Handbook T-XII
CIERMMI Women in Science
Agricultural Sciences and Biotechnology

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ISBN: 978-607-8695-55-3
ECORFAN Publishing Label: 607-8695
HASB Control Number: 2021-10
HASB Classification (2021): 251021-1003

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ECORFAN CIERMMI Women in Science

Volume XII

The Handbook will offer volumes of selected contributions from researchers who contribute to the scientific dissemination activity of the Colegio de Ingenieros en Energías Renovables de Querétaro A.C. in their areas of research in Agricultural Sciences and Biotechnology. In addition to having a total evaluation, in the hands of the directors of the Colegio de Ingenieros en Energías Renovables de Querétaro A.C., the quality and timeliness of its chapters, each individual contribution was refereed to international standards (RESEARCH GATE, MENDELEY, GOOGLE SCHOLAR and REDIB), the Handbook thus proposes to the academic community, recent reports on new developments in the most interesting and promising areas of research in the Agricultural Sciences and Biotechnology.

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CIERMMI Women in Science T-XII
Agricultural Sciences and Biotechnology
Handbooks

Colegio de Ingenieros en Energías Renovables de Querétaro A.C – Mexico. October, 2021

DOI: 10.35429/H.2021.3.1.190

Preface

"It is healthy and necessary to turn our gaze to the earth and, contemplating its beauties, to recognize amazement and humility" Rachel Carson.

Throughout the history of mankind, the role of women has been preponderant for the development of civilization. Their contribution to the development of agricultural processes, from their stage as gatherers to the productive agricultural activity of today, represents the backbone of the rural economy in several regions of the world. Women's participation in this field can be decisive in achieving one of the Sustainable Development Goals related to food security, the purpose of which is to put an end to hunger and malnutrition. However, the role of women not only stands out in the direct productive aspect of communities, but also in the impact that can be generated from the development of science and technology, in this aspect it is noteworthy to mention that the participation of women in scientific fields has been growing in recent decades, in the natural and exact sciences we have been gaining ground approaching parity.

Biotechnology is a flourishing area of science that participates in a wide range of fields such as medicine, veterinary medicine, agriculture, biofuels and environmental remediation, among others. For Mexico it represents a new opportunity for growth and development where women play a prominent role, according to World Bank data this area is growing in countries such as Mexico, Chile and Brazil, not only in research from the academy, but has allowed women to join industrial sectors participating in leadership positions, thus breaking down the predominant cultural barriers in Latin America.

Biotechnology and agricultural sciences converge in shared interests, mutually nourish each other, establishing complementary ties, and substantially contribute tools that allow maximum and efficient use of natural resources, favoring their rational and sustainable use, developing improvements in production processes. The incursion of women in these areas should be a constant growth, the contributions made in this work by fellow researchers are a clear example of our willingness and capabilities, the efforts that each one makes from their own trench will open and consolidate the way for the generations that come behind, the young women and girls who dream of being scientists and improve our world.

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Introduction

The Colegio de Ingenieros en Energías Renovables de Querétaro A.C. (CIER-QUERÉTARO), and its chapters of Renewable Energy, Industrial Maintenance, Mechatronics and Informatics, technical sponsors of the International Interdisciplinary Congress on Renewable Energy, Maintenance, Mechatronics and Informatics, CIERMMI 2021 has as general objective to establish a space for discussion and reflection on issues related to the areas of: renewable energy, industrial maintenance, mechatronics and informatics with the participation of students, teachers, researchers and national and international speakers, promoting the formation and consolidation of research networks. Contributing to provide a space for dissemination and discussion of the presentations of students, graduates, academics and researchers, representatives of various higher education institutions, research centers in our country, as well as educational institutions beyond our borders. Promoting the formation of research networks between different institutions. Offering a space for undergraduate, master's, doctoral and postdoctoral students, in which they can present the progress of the research they carry out in their different educational centers. Providing a space in which study groups and members of academic bodies, linked to the curricular program of renewable energy, industrial maintenance, mechatronics and computer science careers, can present the research work developed within their institution and in collaboration with other national or international educational institutions. Establishing a training space for the attendees, through the development of specific lectures and conferences.

This volume, Women in Science T-XII-2021 contains 10 refereed chapters dealing with these issues, chosen from among the contributions, we gathered some researchers and graduate students from the 32 states of our country. We thank the reviewers for their feedback that contributed greatly in improving the book chapters for publication in these proceedings by reviewing the manuscripts that were submitted.

As the first chapter, *Fonseca, Mendoza, Ramírez and Lopez* present Bio-based antimicrobial packaging: A response to a reduction in the use of plastics and an advance in food safety. A review, as the second chapter, *Lopez, Mendoza, Peña and Fonseca* will talk about Importance of peptidoglycan hydrolases, bactericidal enzymes produced by lactic acid bacteria, in the reduction of antibiotic. as the third chapter, *Narciso & Peña* present Importance of gene expression studies in the understanding of contaminant biodegradation, as the fourth chapter, *Basilio, Díaz and Juárez*, propose Application of homeopathic preparations and biofungicides to prevent and control anthracnose (*Colletotrichum gloeosporioides*) in Haas avocado crops, as a fifth chapter, *Reyes, Roa, Amaya and Balderas*, realizan Biosynthesis of Metallic Nanoparticles and their Applications, as a sixth chapter, *Gómez*, developed Application of beneficial microorganisms rhizobacteria to improve plant production in protected natural areas, as seventh chapter, *Cabrera, Rojas, Alarcón and Tabarez*, discuss Adaptability and rusticity of zebu breeds over pure European breeds in the climates of the Mexican tropics, in eighth chapter, *Tabarez, Viveros, Garcez & Alarcón* present Preparation and use of intravaginal sponges for induction of estrus in hair sheep, as the ninth chapter, *Rosado, Micelli, Gómez and Ruiz*, performed Evaluation of an alternative nixtamalization method in maize landraces from Chiapas and as the last chapter, *Sánchez, Hernández, Neri and Balderrama*, focus on Growth promotion and productivity of tomato using two plant biostimulants: Arbuscular mycorrhizal fungi and seaweed extract.

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Content

Chapter 1 Bio-based antimicrobial packaging: A response to a reduction in the use of plastics and an advance in food safety. A review

Capítulo 1 Empaques antimicrobianos de base biológica: Una respuesta a la reducción del uso de plásticos y un avance en la inocuidad de los alimentos

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DOI: 10.35429/H.2021.3.1.29

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Abstract

Packaging has been developed to facilitate the transport, handling of food and providing a barrier against external factors. However, it has led to an increase in municipal solid waste (MSW) caused by plastics and waste produced during transport and distribution, which has prompted the development of bio-based antimicrobial packaging (BBA). One of the functions of BBAs is to inhibit the growth of microorganisms and reduce environmental contamination by using biodegradable materials, which is why the development of this type of packaging has become of great interest for research. This compilation provides an overview of the importance of BBAs, the methods and materials used for their production. Also, the most studied antimicrobial agents, their effect on the mechanical and barrier properties of packaging, and the advances have been made in BBAs.

Antimicrobial packaging, Biopolymers, Antimicrobial agents

Resumen

Los empaques han sido elaborados para facilitar el transporte y el manejo de los alimentos, además de aportar una barrera contra los factores externos, sin embargo, ha producido el aumento en los residuos sólidos urbanos (RSU) causados por los plásticos y los desperdicios producidos durante su transporte y distribución, lo que ha impulsado al desarrollo de empaques antimicrobianos de base biológica (EAB). Una de las funciones de los EAB es reducir o inhibir el crecimiento de microorganismos, además de disminuir la contaminación ambiental al utilizar materiales biodegradables, por ello en la actualidad el desarrollo de este tipo de empaques ha cobrado gran interés para la investigación. El objetivo de esta recopilación es proporcionar un panorama general de la importancia de los EAB, los métodos y materiales utilizados para su producción, los agentes antimicrobianos más estudiados y su efecto en las propiedades mecánicas y de barrera en los empaques, así como algunos de los avances que se han tenido en el área de EAB.

Empaques antimicrobianos, Biopolimeros, Agentes antimicrobianos

1 Introduction

For several decades, packaging has facilitated the transport and preservation of food. They have to fulfill specific functions such as containment, protection, convenience and communication. This last function refers to how the consumer identifies the product from others and provides information such as the nutritional table and traceability (Coles & Kirwan, 2011; Robertson, 2013; Singh *et al*., 2017). According to FAO, global food losses and waste are estimated at 1.3 million tons per year, implying a high carbon footprint. The industry's demand to reduce these losses led to changes in the manufacture of packaging, which promoted the development of active packaging (AP), which, in addition to fulfilling basic functions, are designed specifically for the needs of the product. Their importance lies in incorporating active substances in the packaging material, thus stabilizing changes that reduce food quality. The AP can be classified into two major systems: those that absorb oxygen, ethylene, moisture, carbon dioxide, flavors/odors; and those that release carbon dioxide, antimicrobial agents, antioxidants and flavors (Vermeiren, Devlieghere, Van Beest, De Kruijf & Debevere, 1999; FAO, 2011; Guillard, Gaucel, Fornaciari, Angellier-Coussy, Buche, & Gontard, 2018).

Active packaging has been studied to retard food spoilage. Such is the case of Hutter and Yildirim (2016), who evaluated the discoloration of ham by using palladium in active films. Their results show that when storing the food in palladium-containing films, the Δa* values increased. This is due to the absence of oxygen by palladium which prevents discoloration caused by the oxidation of nitrosomyoglobin. Another study is Bovi *et al*. (2018), who prepared active films using fructose as a moisture absorber. Their results showed that adsorbed moisture was higher when using 30 % fructose at 20 °C. In addition, they found that fructose packaging minimized condensation inside the packaging, favoring the preservation of strawberries during storage. Jiang *et al*. (2020) studied the effect of adding lemon essential oil to grass carp collagen films. They observed more efficient preservation of pork meat with the oil-added films. Peroxide values were lower in the active films (7.13±0.85 meq kg^{-1}) than the grass carp collagen films (11.35±1.04 meq kg^{-1}), indicating less lipid peroxidation in the frozen meat during storage. Further studies conducted in recent years on AEs, their function, and their food applications are presented in Table 1.1.

Table 1.1 Examples of active packaging and some food applications

Function of packaging	Active agent	Application in food	Author
Oxygen scavenger	Iron powder	Sausages	Gibis & Rieblinger, 2011
Oxygen scavenger	Palladium	Slices of ham	Hutter & Yildirim, 2016
KuEthylene scavenger	Clay nanoparticles	Banana, Strawberry and tomatoes	Tas *et al*., 2017
Ethylene scavenger	TiO_2	tomatoes	Kaewklin *et al*., 2018
Moisture absorber	Cellulose/fructose	Strawberry	Bovi *et al.,* 2018
Moisture-absorbing and antioxidant	Green tea/PVA	Dried eel	Chen *et al*., 2017
Antioxidant	Rosemary extract polyphenols	Fat food simulator	Piñero-Hernández *et al.*, 2017
Antioxidant	Lemon essential oil	Pork	Jiang *et al*., 2020
Antioxidant and antimicrobial	Clove essential oil	Sardine pancakes	Salgado *et al.,* 2013
Antimicrobial	Potassium sorbate or vanillin	Butter cake	Sangsuwan, Rattanapanone & Pongsirikul, 2014
Antimicrobial	Acrylonitrile and acrylamide	Apple and guava	Kumar, Kumar & Pandey, 2018
Antimicrobial	Eugenol	Lateolabrax japonicus	Li *et al*., 2019

Antimicrobial packaging is characterized by inhibiting the growth of microorganisms that can reduce food quality or cause disease by incorporating enzymes, bacteriocins or essential oils that control spoilage microorganisms and pathogens that cause foodborne illnesses (ETAS) (Alvarez, 2000; Cha & Chinnan, 2004).

According to WHO reports, in 2018, 550 million people fell ill due to contaminated food. Furthermore, figures reported in the World Bank show a productivity loss due to ETAS of $92.2 billion per year. In 2019, at the international conference on food safety, they reiterated the importance of safety and implementation of measures in food to reduce the incidence of ETAS. For this reason, antimicrobial packaging is of great importance.

In addition to ETAS and the losses caused by them, there are also contamination problems caused by plastics, which is the main component currently used for the manufacture of packaging in food. In 2015 and 2016 alone, a worldwide increase of 4.2 % in plastic production was reported, equivalent to 335 million tons. In 2018, Sardon and Dove revealed that by 2050 its production would exceed 500 million metric tons, alarming figures due to its persistence and effects on the oceans, wildlife and humans. The increase in the production of plastics, combined with their short lifespan and poor recycling mechanisms, will lead to a scenario where, by 2050, there will be more plastic than fish in the sea, which is why several studies are being conducted for the use of different polymers from biological sources to replace plastics (Jambeck *et al*., 2015; Guillard *et al*., 2018; Sardon & Dove, 2018).

The BBA has been developed to solve this problem. They are formulated with antimicrobial agents that inhibit the growth of microorganisms that cause ETAS and are added to polymeric matrices of biological nature that are degraded to natural compounds such as CO_2, CH_4, and organic compounds. Hence, they turn out to be an attractive alternative due to their ecological, renewable, economic and biocompatible characteristics (Zhong, Godwin, Jin, & Xiao, 2019). For this reason, this chapter aims to give an overview of the impact of BBA, the methods for their production, the main bio-based matrices and antimicrobial agents used in their formulation and their effect on the mechanical properties of the packaging, as well as future advances in antimicrobial packaging technology.

2. Bio-based antimicrobial packaging

The growth of microorganisms causes spoilage in food products. Traditional preservation methods such as drying, heating, freezing, fermentation and salting extend the shelf life of foods, but they must be packaged to avoid contamination and facilitate handling. For this reason, different preservation methods are currently being combined to extend the shelf life of products without changing their composition. Within these technologies, we can define *antimicrobial packaging* as a packaging system that interacts with the product or headspace to inhibit microorganisms present in the product (Han, 2005; Yam & Lee, 2012).

Antimicrobial packaging extends the dormancy period and reduces the growth rate of microorganisms to prolong their shelf life and maintain food safety. The efficiency of antimicrobial packaging is determined by the release of the antimicrobial, which is slow and gradual to maintain its effectiveness over a prolonged time. Antimicrobial packaging only inhibits the growth of microorganisms but must also meet several vital requirements; use materials that are authorized to be in contact with food, must be simple and cost-effective, chemically stable for prolonged use and act as a barrier to gases and water vapor.

3. Methods of preparation of bio-based antimicrobial packages

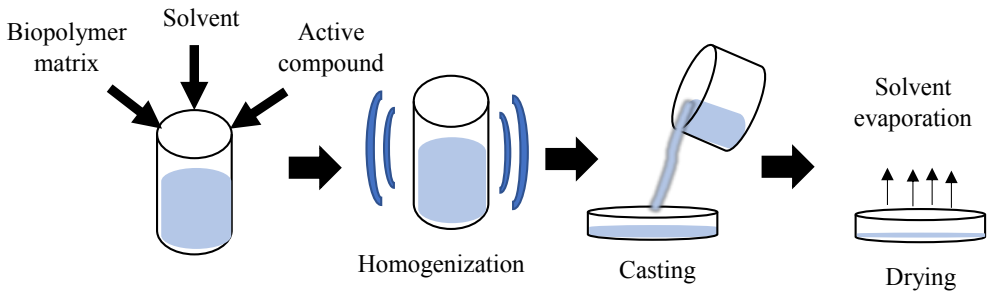
One of the main problems today in the production of bio-based packaging is the determination of the appropriate method for its production at the industrial level. Packaging production has been used for the wet (casting) or dry (thermoforming or extrusion) process for many years. The selection of the production method is crucial because it can change the characteristics of the material. The casting method is a simple process for obtaining edible films. It is regularly used in laboratory studies, but it has limitations in that it formulates films smaller than 25-30 cm and requires 10-24 h of drying. In contrast, dry methods facilitate the production of large-scale packaging at a low cost, which will discuss in more detail in this section (Grumezescu & Holban, 2017; Cerqueira, Pereira, Da Silva Ramos, Teixeira & Vicente, 2017).

3.1 wet process

This process consists of dissolving the biopolymers in suitable solvents, where the additives or functional compounds are added, then these are poured, spread on a surface and dried to evaporate the solvent and thus obtain the film. When drying occurs on the surface of the food, it is called coating or covering. This method is primarily used at the laboratory level because of its simplicity in the equipment required. It has been used to the formation capacity of different bio-based polymers, their physicochemical properties when using other conditions and additives.

The casting method can be synthesized in four steps: dispersion, homogenization, casting and drying (Figure 1.1) (Grumezescu & Holban, 2017; Cerqueira *et al*., 2017). The most commonly used solvents for obtaining films or coatings are water and ethanol. In this technique, higher temperatures can be used for polymer dissolution, as is the case of drying chitosan films using temperatures of 48-58 °C (Kienzele-Sterzer, Rodriguez-Sanchez & Rha, 1982; Cui *et al*., 2017). The method is widely used to prepare bio-based polymer films due to the evaporation of the solvent at room temperature (~25 °C), thus helping to avoid undesired reactions and which may alter the optical characteristics of the films. Some examples of antimicrobial films formed by this method are presented in Table 1.3.

Figure 1.1 Steps of forming films by the casting method



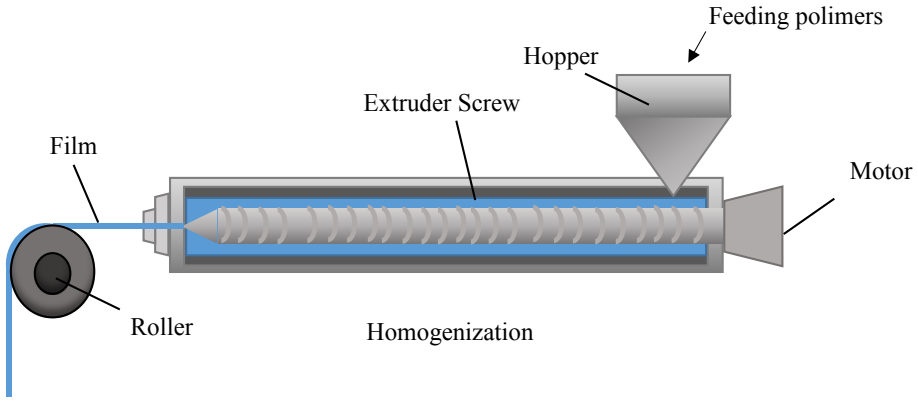
Source: Khan et al. (2018)

3.2 Dry process

The dry process is low cost and is used on an industrial scale. It has been used for materials such as PLA, PHA (polyhydroxyalkanoates), PHB (polyhydroxybutyrate). However, studies for the production of packaging utilizing this technique are minimal. Dry processes include extrusion and thermo-pressing.

Extrusion is a process that uses one or two rotating screws installed in a barrel which increases the pressure, pushes and mixes the ingredients necessary for the manufacture of commercial packaging (figure 1.2). It presents advantages such as a reduction in production time and energy required for solvent removal. However, this process can affect the quality of the films formed by the shear rate and high temperatures of the process (Espitia, Du, Avena-Bustillos, Soares & Mchugh, 2014; Orsuwan & Sothornvit, 2018).

Figure 1.2 Film production by extrusion method.



