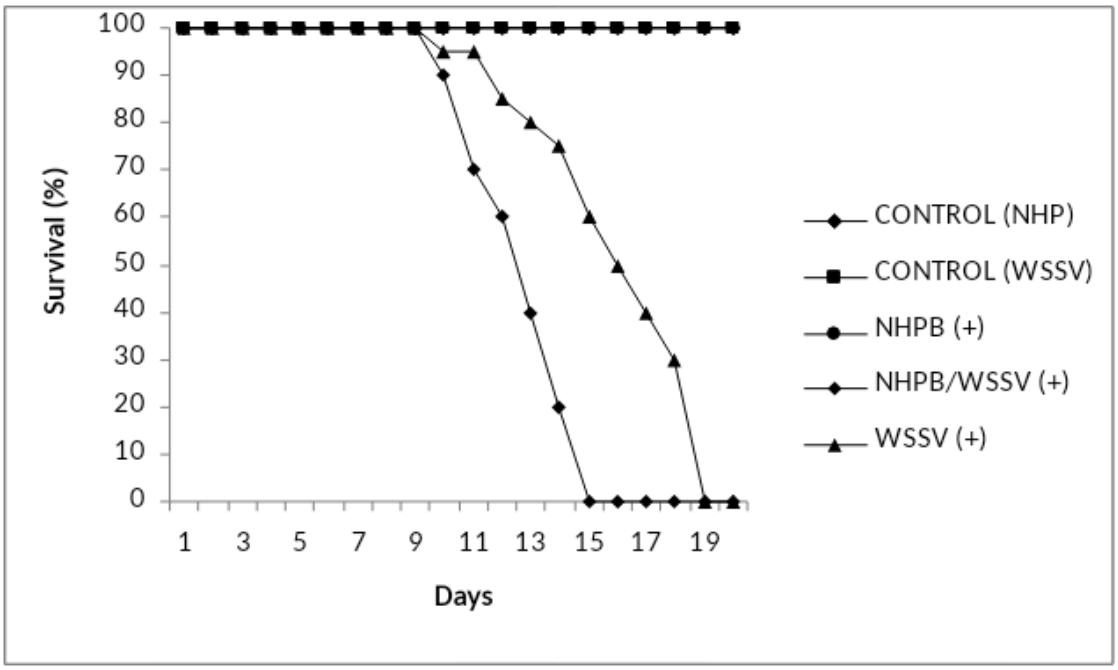

Figure 2. Mortality curves for experimentally infected shrimp with NHPB, WSSV, and NHPB-WSSV. Two controls free of NHPB and WSSV were also considered.

Coinfection of individual hosts by multiple species is a typical pattern in nature and could be even more common than usual in aquaculture given the high stocking densities favoring all kinds of pathogen transmissions.

The results suggest that although NHPB may not cause mortality during a considerable period, this intracellular pathogen can accentuate the mortality caused by WSSV. Previous evidence has documented hepatopancreatic malfunction when infected with NHPB, leading to physiological and behavioral consequences derived from tissue necrosis (Figueroa-Pizano et al., 2014). In this regard, WSSV infection alone was severe, leading to 100% mortality in 19 days; however, the presence of NHPB accelerated the process.

Taking this evidence together with previous reports (Ávila-Villa et al., 2012; Martínez-Córdova et al., 2016), we can conclude that NHPB infections may go unnoticed for long periods but pose a risk of causing severe mortalities if another viral pathogen establishes a clinical coinfection. Although no mortalities were observed in the NHPB group, the infection alone can cause severe mortalities after long periods if no antibiotics are used (Martínez-Córdova et al., 2016), causing chronic detriment that can lead to disastrous results for a farm. Whether this preliminary approach evaluating the shrimp survival

response is evaluated for the first time suggests the severity of NHPB-WSSV coinfection and highlights the relevance of studying coinfections that constitute a realistic scenario in aquaculture.

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Chapter 6

Yellow head syndrome virus, a latent problematic for western aquaculture. A review

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ABSTRACT

In shrimp culture around the world, health is an important issue. Being the viruses the main factor in America in terms of mortalities in shrimp production. Among which the White Spot Syndrome Virus (WSSV), Taura Syndrome Virus (TSV), Infectious Hypodermal and Hematopoyetic Necrosis Virus (IHHNV), Penaeus Vannamei Nodavirus (PVNV) or even Yellow Head Virus (YHV) stand out. According to reports issued by the epizootic international organization (OIE), these are one of the main lines of research in many institutions. With YHV being one of the most important since it is one of the most aggressive pathogens in Thai aquaculture, becoming a threat to aquatic production systems worldwide. Nevertheless, advances in molecular biology, handling skills and sanitary pursuit of the units of aquatic production. Allow us to understand the strategies of replication, infection and distribution of the same unit. The purpose of this review is to summarize the progress of research on the YHV complex and its genotypes and distribution in wild and cultured shrimp around the world. In addition, different methods are used for its detection (presumptive and confirmatory analysis) and strategies against YHV.

Keywords: Shrimp, Virus, YHV.

INTRODUCTION

In aquatic activity, mainly shrimp farming, the spread of viral diseases is one of the most important problems to address since viruses represent one of the most abundant pathogens in the ocean. At present, up to 20 different attacking-peneid viruses have been identified, including White Spot

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Syndrome Virus (WSSV), Taura Syndrome Virus (TSV), Infectious Hypodermal Hematopoyetic Necrosis Virus (IHHNV) and Yellow Head Syndrome Virus (YHV) (Lightner et al., 2012).

Despite efforts such as biosecurity and better management practices in production systems carried out in aquaculture, the dispersion of pathogens (mainly viral) is a reality and one of the main problems in terms of health or performance reduction, since the rapid spread of pathogens is due to activities such as recreational and commercial fishing, as well as the wide range of existing hosts in these community environments. Among the pathogens of importance for shrimp culture are YHV, which has been submitted mainly in Asian countries. In 1992, it was first detected in black tiger shrimp *(Penaeus monodon)* and caused large economic losses in Thailand and Vietnam. In 1995, this virus caused losses of 5000 tons, which represents up to 40 million dollars in the Indo-Pacific region. YHV is closely related to gill-associated virus (GAV), which has caused havoc in *P. monodon* culture systems in Australia since 1996 and has been shown to be a chronic infection of wild organisms of eastern Australia.

Since 2012, YHV has been found on the sanitary code list for aquatic animals by the World Animal Health Organization (the OIE–Office International des Epizooties). Currently, YHV has been found in other Asian countries, such as Taiwan, India, China, the Philippines, Indonesia, Sri Lanka and Malaysia. In Australia, India, Indonesia, Malaysia, Mozambique, the Philippines, Taiwan, Thailand and Vietnam, GAV has been detected, and other genotypes of YHV (YHV complex) have been detected in apparently healthy *P. monodon* (Walker et al., 2001).

YHV in America is considered an exotic disease, and at present, its detection has not been confirmed by pertinent institutions. Despite this in 1995, there was one suspect of YHV in Texas (USA) in a WSSV co-infection, and later between 1995-2000, it was detected together with WSSV in frozen shrimp for importation. Subsequently, the presence of YHV was suspected in Central America between 1999-2000 and in Ecuador from 2000-2001. Nonetheless, Alday (2000) refers to the presence of YHV in a non-YHV-associated pathology, since the sequences of the amplification with five sets of primers allowed the identification of all the complexes of YHV, and only one obtained positive results for YHV.

In Mexico, there are reports of this disease in wild organisms of *Penaeus stilirostrys* and cultured *Penaeus vannamei* in the country's northeast coasts, which, despite not being official reports emitted by OIE, create uncertainty in the productive sector. However, the majority of America, eastern Africa and some specific zones from eastern and southeastern Asia can be considered YHV free, according to established rules by OIE (Lightner et al., 2012).

Description of the etiologic agent of YHD

Yellow head disease (YHD) is caused by an envelope; bacilliform singled stranded positive RNA virus (40-60 nm x 150-200 nm) with a grooved nucleocapsid. This virus contains three important structural proteins: two glycoproteins of the coat, gp64 (63-67 kDa) and gp116 (110-135 kDa), and the viral nucleocapsid protein p20 (20-22 kDa) (Stentiford et al., 2009).

YHV is classified within the genus *Okavirus*, from the *Roniviridae* family, inside of the order *Nidovirals* (Liu et al., 2009); it is known that *Toro*, *Arteri* and *Coronavirus* are related (Jitrapakdee et al., 2003). RNA viruses have developed a wide range of strategies to express their genes, and a very recurrent strategy is the synthesis of subgenomic RNAs (sgmRNAs) (Miller and Koev, 2000). Nidovirals such as *Toro, Arteri, Coronavirus* and *Okavirus* differ evidently in genome size, architecture of the virion and host ranges, but their common ancestor is evident due to the identity of sequences in their replicase proteins and the similitude of the organization of their genomes, the order of the genes and the strategy of replication (Cowley et al., 2000).

In the 5´ end genome of all Nidovirus, over two-thirds of the genome is used for the translation of the polyprotein replicase gene (ORF1a gene); downstream, a small gene group is found, which expresses a cluster of sgmRNAm, and all keep at the 5´ end a leader sequence that is derived from 5´ end genomic RNA and fused to the body of the transcript. Okavirus shows discontinuous transcription similar to *Torovirus*, unlike *Arteri* and *Coronavirus* (Vliet et al., 2002).

On YHV structural proteins, it has been confirmed that in virions, only the gp116 and gp64 proteins are glycosylated, unlike the p20 nucleoprotein (Soowannayan et al., 2010). Jitrakpadee et al. (2003) reported that the proteins gp116 and gp64 are encoded inside ORF 3 of the genome of YHV, which encodes the pp3 polyprotein of 1666 amino acids (aa), which contains six hydrophobic regions and undergoes posttranslational cleavage to produce a polypeptide of 228 aa of unknown function, as well as the envelope proteins gp116 and gp64 of 899 aa and 539 aa, respectively.

Afterward, Soowannayan et al. (2010) described that gp116 forms prominent projections on the coat surface of the mature virions of YHV. However, both gp116 and gp64 proteins are suspected to play a crucial role in host cell entrance. YHV infection in cells of primary cultures of lymphoid organs can be neutralized by antibodies to the gp116 glycoprotein coat but not gp64 antibodies (Assavalapsakul et al., 2005). It has been shown that a deletion of 162 nucleotides that correspond to 54 aa in the Ratchaburi/2006 chain in comparison with the Chachoengsao/1998 chain indicates a loss of six cystein-conserved residues, which triggers a deformation in gp116, which, despite reducing the incorporation of virions inside the cell and eliminating the main sites of neutralization, keeps the virus highly infectious, virulent and able to spread (Sittidilokratna et al., 2008).

Gangnonngiw et al. (2009) reported a kind of recombinant, atypical YHV (A-YHV) that is non virulent in addition to the others already evidenced YHV-1 and YHV-2, and such virulence is more associated with the ORF1b sequence than with the ORF3 sequence despite the large deletion of the last one. The YHV ORF2 gene encodes a basic protein with 146 aa that shows a high level of identity (84.9%) with the GAV nucleoprotein and is homologous to the GAV ORF2 gene. It is clear that this gene is equivalent to the non glycosylated YHV structural protein. On the other hand, a reactive monoclonal antibody for YHV p20 has recently presented binding to the nucleocapsid of YHV virions. The finding that the ORF2 gene encodes the protein N made it possible to distinguish between Okavirus crustaceans

and vertebrates Nidovirus, in which the N protein gene resides in the 3' end region downstream of the genes that encode the glycoproteins and membrane proteins of the virion, while the Okavirus encoding the N protein is upstream of glycoproteins (Cowley et al., 2004). The unusual location of the gene nucleoproteins YHV and GAV could be a consequence of genetic recombination in ancestral Nidovirus, since the high frequency of genetic recombination occurs in virus (+) ssRNA, and it was also proposed that the structural diversity and Nidovirus morphology is due to modular evolution, resulting in an exchange process of complete gene recombination or gene sets (Sittidilokratna et al., 2006).

Recently, Thapanan (2014) reported that the expression of Pm clathrin AP17 in shrimp promotes the entry of virions into cells via endocytosis, increasing the speed of propagation of YHV, and a treatment based on chlorpromazine can inhibit viral replication of YHV in early stages. There are reports of proteins that act to the host benefit, supporting the dispersion of granular hemocytes, the same ones that act as the first line of defense of organisms against YHV (Havanapan, 2016).