Source: Pranata et al. (2019)

Colak *et al*. (2015) used the extrusion technique to prepare sodium caseinate and lysozyme films. Their results showed that increasing the time and temperature in the film extrusion process resulted in the loss in lysozyme activity. However, by using a temperature of 65 °C and glycerol concentration of 20-25 %, they retained 26. 4 % of the initial lysozyme activity, a decrease in antimicrobial activity was also reported by Khalid *et al*. (2018), who prepared films using polycaprolactone and starch as matrix and incorporating pomegranate peel as antimicrobial agent. The reduction in antimicrobial activity of the films was attributed to the degradation of pomegranate peel compounds by heating and shearing in the process. Rodriguez *et al*. (2018) studied the effect on the film preparation method of cellulose acetate, triethyl citrate plasticizer, organic clay and cinnamaldehyde. They used the casting and extrusion method for the formation of the films. Their results showed that the extrusion method allowed a better homogenization of the organic clay than the casting method. The degradation of the quaternary ammonium in the organic clay negatively affected the films' color. The antimicrobial activity showed a 25% reduction in the film. This could be caused for using temperatures close to 200 °C that favored the evaporation of the cinnamaldehyde. Despite this loss, the antimicrobial activity tests showed a reduction of 3.5 logarithmic cycles in the growth of *Escherichia coli*, more significant than that observed for the films made by the casting method.

Thermoforming is a method in which high temperatures and pressures are applied to a mixture of polymers with viscoelastic properties that form a film upon cooling. It has hydrophobic, ionic, covalent, and hydrogen bonding interactions that help to stabilize it. This process consists of placing the biopolymer between a pair of thermostated plates that act as a press where it is necessary to regulate the parameters of temperature, pressure, time, type and content of plasticizer and humidity level (Blanco-Pascual & Gómez-Estaca, 2017).

Although thermo-pressing is not a standard method used to prepare antimicrobial films, this method used Moreno *et al*. (2016) to prepare films with corn starch, bovine gelatin, glycerol, and lysozyme. They showed that these films are more permeable to water vapor and oxygen than those prepared by the casting method that they are less rigid and have greater flexibility. The antimicrobial activity of the films obtained showed that the bactericidal activity against *Listeria innocua* is maintained in both methods. Another author who used this method to elaborate antimicrobial films was Valencia-Sullca *et al*. (2018), who formulated films with a mixture of starch, chitosan, glycerol and polyethylene glycol. Their studies showed that, when using a thermal process such as thermo-pressing, it causes a Maillard reaction due to chitosan giving a yellowish appearance to the films.

In general, both processes have advantages and disadvantages in the production of antimicrobial packaging. For example, dry processes are fast methods where less production time and low cost are needed. However, they require specialized machinery and high temperatures that can reduce the activity of the antimicrobial agents at the time of processing. Unlike films produced by wet casting methods, they use low temperatures for the removal of solvents, which helps to maintain the activity of their compounds and require less specialized equipment; however, they are limited to obtaining films of a size of 20-30 cm with prolonged drying times (up to 24 h).

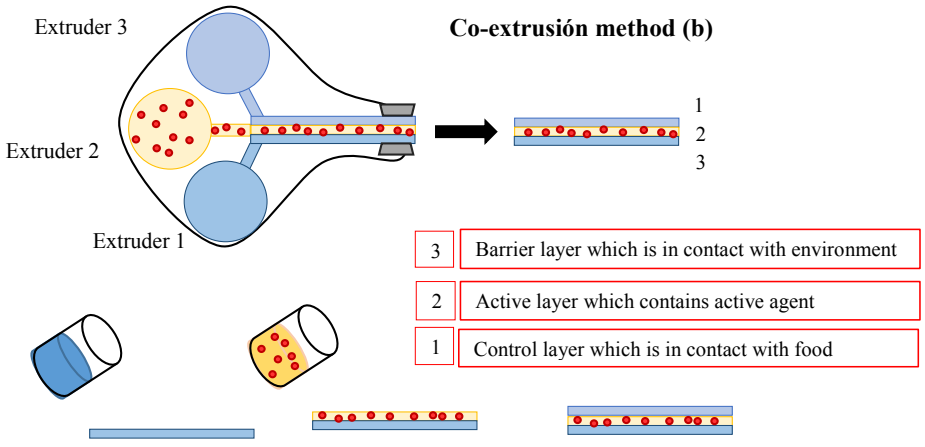
The structure of antimicrobial packaging is also influenced by the type of method used. However, both processes have been used to form unilaminar, bilaminar and multilaminar films that can improve the mechanical and barrier properties of the films.

4. Structure of bio-based antimicrobial packages

The development of bio-based antimicrobial packaging (BBA) is a promising alternative for the substitution of conventional packaging; however, it has disadvantages such as low permeability, which allows the passage of low molecular weight materials that can compromise food quality and safety. Therefore, different studies have proposed different alternatives, such as the combination of polymers or the use of coatings to improve the barrier properties of bio-based packaging without altering its optical and biodegradability properties (Cerqueira *et al*., 2017).

Currently, traditional packaging is rarely manufactured using only one material, especially when talking about flexible packaging. The use of several materials can lead to conform structures that can improve both mechanical and barrier properties, resulting in extending the shelf life of the food. Single or multilayer films containing more than one polymer are used in the industry to improve barrier and mechanical properties, and this type of packaging material has been proposed to control the release of active compounds. Regularly multilayer active films are constituted by three layers, as shown in (figure 1.3.a). They can be elaborated by both wet and dry methods (figure 1.3.b). Thickness, chemical composition and diffusivity are parameters to be considered to design multilayer films (Piergiovanni & Limbo, 2016; Almasi, Jahanbakhsh Oskouie & Saleh, 2020).

Figure 1.3 Methods for the production of multilayer films (a) wet method (casting) (b) dry method (co-extrusion)

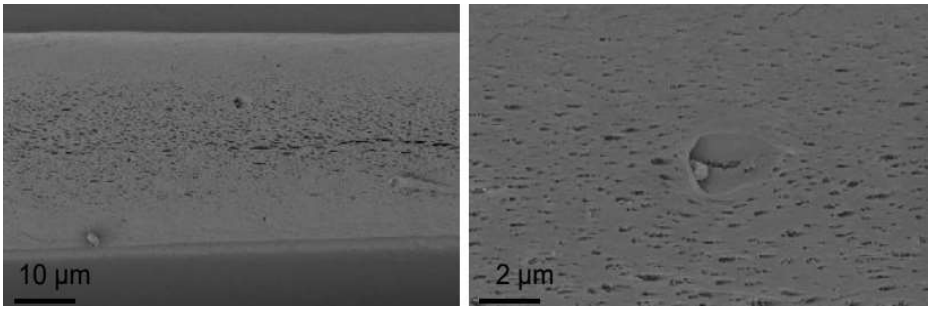


Source: Almasi, Jahanbakhsh Oskouie & Saleh (2020)

Armentano *et al*. (2015) developed antimicrobial films using carvacrol as an antimicrobial agent and combining the polymers PLA (polylactic acid) and PHB (polyhydroxybutyrate). Their results showed that there were changes in the microstructure of the films compared to PLA films where a smooth and uniform surface was shown. In PLA-PHB and carvacrol films, micro-holes (Figure 1.4) were observed on the surface, which could facilitate the release of the antimicrobial.

In terms of mechanical properties, studies showed that the addition of PHB to the PLA matrix did not significantly change the elastic modulus or its elongation percentage. In 2016, Shahmohammadi and Almasi evaluated the morphological, physical and antimicrobial properties of cellulose films produced by *Gluconacetobacter xylinum* strain and ZnO nanoparticles. The film structure was trilayer (two outer layers of cellulose and an inner layer of ZnO and cellulose nanoparticles). The multilayer films showed a smoother surface compared to the unilamellar ZnO and cellulose films. The multilayer film showed lower water vapor permeability and an increase in elastic modulus and breaking point compared to the unilamellar films. A decrease in the antimicrobial activity of the multilayer films was observed in comparison to the free antimicrobial agent incorporated in a monolayer film.

Figure 1.4 Micrograph obtained by field scanning electron microscopy (FESEM) of the PLA-PHB and carvacrol film



Source: Armentano et al. (2015)

Wang *et al*. (2019) formularized and characterized multilayered films of chitosan, sodium alginate and carboxymethyl chitosan. The data obtained on the surface morphology of the multilayered film showed that added ZnO formed aggregates. The solubility and water vapor permeability decreased in the multilamellar films compared to films made only with chitosan and chitosan-alginate. For the antimicrobial activity, the results showed that the addition of ZnO improved the activity of the multilamellar film depending on the proportion in which it was present, also showed that there was an inhibition of the chitosan unilamellar films against *Staphylococcus aureus* and *E. coli*.

As mentioned above, the final properties of the antimicrobial films are modified by the method and the type of structure they present. Another parameter that affects the properties of the packaging and that has to be considered is the choice of the materials used for its elaboration. Among the main components used in the manufacture of antimicrobial packaging are the polymeric matrix, the antimicrobial agent and the type of plasticizer used. The latter can be defined as a low molecular weight volatile compound that is added to polymers to reduce brittleness and impart flexibility. Plasticizers reduce intermolecular forces and increase the mobility of polymer chains, reducing the glass transition temperature of films and improving their flexibility and elongation. Commonly used plasticizers for film and coating production are monosaccharides, oligosaccharides, polyols, lipids and derivatives (Sothornvit & Krochta, 2005; Azeredo *et al*., 2011).

The type of plasticizer used will depend on the polymeric matrix used for the formation of the container, for which the characteristics of each of these polymers, their compatibility and possible interactions must be taken into account. In the following section, some studies and characteristics of the main polymeric matrices studied will be discussed in general terms.

5. Biopolymers used in antimicrobial packaging

Biopolymers have been studied as an alternative to traditional polymers used in food packaging; however, their implementation is not limited to packaging, as they can have applications in medical materials, cosmetics, food additives, water treatment chemicals, and absorbents, among others. Biopolymers are produced by living organisms and can be derived from microbial systems, extracted from plants, or chemically synthesized.

These biodegradable materials can be classified into four large families: the first are polysaccharides that can include starch, cellulose and chitosan; the second family are proteins such as gluten and zein; the third includes the use of oils; and finally, polymers that are produced by natural or genetically modified microorganisms where we can find PHA (polyhydroxyalkanoates) and PHB (polyhydroxybutyrate). Among the most studied characteristics of these polymers when forming films, we can find their barrier and mechanical properties (Rebelo, Fernandes & Fangueiro, 2017; Zhao *et al.,* 2019).

The barrier properties of materials indicate their resistance to diffusion and sorption of substances, a polymer with suitable barriers has low diffusion and solubility coefficients. The diffusion coefficient measures the speed with which the substance penetrating the polymer could move within the polymer matrix. On the other hand, the solubility coefficient gives us the concentration of the substances absorbed by the polymer upon contact with it. Another term used to measure barrier properties is the permeation coefficient, which combines diffusion and solubility coefficients. Permeation is the ability of a permeant to penetrate and pass through material in response to the difference in partial pressures. The barrier characteristics of a polymer are commonly associated with its permeability coefficient values, so low permeability values are found in polymers that allow the low mass transfer, the lower this value, the lower the transfer of the type of fluid being evaluated, for example, in food packaging permeability to water vapor and oxygen are commonly evaluated (Culter *et al.,*2016; Han, 2005).

The mechanical properties of polymers show us their behavior, and these depend on the type of polymer and the additives used. It is necessary to use a force-strain curve to examine the mechanical behavior of packaging materials. This curve shows the force required for deformation to occur. The curve helps us obtain the elastic modulus or Young's modulus, which indicates the ratio between the applied stress and the force produced in the elastic portion of the material's behavior and is found in the linear part of the curve. As this parameter increases, the amount of stress required to achieve a given deformation increases, which means a stiffer material. After the modulus of elasticity, a point known as the elastic limit is reached, which is the maximum stress that the material can withstand, where the deformation that occurred disappears, and the material returns to its original dimension. Beyond this point, we find the plastic region where the material begins to increase in deformation without increasing the required stress. Finally, tensile strength is the maximum resistance of a material subjected to a tensile load (Culter *et al.,*2016).

5.1. Polysaccharides

Polysaccharides are the most studied biopolymers due to their biocompatibility, biodegradability and non-toxicity. This large group of macromolecules is classified according to their structure, chemical composition, application and origin. The latter is the most common and classified into those extracted from plants, algae, lichens, and other bioactive polysaccharides derived from animals. Polysaccharides are used to elaborate edible films or coatings in the packaging area due to their hydrogen bonds that form a network. Polysaccharides present good barrier properties against oxygen. However, they have a poor barrier against humidity due to their hydrophilic nature. Polysaccharides are used in packaging to prolong the shelf life of fruits, vegetables, seafood, or meat products because they reduce dehydration, oxidative rancidity, and browning on the food surface. Cellulose, chitosan and starch are some of the most representative polysaccharides (Liu, Willför & Xu, 2015; Hassan *et al*., 2018).

5.1.1. Cellulose

Cellulose is the most abundant polymer among organic compounds and is made up of glucose units linked by β-1,4-glycosidic bonds. The most commercially exploited source for extracting cellulose is wood. However, it can also be found in plants, algae and those produced by bacteria. It is a crystalline liquid with high strength, flexibility, biocompatibility and biodegradability. Its stability is due to the numerous hydroxyl groups present in its structure that lead to forming a network with intramolecular hydrogen bridge bonds. Cellulose is insoluble in water due to the relatively long length of its constituent cellulose chains and the closeness of their hydrogen bonding. Solvents such as ionic liquids, NaOH solutions, among others, are regularly used for film production. Films made from this polysaccharide tend to show advantages by being tasteless, flexible, odorless, transparent, resistant to fats and oils, have hydrophilic nature and have low oxygen and moisture diffusion (Piergiovanni & Limbo, 2016; Karaki *et al*., 2016; Cazón *et al*., 2017; Mohamen *et al*., 2020).

The most commonly used cellulose derivatives are carboxymethylcellulose, methylcellulose and hydroxypropylmethylcellulose. These present a low barrier to water vapor due to their hydrophilic characteristics. The incorporation of different compounds has been studied to counteract the adverse effects of cellulose derivatives on their barrier properties. Saringat, Alfadol, and Khan (2005) studied the effect of polyethylene glycol and triacetin in hydroxypropyl methylcellulose coatings and observed that the addition of the plasticizer resulted in coatings with lower resistance to attraction and increased water vapor permeability compared to the control. In contrast to the addition of triacetin that decreased water vapor permeability. On the other hand, De Melo Fiori *et al*. (2019) combined carboxymethyl cellulose with polyethylene glycol and sodium-based clay nanofillers. The results showed that the addition of the clay improved the mechanical properties by increasing their strength, elastic modulus and elongation percentage and significantly decreased the water vapor permeability of the films.

5.1.2. Starch

Starches are made up of α-D-glucose units, mainly containing 20-30% amylose (straight-chain polymer) and 70-80% amylopectin (glucose polymer having a branched-chain structure). Starch can be found in wheat, rice, corn, tapioca and potato and is a non-toxic, low molecular weight and renewable material. Another advantage it presents is the formation of transparent films, without color, odor and taste, important characteristics in food packaging (Karaki *et al*., 2016; Hassan *et al*., 2018; Mohamen, El-Sakhawy & El-Sakhawy, 2020). The films or coatings made with starch usually have good barrier properties to CO_2 and O_2. However, Their hydrophilic characteristics, soluble in water and have a poor barrier against water vapor.

Starch film formation starts with the heating of starch granules and water to form a viscous solution. Excess water and high temperatures cause the starch to transform from a semi-crystalline state to an amorphous state, known as gelatinization. This transition process depends on the amylose-amylopectin ratio, water content and dispersion temperature. After gelatinization, the retrogradation process allows the amylose and amylopectin chains to be dissociated in a starch dispersion to reassociate into an ordered structure that gives starch films their final permeability and mechanical properties (Thakur *et al.,* 2019).

The elaboration of films from this polymer has varied according to its components and structure. Ghanbarzadeh, Almasi and Entezami (2010) elaborated films of starch and carboxymethyl cellulose (CMC). Their results showed that the addition of CMC in starch films reduced water permeability. When they increased to 20% CMC, the tensile strength increased to 59% more than pure starch. Concerning color, the films showed a decrease in yellowness values and an increase in lightness. In 2013, Das *et al*. studied the effect of a coating of rice starch with coconut oil and tea leaf extract on tomatoes. Their results showed that the weight loss in coated tomatoes was less than that of the control. It also showed a delay in the ripening effect on the tomato, which extended its shelf life.

Another study combining the properties of starch with different materials is Saberi *et al*. (2018), who developed multilayer films using pea starch, guar gum and a mixture of lipids. The addition of lipids in the starch films decreased the fruit respiration rate, ethylene production, firmness and weight loss, peel peeling, and decay rate of orange fruit, thus extending the fruit's shelf life. Starch films added with antimicrobials have also been used to inhibit the growth of pathogenic microorganisms in ready-to-eat foods. Zhao *et al*. (2019) formulated films with cassava starch, carvacrol, chitosan and gallic acid to inhibit the growth of *Listeria monocytogenes* in ham slices.

5.1.3. Chitosan

It is a polysaccharide that has been widely studied for the formation of active films for its antimicrobial and antifungal activity. Like other polysaccharides, it has low permeability to water vapor and oxygen. It is extracted from chitin, which is present in crustaceans, mollusks, insects, algae and related organisms. Approximately 100 billion tons are produced annually from these sources, making it the second most abundant polysaccharide after cellulose. Chitin consists of N-acetyl-D-glucosamine chains linked by β-1,4-glycosidic bonds. To obtain chitosan, chitin has to go through chemical or enzymatic processes, being the chemical one the most used due to its low cost and capacity for mass production. Chitosan is obtained from the deacetylation of chitin, formed by D-glucosamine and N-acetyl- D-glucosamine units with β-1,4 glycosidic bonds.

When the degree of deacetylation is approximately 50 %, chitosan becomes soluble in acidic media, however, its solubility will depend on the degree of N-acetylation and its molecular weight. (Chang, Tsai, Lee & Fu, 1997; Tripathi, Mehrotra & Dutta 2008; Cazón *et al*., 2017; Muxika *et al*., 2017; Domínguez *et al*., 2018).

The formation of chitosan films has been done by varying different parameters in their preparation, such as the type of plasticizer, the drying method and the addition of antimicrobial agents. In 2005, Suyatma et al. observed the effect of four different plasticizers (glycerol, ethylene glycol, polyethylene glycol and propylene glycol) on the mechanical properties of chitosan films and their storage stability. The use of glycerol, ethylene glycol, polyethylene glycol improved the ductility of chitosan films, however, propylene glycol made the films more brittle. They found that, over time, most of the films showed a decrease in their percentage elongation. They concluded that glycerol and polyethylene glycol were the most suitable because of their storage stability. Regarding the effect of drying type and incorporation of antimicrobial agents, Thakhiew, Devahastin and Soponronnarit (2013), studied the effect on the concentration of active agent and drying method (hot air and low pressure superheated steam drying) had no significant effect on water content, the thickness and water vapor permeability of chitosan and blue ginger films, but had influence on the color, degree of crystallinity, attractive strength, elongation percentage and oxygen permeability.

5.2. Proteins

Proteins are presented in fibrous or globular. They function as structural materials of tissues and perform different functions in living systems. The physicochemical characteristics of proteins depend entirely on the arrangement of amino acid substituents and the concentration in which they are present along the polymeric chain. They have good mechanical and optical properties while providing a suited barrier against aromas, oxygen, organic vapors and selective permeability to other gases. Despite these advantages, films made from proteins can be affected by their high moisture content. Different types of globular proteins have been proposed for film or coating formation, such as wheat gluten and corn zein (Dominguez *et al*., 2018; Hassan *et al*., 2018).

5.2.1. Corn Zein

Zein is a prolamin and the main protein in corn. It is hydrophobic and a thermoplastic material because it is strong, shiny, bacteria-resistant, water-insoluble, antioxidant, and adhesive. It can be dissolved in 70-80 % ethanol, high concentrations of urea, alkalis or anionic detergents. It contains high concentrations of amino acids such as glutamic acid, proline, leucine and alanine. In the food industry, zein is used as a coating material for candies, fresh and dried fruits, nuts, and incorporation into chewing gum. Zein films are usually formed by drying the medium in which they were dissolved, and the use of plasticizers is essential to give the films flexibility as they are brittle. Due to its excellent gas and moisture barrier properties, it is a good alternative for film production. There are several studies on the elaboration of zein films and antimicrobial agents in the literature and the possibility of producing them using thermo extrusion methods. However, it has been seen that this method is not compatible since some biopreservatives lose their activity during the thermal process (Yemenicioglu, 2016; Aguirre- Joya *et al*., 2018; Hassan *et al*., 2018).

These films have been developed in recent years by combining them with antimicrobial agents and different biopolymers to modify their structural characteristics. Vahedikia *et al*., in 2019, incorporated cinnamon essential oil and chitosan nanoparticles into the polymeric matrix and observed that it significantly improved the tensile strength and decreased the elongation percentage of zein films. They also determined a higher crystallinity in the presence of cinnamon essential oil and chitosan nanoparticles. The films showed antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. Another study elaborated with Zein active films was that of Haiying Cui *et al*. in 2020, who added pomegranate peel extract encapsulated in chitosan nanoparticles to zein films. They also applied liquid nitrogen plasma to modify the surface of the films to maintain the release of pomegranate polyphenols from the nanocomposite films. Their results showed higher thermal stability of these films compared to those developed with pure zein alone. The release of the active agent was lower in the plasma-treated films and significantly inhibited the growth of *Listeria monocytogenes* compared to the untreated and control films.

5.2.2. Wheat gluten

Wheat gluten is a globular protein that combines polypeptide molecules. Its properties, such as cohesion and elasticity, facilitate the film formation process. This protein plays an essential role in bread making, just as zein is insoluble in water. The formation of gluten films depends mainly on breaking its disulfide bonds during the thermal process and on the formation of new disulfide bonds during the drying of the films. In addition to these changes, the hydrogen bonds also undergo modifications. The surface of gluten films regularly is glossy, has good insulation to oxygen and limited resistance to water vapor (Micard *et al*., 2000; Aguirre- Joya *et al*., 2018; Hassan *et al*., 2018; Chen *et al.* 2019).

According to studies by Gontard *et al*. (1992), when testing different ethanol concentrations (70-20 mL/ 100 mL) and pH (2-6) in gluten film-forming solutions, an effect on opacity, solubility and water vapor permeability was observed. Permeability can be modified mainly by gluten concentration and pH. As with the other polymers, the preparation and the addition of plasticizers determine the final characteristics of the films. According to the studies of Mangeavel *et al*. (2004), the type of method by which gluten films were prepared affected the tensile strength and elongation percentage. Their results showed that the tensile strength was higher for films formed by thermo-pressing compared to those formulated by the casting method (4.15±0.78 MPa and 0.60±0.15 MPa, respectively) but showed lower values of elongation percentage (179±46% and 732±90%). Regarding the influence of the plasticizer, in both cases, if the plasticizer concentration increased, the value in tensile strength decreased.

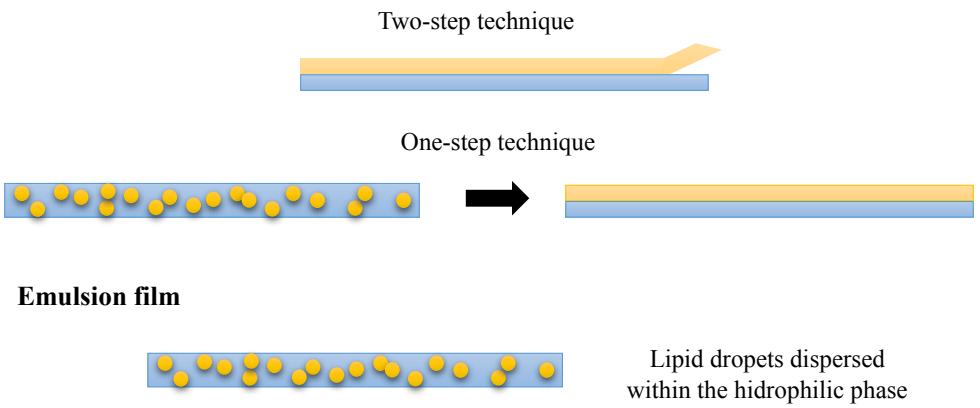
Gluten films added with bacteriocins have also been formulated to inhibit *Listeria innocua*. According to Blanco Massani *et al*. (2014), the addition of 0.1 % Lactocin 705 and lactocin AL705 inhibited the growth of *Listeria innocua* 7 and *Lactobacillus plantarum* CRL691. In other studies, in 2015, El-Wakil *et al*. added TiO_2 nanoparticles and cellulose nanocrystals in wheat gluten films and studied their effect on tensile strength, elastic modulus, and water vapor permeability. They found that the films changed their percentage elongation using 0.6 and 0.8 % TiO_2 and 7.5 % single cellulose crystals. The films with cellulose modified the water vapor permeability, obtaining lower values than films made only with gluten. The addition of TiO_2 also showed a reduction in water vapor permeability in films with 7.5 % cellulose.

5.3. Lipids

Lipids are found in sources such as plants, animals and insects, and for several years have been used to make edible films and coatings used in the food area to preserve fruits and vegetables. Lipids reduce water vapor permeability due to their hydrophobic nature and provide gloss. However, they form brittle and thick films. Their poor mechanical properties mean that they have to be combined with biopolymers such as proteins and polysaccharides. They can be associated in the form of emulsions or bilamellar films. In the emulsion system, the lipid is mixed in the film-forming solution and then formed by the casting method, regularly stabilized with an emulsifier, so that phase separation does not occur and a monolayer film is obtained. In the layer-by-layer system, the same way as the unilamellar films, the lipids are cast on the previously dried layer of the biopolymer.

Figure 1.5 illustrates these two procedures. Their efficiency as a barrier to water vapor depends mainly on the nature of the lipid, the length of the fatty acid chain and the structure of the emulsion that constitutes the dry film. This group comprises monoglycerides, diglycerides, triglycerides, cerebrosides, phosphatides, phospholipids, terpenes and fatty acids. Due to their greater efficiency as a moisture barrier, animal or vegetable waxes are the most widely used in preserving fruits and vegetables (Debeaufort *et al*., 2000; Han, 2005; Cerqueira *et al.,* 2017; Aguirre-Joya *et al*., 2018).

Figure 1.5 Formation of films composed of a lipid and a biopolymer



Source: Han (2005)

5.3.1. Waxes

Waxes are esters of long-chain fatty acids with long-chain alcohol, non-polar, high hydrophobicity, insoluble in aqueous media and soluble in organic solvents. They are usually moldable at room temperature, brittle and do not show good elasticity. Waxes are soluble in hexane, chloroform or benzene. These molecules have no polar constituents or have such a small hydrophilic part that they cannot interact with water, thus preventing the molecules from diffusing, which may explain their efficiency as a barrier to water vapor. Waxes are found in living organisms, such as in bird feathers that repel water, and it has also been observed that some invertebrates produce waxes to keep their skin lubricated and repel water. They are also found on the surface of leaves and fruits. This group can include natural waxes such as carnauba wax, candelilla wax, rice bran wax, and beeswax (Han, 2005; Yurcanisn Bruice, 2007; Wade, 2011; Robersont, 2013; Aguirre- Joya *et al*., 2018).

Waxes can be used for food preservation, such as the films developed by Oregel-Zamudio *et al*. (2017), who combined candelilla wax films with *Bacillus subtilis* strain HFC103 to preserve strawberries. Treatment of strawberries with these films showed a 100 % reduction in fruit decay relative to the control on the sixth day of storage. And a reduction in the severity index, which indicates the damage caused by the presence of mold in fruit, was reported on days 2,4,5 with percentages of 21,41,47, 54 and 56 %, with respect to the control. Other studies conducted by Aguirre-Joya *et al*. (2019) used candelilla wax, pectin, aloe vera mucilage, glycerol, and Larrea tridentata leaf extract to produce a coating. They showed that it reduced the damage caused by *Colletotrichum gloeosporioides* to 22 % and *Alternaria alternata* to 24.5 % in avocados. By 2018, Motamedi *et al*. used a combination of nanoclay and carnauba wax to preserve the quality of "valencia" orange, showing that the application of this coating improved fruit acceptability, nutritional quality and reduced fruit weight loss during storage.

5.4. Polymers synthesized by microorganisms

Biopolymers that are synthesized to aid the survival and function of microorganisms can occur intracellularly, structurally and extracellularly. Intracellularly accumulated polymers, as granules within the cytoplasm of cells, have mechanical properties similar to elastic rubber or crystalline hard plastic. Exopolymers can occur as slime or encapsulate that can be separated from the medium by centrifugation. Microbial biopolymers perform specific functions as a source of energy or as protective agents. They are also released to help microorganisms function, adapt, multiply and survive. They have a wide variety of functions in food, medicine and other applications. They are neutral or acidic in nature. Some produce very high viscosity in aqueous solutions, and others form gels similar to agar and carrageenan. Various physical and chemical parameters influence the production of these biopolymers.

Their low production cost makes them an attractive alternative Table 1.2 presents some microorganisms producing these biopolymers where bacterial cellulose, kefiran, pullulan, gellan and xanthan are produced extracellularly, and PHA is found in those produced intracellularly (Vijayendra & Shamala, 2013; Singh *et al*.,2015).

Tabla 1.2 Examples of polymer-producing microorganisms

Biopolymer	Microorganisms	Reference
Bacterial cellulose	*Acetobacter sp.* *Rhizoium sp.*	Shoda y Sugano, 2005 Castro *et al*., 2012
Kefiran	Microflora kefir	Kooiman, 1968
Pululano	*Aureobasidium pulluslans*	Goksugur *et al*.,2011
Gellan gum	*Sphingomonas paucimobilis* *Pseudomonas elodea*	Bajaj *et al*., 2007 Banik & Santhiagu, 2006
Xanthan	*Xanthomonas campestris*	Kalogiannis *et al*., 2003; Fitzpatrick *et al*., 2013
Polyhydroxyalkanoates	*Bacillus sp.* *Chelatococcus sp.*	Vijatendra *et al*., 2007 Divyashree *et al*., 2009
Polylactic acid from lactic acid bacteria	*Lactobacilus bulgaricus* *Lactobacilus delbrueckii*	Inquinen *et al*., 2011 Lasprilla *et al*., 2012

5.4.1 Polyhydroxyalkanoate (PHA)

Hydroxylalkanoates are bioplastics produced by microorganisms, which are accumulated into granules intracellularly by a wide variety of microorganisms in the presence of a carbon source and a limited supply of nutrients such as nitrogen, phosphorus or oxygen. PHAs are the only family of polymers that act as a carbon or energy source for more than 300 Gram-positive and Gram-negative bacteria species. They have hydrophobic characteristics, so they are insoluble in water. It is a thermoplastic and elastomer, non-toxic, resistant to UV degradation, and pure within the cell. By their chain length can classify PHA into short-chain (3-5 carbons), medium-chain length (6-14 carbons), and long-chain length (more than 15 carbon atoms). The elastic modulus presented by this polymer can vary from 0.008 MPa to 3500 MPa, its elongation percentage has been reported to vary from 2% to 1000%, and its tensile strength generally varies from 8.8 to 104 MPa. Several derivatives have been studied within the PHA group that can count with permeabilities very similar to synthetic polymers such as polyvinyl chloride and polyethylene terephthalate (Laycock *et al*., 2013; Angelina & Vijayendra, 2015; Masood, 2017; Meereboer, Misra & Mohanty, 2020).

Polyhydroxybutyrate (PHB) is one of the most produced PHAs and was discovered by Lemoigne in 1927. In 1923, Lemoigne characterized PHB chemically and observed that it was associated with the sporulation of *Bacillus* spp. This is associated with lipids accumulated by various bacteria as they enter their stationary growth phase and then used as an internal reserve carbon and energy source. PHBs commonly accumulate in response to essential nutrient restrictions, so past studies for the synthesis of this polymer have focused on growth stress conditions (Page, 1995).

Studies on active films with PHB may include incorporating essential oils and other compounds to inhibit bacterial and fungal growth. Narayanan *et al*. (2013) incorporated eugenol at a ≥40 mg / g PHB concentration for bacteria and ≥80 mg / g PHB for fungi in PHB films produced by *Bacillus mycoides* strain DFC1. The films showed inhibition against *Staphylococcus aureus* MTCC 737, *Salmonella Typhimurium* MTCC 98, *Escherichia coli* NCIM 23058, *Bacillus cereus* MTCC 1272, *Aspergillus flavus* MTCC 277, *Aspergillus niger* MTCC 162 and *Penicillium* sp. MTCC 4610. These results agree with those reported by Xavier *et al*. (2015), they formulated PHB films produced by *Bacillus mycoides* DFC1 (isolated from garden soil) and added vanillin as an antimicrobial, and inhibited at a concentration of ≥80 µg/ g PHB for bacteria and ≥50 µg/ g PHB for fungi. The films showed activity against *Escherichia coli*, *Salmonella Typhimurium*, *Shigella flexneri* and *Staphylococcus aureus*. Also showed activity against fungi such as *Aspergillus*, *Aspercegium parasites*, and *Penicillium clavigerum*.

Rech *et al*. (2020) developed films of PHB and essential oil of cinnamon, melaleuca and citronella, having an inhibitory effect against *Aspergillus niger*. The addition of essential oils increased the degree of crystallinity and stability of PHB films. However, it had a plasticizing effect on the films, reducing the polymer's melting temperature and giving it greater flexibility. As explained in this section, biopolymers have different origins.

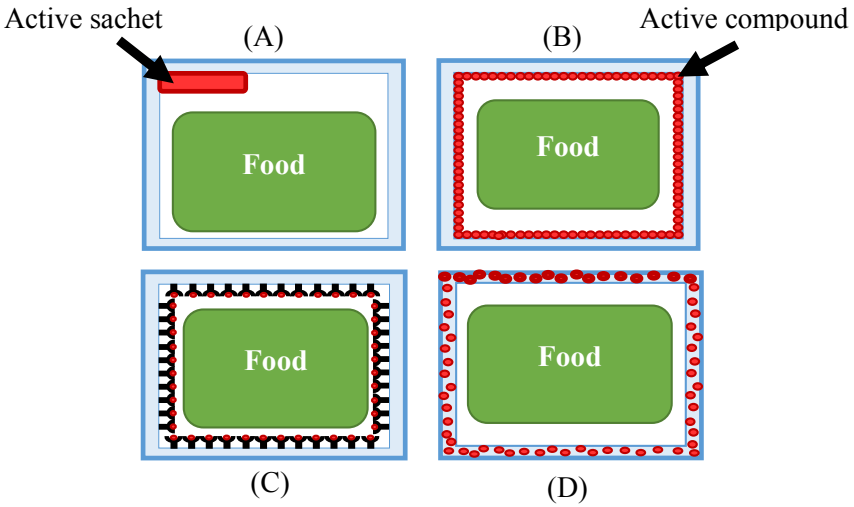
They are found in plants, animals, insects and can even be synthesized by microorganisms. Their properties and characteristics are helpful for food preservation. In the research carried out in the last years, it has been sought not only the individual characterization of the polymer but also the combination between them, with plasticizers and in particular with antimicrobial agents that modify their activity against pathogens present in food. The incorporation and composition of the active agent can also alter the structure of the packaging developed, so it is important to discuss them.

6. Main active agents used in antimicrobial films

The antimicrobial activities of packaging are based on the migration of antimicrobial substances from the package to the food. These substances inhibit or reduce the growth of microorganisms, retains its desired qualities and increasing shelf life. However, there are also packages where there is no migration of the active compound. Some designs of active agent incorporation systems are shown in Figure 1.6.

In Figure 1.6 (A), the active agent is incorporated in a small bag between the packaging and the food. This system is usually used for oxygen and moisture-absorbing agents. In the case of Figure 1.6 (B), a system is presented where the active agent covers the inner surface of the package. It is used for heat-sensitive or incompatible and immiscible compounds with the polymeric matrix. On the other hand, in the system shown in Figure 1.6 (C), the active agent is immobilized on the packaging surface by ionic or covalent bonds that prevent release to the food. Finally, in Figure 1.6 (D), the antimicrobial agent is added to the film-forming mixture. This system presents advantages such as uniform distribution in the polymeric matrix, high resistance to processing conditions and slow release into the food. This last design allows the efficient migration of the antimicrobial, which acts on the cells slowly and gradually, allowing its activity to remain for a prolonged time (Nerin *et al*., 2016; Alsami *et al*.,2020).

Figure 1.6 Active food packaging system designs



Source: Almasi, Jahanbakhsh Oskouie & Saleh (2020)

Note: (A) use of active sachet inside the packaging, (B) coating of an active agent on the polymer, (C) immobilization of active agents on the polymer surface, (D) incorporation of the active agent into the polymer matrix

6.1. Enzymes

Enzymes have many applications in food. They can be added directly to the food or incorporated into films. Lysozyme is an enzyme that sensitive bacteria by breaking down peptidoglycan polymers found in cell walls. Its activity isn't limited only to bacteria as it shows activity against fungi, viruses and protozoa. Lysozyme activity is limited to Gram-positive bacteria as the cell wall components give free access to the enzyme, whereas, in Gram-negative bacteria, the lipopolysaccharide layer of the outer layer is a barrier against lysozyme attack (Mousavi Khaneghah *et al*., 2018).

The utilization of enzymes, especially lysozyme, has been reported for application in developing active films. Fraba, Sanchez-Gonzalez and Chiralt (2014) incorporated lysozyme in two different polymeric matrices (corn starch and pea proteins) and studied the enzyme release at 10 °C and 25 °C. Both films had activity against *Listeria monocytogenes* at a temperature of 10 °C. However, this activity was affected at 25 °C, where pea protein films showed higher activity by reducing pathogen growth by 40 % compared to the control. In 2015, Kaewprachu *et al*. developed catechin-lysozyme gelatin films to maintain the quality of ground pork, compared their preservation efficiency against polyvinyl chloride (PVC) films during storage (7 days at 4 °C). They observed less weight loss and minor discoloration in the lysozyme-catechin films than those wrapped with PVC. Regarding antimicrobial activity, the growth of microorganisms on catechin-lysozyme gelatin films was 4.15 ±0.72 log CFU/g at 7 days of storage, lower than that reported for PVC films that reported growth of 5.87±0.31 log CFU/g.

Khairuddin *et al*. 2017, made an antimicrobial film with wheat gluten, lysozyme and ethylenediaminetetraacetic acid as antimicrobial agents. A reduction of *Escherichia coli* and *Bacillus subtilis* growth to 1.74 and 3.48 log CFU/mL was observed. Wu *et al*. (2018) developed chitosan and lysozyme coatings. They evaluated the effect on the quality of yellow croakers. Films with lysozyme presented growth of 5.86±0.40 log CFU/g, at 15 days of storage, values lower than the maximum allowed (7.0 log CFU/g). Lipid oxidation in chitosan films with or without lysozyme showed a reduction in the thiobarbituric acid index and improved sensory evaluation scores.