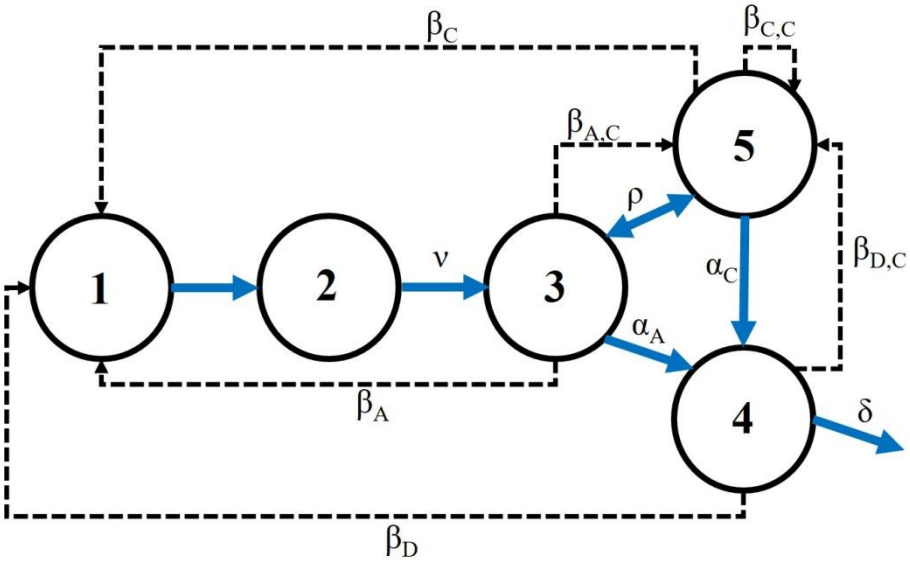

Figure 1. Life cycle graph of the YHV complex in *shrimp P. monodon*. 1) susceptible shrimp, 2) shrimp carrying YHV before becoming apparent signs, 3) shrimp with acute YHV infection, 4) dead shrimp, 5) shrimp with chronic YHV infection. β_D) Transmission coefficient from dead infected - susceptible shrimp, β_A) transmission coefficient from acute infection - susceptible shrimp, β_C)transmission coefficient from chronic infection – susceptible shrimp, $\beta_{D,C}$) transmission coefficient from dead infected carcass - chronic shrimp, $\beta_{A,C}$) transmission coefficient from acute infection - chronic shrimp, $\beta_{C,C}$)transmission coefficient from chronic infection - chronic shrimp, ν) the infection coefficient, ρ)recovery coefficient to an acute to a chronic infection, α_A)mortality rate of an acute infection, α_C) mortality rate of a chronic infection and δ) decay coefficient of dead infected shrimp. Redrawn from Lotz, et al., 2005.

Yellow Head Virus-complex Life Cycle

Experimentally, YHV complexes have been transmitted by *per os* with infected carcasses, dip in contaminated water (Walker et al., 2001), direct injection for research issues and cohabitation with infected organisms (Soowanayan, 2015). Lotz et al. (2005) proposed a hypothetical life cycle for the YHV complex (Figure 1), where the chronic stage is more prominent (4, β_C, $\beta_{D,C}$, $\beta_{A,C}$, $\beta_{C,C}$ and α_C); in

asymptomatic chronic infections, (4) turns back to acute infections (3 and ρ) upon re-exposure, inducing increased complexity. This is a peculiarity of the YHV complex, where the kind of infected hosts that might serve as the source of exposures that would introduce a reversion remains unknown, and none of the parameters in this life cycle of YHV have been estimated. Cowley et al. (2002) reported that the YHV complex occurs by vertical transmission in the wild and in hatcheries, and this transmission develops from mother to offspring because the virions bind to the surface of the egg. Additionally, viruses were detected in the complex (e.g., GAV) in the spermatophore from infected male *P. monodon*, suggesting contamination by the male. Therefore, it has been shown that the complex YHV transmits vertically through contamination of the surface of the fertilized egg.

Transmission routes and host range

Pond water (transport and intake water) that carries YHV is the primary abiotic vector with fast dissemination and transmission to susceptible shrimp and by cannibalism of infected or moribund shrimp. Transmission of the YHV complex can occur by bringing infected but apparently healthy host crustaceans into offspring ponds and its introduction to a naive system through the use of different sources of infection, such as nets, feeding trays and other pond equipment.

Although the YHV complex can remain viable for up to 72 hours in salt water, it has been documented that YHV1 of these complexes can be inactivated by heating (60 °C/15 min) and exposing it to different solutions (chlorine 30 ppm, NaOH 2%/10 min, calcium hypochlorite 30 ppm, iodine compounds 250 ppm/30 min and formalin 3%/10 min). There are no reports of YHV-complex survival using other chemical compounds, but it is likely that these viruses are sensitive to oxidizing agents, SDS, nonionic detergents and lipid solvents (Stentiford et al., 2009).

Previous studies of experimentally infected *P. monodon* with YHV exposed frequent co-infections with secondary opportunistic pathogens such as bacteria and other viruses such as Hepatopancreatic Parvovirus (HPV), WSSV (Wang and Chang, 2000) and Monodon Baculovirus (MBV), playing an important role in mortalities caused by YHV and not for opportunistic pathogens (Stentiford et al., 2009). In these studies, on YHV, as for other shrimp viral pathogens, the infectious doses documented are tissue-purified volumes due to the lack of available crustacean cell lines.

Within YHV, the main hosts are *P. monodon* black tiger shrimp from Asia; however, infections have occurred in *Penaeus japonicus*. On the other hand, being YHV a closely related virus to GAV, the susceptibility of a species to this last one is very related to which presents YHV in the same species (Spann et al., 2000) likewise *Farfantepenaeus merguiensis* and *Penaeus esculentus* are susceptible to the infection and the development of the GAV disease and it is reported that *F. merguiensis* from Thailand has been experimentally infected with YHV and has been maintained as a carrier of latent infection in culture systems (Flegel, 2006). In the same way, *P. vannamei* and *P. stylirostris* are not known as natural hosts, but they are susceptible to infection by injection or feeding with YHV-infected tissue (Lu et al., 1994).

In *P. vannamei,* there are reports that show the presence of shrimp with signs of the disease, such as loss of body color and mortalities of 60-70%, in farms of the central part of Thailand, where it is believed that the origin of these outbreaks comes from a natural reservoir, and in this species, economic losses of up to 3 million dollars have been reported between 2007 and 2008 (Senapin et al., 2010). YHD has caused economic losses estimated at 500 million dollars since its discovery until 2006 (Ligthner, 2007). In other palaemonids, such as *Palaemonetes pugio*, under some conditions, this serves as a reservoir of the virus, while blue crab serves only as a carrier (Ma et al., 2010). In addition, *Metapenaeus affinis* was more susceptible to intramuscular injection and feeding than *Metapenaeus brevicornis*; nonetheless, both species of shrimp are considered carriers that can survive for long periods of time after infection, unlike some species of crabs, in which intramuscular infection by feeding does not proliferate, contrary to what happens with WSSV in these very organisms (Longyant et al., 2006). Currently, the red claw crayfish *Cherax quadricarinatus* is reported as the ideal carrier for YHV because it is susceptible, highly tolerant and transmits this virus without showing any signs of YHD (Soowannayan et al., 2015).

Nomenclature and Taxonomy of YHV

Initially, YHV was classified as a *Baculovirus* by Limsuwan in 1991, and it had a DNA core since its cytoplasmic nature, development, size and morphology were suggested. In addition to being known as a granular yellow head baculovirus, until that moment, it was not a new virus in Thailand but one that had been recently accepted by a dramatic increase in occurrence and severity, with the possible causes being an increase in reservoirs of the virus due to an increase in the activity or intensity of culture farms. Later, Wongteerasupaya et al., 1995 mention that YHV is an RNA virus, similar to *Rhabdovirus* or *Coronavirus*, which was provisionally classified as a *Rhabdovirus*. Currently, YHV is classified inside the order of *Nidovirals* that are mostly recognized for infecting mammals (*Coronavirus*, *Torovirus* and *Arterivirus*) but also some infect birds (*Coronavirus*) or invertebrates (*Ronivirus*).

Nidoviruses cause a large variety of diseases, and they can vary from asymptomatic, persistent and carrier to cause fatal infections (Pasternak et al., 2006). Officially since 1996, the genera *Coronavirus*, *Torovirus* and *Arteriviridae* have joined the family *Coronaviridae,* which was initially considered unrelated to YHV, and recently, the family *Roniviridae* was admitted within the domain of invertebrate hosts.

The taxonomy acquires its name of the singular strategy of Nidovirus to express all genes located downward of the replicase gene of a nested cluster of sgmRNA of 3´coterminal (form latin nidus=nest). However, the most important reason for this unification was found in the replicase gene itself, and phylogenetic analysis of the replicase domain, including the RNA dependence of the RNA polimerase (RdRP) and the helicase, grouped different Nidoviruses, indicating that all of them have a common ancestor (Cowley et al., 2000; Pasternak et al., 2006).

YHV is a member of the family *Roniviridae,* and the name of this family is generated in reference to the form of virions that are shown as rod-shaped and gender *Okavirus* comes of the preference that

this YHV presents in the lymphoid organ named OKA because of its principal affectation tissue (Mayo, 2002).

Genotypes of the YHV complex

YHV and GAV viruses are highly complementary in their genomes, although they present considerable differences that have helped to classify them as geographic topotypes or genetic lines (Wijegoonawardane et al., 2008). Recently, it was reported that at least eight different geographic topotypes of YHV were obtained by analyzing sequences of the ORF1b gene. These genetic lines are distributed in most of *P. monodon´*s natural geographic range in Africa, Asia and Australia. Among the assigned geographic topotypes, genotype 1 (YHV1) includes the reference chain of YHV (GenBank AY052786), which is the only agent capable of causing YHD. Several YHV-causing partial sequences in shrimp have been reported in GenBank, and some of them show tiny variations in nucleotides; however, the Thai sequence (THA-00D-11) and three Taiwan viruses (TWN-00-D1, TWN-00-D2 and TWN-00-D3) are equal.

Genotype 2 includes the reference chain of GAV (AF227196), the Australian virus of healthy or affected shrimp with mid-crop mortality syndrome or MCMS (AUS-97-MCMS3, AUS-97-MCMS1 and AUS-97-MCMS2), some Vietnamese viruses of healthy post larvae (VNM-02-H6, VNM-02-H64, VNM-01-H65 and VNM-01-H77) and Thai viruses (THA-03-HA, THA-03-HB, THA-03-HG and THA-04-HK). The third filogenetic group (genotype 3) comprises all healthy breeder viruses from Thailand and Sarawak and other viruses found in healthy post larvae from Vietnam (subgroup formed by VNM-01-H41, VNM-01-H42, and VNM-02-H70).

Genotype 4 includes detected viruses in three batches of healthy post larvae in Nellore India (IND-02-H5, IND-02-H9, and IND-02-H7). Genotype 5 comprises viruses derived from juvenile shrimps from Malaysia (MYS-03-H4) and Thailand (THA-03-SG21) that showed slower growth, a batch of healthy post larvae sampled in the Philippines (PHL-03-H8) and a virus found in *P. vannamei* in Thailand (YHV ORF 1b gene); the relation between these four viruses from genotype 5 was more dispersed than viruses grouped inside the other lineages (Wijegoonawardane et al., 2008). Genotype 6 is tightly related to genotype 2 (GAV) and comprises viruses found from Mozambique (MOZ-04-H6, MOZ-04-H8, MOZ-04-H9, MOZ-04-H11 and MOZ-04-H12).

In 2015, genotype 7 (YHV7) was reported, and it was identified in *P. monodon* breeders with high mortalities collected in 2012 in Queensland Australia ponds, which came initially from the José Bonaparte Gulf. However, the role of the new YHV7 genotype in this episode of the disease is still unknown (Mohr et al., 2015). There are significant differences between the different genotypes of the YHV complex. YHV7 is a virulent pathogen similar to YHV1 that can cause culture mortality a few days after the appearance of the first signs of the disease (Callinan and Jiang, 2003). Genotype 8 (YHV8) was isolated from China in July 2012 and is related to YHV1, both of which form a monophyletic group.

The complete genome of YHV8 is 26,769 nucleotides with three open reading frames (ORFs) (Dong et al., 2017). YHV1 has 26,662 nucleotides distributed in four ORFs, while GAV is smaller and has a 26,235 nucleotide genome due to significant deletions in its intergenic regions, but 79% of its nucleotide sequence is identical to that of YHV1 (Cowley and Walker, 2002; Sittidilokratna et al., 2008). Nevertheless, GAV has an additional ORF that can be expressed in slight infections on shrimp tissues (Cowley *et al.*, 2004, Cowley and Walker, 2008). The analysis of sequences of genotypes 3, 4 and 5 indicates that their genome organization and transcription strategy are more related to GAV than to YHV1 (Wijegoonawardane et al., 2008). The complete genome of YHV8 was described in co-infection with AHPND (Dong et al., 2017), to which no genotype has been assigned. Recently, Li et al. (2018) established the purification and isolation of intact viral particles of genotype 8 of YHV isolated from China that had not been purified, where the author mentions that the rate of centrifugation for obtaining viral particles is decisive for obtaining full virions.

Detection Methods

To diagnose YHD, as in all diseases in aquaculture, certain basic steps and methods must be considered to detect YHV (etiological agent). These include anamnesis, clinical examination (clinical signs), microscopy (optical and electronic), bacteriology, histology, tests based on antibodies, molecular methods, immunological parameters and bioassays, depending on the pathology (Cuéllar-Anjel, 2008).

Specifically, the history of YHD, as a presumptive diagnosis, has been reported as an excessive consumption of food between 50-70 days of juvenile *P. monodon* culture, preceded by an abrupt cessation of feeding and subsequent mortality of 100%. Between these periods, a yellowish coloration of the hepatopancreas and cephalothorax can be observed, both with soft texture (Lightner et al., 2012). Among the microscopic analyses for YHD that are performed is the hemolymph smear, as a preliminary/presumptive diagnosis of this disease. A smear of hemolymph YHD positive reveals hemocytes with pycnotic and karyorrhexic nuclei without the presence of opportunistic bacteria. Another presumptive diagnosis is the histological analysis of shrimp gill tissue showing severe multifocal to diffuse necrosis with denso-basophilic spherical cytoplasmic inclusions (Flegel, 2006), while acute and chronic shrimp (A and C, respectively, Figure 1) add stomach necrosis (Lightner, et al., 1999).