6.2. Bacteriocins

Bacteriocins are antimicrobial peptides synthesized as metabolites of lactic acid bacteria. They are active against a large number of microorganisms. Bacteriocins are varying in size, structure and specificity. The mechanism of action of bacteriocins is through interaction with cell membranes causing their death. It is an antimicrobial agent investigated for its natural characteristics, which does not modify food's sensory properties. Its incorporation in films has been of great interest due to its activity against Gram-positive bacteria and its resistance to high temperatures and acidic media. Nisin and pediocin are the most widely used in developing active packaging. However, studies aren't limited to these two bacteriocins (O'Connor *et al*., 2015; Martinez, Rodriguez & Suarez, 2016).

Woraprayote *et al*. (2013) incorporated pediocin PA-1 into polylactic acid (PLA) films and sawdust particles by diffusion method. In vitro testing of the films was performed on pork slices using *Listeria monocytogenes* ATCC 19115 as a sensitive strain. The results showed an zone of inhibition of the films from 2.75 to 3.88 mm for PLA films, sawdust particles and pediocin. However, PLA films alone with bacteriocins did not show inhibition against this pathogen. They considered that most probably the sawdust particles promoted the adsorption of the bacteriocin on the films, which increased their efficacy. In pork samples, the films reduced the *Listeria* population by 1.5-2.0 log cycles. Another study using this incorporation method was Woraprayote *et al*. (2018), who impregnated films of polylactic acid and sawdust particles with bacteriocin 7293 produced by *Weissella hellenica* BCC 7293. The films were prepared by extrusion. The maximum concentration absorbed by the films of bacteriocin 7293 was 19.54 $\mu g/cm^2$. Its activity was tested against Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Escherichia coli* and *Salmonella typhimurium*) that are responsible for the rejection of pangasius fillets. The tests showed inhibition for Gram-positive and Gram-negative bacteria in both in vitro and pangasius fish fillets.

In addition to the use of bacteriocins for film production, they have been used to make coatings. Guitian *et al*. in (2019) added enterocin produced by *Enterococcus avium* DSMZ17511 to develop antimicrobial coatings with food-grade agar. The coatings were used to wrap cheeses with low moisture content and artisanal cheeses with high moisture content. The cheese samples were previously inoculated with *Listeria monocytogenes* 01/155. Results showed a reduction of pathogen growth by 1.0-1.5 log cycles compared to control by day 8 and a 2.0 log cycle reduction by day 10 of storage. In artisanal goat cheeses, the reduction of *Listeria* was 5 log CFU/mL after two weeks of testing.

6.3. Essential oils

They consist of volatile compounds such as terpenoids, aliphatic chemicals and terpenes. They are derived from plants and have low molecular weight. Essential oils are containing phenolic compounds such as thymol, eugenol and carvacrol show higher antimicrobial activity against all types of microorganisms (Sanchez-Ortega *et al*., 2014). The use of essential oils in films has been widely studied as they can be extracted from natural sources such as plants.

The activity of a single essential oil can vary depending on the strain tested, as observed in studies done by Hafsa *et al*. (2016), who tested the antimicrobial activity of chitosan films with Eucalyptus globulus essential oil. The films showed a zone of inhibition from 54.53 to 153.37 mm^2 for *Escherichia coli*. *Pseudomonas aeruginosa* showed a smaller zone of inhibition ranging from 27.80 to 118.29 mm^2. In the case of *Staphylococcus aureus*, they showed zones from 10.56 to 61.35 mm^2. Finally, the films showed inhibition zones to *Candida parapsilosis* from 8.43 to 65.94 mm. This strain is the most resistant to this essential oil. In 2017, Kashiri *et al*. extracted essential oils from Zataria multiflora Boiss, a plant that grows in central and southern Iran, and incorporated it into zein films that were subsequently applied on the surface of polypropylene bags. Milk previously inoculated with *Listeria monocytogenes* and *Escherichia coli* was packaged in these bags and stored at 4 °C for 6 days. The packages with the essential oil showed a reduction of 0.65 log CFU/mL on day 1, 1.10 and 0.91 log CFU/mL on day 3 and 6 for *Listeria*. In the *Escherichia coli* strain, the reduction in growth was 0.51, 0.92 and 0.99 log CFU/ ml on days 1, 3 and 6.

Iamareerat *et al*. (2018) formulated cassava starch films with cinnamon essential oil and sodium bentonite. They observed that as the essential oil concentration increased, the zone of inhibition increased of the sensitive strains studied: *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus*. The highest inhibition was observed at a 2.5 % cinnamon essential oil concentration, with a halo of 15.25 mm for *Escherichia coli* and 10.75 mm for *Staphylococcus aureus*. The films were also tested on pork meatballs. The films maintained the microorganisms' growth in these samples below the permitted values (10^6 CFU/g) for 48 h at 25 °C.

The effect of combining essential oils with another active agent has also been studied. Arezoo *et al*. (2019) analyzed the effect of incorporating titanium dioxide nanoparticles and cinnamon essential oil in sago starch films. In vitro tests were performed using *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus*. The results showed a higher inhibition on gram-positive bacteria than gram-negative bacteria. These could be caused because Cinnamaldehyde has a hydrophobic nature that helps to destabilize and rupture the membrane of the bacteria, and when combined with TiO_2, leads to cytoplasmic leakage, which causes the death of the bacteria.

7. Effects of antimicrobials on mechanical and barrier properties

The addition of active compounds in polymeric matrices favors the activity and growth control of microorganisms. Research on their mechanical and barrier properties has shown that their incorporation alters their structure, modifying their final characteristics. Incorporating an active agent could increase the barrier properties or permeability to the water vapor of films that depend on the active agent's type of interaction in the polymeric matrix.

Xavier *et al*. (2015) showed that the incorporation of Vanillin in PHB films presented an increase in the elongation percentage, from 0.91% for pure PHB films to 2.09% for films with vanillin making them more elastic. Kaewprachu *et al*. (2015) observed an increase from 2.61 to 4.25×10^{-6} g mm h^{-1} cm^{-2} pa^{-1} in water permeability by incorporating catenin and lysozyme in gelatin films compared to polyvinyl chloride films. Solubility was also another parameter that increased from 68.07% to 1.78% in these films. Li *et al*. (2017) developed chitosan films with lysozyme and rectorite, a type of layered silicate that can adsorb and stabilize other antimicrobial materials. According to their results, the tensile strength was reduced from 38.39 to 27.8 MPa concerning the chitosan films. The elongation percentage was also affected, obtaining a reduction from 12.44% to 8.42%, respectively. This could indicate a more significant interaction between the chitosan units and the lysozyme and rectorite.

The incorporation of essential oils can favor the reduction of water vapor permeability, as observed in studies done by Lamareerat *et al*. (2018), who had modifications in water vapor permeability in cassava starch and cinnamon essential oil films, reducing water permeability by increasing the essential oil content from 203.09 to 73.21 mm / m^2 day kPa. The mechanical properties of the films as the tensile strength decreased from 1.68 MPa to 0.31 MPa with the addition of essential oil. Opposite case with the elongation percentage, which increased from 92.38% to 281.06% compared to starch-only films. Similar results were reported by Bagde and Vigneshwaran (2019). They immobilized the bacteriocin produced by *Pediococcus acidilactici* strain on cellulose nanocrystals by adsorption method and were added to corn starch films. The water vapor permeability of the films was reduced from 1.9 g mm / Kpa m^2h^{-1} to 1.72 g mm / Kpa m^2h^{-1} concerning the control. The tensile strength increased from 3.1 MPa to 4.33 MPa, which indicated that there might have been increased binding between the polymer molecules and the immobilized bacteriocin.

Another study on the incorporation of essential oils is that of Simsek, Eke and Demir (2020), who studied the effect of essential oil on the physical, water vapor permeability, mechanical, optical and microstructural properties carboxymethyl cellulose films. Their results showed that as the essential oil content increased, the moisture content, solubility and water permeability of the films analyzed decreased. The mechanical properties and tensile strength increased, but the percentage of elongation decreased concerning the film control. Han, Yu and Wang (2018) reported an increase in thickness, oxygen and water vapor permeability, and elongation percentage of films.

Previous reviews have discussed the importance of components in the formulation of bio-based antimicrobial films, their interactions, production methods, and structures present, whether single or multi-layered. However, no emphasis has been placed on the importance and application of this type of packaging today. For this reason, in the following section, we will review their potential applications in the food area and the exponential growth in the research being carried out.

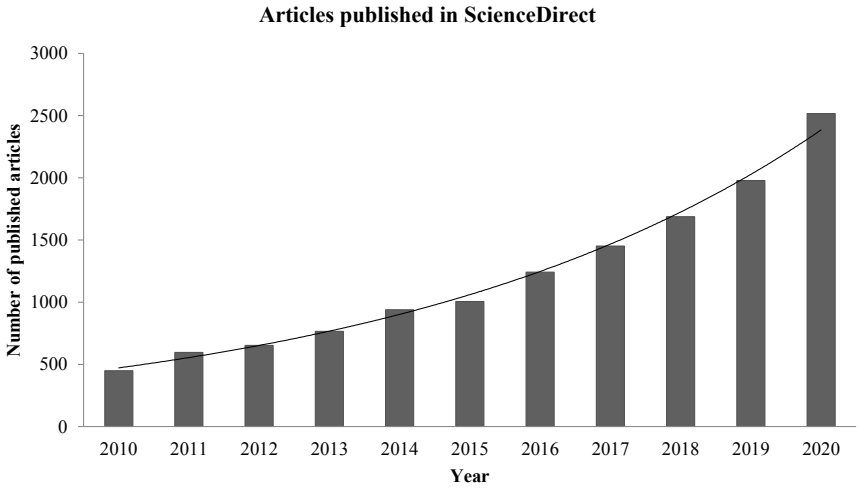
8. Importance of biodegradable antimicrobial packaging and its applications

The packaging industry's approach has been forced to make changes in packaging production, mainly to offer consumers safer products with a longer shelf life, in addition to caring for the environment by using materials from renewable sources such as bio-based biodegradable polymers. BBAs can include edible films or coatings made from proteins, lipids, polysaccharides, polylactic acid (PLA), polyhydroxybutyrate (PHB) and polyhydroxyalkanoate.

These qualities make BBAs relevant in the food industry. Their applications can range from natural products to ready-to-eat products. An example of these is the studies carried out in marine products where the BBAs inhibit the growth of pathogens such as *Salmonella*, *Listeria monocytogenes*, *Clostridium botulinum* and *Aeromonas hydrophila* or their application in bread to inhibit the growth of fungi such as *Penicillium commune, P. solitum, P. corylophilum, P. palitans* and several of the genus *Aspergillus,* which can produce unpleasant aromas in the product (Jideani & Vogt, 2015; Singh *et al.,* 2016).

Graph 1.1 shows the articles published using the keywords "Food packaging, Active packaging, antimicrobial packaging" as a search tool in the ScienceDirect database. An upward trend is observed in the articles published from 2010 to 2020 since the published number of articles was almost five times higher, which shows a constant development in this line of research, which seeks solutions for the different current problems of food safety and ecology.

Graph 1.1 Articles published in Sciencedirect on food packaging, active packaging and antimicrobial packaging



Among the articles published on this platform, we find antimicrobial packaging using active compounds such as silver nanoparticles, essential oils, bacteriocins and different types of polymeric matrices. They can range from simple unilamellar structures to structured films using nanocomposites or combinations of synthetic polymers to help reinforce the structure or change the final characteristics of the film. It can also be noted that the microorganisms most commonly used to test the inhibition of films are *Escherichia coli*, *Salmonella* and *Listeria monocytogenes*, which are some of the most critical microorganisms causing ETAs.

According to studies conducted by Radha *et al*. (2015), the use of clove and cinnamon essential oils at a concentration of 4 % in films made from corn starch inhibited the growth of Gram-positive bacteria such as *Lactococcus lactis, Listeria monocytogenes, Leuconostoc mesenteroides* and Gram-negative bacteria such as *Pseudomonas fluorescens, Shewanella putrefaciens, Salmonella typhimurium* and *Escherichia coli*, using the disk diffusion method. Tests on meat samples showed a reduction in the growth of *Pseudomonas spp.* and *Enterobacteriaceae*.

Another study analyzing the inhibition of ETAs is those of Fatima *et al*. (2018), who used 2 % chitosan nanoparticles in PLA films. They obtained a reduction in the growth of *Listeria monocytogenes* of 67.09 % and 30.46 % for *Escherichia coli*. Ma *et al*. (2018) used PLA combined with PHB in a 3:1 ratio and 5 % cinnamaldehyde, obtaining films with the ability to maintain the growth of *Escherichia coli* and *Salmonella* below permissible levels in salmon for 17 days.

Souza *et al*. (2018) elaborated films with sodium chitosan-montmorillonite incorporating essential oil of ginger as an antimicrobial agent. Their results showed a reduction in the agent's activity when incorporated into a polymeric matrix, reducing the range of activity on different bacteria. The essential oil alone showed activity against bacteria such as *B. cereus, S. aureus* and *L. monocytogenes*. However, when incorporated into the films, these only showed activity against *B. cereus* and *S. enterica*. The application of antimicrobial agents in packaging provides a controlled release that increases the shelf life of different food products. However, their activity can be reduced.

Even with this problem, studies carried out in recent years have established an area of opportunity by meeting the objective of reducing the growth of different microorganisms in foods for a prolonged time. Table 1.3 shows some of the studies carried out in recent years on the preparation of packaging, the type of antimicrobial agent used, the preparation method, the polymeric matrix and the microorganism inhibited.

9. Future advances in antimicrobial active packaging

Throughout this study, an overview of the importance of antimicrobial packaging and bio-based polymers has been given. While antimicrobial agents are essential to conferring the activity of films, these can impact their structure. Another aspect that affects their structure is the method used for packaging formation, which can also affect the effectiveness of antimicrobials. For example, extrusion or thermo-pressing for packaging production offers advantages for mass production, but the shearing process or the use of high temperatures decreases the activity of the films. An innovative process has been proposed by Woraprayote *et al.* (2013), who used sawdust particles to assist the absorption of the active agent after the film is formed, resulting in the incorporated antimicrobial agents not being subjected to the production processes.

The processes of incorporating active agents are not the only area developed in the elaboration of packaging. The structural variations in films have been studied in the formation of multilamellar films and the controlled release processes by combining two release systems. Such is the case of Wu *et al*. (2015), who incorporated nanoliposomes with cinnamon essential oil in gelatin films. The antimicrobial activity of the films without the encapsulated agent was slightly higher in the first three days. However, the films with nanoliposomes showed better pathogen control after being stored for one month. This indicated better antimicrobial stability and prolonged activity. These results agree with those presented by Cui *et al*. in 2017, who encapsulated phages to control *Escherichia coli* in beef. Their results showed inhibition of free phages in the first 6 days, but samples with encapsulated phages showed activity for up to 15 days.

Other techniques that improve the physical properties of the films and achieve a reduction in the migration of the active agent are the implementation of ultraviolet (UV) rays, gamma rays, plasma, and the use of the electron beam. Within this group, plasma-treated films that cause a modification on the surface, where they break covalent bonds and form free radicals, stand out. Kolarova Raskova *et al*. (2018) evaluated the effect of this treatment on polyvinyl alcohol films containing nisin as an antimicrobial agent, showed that the treatment influenced the degree of nisin adhesion.

The use of irradiation is also an innovative technique to modify the release of active agents in packaging. This mechanism is based on its ability to induce crosslinking in the polymeric network, thus improving the physical properties of the films. Irradiation can bind functional groups on the surface, allowing the material to immobilize enzymes or other bioactive species. An advantage, also the incorporation of agents that promote the adsorption of compounds, is that it does not require temperature or hazardous chemicals for crosslinking. Lacroix *et al*. (2002) showed that gamma irradiation could induce crosslinking in calcium caseinate films, which allowed greater control of the release of enzymes and active compounds.

Undoubtedly, antimicrobial packaging of bio-based polymers is an extensive area of research, and new ways to design and produce them are being studied every year. In addition, new compositions and active agents are being used to enhance their characteristics further. The range of foods that can benefit from these developments is extensive; they can reduce foodborne diseases and the waste caused by microorganisms that deteriorate their composition, giving them undesirable characteristics. However, their implementation is not limited to the food area also can be implemented in the medical and cosmetic areas.

Table 1.3 Bio-based antimicrobial films tested on foods

Antimicrobial agent	Polymeric matrix	microorganism to inhibit	Food	Method of preparation	Type of packaging	Author
Silver nanoparticles	Glucomannan-chitosan	*Staphylococcus aureus Escherichia coli Candida albicans*	Slices of bread	Casting	Films	Nair, Alummoottil & moothandasserrry, 2016
Silver nanoparticles	Chitosan	*Botrytis cinerea*	strawberry	-	Coverage	Moussa, Teyel,Alsohim & Absallah, 2013
Silver nanoparticles	Chitosan	Mesophiles, psychrophiles, enterobacteria, molds and yeasts	Cut cantaloupe	-	Coverage	Ortiz-Duarte, Perez.cabrera, Arrés-Hermández & Martínez-Hernández, 2019
Ginger essential oil	Chitosan - sodium montmorillonite	*Bacillus cereus, Salmonella enterica*	Fresh poultry meat	Casting	Films	Souza et al., 2018
Cinnamaldehyde	PLA-PHB	*Escherichia coli, Salmonella*	Salmon	Casting	Films	Ma, Li & Wang, 2018
Chitosan nanoparticles	PLA	*Listeria monocytogenes and Escherichia coli*	Indian prawns	Casting	Films	Fathima, Panda, Ashraf,Varghese & Bindu, 2018
Nisin	cellulose	*Listeria monocytogenes*	Ham	Casting	Films	Yang, Liu, Wu & Lu, 2020
Carbachol, linalon, thymus	Methylcellulose - Hydroxypropyl methyl cellulose	*Aspergillus niger*	Cheddar cheese	Casting	Films	Kuorwel *et al*., 2012
Cinnamon and clove essential oils	Corn starch	*Lactococcus lactis,Listeria monocytogenes, Leuconostoc mesenteroides, Pseudomonas fluorescens, Shewanella putrefaciens, Salmonella typhimurium, Escherichia coli.*	Raw meat	Casting	Films	Radha Krishnan *et al*., 2015

10. Conclusion

Innovations in the area of bio-based antimicrobial packaging have demonstrated the efficiency of these technologies in preserving food. Not only do they help reduce losses caused by spoilage microorganisms, but they also reduce the incidence of ETAs and the use of plastics. For the formation of this type of packaging, food-grade biopolymers of natural origin can be used to obtain films or coatings. However, The production of bio-based antimicrobial packaging is limited because the interactions between their constituents, production method and type of structure have not yet been fully elucidated. In addition, these film production systems are costly and not yet regulated in food systems.

The increase in research studies responds to the demand of increasingly informed consumers for food preserved in biodegradable, innocuous packaging and even minimally processed products. The current challenges are obtaining bio-based films that compete with those derived from petroleum and production on an industrial scale. To this end, the industry must be interested in promoting research on this type of packaging system, highlighting the environmental importance and cost reduction by obtaining products with a longer shelf life.

The development of new technologies and processing methods would be another aspect that to be investigated since the traditional or proposed methods have disadvantages, such as the case of dry methods (thermo-pressing and extrusion) where the activity of antimicrobial agents is reduced, or the casting method, which is limited to laboratory use because films cannot be mass-produced.

The advantages offered by bio-based antimicrobial packaging in the food area are promising, especially in ready-to-eat or minimally processed foods, where shelf life is restricted to a few days. As packaging is designed specifically to the needs of each food, it helps to highlight its qualities and better preserve its sensory and nutritional properties.