To reinforce the aforementioned presumptive analysis, confirmatory dot blot or ELISAs are performed simultaneously using monoclonal antibodies against the gp116 coat glycoprotein of YHV (scFv). However, the limit of detection for the dot-blot assay is 9 ng of YHV, while with ELISA, it is 45 ng; therefore, the dot-blot/scFv test turned out to be more sensitive, simple and fast to use in the field (Intorasoot et al., 2007).

To date, the Manual of Diagnostic Tests for Aquatic Animals of OIE (2019) suggests 2 molecular methodologies for the detection of YHD: reverse transcription PCR (RT-PCR) and *in situ* hybridization (ISH). Among the RT-PCR analyses, 3 different protocols have been proposed, where the first detects

only the YHV1 genotype without detecting the other genotypes (Wongteerasupaya et al., 1995). Multiple nested RT-PCR is the second protocol that distinguishes YHV1 from YHV2 or GAV (Cowley et al., 2004) and identifies the YHV8 genotype in both stages of the test (Liu et al., 2014) while only detecting the genotype YHV7 in the first stage of RT-PCR (Mohr et al., 2015).

The last RT-PCR protocol is also a nested and multiple tests, which detects the YHV1-YHV7 genotypes without differentiating them (Wijegoonawardane et al., 2008b). For this, the sequence analysis of each RT-PCR product must be performed. It should be mentioned that this protocol, unlike the second, is incapable of detecting the YHV8 genotype. In addition, Khawsak et al. (2008) reported that the primers used in multiple nested RT-PCR protocols do not cross with other viruses, such as WSSV, TSV and IHHNV. Although several ISH methods have been reported for YHD, the OIE proposes the protocol described by Tang et al. (2002), with which YHV1 and YHV2 (GAV) can be identified. The OIE recommends that for these molecular methods, fresh tissues of gills, lymphoid organs and hemolymph should be used.

Currently, a real-time PCR (qPCR) test for YHV is not available in the OIE´s manual. Soowannayan et al. (2013) documented the effect of the antiviral tunicamycin against YHV in *P. monodon*, where they used an RT-qPCR assay to determine YHV copy numbers in hemolymph and hemocytes, without emphasizing the importance of this assay to detect this virus. In contrast, Arseneau and Laflamme (2016) implemented a methodology based on RT-qPCR with a detection limit of 170 copies of YHV, referencing analytical details of their assay. Similarly, in 2016, Yang and his team of collaborators designed an isothermal PCR-based system for the detection of at least two YHV complex genotypes, genotype 8 and genotype 1, with which they performed pathogen detection by detecting m $7x10^{7,}$ which was compared to conventional real-time PCR. Thus, Cowley et al. (2019) reported a real-time nested PCR method to identify YHV genotype 7 using ORF1b as a region of the rest of the genotypes that can be identified until 10 copies of the genome are identified mainly in pleopods and gills, with the two methods for the detection of YHV being efficient.

Viral blocking of YHV as a strategy

The viral blocking theory suggests that organisms previously infected with any viral pathogen acquire partial protection against a YHV infection, according to reports by Aranguren et al., (2012) who reported a previous infection with TSV to avoid a YHV infection, which suggests that viral interference exists between TSV-YHV, which could explain the absence of YHD in America, where TSV is present in culture ponds very often. RNA interference (RNAi) is a cell mechanism that is triggered by a double stranded RNA (dsRNA) fragmented by a Dicer enzyme generating small interfering RNA (siRNA), which is incorporated into the complex RISC (RNA-induced silencing complex) targeting the complementary mRNA fragmenting it into siRNA and inhibiting their translation.

The RNAi pathway is well documented, proving its effectiveness in abolishing the viral infections of pathogenic viruses in humans, such as poliovirus (Gitlin et al., 2002), human immunodeficiency virus HIV-1 (Novina et al., 2002), hepatitis C (Randall et al., 2003), and hepatitis B (Gilad et al., 2003). This suggests that this mechanism can be used as a tool for the prevention of viral diseases in aquaculture.

Maningas et al. (2008) was the first group to report the silencing of the transcription and translation of clotting protein (CP) and transglutaminase (TGase), components of the humoral response in the shrimp innate immune system. Inoculation of dsRNA homologous to TGasas and CP inhibited the coagulation of hemolymph in vivo, and susceptibility to infection with *Vibrio penaeicide* and WSSV increased in dsRNA-treated organisms. Currently, treatment with RNAiis is used to determine the function of at least two antimicrobial peptides (AMPs) in shrimp, the antilipopolysaccharide factor (ALF) and crustina. ALF silencing in *P. vannamei* challenged with *V. penaeicidae* and *Fusarium oxysporum* significantly increased the susceptibility of shrimp to these pathogens (De la Vega et al., 2008). This shows the function of these genes in the immune activity of the shrimp.

Shochey et al. (2008) demonstrated the antimicrobial activity of crustins (AMPs) in *P. vannamei* challenged with *Vibrio penaecida* previously injected with dsRNA of crustin isoforms. In this study, they suppressed crustin expression and later increased mortality in crustin-depleted shrimp infected with *V. penaecida*. Amparyup et al. (2009) observed that gene silencing of prophenoloxidase (proPO) in *P. monodon*was more susceptible to *Vibrio harveyi*, while Fagutao et al. (2009) obtained an increase in the mortality of *Marsupenaeus japonicus* shrimp without bacterial or viral challenge when silencing proPO, registering an increase in the bacterial load in the hemolymph. Both studies concluded that the proPO system is an important component in the immune defense of the shrimp.

Therefore, RNAi technology provides an efficient tool to analyze other genes in shrimp, such as the β-integrine gene that inhibits WSSV infection (Li et al., 2007), as well as to explain the function of other genes involved in the molting, growth and reproduction of shrimp, such as gonad-inhibiting hormone (Treerattrakool et al., 2008), among others involved in the antiviral response. Reports of the function of dsRNA in shrimp show that the exposure of *P. vannamei* to dsRNA induces antiviral innate immunity against WSSV and TSV (Mudagandur et al., 2009). Lower replications of YHV were observed in cells of primary culture of shrimp that were transfected with dsRNA directed to nonstructural genes of the virus (Tirasophon et al., 2005), and the inhibition of YHV by dsRNA resulted in significantly lower mortalities in *P. monodon* (Yodmuang et al., 2006; Tirasophon et al., 2007). As a prophylactic strategy, Srisapoome et al (2018), reported the use of a byproduct of paper pulp, known as Lignine Kraft, to treat a YHV infection in shrimp, thus stating that the concentration of 200 mg/L lignin Kraft does not affect the health of organisms; however, it does not work as a preventative. By direct injection, there is no difference between the concentration of hemocytes, but if it favors phagocytosis, lignin Kraft pre-incubated for two hours with YHV and then delivered via promotes mortality rates by 50-60% at 14 days

of exposure. They conclude that the use of lignin byproducts for the treatment of YHV in shrimp should be reviewed in more detail.

CONCLUSIONS

Once the main features of this pathogen are known, it is easy to deduce that it is a highly fickle enemy, with an impressive capability of change that greatly limits the existing mitigation strategies. In Mexico, the attack of viral pathogens mainly in the northeast has left considerable sequels, so the presence of a character of this nature would have serious effects on production systems. Therefore, it is considered crucial to develop strategies that allow us to better understand the conditions of propagation and their effects on the environmental conditions of Mexican shrimp culture.

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Chapter 7

Probiotic effects in tilapia *Oreochromis niloticus* culture based on growth performance, survival and water quality

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ABSTRACT

The intensive production systems in aquaculture have generated serious problems related to the occurrence of diseases and to a deterioration of the environment, resulting in huge economic losses for fish farmers. The objective of this study was to analyse the effect of a microbial consortium with probiotic potential on the growth, survival and water quality of the tilapia *Oreochromis niloticus* culture in Tabasco, Mexico. A microbial consortium *Rhodopseudomonas palustris*, *Lactobacillus plantarum*, *Lactobacillus casei* and *Saccharomyces cerevisiae* was applied every week by immersion in earthen ponds during a growing cycle. The experiment took place in three treatments: 1) ponds with no probiotcis, 2) ponds with a dose of 4l/ha and 3) ponds with a dose of 10l/ha. Each treatment was applied three times. The growth parameters and survival of the tilapia and physicochemical parameters of the water and sediment were recorded. The EM1 and EM2 treatments significantly ($p<0.05$) increased the survival (73.0±2.51% y 79.1±1.00%, respectively) compared with the (64.0±4.04%). However, it was no significant ($p>0.5$) effect on growth by the end of the culture. The water quality improved significantly ($p<0.05$) within recommended parameters for the specie with the EM1 and EM2 treatments; although only decreased significantly ($p<0.05$) the percentage of organic matter in the sediment with EM2 treatment (0.89±0.36%). The results of this study showed a beneficial effect of the microbial consortium in water quality and survival of tilapia culture. However, the dose represented an important factor in the response of fish.

Keywords: *Oreochromis niloticus*, Probiotics, Growth, Water quality.

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INTRODUCTION

Aquaculture has become an important economic activity in many countries. However, the more intensive production systems have resulted in aquatic organisms being subjected to stressful conditions generated by a greater organism density and by feed and fertilizer inputs. Such conditions have caused serious problems related to the occurrence of diseases and to a deterioration of the environment, resulting in huge economic losses (Sahu et al., 2008; Al-Dohail et al*.,* 2011).

Veterinary medicines such as antibiotics and chemotherapeutics have long been applied to prevent and cure different types of diseases in fish farming (Aly et al*.,* 2008a). However, the abuse of these wide-spectrum antimicrobial agents has generated a variety of problems, including resistance to pathogenic agents, hormonal alterations in fish and negative effects (intoxication, cancer) on the health of consumers (De Schrijver; Ollevier, 2000; Al-Dohail et al*.,* 2011).

The use of clean technologies, including probiotics, in aquaculture is recognized as an alternative therapy in the management and control of fish health (Panigrahi et al*.,* 2010). Probiotics have been defined as "live microorganisms that benefit the host by changing the microbial community of the environment, or of that associated with the host, providing a better use of the feed or improving its nutritional value, stimulating a response to diseases, and improving the quality of the environment" (Verschuere et al*.,* 2000).

Using probiotics in fish farming has produced advantages such as a greater nutritional value (De Schrijver; Ollevier, 2000; Sahu et al., 2008), improved immune responses (Lallo et al*.,* 2008; Panigrahi et al*.,* 2010; Al-Dohail et al., 2011), an increase in survival (Aly et al*.,* 2008b; Ramakrishnan et al*.,* 2008), the inhibition of pathogenic bacteria (Skjermo; Vadstein, 1999; Huys et al*.,* 2001) and better water quality (Kristensen et al*.,* 1995; Makridis et al*.,* 2000; Balcázar et al*.,* 2006). However, most of the research carried out with probiotics and fish has taken place in the laboratory, for which reason its efficiency at the industrial level has been severely questioned (Gómez et al*.,* 2007; Kesarcodi-Watson et al*.,* 2008).

The tilapia *Oreochromis niloticus* is among the most successful freshwater species in fish farming, and this is due to its capacity to adapt to different production environments and to its high level of acceptance on the international market (Ingle de la Mora et al*.,* 2003). However, the presence of diseases and deficient water quality have caused the producers of this species to experience considerable economic losses. In view of this, the present study analyzed the effect of a microbial consortium of microorganisms (EM®) with probiotic potential on the quality of the water and sediment and on the growth and survival of the tilapia *O. niloticus* in a semi-intensive system located in a humid tropical area.

MATERIALS AND METHODS

Obtaining microorganisms and their culture. A microbial consortium (EM™ Technology, Japan) of worldwide distribution was used. The product contains three types of dormant efficient

microorganisms (EM): a photosynthetic bacterium (*Rhodopseudomonas palustris* at $2x10^3$ CFU ml^{-1}), lactic bacteria (*Lactobacillus plantarum* and *Lactobacillus casei* at $5x10^4$ CFU ml^{-1} each) and yeast (*Saccharomyces cerevisiae* at $4x10^3$ CFU ml^{-1}). The microorganisms were inoculated on a substrate of molasses and water to become active and were kept fermenting for seven days at a temperature of 36.5 to 37 °C, following the method suggested by the manufacturers (EMRO, 2008).

Tilapia production farm. The study took place in a commercial semi-intensive culture farm located in the municipality of Centro, Tabasco, Mexico (92°49'40" W, 17°57'34" N). The predominant climate in the area is humid warm with abundant summer rains (May-August) and a dry season (February-April). The field work started in February and ended in August 2019. The experiment was carried out in 0.25 ha earthen ponds, with a culture cycle of 180 days. The fry, all the same size, were obtained from a certified laboratory from one lot of breeders. They were placed in the ponds simultaneously at a density of 9 fish/m^2. The culture system was managed following the operational program of the farm technicians.