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Chapter 2 Importance of peptidoglycan hydrolases, bactericidal enzymes produced by lactic acid bacteria, in the reduction of antibiotic

Capítulo 2 Importancia de las hidrolasas de peptidoglucano, enzimas bactericidas producidas por bacterias acido lácticas, en la disminución de la resistencia a antibióticos

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DOI: 10.35429/H.2021.3.30.48

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A. Marroquín, J. Olivares, D. Ventura and L. Cruz (Coord) Agricultural Sciences and Biotechnology. Handbooks-©ECORFAN-México, Querétaro, 2021.

Abstract

The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) highlight in their global action, the problem of antimicrobial resistance (AMR), focusing on the concern about the effect every time minor of antibiotics, which is considered a threat in human medicine, veterinary, food sector and environment. In recent years, the interest in generating new technological alternatives for this problem has increased; this is the case of bioactive metabolites obtained from bacteria, viruses and fungi, such as protein molecules with bactericidal activity, as bacteriocins and enzymes and non-protein origin diacetyl and reuterin. Peptidoglycan hydrolases (PGH), also called autolysins, are enzymes involved in various cellular functions. These enzymes can hydrolyze the peptidoglycan bonds in a controlled way, and they are classified as N- acetylmuramidases, N-acetylglucosaminases, N-acetylmuramoyl-L-alanine amidases and peptidases. PGH are secreted by the pathway dependent on the General Secretion Pathway (Sec) or by the Double Arginine Translocation System (TAT) and have molecular weights in a range of 27 kDa to 137 kDa. Their importance liesin being used as bactericidal compounds, inhibiting the growth of bacteria of clinical relevance, which are currently a global public health problem.

Antibiotic resistance, Bactericidal enzymes, Peptidoglycan hydrolases

Resumen

La Organización Mundial de la Salud (OMS) y la Organización de las Naciones Unidas para la Alimentación (FAO) destacan en su plan de acción mundial el problema de la resistencia a los antimicrobianos (RAM), centrándose en la preocupación por el efecto cada vez menor de los antibióticos, lo cual se considera una amenaza en medicina humana, veterinaria, sector alimentario y medio ambiental. En los últimos años se ha incrementado el interés por generar nuevas alternativas tecnológicas para esta problemática; tal ha sido el caso de metabolitos bioactivos obtenidos de bacterias, virus y hongos, como proteínas con actividad bactericida como, bacteriocinas, diacetilo, reuterina y enzimas. Las peptidoglucano hidrolasas (PGH) también denominadas autolisinas, son enzimas involucradas en diversas funciones celulares, estas enzimas hidrolizando de manera controlada los enlaces del peptidoglucano y se clasifican en N-acetilmuramidasas, N-acetilglucosaminasas, N-acetilmuramoil-L-alanina amidasas y en peptidasas. Las son secretadas mediante la vía dependiente de Secreción (Sec) o mediante el sistema de translocación doble de arginina (TAT) y tienen pesos moleculares en un rango de 27 kDa a 137 kDa.

Resistencia a antibioticos, Enzimas bactericidad, Peptidoglucano hidrolasas

Introduction

Lactic acid bacteria (LAB) constitute a heterogeneous group of microorganisms represented by several genera, with metabolic, morphological and physiological characteristics in common (Gálvez *et al*., 2007). They have diverse applications, due to the relevant role they play in fermentation in the food industry. Hence, they are widely used for the preservation of various food products, their usefulness is based on providing sensory characteristics such as flavor, odor, texture and consistency, in addition to increasing its nutritional value and safety (Carr *et al*., 2002; Azadnia *et al*., 2011). The end products of LAB metabolism involved in antibacterial capacity are organic acids as lactic, acetic and propionic acid; in addition can also produce hydrogen peroxide, carbon dioxide, reuterin, reutericicline, 2-pyrrolidone, 5-carboxylic acid, bacteriocins, peptidoglycan hydrolases (PGH), among others (Hernández *et al*., 2005). As a consequence, LAB historically have been used to preserve food, in addition to being GRAS (generally recognized as safe). Amog these antibacterial compounds, PGH are enzymes involved in diverse cellular functions since they hydrolyze in a controlled way the peptidoglycan bonds, then they can be used as bactericidal compounds, inhibiting the growth of bacteria thatrepresent a public health problem, due to this they have generated great interest (Turner *et al*., 2004).

1. Antibiotics

Molecules of natural, synthetic or semi-synthetic origin, capable of inducing the death or inhibiting the growth of bacteria and fungi, and the replication of viruses are defined as antimicrobials, within this category are antibiotics effective to stop the growth (bacteriostatic) or produce death (bactericidal) in bacteria by various mechanisms, exerting a specific action on some structure or function (Vignoli and Seija, 2008).

In the twentieth century, the use of antibiotics increased as treatment and prevention of diseases caused by infectious agents in humans, but also in the agricultural, veterinary and food sector (Alós, 2015).

2. Classification of antibiotics

Antibiotics have been divided into three major groups (Table 2.1), according to their spectrum, mechanism of action (Figure 1) and pharmacokinetics-pharmacodynamics (Seija & Vignoli, 2008).

Table 2.1 Classification of antibiotics

Classification	Subclassification	Diana
Spectrum	Broad	Active against a large number of differentspecies and genera.
	Reduced	They are active against a limited number of species.
Mechanism of action	Attack on the bacterial wall.	
	Protein synthesis inhibitors.	
	DNA replication inhibitors.	
	Cytoplasmic membrane inhibitors.	
	Metabolic pathway inhibitors.	
Pharmacokinetics/ pharmacodynamics	Beta-lactams	Inhibits last stage of bacterial cell wallsynthesis.
	Penicillin	It prevents cell wall synthesis by inhibitingthe enzyme transpeptidase.
	Cephalosporins	Interferes with peptidoglycan synthesis.
	Monobactam	Interferes with cell wall synthesis.
	Carbapenems	Inhibits the synthesis and assembly of thelast stage of cell wall peptidoglycan.
	Beta-lactams associated with inhibitors of beta-lactamases	Inhibits bacterial beta-lactamase enzymes.
	Glycopeptides	Inhibits the synthesis and assembly of thesecond stage of cell wall peptidoglycan.
	Aminoglycosides	It interferes with the correct reading of thegenetic code.
	Macrolides	Interferes with a block in transpeptidationand translocation reactions.
	Quinolones	Inhibit DNA and RNA synthesis by interacting with DNA gyrase and topoisomerase IV.

Source: Seija & Vignoli (2008)

3. Worlwide problem of antibiotic use

Antibiotic resistance (AR) is a natural expression of bacterial evolution and genetics, first reported in 1912 (Cabrera *et al*., 2007). Some factors that favor AR are the inappropriate use of antimicrobials (Alós, 2015), the low quality of active compounds, the lack or deficiency of infection prevention and control programs, the inability of laboratories to detect resistance, as well as inadequate surveillanceand insufficient regulation of antibiotic use (Fariña, 2016). These factors are particularly important in countries where legislation is inadequate, surveillance and monitoring of antimicrobial use is lacking, and prevention and control of antimicrobial resistance (AMR) is also weak (WHO, 2017). As this problem has increased, epidemiological surveillance has categorized bacteria that are resistantto multiple antimicrobial agents, naming them by the absence of sensitivity into multidrug-resistant, extremely resistant, and resistant to all antimicrobials. According to Magiorakos *et al*. (2012), "Multiple Drug Resistance (MDR) is defined as the absence of sensitivity to at least one drug in three or more of the antibiotic categories; Extensively Drug-Resistant (XDR) refers to the absence of sensitivity to at least one agent in all but two or fewer of the antimicrobial categories; and resistance to all antimicrobials is defined as resistance to all antibiotic categories."

In recent years, the incidence of multidrug-resistant microorganisms has increased considerably. In addition, ineffective treatments must be applied that prolong the time of agony of the sick, forcing the administration of expensive drugs and increasing the time of hospitalization and risk of mortality (Fariña, 2016).

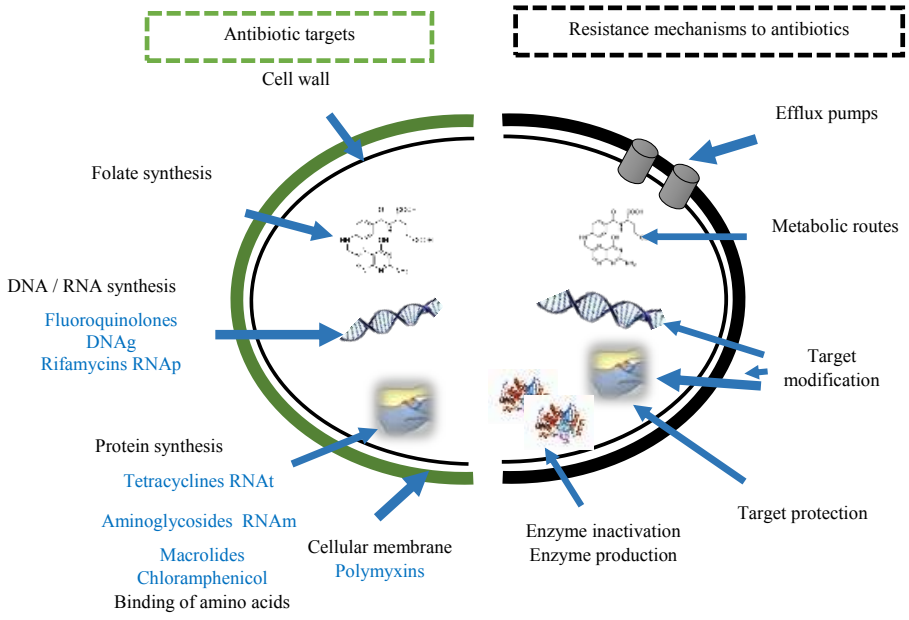
4. Mechanism of bacterial resistance to antibiotics

AR is the result of mutations and the exchange of genetic material by transferring resistance genes through mechanisms such as transformation, conjugation, transduction, and transposition. The transfer of genes from free DNA of a previously lysed bacterium to another is called transformation, while the transfer of genetic material contained in plasmids from one bacterium to another via pili is defined as conjugation (Levy, 2004, Prescott *et al*., 2002). Transduction is described as the transfer of genetic material from one bacterium to another by a phage. The last mechanism is transposition, which occurs by a displacement of a section of DNA between one genetic location (donor site) and another (acceptor site) (Levy, 1998; Levy, 2004; Prescott *et al*., 2004).

Bacteria have developed several mechanisms to resist the action of antibiotics as shown in Figure 2.1, among them are active expulsion, decreased cell wall permeability, enzymes production and binding to an essential protein (Couvalin, 1988; Prescott *et al*., 2004). The production of enzymes such as beta-lactamases are enzymes that hydrolyze beta-lactam antimicrobial agents. In gram-negative bacteria, beta-lactams enter the cell through porins and find beta-lactamases in the periplasmic space. Beta-lactamases destroy beta-lactam molecules before they have a chance to reach their target penicillin-binding proteins (PBPs). In contrast, in gram-positive bacteria, beta-lactamases are secreted extracellularly into the surrounding environment. It destroys beta-lactam molecules before they have a chance to enter the cell (Prescott *et al*., 2004).

Gram-negative bacteria can produce adenylating, phosphorylating or acetylating enzymes that modify an aminoglycoside to inactivate it. For example, chloramphenicol acetyltransferase is produced by gram-negative bacteria that modify chloramphenicol to inactivate it. Gram-negative bacteria can become resistant to beta-lactam antibiotics developing permeability barriers. Altered porins usually cause this in the outer membrane that no longer allows the entry and transit of antibiotic molecules into the cell. When beta-lactams cannot reach PBPs, the cell is resistant (Couvalin, 1988).

Figure 2.1 Antibiotic targets of action and resistance mechanisms



Source: Mandigan (2009)

PBPs in both gram-positive and gram-negative bacteria can be mutated so that beta-lactams cannot bind to them; therefore, the cell is resistant to antimicrobial agents. Mutations in the chromosomal genes for DNA gyrase and topoisomerase IV confer resistance to quinolones.

A wide variety of efflux pumps provide antimicrobial resistance to both gram-positive and gram-negative bacteria. The active efflux of antibiotics is mediated by transmembrane proteins inserted in the cytoplasmic membrane, and, in the case of gram-negative organisms, it also involves components in the outer membrane and periplasm. These proteins form channels that actively export an antimicrobial agent out of the cell as quickly as it enters. Some microorganisms develop an altered metabolic pathway that bypasses the reaction inhibited by the antimicrobial. Mutations that inactivate thymidylate synthetase block the conversion of deoxyuridylate to thymidylate. These mutants require exogenous thymine or thymidine for DNA synthesis and are therefore resistant to antagonists of the folate pathway such as sulfonamides and trimethoprim, to name a few (Couvalin, 1988).

5. Examples of recent cases of antibiotic resistance

Resistance to antibiotics has increased significantly in the last decade in the world, with the following cases standing out:

a) In Poland, between 2014 and 2015, *Acinetobacter baumannii* strains were isolated from blood cultures in hospitalized patients with pneumonia showing resistance to fluoroquinolones, amikacin, trimethoprim/sulfamethoxazole, imipenem, meropenem, cephalosporins and tetracyclines (Hernandez *et al*., 2018).

b) In other study, 46 potentially pathogenic *Pseudomonas aeruginosa* strains were isolated from agricultural water samples, which showed high rates of resistance to ampicillin, ceftriaxone, chloramphenicol, cefotaxime cephalothin, nitrofurantoin, kanamycin, streptomycin and tetracycline (Gutierrez *et al.,* 2017).

c) In Michoacán, Mexico 34 samples of *Escherichia coli* were isolated from cows with mastitis showing resistance to amikacin, ampicillin, levofloxacin, cephalothin, cefotaxime, ceftriaxone, chloramphenicol, gentamicin, netilmicin, nitrofurantoin, cefepime, trimethoprim sulfamethoxazole, tetracycline, kanamycin and streptomycin (Jiménez *et al*., 2017).

d) In another case in Mexico at the National Institute of Pediatrics, from 149 cultures of urine culture, blood culture, non-surgical wound and vaginal exudate samples, strains of Enterococcus faecalis and Enterococcus faecium were isolated in the period from January to December 2016; *Enterococcus faecalis* and *Enterococcus faecium* strains, which showed resistance to ampicillin, streptomycin, penicillin, vancomycin, gentamicin, erythromycin and quinupristin and dalfopristin (Arredondo-García *et al*., 2018).

e) At the National Institute of Rehabilitation "Luis Guillermo Ibarra Ibarra" in Mexico City, 11 strains of *Staphylococcus aureus* and 12 strains of coagulase-negative *Staphylococcus* from surgical wounds, bronchial secretions, blood cultures, catheter tips and bone cultures, collected during the period from February to July 2018 were studied showing methicillin resistance (MRSA) (Garcia *et al.,* 2019).

6. Worlwide action plan on antibiotic resistance

To guide and promote research and development (R&D) of new alternatives to the global problem of AR, the WHO in 2017 published the first list of priority AR pathogens, which includes 12 families of bacteria, using specific criteria to list them such as the degree of lethality of infections, whether or not treatment requires long hospitalization, the frequency of contagion in the community, consider whether or not they can prevent infections, number of therapeutic options that exist, and presence of R&D of new antibiotics to treat infections (WHO, 2017).

The list is divided into three categories, critical, high or medium priority. The critical priority group includes multidrug-resistant bacteria that can cause serious and lethal infections. The second and third tier encompasses other bacteria that manifest increasing drug resistance and cause common diseases (Table 2.2) (WHO, 2017).

Table 2.2 WHO list of priority multi-resistant pathogens for R&D of new antibiotics

Priority 1: Critical	Priority 2: High	Priority 3: Medium
Carbapenem-resistant *Acinetobacter baumannii* Carbapenem-resistant *Pseudomonas aeruginosa* Carbapenem-resistant *Enterobacteriaceae*, producers of extended- spectrum beta-lactamases.	*Enterococcus faecium*, resistant to vancomycin *Staphylococcus aureus*, methicillin-resistant, intermediate susceptible and vancomycin resistant *Helicobacter pylori*, resistant to clarithromycin *Campylobacter spp.*, resistant to fluoroquinolones *Salmonellae*, resistant tofluoroquinolones *Neisseria gonorrhoeae*, cephalosporin-resistant, resistant to fluoroquinolones	*Streptococcus pneumoniae*, notpenicillin sensitive Ampicillin-resistant *Hemophilusinfluenzae* Fluoroquinolone-resistant *Shigella spp.*

Source: WHO (2017)

7. Development of the presence of antimicrobial resistance in Mexico

In 1973, 493 strains of *Salmonella typhi* were isolated and studied during an outbreak in the laboratories of the Hospital de Infectología del Centro Médico La Raza (IMSS), showing resistance to chloramphenicol (CM), tetracycline (TC), streptomycin (SM) and sulfonamides (SU) (Olarte *et al*., 1973).

In 1981-1982, 22 strains of enterotoxigenic *Escherichia coli* with resistance to ampicillin, tetracycline, streptomycin and kanamycin were isolated from children at the University Hospital of Puebla (Martínez *et al*., 1987).

Other studies in Mexico City over three decades, 1960, 1970 and 1980, have isolated strains of *Shigellaspp.*, *Salmonella spp.* and *E. coli* (Estrada-Garcia *et al*., 2005; Santos *et al*., 1989) that showed resistance to ampicillin, and decreased resistance to furazolidone.

Studies conducted in 1996 for *Enterococcus sp.* in Mexico showed high levels of resistance to gentamicin and *Enterococcus faecalis* showed resistance to ampicillin and imipenem. Also, in 2007, resistance to vancomycin was reported in these pathogens (Sifuentes-Osornio *et al*., 1996).

In 1998, it was observed that strains *of Streptococcus pneumoniae* had begun to show resistance to penicillin and to cephalosporins, macrolides, ciprofloxacin, trimethoprim-sulfamethoxazole, chloramphenicol and tetracyclines (Silva *et al*., 1998).

BLEE-producing strains of *Klebsiella pneumoniae*, *E. cloacae, E. coli* and *Serratia marcescens* were isolated from several hospitals in Mexico between 2001-2008. In other studies, *Streptococcus pneumoniae* strains were found to have increased resistance to erythromycin, chloramphenicol, trimethoprim/sulfamethoxazole and vancomycin. In addition, reports have shown increasing resistance to trimethoprim/sulfamethoxazole and erythromycin in 10 Latin American countries, including Mexico (Quinones-Falconi *et al*., 2010; Bautista-Márquez *et al*., 2013).

In 2009, multidrug-resistant *Salmonella typhimurium* strains and the production of an AmpC-type beta-lactamase were reported in Yucatan (Wiesner *et al*., 2009; Zaidi *et al*., 2007).

Likewise, methicillin resistance has been described in *Staphylococcus aureus* strains. In 2010, the first report of resistance to linezolid was made in Mexico (Villaseñor-Sierra *et al*., 2012).

In 2011, increasing resistance to clarithromycin was reported in *Helicobacter pylori* in Mexico City (Ayala *et al*., 2011) and in 2012 antimicrobial resistance in *Campylobacter spp.* isolated in Sonora, San Luis Potosi, Michoacan and Yucatan (Zaidi *et al*., 2012).

8. Alternative for controlling antibiotic use

The WHO and FAO proposals seek to promote R&D in projects aimed at solving the problem of AR.

Therefore, work has been proposed derived from the use of microorganisms with antimicrobial activity,such as fungi such as *Aspergillus ochraceus* 3MCMC3 isolated from *Rhizophora mangle* roots that inhibit the growth of *Escherichia coli* ATCC 10536*, Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhimurium* ATCC 14036, and *Salmonella typhimurium* ATCC 14036, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhimurium* ATCC 14028, *Bacillus cereus* ATCC 9634 and *Staphylococcus aureus* ATCC 25923 (Castillo-Machalskis *et al*., 2007).