Evaluation of the probiotic in tilapia culture. The experimental design consisted of adding different doses of the microbial consortium with probiotic potential to the water during a culture cycle. The product was added weekly after changing the water, as follows: treatment 1 (C, control) ponds with no product, treatment 2 (EM1) ponds with a dose of 4 L ha^{-1} and treatment 3 (EM2) ponds with a dose of 10 L ha^{-1}. Each treatment was applied three times. Feeding of the fry started on the first day they were placed in the ponds and continued until they reached a commercial size (180 days), following the feeding program established for the gray tilapia (El Pedregal Silver Cup, Mexico). The farm technicians adjusted the feeding rate weekly in accordance with estimations of body weight increase and survival rate.

Environmental parameter data. Water quality was monitored every two weeks during the day, from 7:00 to 9:00 am. The parameters pH, temperature, dissolved oxygen and salinity were recorded at a depth of 50 cm with Hanna HI 95928 equipment (USA). The concentrations of total ammonium nitrogen (TAN) and nitrate (NO_3-N) were estimated using a Hanna HI 9828 (USA) ammonium recorder. Water transparency and depth were recorded *in situ* with a Secchi disk. Sediment was collected at a depth of 15 cm with a PVC corer (Kristensen et al*.,* 1995). The samples were taken to the laboratory where the variables pH, organic matter, total nitrogen and extractable phosphorus were recorded (NOM-021-RECNAT, 2000).

Growth parameter data. Fifty fry were collected randomly every 30 days during the culture cycle to record the weight (g) with an electronic scale (±5 g) and the total length (cm) with an ichthyometer (±1 mm). The fish were returned to the tank after the biometrical data were recorded. A total of 3,150 fish were weighed and measured [measured organisms x number of replicates per treatment x (days of culture measuring $frequency^{-1}$)]. Weight gain (g $tilapia^{-1}$), weight gain (%), daily weight gain (g day^{-1}), specific growth rate (SGR, % day^{-1}) and survival (%) were estimated at the end of the culture cycle (Ali et al*.,* 2010).

One-way analysis of variance (ANOVA) was applied to the environmental, growth and survival parameters of the tilapia. The $p<0.05$ values were considered significantly different. When the means of the treatments presented significant differences, an *a posteriori* analysis and a Tukey test were applied to identify the nature of the differences ($p<0.05$) (Daniel, 2008). A multiple discriminant analysis (MDA) was carried out to determine whether the treatments were related to the environmental parameters (Zar, 2010). All statistical analyses were carried out with STATISTICA version 7.0 for Windows (StatSoft, 2010).

RESULTS AND DISCUSSION

Environmental parameters in the tilapia culture. Significant among-treatment differences ($F=33.38$, $p<0.05$) were estimated for six of the seven environmental parameters in the water column (Table 1). The lowest concentration of dissolved oxygen was recorded in treatment C (4.63 ± 2.84 mg L^{-1}), while no significant differences (Tukey, $p>0.05$) were recorded for treatments EM1 and EM2. The temperature values in treatments C and EM1 were significantly (Tukey, $p<0.05$) greater than those in treatment EM2, whereas the transparency values in treatment EM2 were significantly smaller (Tukey, $p<0.05$) than those in treatments C and EM1 (Table 1).

Table 1. Environmental parameters in the control and treatment tanks during farming of the tilapia *O. niloticus*.

	Treatment		
Parameters	**C**	**EM1**	**EM2**
Water			
Dissolved oxygen (mg L^{-1})	4.63 ± 2.84^{a}	4.80 ± 3.19^{a}	5.18 ± 1.76^{a}
Temperature (°C)	29.9 ± 1.74^{a}	30.5 ± 2.22^{a}	28.7 ± 2.26^{b}
pH	4.24 ± 3.58^{a}	7.84 ± 0.62^{b}	8.05 ± 1.04^{b}
NO_3-N (mg L^{-1})	0.30 ± 0.05^{a}	0.37 ± 0.06^{b}	0.28 ± 0.14^{a}
Total ammonium nitrogen (mg L^{-1})	0.35 ± 0.20^{a}	0.04 ± 0.03^{b}	0.12 ± 0.19^{b}
Transparency (cm)	12.5 ± 11.5^{a}	9.00 ± 4.34^{a}	29.6 ± 28.9^{b}
Depth (cm)	75.2 ± 45.6^{a}	95.5 ± 24.9^{b}	105 ± 27.5^{b}
Sediment			
pH	7.38 ± 0.19^{a}	7.02 ± 0.71^{b}	7.29 ± 0.42^{a}
Extractable phosphorus (mg kg^{-1})	9.98 ± 6.18^{a}	9.17 ± 4.62^{a}	25.6 ± 10.6^{b}
Total nitrogen (%)	0.16 ± 0.03^{a}	0.07 ± 0.02^{b}	0.08 ± 0.02^{b}
Organic matter (%)	1.60 ± 0.56^{a}	0.89 ± 0.36^{b}	1.38 ± 0.23^{a}
n	3	3	3

Means on a same line with a different superscript are significantly different ($p<0.05$).
Average ± standard deviation.
C: Control, EM1: Probiotic dose 4 L ha^{-1}, EM2: Probiotic dose 10 L ha^{-1}.

Treatments EM1 and EM2 recorded the greatest depths during the culture cycle and were significantly different (Tukey, $p<0.05$) from treatment C, which presented the minimum depth (Table 1). Regarding pH, treatment C had the lowest value and was significantly different (Tukey, $p<0.05$) from treatments EM1 and EM2.

The NO_3-N concentrations presented no significant differences (Tukey, $p>0.05$) in treatments C and EM2, and these were significantly different from treatment EM1, where the greatest concentration was recorded (0.37 ± 0.06 mg L^{-1}). In the case of the TAN, the greatest concentration was recorded in treatment C (0.35 ± 0.20 mg L^{-1}) and was significantly different (Tukey, $p<0.05$) from those in treatments EM1 and EM2, where the lowest TAN concentrations (0.04 ± 0.03 mg L^{-1}; 0.12 ± 0.19 mg L^{-1}) were recorded.

Regarding the physicochemical parameters in the sediment (Table 1), treatments C and EM2 presented no significant differences (Tukey, $p>0.05$) with respect to the pH values, whereas the lowest value was recorded in treatment EM1 (7.02 ± 0.71) and was significantly different (Tukey, $p<0.05$) from those recorded in the other treatments. The extractable phosphorus content was significantly different (Tukey, $p<0.05$) in treatments C and EM1 compared with treatment EM2, which had the greatest content (25.6 ± 10.6 mg kg^{-1}). Treatment C presented the greatest percentage of total nitrogen ($0.16\pm0.03\%$) and was significantly different (Tukey, $p<0.05$) from treatments EM1 and EM2 ($0.07\pm0.02\%$; $0.08\pm0.02\%$). Finally, treatment EM1 recorded the lowest percentage of organic matter ($0.89\pm0.36\%$) and was significantly different from treatments C and EM2 ($1.60\pm0.56\%$; $1.38\pm0.23\%$).

According to the MDA, the environmental parameters recorded in the water were not significant, while the sediment presented two significant variables ($\lambda=0.05$; $F_{20.210}=35.9$; $p<0.05$; $R^2=0.93$): total nitrogen ($\lambda=0.08$; $p<0.05$) and extractable phosphorus ($\lambda=0.08$; $p<0.05$). Figure 1 shows the distribution of the corresponding observations between the first and second functions based on the discriminant space. Treatments EM1 and EM2 overlapped in comparison with treatment C, which remained isolated. Additionally, 95% of the canonical variables were correctly classified. According to the values of the standardized coefficients for the variables in the first discriminant function, the discriminant effect among the three treatments in the tilapia culture indicated that the total nitrogen had a canonical load of 73% ($\lambda_p=0.57$; $F_R=39.5$; $p<0.05$; $T=92\%$; $R^2=8\%$). while in the second discriminant function, the extractable phosphorus presented a canonical load of 65% ($\lambda_p=0.58$; $F_R=36.5$; $p<0.05$; $T=55\%$; $R^2=45\%$). The other variables did not present a significant canonical load. The use of probiotics in aquaculture has increased in recent years as a result of their potential benefits on fish health and on improved environmental quality (Balcázar et al*.,* 2006; Gómez et al*.,* 2007). The use of efficient microorganisms (EM1 and EM2) in this study produced a positive effect on water quality, as the water parameter values lay within the recommended range of values for the semi-intensive culture of the tilapia *O. niloticus* (Ingle de la Mora et al*.,* 2003), in comparison with the control treatment (C). However, optimum growth conditions for this

species were maintained by using the greatest dose of the commercial mixture of microorganisms (EM2) and not the dose recommended by the manufacturers (EM1).

Several authors have mentioned that factors such as the dose, the frequency of probiotic use and the type of organism that is farmed are considered important for the product to be successful, as the concentration has an effect on the action mechanisms of the microorganisms (Balcázar et al., 2006), and it is possible to observe a positive effect on environmental quality or a negative effect on fish health (skin infection) (Gómez et al., 2007; Ali et al., 2010).

In this study, the increase in the concentration of oxygen and the decrease in nitrate and total ammonium nitrogen in treatment EM2 coincide with other results that have shown that phototrophic bacteria (*R. palustris*) produce oxygen from carbon dioxide, with hydrogen sulfide as an electron donor, and decrease the amount of this gas that is toxic to farmed fish (Çetinkaya et al., 1999). Additionally, these bacteria have the capacity to use ammonium and nitrate as a source of nitrogen, reducing the concentration of these compounds in the water of culture systems (Kyum et al., 2004). In addition, other studies have indicated that the use of the yeast *Saccharomyces cerevisiae* decreases nitrogenous compounds (ammonium and urea) (Kesarcodi-Watson et al., 2008) and improves water quality.

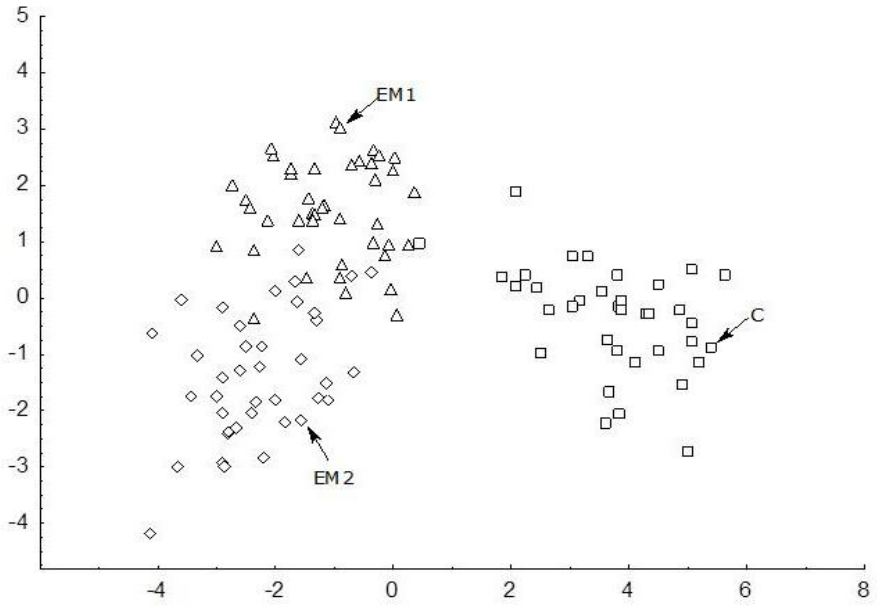

Figure 1. Discriminant analysis of the environmental parameters that explain the variability in the treatments (overlapping area: 95% confidence interval). C: Control, EM1: Probiotic dose 4 L ha^{-1}, EM2: Probiotic dose 10 L ha^{-1}. (X= Discriminant function 1; Y= Discriminant function 2).

Regarding the pH values, treatments EM1 and EM2 were observed to be moderately alkaline compared with treatment C, which was acidic. Some authors have mentioned the importance of controlling pH in culture systems due to its direct relationship with the synthesis of ammonium, nitrites and nitrates and to its effect on fish growth (Lallo et al., 2008). Previous studies have reported that efficient microorganisms such as those used in this study (*R. palustris*, *L. plantarum*, *L. casei* and *S. cerevisiae*) produce compounds such as quinones, biotin (photosynthetic bacteria) (Qi et al., 2009), lactic acid,

hydrogen peroxide, acetaldehyde, diacetyl, bacteriocins (lactic bacteria) (Verschuere et al., 2000), vitamins, glycerol, ethanol and carbon dioxide (yeast) (Irianto; Austin, 2002; Kesarcodi-Watson et al., 2008) that lower the pH in water due to their hydrophilic characteristics (presence of hydroxyl groups).

In aquaculture, productivity is a condition that is necessary to generate an ideal environment for fish development, but an increase in phytoplankton has been reported to cause problems of low dissolved oxygen concentration and mortality of farmed organisms (CNP, 2012). An increase in transparency was recorded in treatment EM2 compared with treatments C and EM1. This coincides with authors (Çetinkaya et al., 1999; Kyum et al., 2004.) who have reported that phototrophic bacteria may regulate phytoplankton growth through competition for nutrients (Qi et al., 2009), as is the case for the bacterium *R. palustris*, present in the commercial mixture of microorganisms that was used in this study.

The temperature and the depth of the water in the ponds have been associated with the environmental conditions and the operational conditions of the management of the farm (CNP, 2012). However, the significant changes recorded for these parameters did not influence the effect of the probiotics in the ponds of this study, as these variables did not have a considerable canonical load on which the efficiency of the product might have depended.

Farming systems characteristically generate high percentages of organic matter in the sediment. This material is closely related to the aerobic and anaerobic processes carried out by the microorganisms responsible for remineralizing nutrients in water and making them available to the microbial communities present in the ponds (Kristensen et al., 1995). In this study, the organic matter content in treatments EM1 and EM2 decreased compared with treatment C. It was also observed that the treatment with the greatest dose of microorganisms (EM2) had a greater percentage of total nitrogen and a lower content of extractable phosphorus in the sediment. These two variables were significant in the multiple discriminant analysis model and contributed to the behavior of the environmental parameters related to the treatments, as they had the greatest canonical loads. These results coincide with some authors' reports (Balcázar et al., 2006; Qi et al., 2009), which indicate that the use of lactic bacteria and microorganisms with probiotic potential in fish farming has produced significant decreases in the content of organic matter, as well as increases in labile substrates in the water. Studies have shown that organic phosphate is produced and organic nitrogen is available in ionic form during the fermentative processes of lactic bacteria (Lallo et al., 2008), which may be assimilated by the different microbial populations.

Growth and survival parameters in the tilapia culture. The three treatments in the semi-intensive tilapia culture were allowed the same time to harvest (180 days). The growth of the tilapia was significantly different in the three treatments ($F=20.96$; $p<0.5$) (Table 2). The lowest weight at the end of the culture cycle (harvest size) was recorded in treatment C, while the greatest values were obtained in treatments EM1 and EM2. However, the weights recorded in the three treatments presented no significant differences (Tukey, $p>0.05$).

The greatest total length at the end of the culture cycle was recorded in treatment EM2, followed by treatment EM1 and finally treatment C (Table 2). This last was significantly different (Tukey, $p<0.05$) from treatments EM1 and EM2, which presented no significant differences (Tukey, $p>0.05$).

In contrast, no significant differences (Tukey, $p>0.05$) were observed among the treatments (C, EM1 and EM2) with respect to the parameters weight gain (g), weight gain (%), daily weight gain (g day^{-1}) and specific growth rate (% day^{-1}). However, treatment EM2 recorded the greatest values (Table 2). Tilapia survival at the end of the culture cycle was significantly different ($F=20,57$, $p<0.05$) among the treatments (Table 2), as it increased significantly (Tukey, $p<0.05$) in treatments EM1 and EM2 compared with treatment C.

The use of probiotics in aquaculture has been associated with an efficient process of absorption and assimilation of farmed organisms' feed, as the microorganisms are capable of c76olonizing the gastrointestinal tract, where they secrete nutrients and digestive enzymes that improve the metabolic processes and immunological responses of the hosts (Balcázar et al*.,* 2006; Aly et al*.,* 2008a; Sahu et al., 2008).

Table 2. Growth parameters of the tilapia *O. niloticus* with respect to the treatments at the end of the culture cycle.

	Treatment		
Parameters	**C**	**EM1**	**EM2**
Final weight (g)	393±28.6^{a}	409±5.49^{a}	414±54.8^{a}
Final total length (cm)	23.7±0.44^{a}	28.1±0.48^{b}	28.7±1.06^{b}
Weight gain (g tilapia^{-1})	346±26.5^{a}	353±7.51^{a}	366±54.3^{a}
Weight gain (%)	636±44.0^{a}	741±45.5^{a}	773±107^{a}
Daily weight gain (g day^{-1})	122±45.6^{a}	126±5.27^{a}	163±23.3^{a}
SGR (% day^{-1})	595±6.51^{a}	599±1.36^{a}	599±14.4^{a}
Survival (%)	64.0±4.04^{a}	73.0±2.51^{b}	79.1±1.00^{b}
N	3	3	3

Means on a same line with a different superscript are significantly different ($p<0.05$).
Average ± standard deviation.
SGR= Specific growth rate.
C: Control, EM1: Probiotic dose 4 L ha^{-1}, EM2: Probiotic dose 10 L ha^{-1}.

The present study proved that the addition of a commercial mixture of efficient microorganisms (*R. palustris*, *L. plantarum*, *L. casei* and *S. cerevisiae*) to the water increased the final weight of the tilapia, with treatment EM2 recording the greatest value. Studies carried out with commercial probiotics that include *L. acidophilus* and *S. cerevisiae* for *Cyprinus carpio* (Ramakrishnan et al*.,* 2008) and *Bacillus pumillus* for *O. niloticus* (Aly et al*.,* 2008b) recorded greater significant weights at the end of the culture cycle. However, the present study recorded no significant differences among the evaluated treatments. In contrast, the total lengths at the end of the culture cycle were significantly different in treatments EM1 and EM2 compared with treatment C, and the treatment with the greatest dose recorded the greatest total length

values. Several authors have reported that the use of probiotics in fish farming significantly improves growth due to an input of nutrients and sources of energy that lead to an increase in the size of the organisms (Mazurkiewicz et al., 2005; Ali et al., 2010). The results of the present study, however, showed a beneficial effect only with respect to the total length.

This study recorded no significant differences in the growth parameters of the tilapia (weight gain, daily weight gain and specific growth rate) with the addition of the commercial mixture to the water. However, the greatest values of these variables were recorded for treatments EM1 and EM2 compared with treatment C. Similar results were obtained for the growth parameters when using this same commercial probiotic for *Oreochromis* sp. under laboratory conditions (Ladino-Ojuela, Rodríguez-Pulido, 2009). The authors of that study discussed that it was possible that beneficial effects were not observed due to the time of evaluation (15 days). However, the experimental time in the present study was 180 days in a commercial farm. Previous studies have mentioned different methods for using probiotics in aquaculture and have indicated that the best method is with feed, as microorganisms can then enter, colonize and multiply in the digestive tract (Makridis et al., 2000; Irianto, Austin, 2002).

Survival at the end of the culture cycle increased in treatments EM1 and EM2 compared with treatment C. Various studies have confirmed an increase in survival with the use of probiotics (Ali et al., 2010; Al-Dohail et al., 2011). Of the different identified probiotic action mechanisms (Lallo et al., 2008; Panigrahi et al., 2010), two have been linked to an increase in farmed fish survival. The first is their capacity to produce antiviral effects, counteracting problems caused by pathogenic agents and improving the immune system.

Notwithstanding that the action mechanisms of the probiotics have not been completely explained and different responses are obtained in different culture systems, the results of this study indicate that the efficient microorganisms included in the commercial mixture had a positive effect on water quality and survival. However, this effect was recorded with the greatest dose and not with the dose recommended by the manufacturer. It is thus necessary to carry out studies to determine 1) the optimum dose to be provided, 2) an emergency dose in case of environmental changes, 3) the colonization of the efficient microorganisms in the digestive tract of the tilapia, 4) the presence of antagonistic effects, 5) whether there is an increase in the immune response and 6) the effect of providing EM® in the feed.

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Chapter 8

Structure and carbon stock in relation to the biomass of *Nymphaea elegans* and *Sagittaria longiloba* in three temporary lagoons in the arid northwest of Mexico

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ABSTRACT

There are relatively few published studies on the effects of flood frequency and timing on wetland plants, representing under-researched areas in linking water regime to plant growth response. In the present study, the structural development and carbon stock in relation to the biomass production of *N. elegans* and *S. longiloba* were recorded under arid subtropical environmental conditions in three temporary lagoons in northwest Mexico. By means of sampling the plants in the field and processing in the laboratory, physical-chemical measurements of surface water and sediment were performed. Among the main results, it was identified in the study area that *N. elegans* and *S. longiloba* maintain a life period of ~2.5 months related to the presence of surface water (Flood pattern) and moisture content in the soil, with maximum stocks of biomass carbon of 165.5 g/C/plant in *N. elegans* and 75.5 g/C/plant for *S. longiloba*. That is why it is recommended to restore and maintain the natural hydrological regime that feeds the studied lagoons with water and allows the development of *N. elegans* and *S. longiloba* in a higher density and life period according to seasonal rainfall, which allows maintaining the environmental services they provide to the ecosystem.

Keywords: macrophytes, wetland, climate change

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INTRODUCTION

The increase in greenhouse gas emissions and the associated impacts on global warming (IPCC, 2013) have led to an urgent need to identify and protect ecosystems with high carbon storage capacity (Canadell; Raupach, 2008), such as freshwater wetlands. Wetlands represent 3% of the world's soils but account for approximately 21% of the global soil organic carbon stock (Scharlemann et al., 2014). In addition, they offer many ecosystem services to humankind, including water quality improvement, flood mitigation, coastal protection and wildlife protection (Mitsch et al., 2009). Within these ecosystems, CO_2 from the atmosphere is taken up via photosynthesis, most of which is temporarily stored in the plant foliage, whereas the remainder is sequestered for a long period in biomass and soils (Ahalya; Park, 2018). Among the various methods to quantify carbon, the observation and field sampling approach is the best and most accurate, although it is costly and time-consuming, as continuous sampling data are required (Lu, 2006). Various studies also report pH, soil texture, temperature, rainfall pattern, and hydrology as factors responsible for retaining sequestered carbon in wetland soils (Perera; Amarasinghe, 2019). An important limitation is that wetland field research has mainly focused on study sites located in humid regions in temperate and tropical latitudes (Alongi et al., 2020), with few studies in arid regions (Adame et al., 2018) such as northwestern Mexico, where differences in rainfall, evapotranspiration, and soil conditions could influence carbon capture and storage (Schile et al., 2017).

There are relatively few published accounts concerning the effects of flood frequency and timing on wetland plants, representing under-researched areas in linking water regime to plant growth response (Kenow et al., 2018). In recent decades, interest in the study of the plants *Nymphaea elegans* Hook and *Sagittaria longiloba* Engelm has increased since both species typically grow in a great diversity of aquatic and marshy habitats and predominate in freshwater swamps and marshes with little runoff (Zepeda-Gómez; Lot, 2005). In addition, since lentic ecosystems are being reduced by anthropogenic activities (droughts and water channeling, among other causes) (Zepeda-Gómez; Lot, 2005), the natural habitat of aquatic plants *N. elegans* and *S. longiloba* is highly threatened, and they are considered highly fragile. In this context, the present project studies the structural development and carbon stock in relation to the biomass production of *N. elegans* and *S. longiloba* under arid subtropical environmental conditions in three temporary lagoons in northwestern Mexico. Derived from the conditions of the arid region and the study site as a temporary lagoon, the following hypotheses were structured: (*i*) the species under study (*N. elegans* and *S. longiloba*) will increase their biomass progressively in response to the flood pattern above ground level, (*ii*) they will complete their life cycle until fructification before the water level is lower than ground level, and likewise, (*iii*) the *N. elegans* plant will have the highest carbon stock due to its structure with higher biomass production in leaves and stems in relation to *S. longiloba*.

MATERIALS AND METHODS

Study site

The proposed lagoon system is administratively located in the state of Sonora, Mexico (Fig. 1). This system has the RAMSAR (2007) designation that includes the entire area of the coastal zone of high importance for the hibernation of migratory waterfowl and shorebirds, in addition to some bodies of fresh water that fulfill their function in connectivity with the coast. It is an area of reproduction, breeding, feeding and refuge for aquatic invertebrates and birds of national and international importance.

The regional climate is dry and semidry and very dry with very low levels of rainfall throughout the year (BW(h')hw - BS0(h')hw) (García; CONABIO, 1998), with an average annual temperature of 24°C and average annual rainfall of 270.4 mm. The month of August is the warmest (38.6°C), and the month of January is the coldest (7.8°C), while the rainy season occurs during the months of July and October, with August and September being the months rainiest with 69.1 mm and 66.6 mm, respectively (SMN, 2021).

Figure 1. Study sites in basin delimitation (site = lagoon).

Plant material

In three temporary freshwater lagoons (sites 1, 2 and 3), three field monitoring campaigns were carried out (separated by 20 days between each monitoring event) for the collection of samples (Sept 11, Oct 01, and Oct 21, 2021). The first monitoring was performed twenty days after the presence of flooding in the study lagoons. Twenty days after the third monitoring event, the flood pattern was below ground level.

Physicochemical of water and sediment

In each monitoring, salinity (PSU), redox potential (mV), temperature (°C) and pH were measured in surface water (first 20 cm) using a Hanna HI9828 multiparameter. In addition, three sediment samples were collected at each site (n=27) in the first 20 cm of soil (n=18) with a nucleator (0.0033 m^2) to determine the texture according to the Bouyucos method (Klute, 1986), pH by electrometry in 1:2 relation to water, and organic matter (OM) content by ignition according to Heiri et al. (2001). In addition, three sediment samples with known volume were collected in each sample unit (n= 27) to determine the apparent density and moisture content of the soil according to Moreno-Casasola and Warner (2009). The moisture content of the soil is the percentage of water that is capable of storing one gram of soil; if the value was 100%, it would mean that 1 g of soil stores 1 g of water (Infante, 2011).