Another example are the studies of bacteriophages ØA392 against infections caused by imipenem- resistant *Pseudomonas aeruginosa* (Wang *et al*., 2006) or the phages Φ H5 and Φ A72 that inhibit the growth of *Staphylococcus aureus* in milk (García-Suarez *et al*., 2008). Also, LAB with antimicrobial activity such as *Lactobacillus acidophilus* ATCC 33200, *Limosilactobacillus fermentum* ATCC 9338, *Lacticaseibacillus casei* ATCC 27139, *Lactiplantibacillusplantarum* ATCC 10776 have been used, *Lactobacillus bulgaricus* ATCC 11842 and *Lactobacillus helveticus* ATCC 15807 that have bactericidal activity against *Escherichia coli, Salmonella enteritidis* and *Shigella dysenteriae*, which were isolated and identified from clinical samples (Larre *et al*., 2007).

9. Proteins of lactic acid bacteria with biological activities

In addition to their technological function, LAB have the ability to inhibit the growth of certain altering and/or harmful microorganisms in food or even within the community. LAB have a primary antimicrobial effect, this is due to the competition for the substrate and the production of various metabolites such as organic acids; as well as ethanol, CO_2, H_2O_2, diacetyl, acetaldehyde and other oxygen metabolites. Also, LAB produce ribosomal antimicrobial protein compounds such as bacteriocins and enzymes such as PGH (Cintas *et al*., 2000).

The reserve of organic acids mainly lactic and acetic acid reduces the pH of the environment, this causes the inhibition of Gram-positive and Gram-negative bacteria, such is the case of *Limosilactobacillus fermentum* (QDC32) *Lacticaseibacillus casei* (QDC31), which have an inhibitory effect against *Salmonella typhimurium*. This effect is attributed to the penetration of lactic acid in a non-dissociated form in the cellular membrane, which decreases the pH in the cellular interior and provokes the dissociation, giving place to the liberation of H+ and the corresponding anion; so that, both ions interfere in the metabolism and inhibit the cellular growth, since all the efforts of the cell are to expel the ions (Requena, 1995; Urrego *et al*., 2005).

In the case of heterofermentative LAB, one of the final products of fermentation is CO2 and sometimes it is obtained by decarboxylation of amino acids, this product promotes an anaerobic environment,reduces the pH and helps to destroy the integrity of the cell wall (Ouwehand, 1998; Mora-Villalobos. *et al*; 2020). Another metabolite with antimicrobial activity is diacetyl, which is a fermentation product of citrate,has been shown to have antimicrobial activity at the level of 200 µg/ml for yeasts and Gram-negative bacteria and at 300 µg/ml for non-lactic Gram-positive bacteria (Axelsson, 2000; Ouwehand, 1998).

In addition, they can produce hydrogen peroxide when oxygen is present, leading to peroxidation of membrane lipids by hydroxy radicals and a consequent susceptibility of the cell (Ouwehand, 1998). Among the protein compounds of ribosomal synthesis are bacteriocins which are peptides that are excreted into the extracellular medium and, in some cases, have a broad spectrum of action and activity against pathogenic bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, Clostridium botulinum* and *Salmonella* (Yang *et al*., 2012; Benmechernene *et al*., 2013). The main substances produced by LAB that exhibit antimicrobial activity are listed in Table 2.3.

From the molecular point of view, antimicrobial peptides exert their action on some of the following bacterial structures or functions, either by inhibiting the synthesis of the bacterial wall, altering the integrity of the cytoplasmic membrane, preventing protein synthesis or blocking the synthesis or functions of nucleic acids (Calvo & Martínez-Martínez, 2009). In addition to the production of peptides with antimicrobial activity (Lorenzen & Meisel, 2005), peptides with other biological activities such as immunomodulant (LeBlanc *et al*., 2002), anticancer (De Moreno de LeBlanc *et al.,* 2005), hypocholesterolemic (Kawase *et al.,* 2000), mineral carrier (Lorenzen & Meisel, 2005), regulator of intestinal and nervous system activity (Rokka *et al*., 1997) and antioxidant (Hernández-Ledesma *et a*l., 2005).

Table 2.3 Metabolites with antibacterial activity produced by LAB

Metabolite	Producing microorganism	Reference
Diacetyl	Most of the BAL	Montville & Winkowski, 1997
Reuterin	*L. reuteri* *L. coryniformis*	Magnusson & Schnürer, 2001
BLIS: Bacteriocin-likeand inhibitory substances	Most of the BAL	Montville & Winkowski, 1997
Bacteriocins	Most of the BAL	Nes, *et al*., 1996
Cyclic dipeptides:Cyclo-PhePro Cyclo-PheOHPro Cyclo-GlyLeu	*L. coryniformis* *L. plantarum* *L. pentosaceus*	Magnusson & Schnürer, 2001
Hydroxy acids 3-hydroxy-tetradecanoicacid 3-hydroxy-decanoic acid 3-hydroxy-5-cis- dodecanoic acid 3-hydroxydecanoic acid	*L. plantarum*	Magnusson *et al.,* 2003
Bioactive peptides	Most of the BAL	Visser *et al*., 1986.

10. Peptidoglucan hydrolases (PGH)

Enzymes with lytic activity as antibacterial agents have been used in the food industry, due to the advantages they provide to the product and the consumer. Among the advantages are the elimination of bacteria, their biocontrol and the treatment of infectious diseases caused by these microorganisms. Several authors report that LAB is an important source of PGH production, the most reported genera are *Pediococcus* and *Lactobacillus* (Turner *et al*., 2007).

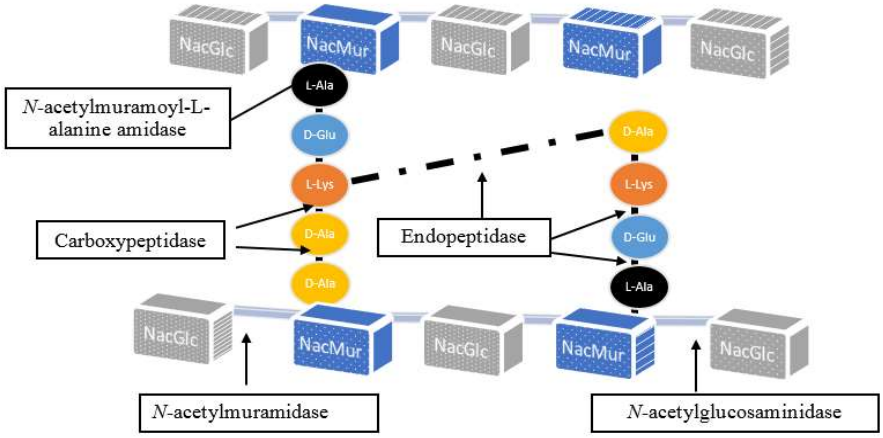
PGHs are enzymes involved in various cellular functions that require the cell wall; for example, during growth, cell division, regulation of cell wall growth, exchange of peptidoglycan units during growth, separation of daughter cells during division, flagella formation (in some cases) and autolysis, which is generally induced under adverse conditions such as lack of nutrients (Lortal & Chapot-Chartier, 2005;Vollmer *et al*., 1997). This regulation is performed by PGHs through the hydrolysis of covalent bondsof the PG, are located in the cell wall and act specifically on the basis of their incision site in the PG (Vollmer *et al*., 2008).

The characterized PGHs have a modular structural organization with two domains: a catalytic domain containing the active site of the enzyme and a cell wall binding domain (Turner *et al*., 2004; Lortal & Chapot-Chartier, 2005).

11. Classification of peptidoglycan hydrolases

The classification of PGHs depends on the type of bond they hydrolyze in the PG, as shown in Figure 2.2. N-acetylglucosaminases hydrolyze the β-1,4 bond of the glucan chain, leaving a reducing N-acetylglucosamine end. N-acetylmuramidases hydrolyze the β-1,4 bond of the glucan chain, leaving a free reducing N-acetylmuramic acid end, also called lysozymes. In case of forming a 1,6-anhydro ring on the N-acetylmuramic they are called lytic transglucosylases. N-acetylmuramoyl-L-alanine amidases break the amide bond between the N-acetylmuramic acid and the L-alanine of the peptide. Peptidases are able to hydrolyze the last amino acid of the carboxyl end of the peptides also called carboxypeptidases or to completely break the bridges formed by the peptides and are called endopeptidases (Layec *et al*., 2008; Vollmer *et al*., 2008).

Figure 2.2 Classification of peptidoglycan hydrolases according to their specificity



Source: Layec et al.,2008.

12. Catalytic domains of peptidoglycan hydrolases

The specificity of PGHs depends on their catalytic domain, as mentioned above in most cases they are composed of two domains: a catalytic domain containing the active site of the enzyme and a cell wall binding domain composed of several amino acid repeats (Diaz *et al*., 1991; Joris *et al*., 1992; Layec *et al*., 2008). BALs belong to the phylum Firmicutes, which is characterized by extensive PGH expression, so far 14 catalytic domains and 27 surface association domains have been described (Finn *et al*., 2006).

13. Catalytic domains

Catalytic domains are specialized for cleavage of a specific peptidoglycan bond, 14 catalytic domains have been described and are listed in Table 2.4. PGHs are composed of a single catalytic domain, however, enzymes have been studied that exhibit multiple distinct or identical catalytic domains associated with one or more substrates/binding domains. An example is the major PGH of *S. aureus*, a bifunctional autolysin named Atl (Oshida *et al*., 1995). Atl is initially produced as a 138 kDa protein with an amidase domain and a glucosaminidase domain after proteolytic processing that generates two major PGHs: a 62 kDa N-acetylmuramyl-L-alanine amidase and a 51 kDa N-acetylglucosaminidase, conferred by glucosaminidase and amidase_2 (Oshida *et al*., 1995; Komatsuzawa *et al*., 1997).

Table 2.4 Catalytic domains of peptidoglycan hydrolases

***N-acetylmuramoyl-*L-alanine amidase**	**Endopeptidase**	**Carboxypeptidase**	***N-acetylglucosami-nidase***	***N-acetylmurami-dasa***
Amidase 2 (PF01510) Amidase 3 (PF01520) Amidase 5 (PF05382) CHAP (PF05257)	Peptidase M23 (PF01551) CHAP (PF05257)	Peptidase_S66 (PF02016) VanY (PF02557) Peptidase_S11 (PF02113) Peptidase_S13 (PF00768)	Glucosaminidase (PF01832)	Glico_hydro_25 (PF01183) SLT (PF01464) Transglucosylase (PF06737)

Source: Layec et al., 2008

Lactococcus lactis has three known N-acetylglucosaminidases (AcmA, AcmB and AcmC) and one hypothetical one (AcmD), two of which (AcmA and AcmD) have three peptidoglycan-binding LysM domains (Huard *et al*., 2003; Huard *et al*., 2004). The presence of three LysM domains has been shown to be optimal for AcmA activity because variant proteins with fewer or more LysM domains exhibit lower activity (Steen *et al*., 2005).

The level of hydrolytic enzyme activity is not only a result of the efficiency of the catalytic domains, but is also controlled by the cell wall binding domains (Layec *et al*., 2008).

14. Cell wall binding domains

Cell wall binding domains are of great importance for the catalytic efficiency of PGHs. One of their main functions is the binding of proteins to the cell wall and the targeting of the enzyme to its site of action (Braun *et al*., 1997; Janecek *et al*., 2000). Their number may be essential for efficient binding of PGHs to the cell wall. For example, the copy number of the choline-binding domain (ChBD/CW_binding_1), LysM and SH3, has been shown to be important for high catalytic efficiency of PGHs, since their inactivity leads to strongly reduced (Eckert *et al*., 2006) or even deficient PGH activity (Sass & Bierbaum, 2007).

N-acetylmuramidase LytC from *S. pneumoniae* contains a choline-binding domain by which it binds to the teichoic acid of the cell wall, which is essential for its activity (Monterroso *et al*., 2005).

Another case is the G5 domain, which induces binding to N-acetylglucosamine, is found in certain hydrolases that can cleave oligosaccharides in their environment to provide carbon sources (Clarke *et al*.,1995) or the SH3 domain which is associated with the survival of pathogens within the invaded cell (Layec *et al*., 2008).

Table 2.5 shows 27 surface domains that can be found in bacteria such as *Pediococcus, Lactobacillus, Oenococcus*, among others.

Table 2.5 BAL cell wall binding domains

Binding domains	Joint sites	XFAM access number
Big_4	Variety of bacterial surface proteins.	PF07532
CBM_5_12	Carbohydrate Binding Modulus (CBM)	PF02839
ChW	Proteins containing ChW repeats (tryptophan)	PF07538
Collagen	Triple helix repeat proteins	PF01391
CpL_7	The CW_7 repeats form a cell wall binding motif.	PF08230
Cu_amine_oxidN1	Oxidation of primary amines to aldehydes	PF07833
Cw_binding_1	Repeats in P15057 recognition of choline-containing cell walls	PF01473
Cw_binding_2	SlpA and Cwp2 domains for the binding of PSII, acell wal component.	PF04122
DUF1142	Prophage tail proteins that probably act asendopeptidases.	PF06605
DUF1958	Prokaryotic penicillin binding protein 4.	PF09211
Erfk_YbiS_YhnG	YkuD, ErfK / YbiS / YcfS / YnhG Protein	PF03734
FG_GAP	Extracellular FG-GAP repeat found in alphaintegrins.	PF01839
GBS_Bsp_like	GBS Bsp-like repeat, group B streptococcus(GBS) protein.	PF08481
G5	G5 domain, extracellular proteins, PG metabolismproteins	PF07501
LysM	LysM domain (lysine motif) bacterial cell walldegradation.	PF01476
PBP5_C	Penicillin-binding protein 5, C-terminal domain,D-alanyl-D-alanine carboxypeptidase.	PF07943
PG_bindling_1	Putative peptidoglycan binding domain, it iscomposed of three alpha helices.	PF01471
fago bolin	SPP1 phage holin, holin proteins of the bacteriophage group dsDNA *Siphidoviridae*	PF04688
SH3_2	SH3 variant domain, protein involved intransduction.	PF07653
SH3_3	Bacterial SH3 domain, hypothetical bacterialproteins of unknown function	PF08239
SH3_4	Bacterial SH3 domain, hypothetical bacterialproteins of unknown function	PF06347
SH3_5	Bacterial SH3 domain, hypothetical bacterialproteins of unknown function	PF08460
SLpA	Protein A domain of the surface layer,bacterial cell surface proteins	PF03217
SLH	Cell wall pyruvoylated polymers: teichoic acids, teicuronic acids, lipoteichoic acids or lipoglycans.	PF00395
SPOR	Bacterial SPOR domains bind to the peptidoglycan	PF05036
TMP	WXXh motif repeat where X can be any residueand h is a hydrophobic residue.	PF05017
YSIRK_signl	Motif SIRKxxxGxxS transmembrane domain	PF04650

Source: Modified from Layec et al.,2008

15. PGH production by lactic acid bacteria

Several authors have reported that LAB are an important source of PGH and can be used to control pathogens in the food industry and in hospitals, due to their antimicrobial activity. The genus with the most reported production of PGH is *Lactobacillus* (Cibik & Chapot-Chartier, 2004; Turner *et al*., 2004; Yokoi *et al*., 2005; Donovan & Foster-Frey 2006). Furthermore, it has been found that one species can produce two or even three enzymes with this lytic activity (Baker *et al*., 2006).

Studies of this type of enzyme begin with lysozyme which is a muramidase (N-acetyl muramidase, E.C. 3.2.1.17), discovered by Alexander Fleming, catalogued as a food additive, with a molecular weight of 14.31 kDa and is composed of a sequence of 129 amino acid residues, with an isoelectric point (pI) of 10.7. This enzyme prevents the growth of *Oenococcus oeni, Clostridium tyrobutyricum, Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis*, (Jollés & Jollés, 1984).

In *Leuconostoc mesenteroides subsp*. *mesenteroides* isolated from dairy products, a glucosidase and an N-acetyl-muramyl-L-alanine amidase with lytic activity of 41 and 52 kDa are expressed according to specificity analysis (Cibik *et al*., 2001).

It has also been reported the characterization of a PGH produced mainly during Stationary phase of *Clostridium perfringens*, named as ACP, which has a modular structure with three domains: a signal peptide domain, an N-terminal domain with repeated sequences and a C-terminal catalytic domain with a molecular weight of 122.388 kDa with a pI of 8.79 (Camiade *et al.,* 2010).

In another study, Donovan et al. 2006 investigated *Streptococcus agalactiae* bacteriophage B30 endolysin with lytic activity against the three main pathogens causing mastitis in dairy cattle, i.e. *Streptococcus agalactiae*, *Streptococcus uberis* and *Staphylococcus aureus* (Baker *et al*., 2006; Pritchard *et al*., 2006; Donovan *et al*., 2006).

Lactobacillus gasseri JCM11 31 (*Lactobacillus acidophilus*) has two extracellular proteins of 55 and35 kDa with autolytic activity (by zymogram analysis), the optimum pH for lysis was in the range of 6.0 to 7.0 (Yokoi *et al*., 2004).

In 2005, the activity of PGH produced by *Lactococcus lactis, Streptococcus thermophilus, Lactobacillus helveticus, Lactobacillus delbrueckii subsp. lactis, Lactobacillus acidophilus, Leuconostoc citreum and Lacticaseibacillus casei* with molecular weights of 18-55 kDa was reported (Lortal & Chapot-Chartier, 2005).

Two PGHs with muramidase activity have been identified in *Enterococcus hirae* ATCC9790; one of them, named SF muramidase, which has been shown to be an exoenzyme that progressively degrades glucan strands from its GlcNAc end and pesticin which is an endo N-acetylmuramidase (Vollmer *et al.,*2008).

In *Staphylococcus lugdunensis* a PGH (ALT) with N-acetylglucosaminidase and N-acetylmuramoyl-L- alanine amidase activities was identified with a molecular weight of 140.69 kDa, being the main autolysin of *Staphylococcus aureus* and *Staphylococcus epidermidis* (Bourgeois *et al*., 2007).

In 2013 García-Cano et al. reported PGHs isolated from *Pediococcus acidilactici* ATCC 8042 with lytic activity against *Staphylococcus aureus* with molecular weights of 110 and 99 kDa in zymograms with substrate *M. lysodeikticus* ATCC 4698 (García-Cano *et al*., 2015). Likewise, a PGH from *Enterococcus faecalis* (AtlD) with molecular weight of 62 kDa with antibacterial activity against *Listeria monocytogenes, Staphylococcus aureus* and *Enterococcus* strains of clinical origin was reported (Serrano-Maldonado *et al*., 2018).

Cibik and Chapot-Chartier (2004) evaluated PGH enzymes from *Lactobacillus pentosus*, where they identified PGH in membrane proteins with molecular weights of 31, 58 and 112 kDa in the stationary phase of growth (16 h) . In cytosol proteins it was presented activity in 31 kDa and, in the crude extract were found enzymes with PGH activity with an approximate molecular weight of 31, 43, 58, 77, 95 and 112-kDa.

16. Recombinant peptidoglycan hydrolases

Recombinant PGH have been reported, where their expression, activity, as well as their physicochemical, biochemical, reaction and specificity properties have been evaluated. Some cases are mentioned below. Cloning of a PGH from *Lactobacillus gasseri* JCM11 31 T was performed in the *E. coli* XL1-Blue system using the plasmid vector pUC118. Two recombinant plasmids, holgaY and lysgaY, wereproduced. The PGH gene inserted into the LysgaY plasmid encoded for a protein of 310 amino acids, whose molecular weight was calculated to be 33.7 kDa and a pI 8.75.

The gene inserted into theholgaY plasmid, on the other hand, coded for a protein of 143 amino acids with calculated molecular mass and pI of 15.7 kDa and 9.25, respectively. Sequencing of the gene revealed significant homology with hypothetical muramidases from the phage *Lactobacillus* Badh, Lj965, Lj928, LL-H, mv4 and mv1(Yokoi *et al*., 2005).

The AtlL protein from *Staphylococcus lugdunensis* was characterized for the first time and cloned in *E.coli.* The atlL gene encodes a bifunctional protein with lytic activity N-acetylmuramoyl-L-alanine amidase and N-acetylglucosaminidase on peptidoglycan. Cloning was performed in the pBAD / His B expression system (Invitrogen), which was used to subclone and express the gene fragments in *E. coli.* The atlL gene encoded a protein of 1279 amino acids with a calculated molecular mass of 140.69 kDa (Bourgeois *et al*., 2009).

In another work, the pET system (pET System, Novagen) was used with the Escherichia coli strain BL21 (DE3) for the cloning and expression of the 99 kDa bifunctional PGH of Pediococcus acidilactici ATCC M8042.

CHAP k lysine CHAP k from bacteriophage K, was heterologously expressed in *Escherichia coli* BL21(DE3) using the pET28a vector, the gene encoded a protein with a calculated molecular mass of 19,701kDa, with a purity of 95% (Shan *et al*., 2020).

17. Applications of peptidoglycan hydrolases

It has been described concisely the mechanism of these enzymes mainly in the most studied which is the lysozyme, this enzyme uses its mechanism of action against Gram positive bacteria destroying the cell walls by hydrolysis of the β 1-4 bond between the N-acetyl-muramic acid and N- acetylglucosamine of the peptidoglycan, thus weakening the cell wall and causing the consequent cell lysis.

In the food industry, lysozyme is used in foods such as meats, sausages, fish, vegetables, fruits, wine and milk powder and is also used in cosmetics and in the pharmaceutical industry (Maidment, 2009; Nakimbugwe *et al*., 2006).

Egg lysozyme is listed as a food additive with the code E-1105, it has been used as a contact preservative for food surface such as fresh vegetables, fish, meat, fruits, shrimps and other foods (Mine *et al*., 2004). Lysostaphin is an enzyme produced by *Staphylococcus simulans* which has a bactericidal effect on *S. aureus* and this confers applications in food, veterinary and human medicine (Fedorov *et al*., 2003; Szweda *et al*., 2012; Turner *et al*., 2007).

18. PGHs with antimicrobial activity against resistant microorganisms

Antibiotic resistance represents a threat to health, food safety and development, the use of some enzymes with lytic activity as antimicrobial agents has increased in the food industry (Donovan *et al*., 2006; Garcia *et al*., 2019). Table 2.6 lists some HGPs that have shown activity against resistant microorganisms.

Table 2.6 PGH reported with antimicrobial activity

PGH	Isolated microorganism	Inhibited microorganism	Reference
Lysostaphin	*Staphylococcus simulans*	*Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA)	Fedorov *et al*., 2003
HolgaY and lysgaY	*Lactobacillus gasseri* JCM 1130	*Lactobacillus gasseri, Streptococcus cremoris and Lactococcus lactis*	Yokoi *et al*., 2004
N-acetyl-muramidase	Bacteriophage B30	*Streptococcus agalactiae, Streptococcus uberis and Staphylococcus aureus*	Baker *et al*., 2006
AtlL	*Staphylococcus lugdunensis* ATCC 43809	*Micrococcus lysodeikticus, Bacillus subtilis and Staphylococcus lugdunensis*	Bourgeois *et al*., 2009
Endolysin	Profago LambdaSa2	*Streptococcus pyogenes, Streptococcus dysgalactiae, Streptococcus uberis, Streptococcus equi*	Donovan *et al*., 2006
Acp	*Clostridium perfringens*	*Micrococcus lysodeikticus* ATCC4698, *Bacillus subtilis*, *Clostridium difficile* and *Clostridium perfringens*	Camiade *et al*., 2010
N-acetylglucosamidase	*Pediococcus acidilactici* 99 kDa	*Streptococcus pyogenes, Enterococcus faecium, Lactobacillus paracasei, Listeria monocytogenes, Pediococcus acidilactici ATCC 8042, Enterococcus faecalis and Staphylococcus aureus ATCC 6538*	García-Cano *et al*., 2015
AtlD	*Enterococcus faecalis*	*Enterococcus faecium*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Micrococcus lysodeikticus* ATCC4698, *Enterococcus faecium*, *Enterococcus faecalis* ATCC, Enterococcus *faecalis*, *Listeria innocua*, *Staphylococcus aureus* ATCC 6538	Serrano-Maldonado *et al*.,2018.

19. Conclusions

The overuse of antibiotics is a global public health problem that must be addressed. Therefore, an alternative to this problem is the use of therapies with lytic enzymes such as PGH produced by BAL, which are efficient because they target one of the main structures of the cell necessary for life, the cell wall.

In addition, new bactericidal compounds of natural origin can be designed to replace antibiotics in the health, agricultural and agri-food sectors.

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Chapter 3 Relevance of gene expression studies to understand pollutants biodegradation

Capítulo 3 Importancia de los estudios de expresión génica en la comprensión de la biodegradación de contaminantes

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Abstract

Nowadays, pollution is a global problem that affects the environment and human health. The primary pollutants are hydrocarbons, plastics, heavy metals and pesticides, all of which are essential for basic human needs. For this reason, research into environmentally friendly and viable degradation methods has become key, e.g., biodegradation. Biodegradation is a technology that uses the enzymes or metabolism of an organism to hydrolyze pollutants but is limited by factors such type of substrates, environmental conditions and organism physiology. The study of gene expression, i.e., protein production from genetic information at a specific time and condition of biodegradation, provides valuable information about the genes and enzymes expressed during the degradation process, the response to stress and the pathways involved. This information can be applied to increase biodegradation efficiency, find new enzymes, improve enzyme activity, or optimize metabolic pathways. Gene expression studies can be performed by applying omics technologies. This chapter aims to describe the importance of studying the gene expression of organisms used in the pollutant biodegradation process.

Gene expression, Biodegradation, Pollutants

Resumen

Hoy en día, la contaminación es un problema global que afecta el medio ambiente y la salud humana. Los principales contaminantes son hidrocarburos, plásticos, metales pesados y pesticidas, todos ellos esenciales para las necesidades básicas de los seres humanos. Por esta razón, las investigaciones de métodos de degradación viables y amigables con el medio ambiente han adquirido relevancia, por ejemplo, los estudios de biodegradación. La biodegradación es una tecnología que utiliza las enzimas o el metabolismo de un organismo para hidrolizar contaminantes, pero se ve limitada por factores como el tipo de sustrato, las condiciones ambientales y la fisiología del organismo. El estudio de la expresión génica, es decir, de la producción de proteínas a partir de la información genética en un momento y condición especifica de biodegradación, proporciona información valiosa sobre los genes y enzimas expresadas, la respuesta a estrés y las vías metabólicas involucradas en el proceso de biodegradación. Está información puede ser utilizada para incrementar la eficiencia de la biodegradación, encontrar nuevas enzimas, mejorar la actividad enzimática u optimizar vías metabólicas. Los estudios de expresión génica se pueden realizar aplicando tecnologías ómicas. Este capítulo tiene como objetivo describir la importancia de estudiar la expresión génica de los organismos utilizados en los procesos de biodegradación de contaminantes.

Expresión génica, Biodegradación, Contaminantes

1 Introduction

Environmental pollution is defined as introducing the environment (air, water or soil) of substances harmful in higher than usual concentrations that reduce the quality (Manisalidis *et al*., 2020). Some critical substances toxic, named pollutants or xenobiotics, are hydrocarbons, synthetic polymers (plastics), heavy metals and pesticides. When contaminants found into air, water or soil, they can produce various adverse environmental impacts and human health effects. Xenobiotics are necessary for basic human needs to make medicines, plastics, detergents, chemicals, herbicides, combustibles and various other products (Mishra *et al*., 2019).

Therefore, the research into pollute degradation methods that are environmentally friendly and economically viable, such as biodegradation, is relevant. Biodegradation is a promising technology, but now it is limited by scarce evidence of gene expression changes in the organisms used. This work aims to describe the importance of studying the gene expression of organisms in the biodegradation pollutants process.

The following sections first show the environmental impacts caused by the primary pollutants (hydrocarbons, plastics, heavy metals and pesticides). Then, the biodegradation is defined as well as their process and some microorganisms used. Subsequently, is presented the general process of gene expression. After, the use of science omics in the study of gene expression and its application in biodegradation research is exposed. Finally, some examples of successful biodegradation research using gene expression studies are shown.

2 Environmental impacts

The list of pollutants in the world is highly extended. Among the most abundant and important contaminants are hydrocarbons, plastics, heavy metals and pesticides, due to their environmental and human health impacts (Kour *et al.,* 2021). As illustration of its importance, the study of Polidoro *et al.* (2016) in coastal streams and sediments of America Samoa showed a presence of approximately 0.12% w/w of heavy metals (Cd, Co, Cr, Cu, Hg, Ni, Pb, Sn and Zn) in sediments samples, and presence closely to 20 ppb of pesticides (benthiocarb, diazinon, ethion, fenitrothion and parathion), 0.2 ppb of total PAH´s and 9 ppb of phthalates (diethyl phthalate) in water samples. We will refer to these four pollutants in the subsequent.

The anthropogenic sources of pollutants include household, agricultural, industrial, transportation and others (Mishra *et al*., 2019). Hydrocarbons, for example, can be introduced in ecosystems by spills, pipes in bad conditions, lousy manipulation practices, or runoff of rain and rivers. Due to poor waste management and environmental education, the plastics arrive in the ecosystems by sewage and industrial water discharge or even by human hand. On the other hand, heavy metal and pesticide contamination are principally from metallurgical industrial, farming activities and illegal dumping effluents (Thakare *et al*., 2021). Table 3.1 shows some important pollutants nowadays, prevalence (air, water or soil), the primary source and the more relevant environmental and health human impacts.

Table 3.1 Main environmental and health human impacts caused by relevant pollutants

Pollutant	Example	Prevalence	Source	Environment and human health impact	Reference
Hydrocarbons	Naphthalene Fluorene Phenanthrene Anthracene Xylene Toluene	Water: sediment, column and surface Soil Air	Petrochemical industry Oil leaks Industrial activities as asphalt production Vehicle exhaust gases Forest fires	Toxic effects on flora and fauna Bioaccumulation along the food chain Carcinogenic, teratogenic and mutagenic They have been found in the human liver, kidney, lung, plasma and tissues Cardiovascular disorders	Marris *et al.,* 2020 Ahmed and Fakhruddin, 2018 Alegbeleye *et al*., 2017
Synthetic polymers (plastics)	Polyethylene (PE) Polyethylene terephthalate (PET) Polyvinyl chloride (PVC) Polystyrene (PS) Microplastics (<5 mm)	Water: sediment, column and surface Soil	Public waste: food packaging, bottles, textile fibers, hygiene products, detergents Sewage, river and stormwater discharges Aquatic transportation Commercial and recreational fishing equipment	Fauna trapped or suffocated Ingestion and bioaccumulation in organisms Toxic to reefs Adverse effects on growth, photosynthesis, reproduction and immune system of organisms Release of toxic compounds	Ganesh-Kumar *et al.,* 2019 Chae *et al.,* 2018 Rhodes, 2018
Heavy metals	Mercury Copper Zinc Nickel Lead Cadmium Chromium Cobalt Arsenic	Water: underground and surface Soil Air	Metallurgical and glass industry Carbon-burning and other fuels Vehicle exhaust gases Municipal and industrial wastewater Paints Volcanic eruptions	Decrease in biodiversity Damage to the membrane, proteins and DNA of organisms Interference with enzymatic activity impacting germination, development, photosynthesis and reproduction of organisms Through plants, they can reach humans Carcinogenic, neurotoxic and nephrotoxic Affect prenatal development and childhood Cause of cardiovascular disease, immune and reproductive disorders and alteration in blood composition	Thakare *et al*., 2021 Zwolak *et al*., 2019 Vareda *et al*., 2019 Vardhan *et al*., 2019
Pesticides	Carbamates Organophosphates Organochlorines Pyrethroids Triazines	Water: underground, surface Soil Air	Agriculture and irrigation Pest control activities (insecticides, fungicides, herbicides, rodenticides) Maintenance of private gardens Seeping Industrial wastewater Volatilization	Disruption depredator-prey interaction affect earthworms, parasitoids, and pollinators (bees, beetles and birds) They affect small fish directly and indirectly by decreasing their food (algae and plankton) Interference with soil fertility, properties of microflora, nitrogen fixation, nitrification, ammonification and mineralization Mutagenic, carcinogenic and neurodegenerative Cause of tumors, nervous system disorders, pulmonary dysfunction, immune system deficiency, cardiovascular, respiratory, kidney, endocrine, reproductive, and blood disorders	Hassaan *et al.,* 2020 Kaur *et al*., 2019 Yadav and Devi, 2017

The excessive use of xenobiotics has caused several environmental and healthy human impacts, due to their toxicity and non-biodegradable nature (Kour *et al.,* 2021). Consequently, searching for new economic and ecologic degradation processes is necessary; in this sense, an alternative can be biodegradation.

3 Biodegradation

Biodegradation is a biochemical process that refers to the broken down of various pollutants into more minor compounds caused by the metabolic potential of different organisms (Kour *et al.,* 2021; Alshehrei, 2017). Biodegradation is often used to describe a variety of microbial processes such as mineralization, detoxication or cometabolism (Riser-Roberts, 2020). Bacteria, archaea and fungi are typical biological factors (Abatenh *et al*., 2017). On the other hand, bioremediation is the application of biodegradation to hydrolyze environmental contaminants (in soil, sediments, groundwater) to reduce levels below concentration limits established by regulatory authorities (Singh *et al*., 2014; Kensa, 2011). Bioremediation is then an efficient, cost-effective and eco-friendly cleanup tool (Kour *et al.,* 2021). Bioremediation can divide into two types: phytoremediation and microbial bioremediation (An *et al*., 2020).

It is well known that microorganisms are capable of degrading a wide range of organic compounds (Riser-Roberts, 2020). Microbial biodegradation has received significant attention as an efficient biotechnological strategy to decontaminate the environment (Kour *et al.,* 2021). Microorganisms such as bacteria, fungi, algae are reported for their ability to degrade pollutants (Table 2). Still, biodegradation efficiency depends on many factors such as the type of pollutants, environmental conditions, and microorganisms used, which have to be deeply studied.

In the case of hydrocarbons biodegradation, there are many fundamental factors for a successful process. For example, hydrolysis rates are strongly influenced by substrate characteristics (availability, volatilization, type and length of hydrocarbons), involved microorganisms (cell metabolic pathways) and environmental conditions (pH, temperature, salinity) (Varjani, 2017).

On the other hand, the polymers synthetic biodegradation is determined by polymers characteristics such as functional groups increasing hydrophobicity, the molecular weight, density, branching, amount of crystalline or amorphous region and form (films, pellets, fibers) (Alshehrei, 2017). The plastic films compared with pellets and fibers, facilitate the adherence of cells, leading to considerable changes in the plastics morphology (Taniguchi *et al.*, 2019).

Heavy metals can be effectively remediated using plants and microorganisms (bacteria, fungi, microalgae) with tolerance to toxicity and capable of converting heavy metals into a less hazardous state (Table 2) (Thakare *et al*., 2021;Ojuederie and Babalola, 2017). Factors that influence the heavy metals bioremediation efficiency are biomass concentration, temperature, pH, metal ion concentration, redox potential and climatic conditions (Jacob *et al*., 2018).

Microorganisms (bacteria, fungi, actinomycetes) and plants have been used to help remove or detoxify pesticides. The factors that affect the degradation of the pesticides are the molecular weight, the structure, type and number of substituents of pesticide molecule, besides environmental factors as temperature, pH, salinity and viscosity (Ye *et al*., 2018; Parte *et al*., 2017).

Table 3.2 shows some reports about microorganisms with degradation capacity and the general biodegradation mechanisms. Even though the list is extensive, it is essential to mention that there are still pollutants without biodegradation studies.

Table 3.2 Examples of pollutant-degrading organisms and principal degradation mechanisms

Pollutant	Organism	Substrate	Reference	Mechanisms
Hydrocarbons	*Acinetobacter* sp.	Total petroleum hydrocarbons	Cai *et al.,* 2021	The initial attack is generally through attachment to the substrates or production of biosurfactants/bioemulsifiers. The intracellular attack is an oxidative process (oxygenases and peroxidases), then peripheral degradation pathways convert HC into intermediates of central metabolism: β-oxidation and tricarboxylic acid cycle (Varjani, 2017).
	Pseudokirchneriella subcapitata	1-methylphenanthrene and 3,6-dimethylphenanthrene	Luo *et al*., 2020	
	Klebsiella pneumoniae	Alkanes C_{10}-C_{20} in petroleum	Ozyurek and Bilkay, 2017	
	Pseudomonas aeruginosa	Total petroleum hydrocarbons	Varjani and Upasani, 2016	
	Bacillus sp.	Anthracene, naphthalene, benzene, toluene, xylene	Bisht *et al*., 2014	
	Aspergillus terreus	Naphthalene and anthracene	Ali *et al.,* 2012	
Synthetic polymers (plastics)	*Tenebrio molitor*	Polyvinyl chloride	Peng *et al.,* 2020	Microorganisms attack the polymer surface, and the extracellular enzymes secreted cause the main chain to cleave. The lower molecular weight compounds formed can be used by the microorganisms as carbon and energy source (Alshehrei, 2017).
	Streptomyces albogriseolus	Polyethylene	Shao *et al*., 2019	
	Zophobas atratus	Polystyrene	Yang *et al*., 2019	
	Brevibacillus sp. *Aneurinibacillus* sp.	Polypropylene	Skariyachan *et al.,* 2018	
	Aspergillus nidulans	Polyethylene terephthalate and polybutylene succinate, polycaprolactone, polylactic acid	Peña-Montes *et al.,* 2017	
	Ideonella sakaiensis	Polyethylene terephthalate	Yoshida *et al*., 2016	
Heavy metals	*Bacillus cereus*	Cd, Cu, Ag, Zn	Al Azad *et al*., 2020	Two mechanisms exist the neutralization of metals to non-toxic forms by the enzymatic attack (oxidoreductases, oxygenases, peroxidases) and bioaccumulation of heavy metals inside cellular components with non-apparent toxic effect (Ojuederie and Babalola, 2017).
	Saccharomyces cerevisiae	Pb, Cd, As, Hg	Massoud *et al*., 2019	
	Mixed culture: *Desmodesmos* sp., *Chlorella* sp., *Scenedesmus* sp.	Al, Cu, Fe, Mn, Zn	Aslam *et al*., 2019	
	Robinia pseudoacacia	Zn. Cd, Pb	Fan *et al*., 2018	
	Gemella sp., *Micrococcus* sp. *Hafnia* sp.	Cd, Cr, Pb	Marzan *et al*., 2017	
	Penicillium simplicissimum	Mo, V, Mn, W, Zn	Anahid *et al*., 2011	
Pesticides	*Chlamydomonas reinhardtii*	Trichlorfon (TCF)	Wan *et al*., 2020	Degradation by extracellular enzymes is through oxidized, dehydrogenation, reduction or hydrolysis. Degradation by intracellular enzymes begins with the adsorption of pesticides on the surface of microbial cells. The membrane permeability determines its penetration of the cell and can be degraded by mineralization or partially degraded by co-metabolism (Ye *et al*., 2018).
	Aspergillus flavus	Malathion	Derbalah *et al.,* 2020	
	Pseudomonas nitroreducens	Chlorpyrifos	Aswathi *et al*., 2019	
	Pleurotus ostreatus	Aldrin, dieldrin	Purnomo *et al*., 2017	
	Stenotrophomonas sp.	1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane (DDT)	Pan *et al*., 2016	
	Trichoderma viride FRP3	Glyphosate	Arfarita *et al.,* 2016	

Several microorganisms with biodegradation capability have been reported; it is known that the expression of their specific enzymes affects the rate of contaminant degradation (Abatenh *et al*., 2017). Understanding the conduct of liable degrader is an effective approach to evaluating the efficiency of various biodegradation approaches (Aydin *et al.,* 2016). Therefore, gene expression studies provide valuable biological information that can be used to improve the biodegradation process.