Biomass estimation

In each monitoring, 3 plants of each species (*N. elegans* and *S. longiloba*) were taken in each lagoon by means of botanical presses to transfer them to the laboratory. Leaf length and width and stem length were measured; subsequently, they were dried in each of its morphological components (leaf, stems, roots, rhizome, flower, and fruit) in an oven at 65°C to obtain biomass in dry weight.

Carbon stock

Derived from the lack of allometric equations to estimate carbon from the biomass of *N. elegans* and *S. longiloba*, mean carbon concentrations in the litter have been reported to be 38-49% (Kauffman et al., 1995). A conversion factor of approximately 0.45 is recommended.

Carbon ($g \bullet C_{org} \bullet plant$) = (Biomass * conversion factor (0.45))/plant (Kauffman; Donato, 2012).

Statistical analysis

Data were analyzed using the Kolmogorov–Smirnov test and Levene's homogeneity of variances. Differences in physicochemical data sets of water, sediment, plant structure, and carbon stock were identified at the 5% level of significance using Tukey's one-way ANOVA (Steel; Torrie, 1996).

Physical and chemical properties of water and sediment

The pH values showed significant differences between the monitoring periods, in a range of 9.1 to 9.7. In relation to the temperature values, a maximum of 32±0.15°C was identified in the first monitoring. In addition, the salinity showed values of freshwater systems with a slight increase in monitoring 3 with 0.61±0.03 UPS, and the values of ORP recorded more reductive conditions in monitoring 3 with -181±1.3 mV (Table 1). The physical chemicals of the sediment showed low variation during the study period. The bulk density of the soil registered average values of 1.03±0.02 g cm^{-3}, while

the content of organic matter presented an average value of 6.22±0.32%. In addition, the pH registered an average value of 6.7±0.04. The texture values in the sediment did not show significant differences, with higher clay contents from 49.9±5.4 to 54.3±1.3% (Table 2).

Table 1. Physical chemical of surface water in each monitoring.

	Monitoring 1	Monitoring 2	Monitoring 3	F	p
pH	9.3±0.06	9.1±0.03	9.7±0.01	16	0.01
Temp (°C)	32±0.15	31±0.16	22±0.13	8.5	0.09
Sal (PSU)	0.14±0.01	0.33±0.02	0.61±0.03	12.3	0.4
ORP (mV)	-140±3.7	-145±2.5	-181±1.3	22	0.06

PSU= practical salinity units, mV=millivolts, $p<0.05$

Table 2. Physical chemical in sediment in each monitoring.

		Monitoring 1	Monitoring 2	Monitoring 3	F	p
	BD (g/cm^3)	1.1±0.011	1.1±0.019	0.9±0.024	12	0.4
	OM (%)	6.7±0.5	6.2±0.3	5.8±0.2	1.8	0.6
	pH	6.7±0.02	6.9±0.08	6.6±0.02	9.9	0.1
Texture (%)	Sand	37.4±3.8	36.3±5.3	26.8±2.3	2.1	0.18
	Silt	12.5±1.6	13.8±1.1	18.9±1.5	5.9	0.06
	Clay	50.1±2.3	49.9±5.4	54.3±1.3	1.4	0.7

BD: Bulk density, OM: Organic matter

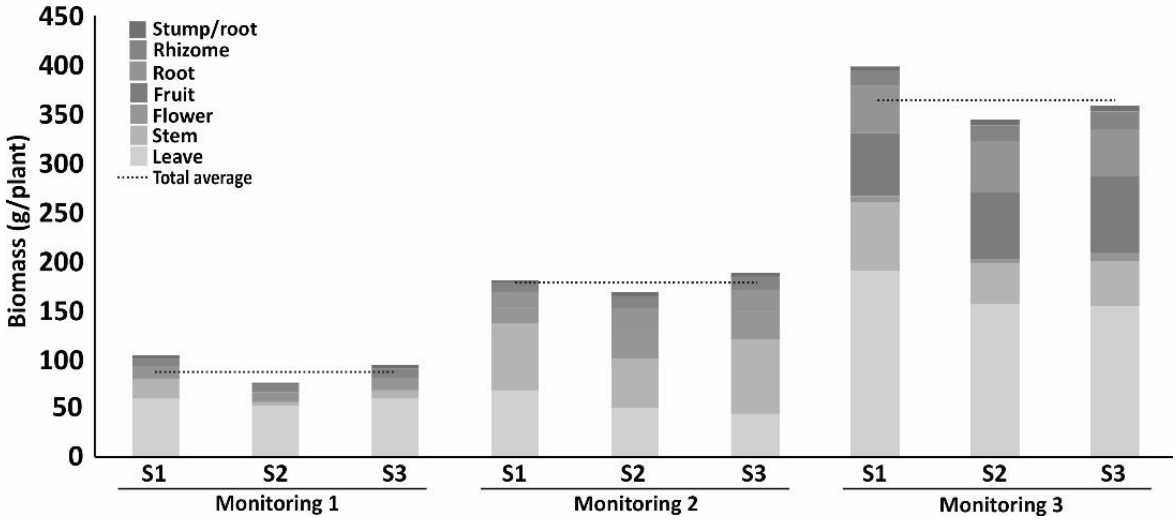

Figure 2. Biomass of the *N. elegans* plant by component (S=Site).

Biomass production

The structure of the N. elegans plant increased over time with a length of 12.7 cm and width of 13.5 cm on average in the first monitoring, up to 22.9 and 20.1 cm in the third monitoring with significant differences ($N=381$, $F= 8.7$, $p<0.05$ and $N=381$, $F=9.9$, $p<0.05$, respectively). The average length of the stem was 54.1 cm during the first monitoring up to a maximum of 85.2 cm during the third monitoring

(N=396, F=12.1, p<0.05). Biomass production in N. elegans temporarily showed a gradual increase, with 92 g/plant in the first monitoring, 179.8 g plant^{-1} in the second and 367.8 g-plan^{-1}t in the third. The component that contributed the most biomass was the leaf, with a maximum biomass of 168.1 g plant^{-1}. The plants presented low flowering from the first monitoring; however, the maximum occurred in the second monitoring with 25.2 g plant^{-1} of flowers in dry weight. In relation to the fruits, they only appeared in the third monitoring, with a maximum average of 71.3 g plant^{-1} (Fig. 2).

The structure of the *S. longiloba* plant increased over time with a length of 18.6 cm and width of 13.9 cm on average in the first monitoring, up to 26.1 and 18.5 cm in the third monitoring without significant differences (N=87, F= 9.6, p=0.4 and N=87, F=12.7, p=0.08, respectively). The average length of the stem registered at 38.7 cm during the first monitoring up to a maximum of 64.4 cm during the third monitoring (N=109, F=22, p=0.01). Biomass production in *S. longiloba* temporarily showed a gradual increase, with 8.19 g/plant in the first monitoring, 8.9 g/plant in the second and 33.2 g/plant in the third. The component that contributed the most biomass was the stems, with a maximum biomass of 21.2 g plant^{-1} on average. The plants showed low flowering from the first monitoring; however, the maximum occurred in the third monitoring with 0.72 g plant^{-1} of flowers in dry weight. In relation to the fruits, they only appeared in the third monitoring, with a maximum of 4.43 g plant^{-1} (Fig. 3).

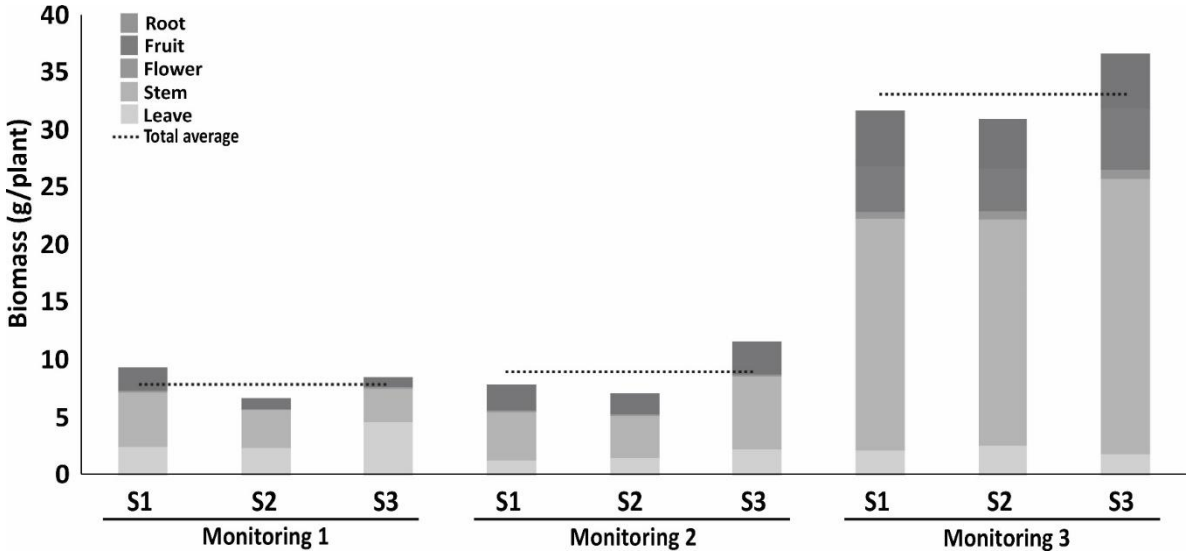

Figure 3. Biomass of the *S. longiloba* plant by component (S=site).

Carbon stock estimation

The carbon stock in relation to the biomass of the *N. elegans* plant registered maximum average values of 165.5 g/C/plant, where the leaf component had the highest concentration with 75.5 g/C/plant (Table 3 and Fig. 4). The carbon stock in relation to the biomass of the *S. longiloba* plant registered maximum average values of 14.9 g/C/plant, where the leaf component had the highest concentration with 9.55 g/C/plant (Table 4 and Fig. 4).

Table 3. Carbon stock (g) in the monitoring site of the *N. elegans* plant.

	Plant	Leave	Stem	Flower	Fruit	Root	Rhizome	Stump	Total
Monitoring 1	S1	27.8	8.2	0.9		5.2	3.3	1.6	46.9
	S2	23.5	1.9	0.8		4.2	3.7	1.0	35.0
	S3	27.5	3.9	1.0		4.4	4.1	1.4	42.3
Monitoring 2	S1	31.3	30.1	7.1		7.2	4.8	1.2	81.6
	S2	22.7	22.8	14.3		9.4	5.5	1.5	76.1
	S3	20.6	33.9	12.8		9.8	6.4	1.5	85.0
Monitoring 3	S1	85.8	31.7	2.8	28.9	21.3	7.3	2.1	179.9
	S2	70.6	18.7	1.8	30.9	23.7	7.3	2.2	155.3
	S3	70.0	20.5	3.4	34.9	22.0	8.1	2.4	161.4

S=site; Values presented in grams.

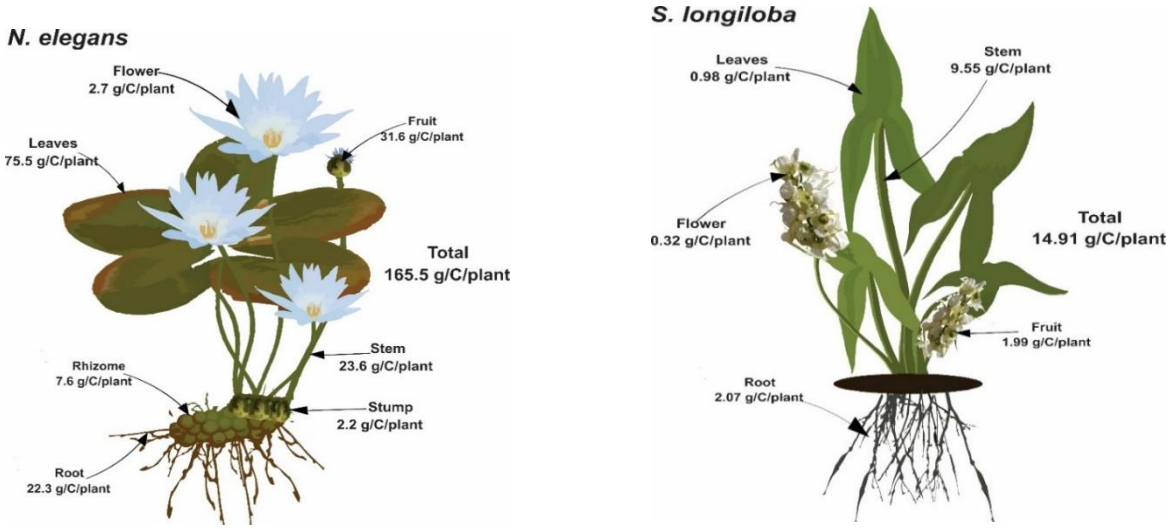

Figure 4. Carbon stock in relation to biomass by morphological component of *N. elegans* and *S. longiloba.*

Table 4. Carbon stock (g) in the monitoring site of the *S. longiloba* plant.

Monitoring	Plant	Leave	Stem	Flower	Fruit	Root	Total
Monitory 1	S1	1.10	2.12	0.10		0.90	4.22
	S2	1.03	1.49	0.08		0.40	3.01
	S3	2.08	1.26	0.12		0.37	3.83
Monitory 2	S1	0.57	1.87	0.10		1.03	3.58
	S2	0.67	1.64	0.08		0.82	3.20
	S3	0.98	2.87	0.08		1.31	5.23
Monitory 3	S1	0.97	9.06	0.26	1.80	2.18	14.27
	S2	1.15	8.82	0.37	1.69	1.93	13.96
	S3	0.81	10.77	0.33	2.50	2.09	16.49

S=site; Values presented in grams.

DISCUSSION

There are few published studies on the effects of aboveground flood pattern frequency and duration on wetland plants, representing underinvestigated areas, particularly the water regime with plant growth response (Webb et al., 2012). Hypothesis (i) that *N. elegans* and *S. longiloba* increase their biomass progressively in response to the flood pattern above ground level is confirmed, completing maturity, flowering and fructification (~2.5 months) earlier than the flood pattern decreases below ground level and the environmental temperature dries out the soil, which is why it is recommended to restore and maintain the natural hydrological regime that feeds the studied lagoons with water and allows the development of *N. elegans* and *S. longiloba* in higher density and life period according to the seasonal rainfall.

The two species under study (hypothesis ii) completed their life cycle to fructification before the water level decreased below ground level, although *N. elegans* requires flood levels above ground level to survive, *S. Longiloba* develops in environments with a low flood pattern but without desiccation, as recorded by Keddy and Ellis (1985) with the early establishment of *S. latifolia* seedlings along a water level gradient ranging from 10 cm above to 5 cm below the substrate surface, also Kenow and Lyon (2009) observed that *Sagittaria* species germinated and flowered when grown in substrates of moist, shallow (2–3 cm) and submerged (15 cm), but not on substrates that were allowed to dry.

Although the *Sagittaria* genus is considered a noxious weed in the Asian continent (Ozaki et al., 2018), since the plant has a broad ecological range in which it is able to survive and dominate (Ndlovu, 2020), for arid regions, its distribution decreases due to high temperature and low rainfall conditions, such as the study area of the present investigation with ~300 mm of rainfall per year with a life cycle duration of *N. elegans* and *S. longiloba* of ~ 2.5 months related to the duration of the flood pattern above ground (N. elegans) and the presence of moisture in the soil (*S. longiloba*).

In terms of promoting favorable conditions for the natural establishment of *Nymphaea* and *Sagittaria* in arid zone conditions, this work together with other studies (Keddy; Ellis, 1985; Marburger, 1993; Kenow; Lyon, 2009; Kenow et al., 2018) corroborates the importance of maintaining natural hydrological regimes that allow flood levels and substrates with a relatively high moisture content that allow the survival of these aquatic species that can have ecological benefits since they are plants pollinated by bees, hoverflies and other insects (Ozaki et al., 2018; Tanaka, 1985; Huang et al., 2006). The underground tissues of *N. elegans* and *S. longiloba* (roots, tubers and rhizomes) improve the permeability of the medium (Todunovics et al., 2005; Licata et al., 2019) and diffuse ions to drive microbial processes (Alufasi et al., 2017; Wang et al., 2018), fix and store carbon in wetland soils through roots (Qadiri et al., 2021), and serve as food for local herbivores.

The established hypothesis that mentions (iii) the *N. elegans* plant with the highest carbon stock is confirmed, since the biomass carbon contents were 14.5 times higher in the *N. elegans* plant than in *S. longiloba*. Carbon sequestration is one of the most important ecosystem services provided by wetlands,

and it occurs in wetlands at a greater rate than in any other ecosystem on the planet (Mitsch; Gosselink, 2015). To provide an accurate estimate of the carbon storage and sequestration rates of wetlands, carbon sequestration and emissions from different wetland types should be thoroughly evaluated (Bernal; Mitsch, 2012; Craft et al., 2018); furthermore, it is an important factor in wetland restoration for mitigating global warming (Yeong et al., 2022).

The hydrology of the area is fundamentally influenced by the rainy season between July and September. However, it is important to mention that the river drainage system for these temporary lagoons is conditioned by the highway. In turn, the growth of the species of *N. elegans* and *S. longiloba* can be impacted by these anthropic aspects in addition to the adverse effects associated with climate variability and change in the area (e.g., prolonged drought events).

CONCLUSIONS

In the present study, it was recorded in a region of arid zones (Northwest Mexico) that the species of *N. elegans* and *S. longiloba* maintain a life span of ~2.5 months related to the presence of flooding (Flood pattern) and contents of soil moisture, with maximum biomass carbon stocks of 165.5 g/C/plant for *N. elegans* and 75.5 g/C/plant for *S. longiloba*. For this reason, it is important to determine the stock and stores of carbon as "baseline" information to know the potential of the ecosystems that they must capture and fix it in parts of the plant (biomass) and the soil, in this way justifying the conservation and restoration of this type of ecosystem that contributes to the mitigation of climate change. In addition, further research on carbon stores in dryland wetlands is recommended. Given the present limits on our ability to optimize wetland creation and restoration for specific carbon and greenhouse gas emission goals, it is wise to prioritize the conservation of existing wetland carbon stocks over restoration and management.

Unfortunately, for both species, the ecological factors that determine the growth and development of these plants in Mexican environments have not yet been sufficiently addressed, and management and conservation programs are required to rescue and simultaneously promote their biological importance among the population, cultural and economic characteristics of these plants.

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Chapter 9

Plant galleries as a strategy for environmental education in México

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ABSTRACT

There is a global crisis in environmental education, hence, educational institutions at all levels must commit to fostering a paradigm shift in their students by creating proposals that help prevent and/or mitigate the environmental impact generated by economic activities. In this sense, this contribution is made on the use and benefit of vegetable galleries as a basis for conscious agricultural practices, and concomitantly, as a strategy for soil recovery, water use, and environmental conservation. Thus, creating social and economic well-being for thecommunities, an objective pursued by education.

Keywords: agricultural practices, environmental conservation, education, soil recovery.

INTRODUCTION

The Food and Agriculture Organization of the United Nations (FAO) is a technical organization created by the United Nations (UN) with the purpose of combating hunger and poverty in the world;

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however, by 2055, it foresees a world population of 10,000 million people (FAO, 2020). The World Bank reports that as of 2019, 8.9% of the world's population was undernourished, facing unprecedented pressures and challenges while suffering strong economic impacts (World Bank, 2022). Under this chaotic scenario, solutions must be created from education based on the concept of sustainability, favoring food production and planet health (FAO, 2021). Sustainable agriculture is an approach in which the use of resources is balanced with productivity, which is achieved through the capacity to innovate, coordinate, and manage soil, water, and organisms within the climatic and economic limits of the environmental system (Quam et al., 1999). For this reason, paradigms must be broken, and stakeholders must work as true agents of change. This can only be achieved through comprehensive environmental education, in which well-structured and innovative strategies must be proposed and applied to lay the foundations for true sustainability. These goals can be accomplished by starting in regions implementing small changes in food production and increasingly gaining a foothold in all economic activities.

The success of bioeconomics, whose main task is to investigate the impact of human enterprises on the environment (Mohammadian, 2000), is supported by biotechnology. Biotechnology plays a fundamental role in solving production and processing problems in agricultural and livestock products in a sustainable manner through the creation of new production techniques aligned with the concept of sustainability. In this way, an increase in yield and profitability in production is achieved by reducing or mitigating pests, improving adverse abiotic conditions such as drought and cold, and taking better advantage of food production areas. All the above are the basic foundations of agroecology, which also promotes the reduction or prohibition of the use of agrochemicals to develop a rational use of the environment, thus favoring the conservation of biodiversity (Muñoz; Montico, 2021; Bravo, 2013; Gazó, Sharry, 2015). As part of agroecological education, the reuse of nutrients, with the use of organic fertilizers and/or biofertilizers from bioprocesses, is proposed as an effective measure to achieve rural sustainability (Gálvez; Huerta, 2017). World organic production statistics show that in Mexico, 1,853,653 hectares (Ha) are registered as organic spaces where the wild harvesting of medicinal and aromatic plants, diverse fruit species, silvopastoral systems, and beekeeping are used in natural conditions. However, hard data only report 215,634 Ha as organic production systems with 45,954 producers and 544 processing plants (Wille et al., 2022). In this sense, while training as part of an environmental education process has a long way to go worldwide, it is important to highlight the educational activity in this aforementioned panorama, and with this, a question arises: What would be the educational strategy to make food production truly sustainable?

The Mexican Ministry of Public Education (SEP) in its 2022 curriculum framework and syllabus of Mexican basic education for children and adolescents encompasses new content related to environmental and financial education (favoring one type of economy over others, such as the popular and solidarity economy) and the use of digital information and communication technologies. It also highlights forming part of various educational processes linked to the community-territory to develop

projects aimed at social justice and solidarity with the environment (SEP, 2022), a consideration that often remains only in the inkwell and in the trunk of good intentions.

In Mexico, the specific subject concerning plant galleries as an ecological strategy is rarely considered in environmental education or training programs. Nonetheless, our contribution presents the benefits of these systems with the purpose of promoting their development to a greater extent, not only for regions that need the effectiveness of windbreaks against erosion or protection but also to understand the integration of tree coverage in a holistic manner. The resultant creation of vegetation corridors with different strata can serve as settlement areas for diverse organisms; they have the capacity to regulate soil temperature and create a single system through the agroecological matrix (Martínez, 2018; Griffon et al., 2010). The objective this document is encourage on the use and benefit of vegetable galleries as a basis for conscious agricultural practices, as a strategy for soil recovery, water use, and environmental conservation.

MATERIALS AND METHODS

A compilation of information in the databases of the Worldwide Web was carried out using scientific articles published in indexed and refereed journals as sources of consultation, as well as national and international library resources and repositories (Michán, 2011). Subsequently, a structured and systematic interpretation and integration of the information was carried out, followed by the drafting of this document (Tinto, 2009).

RESULTS AND DISCUSSION

Environmental education must play a decisive role in food production to reduce the environmental impacts created. An example of this scenario is the case of Central America, where almost all agricultural systems are managed alternating trees, crops, and/or pastures as agroforestry or agrosilvicultural systems. This integration favors the producer since it provides timber, forage, and fruit, in addition to offering shade and windbreak protection. It also increases the biological diversity of the area since its foliage, roots, and leaf litter form ecological niches for a great variety of macro- and microorganisms, in addition to improving soil fertility by increasing organic matter (Beer et al., 2003; PASOLAC, 1999). It is worth mentioning the various benefits of living barriers in environmental training programs, emphasizing that they function as a natural repellent by being a biological control against various pests and parasites (Smith; Liburd, 2012; San Román; Cárdenas, 2016). Among the precedents that reinforce these practices are those carried out by González et al. (2006), who evaluated marigold (*Tagetes erecta*) and sorghum (*Sorghum spp.*) as an alternative for biological and physical control against the white fly (*Bemisia spp.*) in eggplant (*Solanum melongena L.)* crops. They resolved that this barrier was highly effective in reducing whitefly populations and that it should be promoted as an alternative within

integrated pest management practices. The efficiency of the combination of four live barriers, corn (*Zea mays*), sorghum (*Sorghum bicolor*), sunflower (*Helianthus annuus*), marigold (*Tagetes erecta*), and the entomopathogenic fungus (*Paecilomyces farinosus*), was evaluated on vegetable crops in Oaxaca with the same pest (*Bemisia tabaci*) to propose integrated management. The results indicated that the combination of *P. farinosus* with corn barriers produced a yield of 4,721 kg ha^{-1} in chili crops (*Capsicum annuum L.*) and 7,227 kg ha^{-1} saladette tomato *(Solanum lycopersicum*) without being significantly different from the control treatment with a commonly used endosulfan pesticide (Ruíz; Aquino, 1999).

Repellent plant barriers have an unpleasant odor for some insects, and some of these plant species are borage (*Borago officinalis*), sage (*Salvia spp*), marjoram (*Origanum majorana*), thyme (*Thymus spp*), nettle (*Urtica dioica*), milfoil (*Achillea millefolium*), wormwood (*Artemisia absinthium*), basil (*Ocimum basilicum*), buttercup (*Ranunculus acris*), calendula (*Calendula officinalis*), dandelion (*Taraxacum officinale*), and mint (*Menta spp*) (Cucchi, 2020). In the state of Hidalgo, Mexico, 124 plant species are used, from which 186 products are obtained in the form of infusions and fumes to combat 29 types of pests of both vertebrates and invertebrates (Villavicencio et al., 2010). Other vegetable crops also share this anti-pest action, some of them being chard (*Beta vulgaris* var. *Cicla*), garlic (*Allium sativum*), celery (*Apium graveolens*), coriander (*Coriandrum sativum*), chives (*Allium schoenoprasum*), spinach (*Spinacia oleracea*), turnip (*Brassica rapa rapa*), parsley (*Petroselinum crispum*), leek (*Allium ampeloprasum* var. *Porrum*), radish (*Raphanus sativus*), and carrot (*Daucus carota*) (Cucchi, 2020). The dried flowers of the pyrethrum daisy (*Chrysanthemun cinerariaefolium*) contain active components such as pyrethrins, cinerins, and jasmolins as natural insecticides. The canary bird bush (*Crotalaria agatiflora*), turmeric (*Curcuma domestica*), and neem (*Azadirachta indica*) are some other species that have insecticide, fungicide, nematicide and repellent qualities, as well as herbicide power (Castro et al., 2018). However, there are plant species that function in an opposite manner, hosting pests. Such is the case for leguminous plants such as gliricidia (*G. sepium*) and the river tamarind (*Leucaena leucocephala*), which can host the green leafhopper (*Empoaska kraemeri*). This leafhopper is considered a pest that greatly affects bean crops; therefore, in places where it is present, these trees should not be included as living barriers (Pérez, 2009). In general, applications of agroecological systems favor the producer; however, it is also necessary to point out that the added value of lands with these systems increases between 23 and 52% over any traditional land or plot, even considering suburban land (Giraldo; González, 2018). Figure 1 shows an agroecological model based on an environmental education and sustainable production strategy that generates economic, social, and environmental benefits by integrating forestry systems (windbreaks with different strata and timber or fruit tree species), aromatic plants, vegetables, apiaries, and other livestock production systems (tilapia farming and sheep rearing). This system is not utopian, and its creation and functioning are possible if it is considered a "living organism", characterized by a structural conformation where all representatives and their dynamics are controlled through self-regulated biological mechanisms (Muñoz; Montico, 2021).

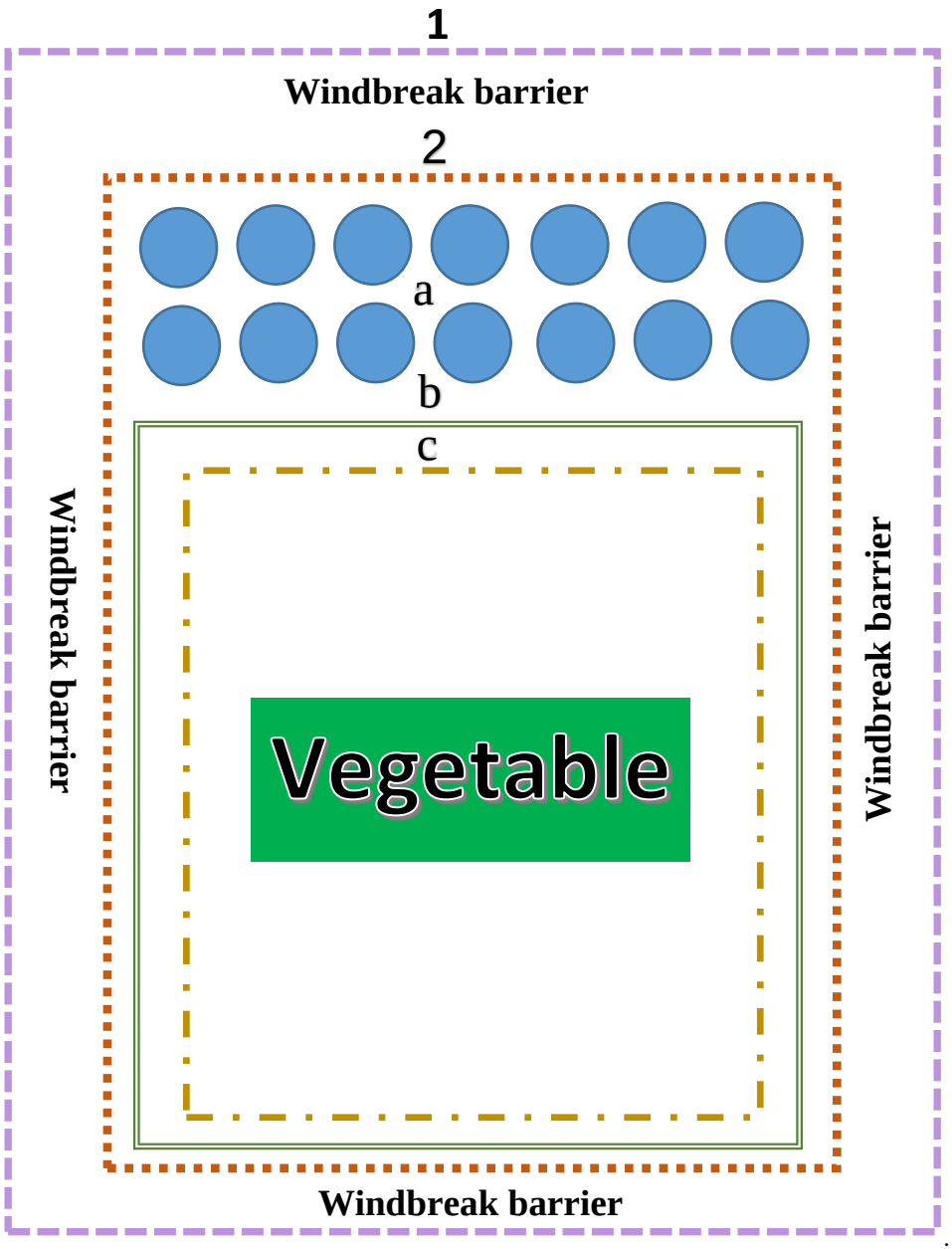

Figure 1. Integrated sustainable production system (without scale). Drawing own design

1. First stratum as a windbreak barrier of trees with an average height of 10 m; some species may be: rosy trumpet tree (*Tabebuia rosea*), cypress (*Cupresuss spp*), cottonwood (*Populus spp*), and barcino (*Cordia spp*).

2. Second stratum is a barrier of trees with an average height of 5 m; these can be citrus (*Citrus spp*), mango (*Mangifera spp*), mesquite (*Prosopis spp*), and acacia (*Acacia spp*)

a: Cultivation of tilapia (*Oreochromis niloticus*) in an open system to irrigate the entire agroforestry system with surplus water from water replacement.

b: Barrier of anti-pest plants or shrubs: basil (*Ocimum spp*), lavender (*Lavandula spp*), citronella (*Cymbopogon spp*), and sage (*Salvia spp*).

c: Organic agricultural crops (Vegetables)

Notes:

- The entire premises have common grass (Cynodon spp).
- Barriers 1 and 2 can be fenced with barbed wire and can be used for raising sheep (*Ovis spp*).
- In addition, apiaries, and hummingbird nests (*Colibri spp*) can be implemented to promote pollination in the area.

To successfully develop this activity, it is essential to carry out training in agricultural and forestry practices in the region combined with the criteria used by local farmers without creating conflict in social, cultural and/or economic aspects. Agroecology, as the basis of environmental education, generates transformations in soil management, ensuring suitable scenarios that maintain productive capacity (León; Acevedo, 2021). Additional factors must be balanced to avoid competition among all species (Beer et al., 2003); these factors include sources of water to maximize its availability, nutrients, and light.

Within the environmental education programme, it is very important to consider the agro-ecological requirements of the site, such as plant characteristics, soil preparation, planting seasons, planting and density systems, design, and layout of the production system (Martínez; Padrón, 2009). The target market to which the products are directed must also be very clear given the functional classification of trees based on the use product in agroforestry systems, such as timber trees, service trees, fruit trees, fodder trees, firewood, and charcoal trees (Beer et al., 2003). In the environmental education program, students should be instructed to design a well-planned integrated system. Windbreaks should not be considered simple rows of trees or bushes of different heights arranged in the opposite direction to the wind direction but as preventive systems against the loss of soil fertility due to wind erosion (Amargos, 2015). Moreover, windbreaks can also be viable as sources of fruit and bee keeping, promoting sustainable agricultural production with benefits to both biodiversity and landscape (Mekonnen, 2016). The following species are considered ideal for this purpose: common poplar (*Populus × canadensis*) and columnar poplar (*Populus nigra 'Afghanica'*), which are the most effective due to their growth rate, environmental tolerance, and low costs (Hansen et al., 2022).

Environmental training programs carried out in Kyrgyzstan, Central Asia, demonstrate the implementation of agroecological production systems with the integration of rows of poplar trees (*Populus nigra* var. *pyramidalis*) as windbreaks protecting cotton (*Gossypium hirsutum* L), corn (*Zea mays* L), and rice (*Oryza sativa* L), where crops achieve higher production rates with a more efficient use of water than in traditional agricultural systems (Thevs et al., 2021).

In training programs on integrated agricultural production systems with the use of living barriers as a strategic potential for landscape restoration, three factors are considered: a) the species must be native to the area, since they have better survival and development under local environmental conditions, b) adequate spatial configuration of the galleries, since these systems can act as structures that can retain the soil, favor the formation of terraces, and reduce surface runoff, in addition to favoring infiltration in low-lying areas, and c) adequate management (irrigation, pruning) to promote greater survival and development by mitigating the stress to which these living barriers are naturally subjected (12). On the other hand, wind erosion rates are determined by the following factors: a) soil classification, b) climatic conditions of the site, c) the windward disposition of the terrain, d) the amount and type of vegetation cover, and e) terrain dimensions (Brandle et al., 2009).

The importance of education on the benefits of this system makes it very versatile; for example, in very cold places, windbreaks provide valuable protection in autumn and winter by reducing soil erosion, abrasion, and desiccation of crops due to low temperatures and snow (Quam et al., 1999). On the other hand, in temperate to tropical zones, living barriers favor the creation of microclimates by reducing wind speed, achieving heat exchange in the form of a balance in radiation, water vapor and carbon dioxide between the soil, vegetation and atmosphere (Golberg et al., sf). Windbreaks are key structural elements in the rural environment and influence the functionality of landscapes in multiple ways, for example, the strategic use of green curtains for odor mitigation in livestock production (Brandle et al., 2009). Vargas (2020) carried out an investigation with living barriers using elderberry trees (*Sambucus nigra L.*) with a barrier distance between trees of 5 m and a distance between rows of 10 m to mitigate the effects of odors, insects, and noise from the development of pig farming activities. Thus, the effectiveness of living barriers in counteracting harmful effects on the human population in the vicinity of such farms was confirmed.

Another benefit of these techniques, which makes their promotion very necessary, is the positive effect they have against loud sounds. This has been investigated mainly in urban areas, where it was observed that there is a significant reduction in noise (>40 dB) thanks to the implementation of broadleaf shrub barriers in combination with coniferous trees (50 to 100 m) (Karbalaei et al., 2015). In other areas, such as cemeteries, a windbreak curtain of native species can be placed around the periphery (Higgins, 2013), as it minimizes the presence of odors and floating particles. These windbreak curtains are also beneficial in the sense that they have the capability of regulating temperature by capturing carbon dioxide (CO_2) to transform it into oxygen O_2 (Dueñas; Villa, 2019). It is very common that cypress trees (*Cupressus sempervirens*) are used in such places since they are long-lived, suitable for almost any climate and soil, present a beautiful landscape and can reach up to 30 m in height (Gutiérrez, 2005). An additional strategy that is highly recommended in these funeral gardens or cemeteries is to promote the development of microorganisms such as *Mycorrhizae* and *Azospirillum spp.* as nitrogen-fixing species and stimulants of plant growth (Dueñas; Villa, 2019).

An evaluation of the capacity to retain atmospheric dust particles was implemented using four tree species, bottle tree (*Brachychiton populneus*), oak (*Quercus ilex* subsp. *Ilex*), olive (*Olea europaea*) and nettle tree (*Celtis australis*) in Valencia, Spain, due to the high atmospheric pollution in urban areas. The capacity to retain atmospheric dust varied significantly among species, with oak having the highest rate of particulate matter capture. This is probably because the species is evergreen and has an elevated abundance of trichomes (a variety of villi) on the underside of its leaves (Ferriol et al., 2014). The results of this investigation are encouraging for the implementation of such environmental strategies in all cities of the world with native species that have the aforementioned characteristics. Moreover, while the use of crop-dusting on traditional agricultural crops continues to be used, it is not the most effective, as only 1% of the product supplied fulfills its purpose. Most (99%) is lost by wind action or seepage to the

subsoil, a situation that affects the environment with known contamination factors (Bejarano, 2017). One mitigation strategy against this environmental impact is the use of plant galleries, as they buffer the harmful effect of atmospheric pollution and are capable of capturing particles down to 2.5 μm (microns) through different mechanisms. One of these is the direct interception of floating particles through the stomata in their leaves, improving air quality (Mănescu et al., 2015; Hansen et al., 2022). The nature of the particles not only involves pesticides, but they may also carry suspended colloids (PM) as a complex mixture of chemicals and/or biological elements, such as metals, salts, and carbonaceous materials. In addition, they can also be volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), and endotoxins (Billet et al., 2007).

In recent years, as an alternative to mitigate air pollution and the challenges of climate change, the innovation of green roofs, vertical gardens and organic hydroponic agriculture has been implemented in urban areas, which has efficiently improved air quality in European cities (Pava, 2020; Köhler; Kaiser, 2021).

Environmental education has been implemented in developed countries at all school levels for decades, which has generated changes in their production and environmental paradigms. However, in Mexico, this is not the case since traditional agricultural production practices are dogmas fostered by the corruption of large companies and the government.

It is expected that by 2023, the agro-food sector in Mexico will be considered a development priority, as stated by the National Agricultural Council (CNA) in its outlook for the Mexican rural area. The ongoing conflict with environmental protection is not encouraging, which is why it is essential to apply environmental education at all levels and to extend it into production chains. At the same time, the National Development Plan (2019 to 2024) must be complied with in environmental issues or at least put on track and not have it remain in the inkwell of good intentions and become demagoguery.

Undoubtedly, ensuring food for the population is a priority for the Mexican government, but this must be done under new sustainable production schemes and curricula, for which it is necessary to establish adequate and innovative public and private policies that truly guarantee and execute these programs.

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