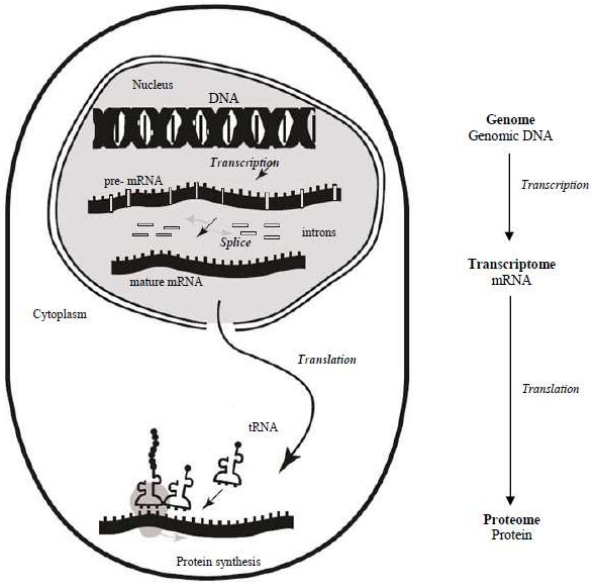
4 Gene expression studies and their regulation

4.1 Gene expression

Gene expression is the relationship between deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and proteins, and it is named the central dogma of molecular biology. Expression of the genes changes under stress situations as the presence of toxic compounds (Qian *et al*., 2021). Genes contain the information necessary for cells to survive and reproduce (Shafee and Lowe, 2017). The genetic information is encoded in the DNA. During the gene expression process, this information is copied from DNA into messenger RNA (mRNA) during transcription. Then, proteins are built from mRNA; this process is called translation (Figure 3.1) (Selzer *et al*., 2018).

DNA is a double-stranded molecule, one of the strands encodes information that the RNA polymerase reads to produce protein-coding mRNA. This strand runs in the 5´to 3´direction where the numbers refer to the backbone's carbon atoms' ribose sugar (Shafee and Lowe, 2017). RNA polymerase starts to copy in promoter DNA sequence and finished in the terminator sequence (Paniagua *et al*., 2003). The organization of genes is different in prokaryotes and eukaryotes. The most striking difference is that prokaryotic gene information is encoded on a continuous DNA stretch, whereas in eukaryotes, coding exons are interrupted by non-coding introns. Therefore, eukaryotic transcription of DNA to mRNA (derived only from exons) requires several steps (Selzer *et al*., 2018). Exon regions are retained in the final mature mRNA molecule, while intron regions are spliced out during post-transcriptional processing. One spliced together; exons form a single continuous protein coding region. Eukaryotic post-transcriptional processing adds a 5´cap to the start of the mRNA and a poly-adenosine tail to the end. These additions stabilize the mRNA and direct its transport from the nucleus to the cytoplasm (Figure 1). In comparison, prokaryote genes are often grouped into a polycistronic operon transcribed in the same mRNA (Shafee and Lowe, 2017). Three mRNA bases (named codon) determine the incorporation of an amino acid into the protein chain. The protein synthesis or translation starts with the codon AUG (adenine-uracil-guanine) that codifies the amino acid methionine. The amino acids are supplied by the specific transfer RNA (tRNA). The tRNA is united to the small ribosomal subunit, and then the large ribosomal subunit is joined. The tRNA supplies the following amino acid, and the amino acids are bonded with the help of the enzyme peptidyl transferase. The end of the protein synthesis is determined by terminato sequence (UAA-UAG-UGA), and finally the protein is liberated (Figure 1) (Paniagua *et al*., 2003). Thus, the flow of genetic information generally proceeds from the genome (entirety genomic DNA) over the transcriptome (total pool of mRNA) to the proteome (entire pool of proteins).

Figure 3.1 The general process of eukaryotes gene expression



Reference: Selzer et al., 2018

4.2 Gene expression regulation

The gene expression is managed by networks of regulatory mechanisms and is acquired during a life span due to specific changes. (Mazaira *et al*., 2018; Tomanek *et al*., 2020). Gene expression can be regulated in response to extracellular and intracellular signaling to cope with cells' metabolic needs and adapt to the changing environment (Li *et al.,* 2018). There are different steps where gene expression can be regulated: chromatin remodeling, transcription, post-transcription, translation and post-translation (Liang *et al*., 2019; Spriggs *et al*., 2010).

Gene expression in eukaryotes is a highly complex and tightly regulated process. The first level is the organization of the genome into chromatin (Shandilya and Roberts*,* 2012). DNA in eukaryotic cells is packaged into chromatin. Different chromatin modifications regulate chromatin structure, which modulates the accessibility of DNA and subsequently contributes to the regulation of gene expression (Li *et al*., 2018).

In eukaryotes, all protein-coding genes are transcribed by RNA polymerase II (Pol II). The regulation of Pol II transcription requires various transcription activators and repressors (Soutourina, 2018). Multiple genes with different functions are temporally regulated by transcriptional "on" and "off" switches that express specific genes that cells need in response to external stimuli (Carpenter *et al.,* 2014).

The regulation also involves post-transcriptional points occurring at the level of splicing, capping, polyadenylation, stability, and export of each mRNA. These mechanisms, modeling the duration of the new response to an abrupt environmental change for adapting the system in a timely and efficient manner (Carpenter *et al*., 2014).

Translational control of gene expression is essential in stress responses, as it allows a rapid change in the proteins without any lag. At the same time, new mRNA is transcribed and processes together with reprogramming protein synthesis to elicit an appropriate response to the type of stress-induced (Spriggs *et al*., 2010). Post-translational modification alters the functional diversity of the proteome by phosphorylation, dephosphorylation, ubiquitination or sumoylation. This mechanism is critical for the rapid reprogramming of cells for defense signaling (Withers and Dong, 2017; Kang and Han, 2011).

Eukaryotic genes typically have more regulatory elements to control gene expression compared to prokaryotes. The regulation process of prokaryotes cells is often regulated at the transcriptional level. The operator sequence next to the promoter is the main regulatory element. Repressor bound to the operator sequence physically obstructs the RNA polymerase, preventing transcription. Various transcriptional regulators usually control promoters of target genes in response to environmental stimuli (Eckweiler *et al*., 2018). Riboswitches are another important regulatory sequence commonly present in prokaryotic. These sequences switch between alternative secondary structures in the RNA depending on the concentration of key metabolites (Shafee and Lowe, 2017).

Microorganisms have an impressive metabolic ability and can easily grow in a wide range of environmental conditions (Abatenh *et al*., 2017). Once they are exposed to pollutants, the microorganisms try to adapt to these pollutants by modulating their gene expression (Paliwal *et al*., 2012). These specific genes could be turned on or turned off depending on environmental conditions (Baez-Rogelio *et al*., 2017). Different states of contamination affect gene expression.

The expression of genes involved in biodegradation may be fundamental to obtain the beneficial effect (Baez-Rogelio *et al*., 2017). Expression patterns provide valuable information for deducing the physiological roles played by the microorganisms (Yang *et al*., 2017).

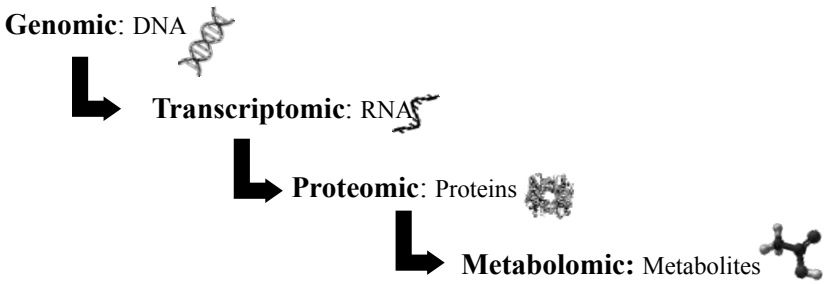
The application of advanced molecular biology techniques has recently provided a new perspective for a better interpretation of the biodegradation process, for example, the omics sciences. The omics sciences study the whole genome, transcriptome, proteome, and metabolome of the organisms involved in a metabolic process such as biodegradation (Wang *et al*., 2019).

5 Omics sciences: a tool for gene expression study

A better understanding of gene expression of the microorganism used to remediate contaminated sites is required to overcome biodegradation limitations and achieve successful implementation (Rodríguez *et al*. 2020). Omics sciences can help in the study of gene expression. The suffix "-omics" mainly indicates studies on a genome-wide scale of a specific biological system. Omics are principally used to study the whole genes (genomics), RNA transcripts (transcriptomics), proteins (proteomics) and metabolic products (metabolomics) (Marvasi *et al.*, 2019; Zampolli *et al.,* 2018).

The integration and interaction of the different components of the omic studies have given rise to the holistic view of the biological processes as the orchestration of biomolecules network of genes, transcripts, proteins and metabolites (Bedia, 2018). Data generated through each omic are analogous to an information cascade in a cell (Figure 3.2) (Rawat and Rangarajan, 2019).

Figure 3.2 Schematic representation of the omic cascade



Reference: Pandey et al., 2019; Bedia, 2018; Jansson and Baker, 2016

The omic technologies have considerably impacted life and environmental sciences and have generated important insights to increase our understanding in many research areas. Genomics, transcriptomics, proteomics and metabolomics offer remarkable promise as tools to address questions regarding the mechanism involved in the pollutant biodegradation process (Rodríguez *et al*. 2020).

Genomic. Omic science that studies the entire DNA sequence from a given sample obtains information on the entire genome, including phylogenetic and functional genes (Jansson and Baker, 2016). Genomic is static by nature and does not give information about the biological mechanisms encoded within it (Bedia, 2018). When genomic is applied to environmental investigations is based on scanning the genome of a single organism with degradative capacity (Rawat and Rangarajan, 2019).On the other hand, metagenomic, also known as environmental genomics, analyzing the genome of an entire collective microbial in an environmental sample (Desai *et al*., 2010). The major difference between genomic and metagenomic is that genomic determines gene sets of an organism. In contrast, metagenomic involves analyzing the genome sequence of an entire community inhabiting the same environment (Pandey *et al*., 2019).

The whole-genome sequence from microorganisms pertinent to bioremediation has been helpful to determine the gene pool of enzymes involved in the degradation of pollutants (Desai et al., 2010). Metagenomic is employed to know the taxonomic community composition and predict the degradation of contaminants (Chandran *et al*., 2020). The most widely used techniques in genomic are quantitative polymerase chain reaction (qPCR), real-time polymerase chain reaction (real-time PCR), DNA microarrays and next-generation sequencing (NGS) (Pandey *et al*., 2019: Bedia, 2018).

Transcriptomic. Transcriptomic detect, quantify, study and analyze the transcriptome that is the complete set of mRNA expression (coding or non-coding proteins) in an organism under specific circumstances (dos Santos *et al*., 2016; Pandey *et al*., 2019). The transcriptome is more dynamic than the genome due to the continuous transcription processes that reflect cell's activity and their response to external stimuli (Bedia, 2018).

Transcriptomic in environmental science is used to identify and decipher the mRNA expression profiles of genes upregulated or downregulated in microorganisms exposed to pollutants (Desai *et al*., 2010). But transcriptomic data alone cannot provide information about the activity of degradative enzymes (Rawat and Rangarajan, 2019).

It is accepted that there is a strong correlation between mRNA levels and protein abundance; however, some studies indicate that they are different. This difference results from factors such as protein regulation, post-transcriptional regulation, or possible functional requirement for protein binding. The integration of transcriptomics and proteomics allow to understand this discrepancy (Rodríguez *et al*. 2020). Principal methodologies used to obtain the transcriptional profile are RNA microarrays, RNA sequencing (RNA-Seq), quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and next-generation sequencing (NGS) (Rodríguez *et al*. 2020).

Proteomic. Omic technology is the branch of science that studies the proteome, the entire set of proteins expressed in an organism in a specific place and time (Rodríguez *et al*. 2020; Pandey *et al*., 2019). Proteomic aims are to provide information about proteome profile, protein phosphorylation, protein trafficking, comparative expression analysis of two or more samples, identification of post-translational modifications and the study of protein interactions (Bedia, 2018).

In environmental studies, proteomic provides information about changes in the protein profiles as a result of the exposition of organisms to pollutants; this identification can facilitate the understanding of which genes are involved in bioremediation processes and how they are regulated (Rodríguez *et al*. 2020). Metaproteomic studies the complete protein profile content of microbial communities residing in a given habitat (Chandran *et al.,* 2020; Pandey *et al*., 2019).

Proteomic provides information about the mechanisms of adaptation, metabolic pathways, the physiological responses of microbes to pollutants, temperature and other stressors during the biodegradation process (Malla *et al*., 2018). In the protein analysis process, there are two key steps. The first is the extraction and separation by two-dimensional gel electrophoresis (2DE-GE), two-dimensional difference gel electrophoresis (2D-DIGE), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or high-performance liquid chromatography (HPLC). The second key step is the identification of proteins by mass spectrometry (MS) based approaches. Four types of MS-bases approaches are generally used: quadrupole (Q), quadrupole ion trap (QIT), linear ion trap (LIT or LTQ), time of flight mass analyzer (TOF) and Fourier transform ion cyclotron resonance mass analyzer (FTICR). Also, MS ionization techniques have improved, such as electrospray ionization (ESI) and matrix-assisted laser desorption (MALDI). Nowadays, a diversity of mass analyzers exists in single (MS) or tandem (MS/MS) (Rodríguez *et al*. 2020; Bedia, 2018: Horgan and Kenny, 2011).

Metabolomic: Metabolomic is the quantitative and qualitative study of the metabolome or global metabolite profile produced by an organism in response to defined conditions and time (Rodríguez *et al*. 2020). Metabolome consists of a mixture of thousands of molecules (primary and secondary metabolites), for example, sugars or lipids (Bedia, 2018; Desai *et al.,* 2010). The metabolome study provides information about the biochemical activity of microorganisms and allows the establishment of a relationship between the genetic and phenotypic profiles (Rodríguez *et al*. 2020).

Metabolomic differs from genomic, transcriptomic, and proteomic. A direct connection between metabolites and genes cannot be established because the cell metabolism changes if there is a slight change in the environment (Pandey *et al.,* 2019). In the environmental area, metabolomic allows understanding the dynamics of the microbes and their functional contributions to the environments in which they live and explores the functional roles of these metabolites (Malla *et al.,* 2018). Metabolomics approaches have been used to investigate the responses of microorganisms to various environmental stressors such as heavy metals or temperature and to provide information about the regulatory events in a cell (Chandran *et al*., 2020; dos Santos *et al*., 2016).

Extraction and separation of metabolites can be performed by mass nuclear magnetic resonance spectrometry (NRM) or mass spectrometry (MS) based approaches, for example, liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE) (Rodríguez *et al*. 2020). The main MS ionization methods are electrospray ionization (ESI) and electronic impact (EI). Nowadays, a diversity of mass analyzers exists in single (MS) or tandem (MS/MS) (Bedia, 2018).

The information generated by a single omics study is crucial in environmental research but is not always enough to understand a complex biological process as microbial biodegradation (Rodríguez *et al*. 2020). Combining omics enables a better understanding of the mechanisms occurring in the biodegradation process and provides much richer information for predictive models (Rawat and Rangarajan, 2019; Jansson and Baker, 2016).

Integration of genomic and transcriptomic technologies is required to decipher the biodegradation pathways and understand how the contaminant's presence regulates the gene expression. Besides, it is possible to elucidate if the involved genes are constitutive, inducible, downregulated or upregulated (Rodríguez *et al*. 2020).

Combined genomic and proteomic data can provide important information about the microbial enzymes associated with the biodegradation pathway (Pandey *et al*., 2019). Studies of combined of transcriptomic and proteomic are especially favorable for analyzing the differences in gene expression and their regulation in response to environmental conditions (Aydin *et al*., 2016). The omics studies mentioned, genomic, transcriptomic and proteomic; will help to discover novel genes, proteins, and underlying pathways for the biodegradation of pollutants (Pandey *et al.,* 2019).

Thus, a multi-omics approach would enable answering biological questions such as which genes are expressed into RNA (transcriptomics) and translated into proteins (proteomics) and which metabolites are present (metabolomic) under specific conditions (Jansson and Baker, 2016).
If the aim is to understand the cellular gene expression, transcriptomics and proteomics should be employed (Dangi *et al.,* 2018)

6 Successful biodegradation research applying the study of gene expression

Recently, different omics-based approaches are being used for bioremediation (Pandey *et al.,* 2019)**.** These studies provided relevant information that could be used to understand the biological process, additionally, can help in the implementation and improvement of bioremediation technologies. Table 3.3 displays some research examples where gene expression studies using omic tools were applied, besides the relevant contributions to the scientific community.

Table 3.3 Successful biodegradation research applying the study of gene expression

Pollutant	Organism used	Omic studies	Results	Contributions	Reference
Hydrocarbons (HC) Monocyclic aromatic hydrocarbon (MAH): aniline	*Delftia* sp. K82 isolated from the Gyeonggi province of Korea in 1992	Genomic: Next-generation sequencing (NGS) Transcriptomic: NGS Proteomic: Liquid chromatography-tandem mass spectrometry (LC-MS/MS) For transcriptomic and proteomic, two cultures were prepared: Luria-Bertani media (LB) and aniline media (ANI)	Genomic: 6327 genes 6117 protein-coding genes Transcriptomic: 3919 genes were identified as differentially expressed genes Proteomic: ANI media 472 proteins LB media 409 proteins	Enzymes of the aniline degradation pathway and aniline-induced novel proteins were identified. ANI cultured was composed of 14 aniline degradation enzymes (11.9% of all proteins). Aniline oxygenase complex was induced more than 2-fold in aniline presence, transcriptionally and translationally. Among 95 proteins belonging to the cell wall, 12 were significantly induced in aniline media. Membrane proteins play essential roles in the protection against extracellular stress. *Delftia* sp. K82 has two different complete aniline degradation pathways.	Lee *et al.,* 2021
Synthetic polymer (plastic) Forms of polyethylene (PE): 1. PE4K: commercial powder of PE 2. PE4K-OX: PE4K thermo-oxidized during 14 days at 120°C 3. PEfi: PET films 4. PEfi-OX: oxo-degradable film, which has been stored at room temperature for 10 years	*Rhodococcus ruber* C208 (environmental strain)	Transcriptomic: NGS Metabolomic: Lipidomic strategy by nano-electrospray ionization mass spectrometry (nano-ESI MS) Condition reference: Mannitol	Transcriptomic: Genes were upregulated in PE conditions (PE4K 28, PE4K-OX 33, PEfi-OX 22) 11 transcripts were commonly overexpressed in the presence of the three types of PE 39 transcripts could be directly assigned to alkane degradation and β-oxidation 34 transcripts encoding putative cytoplasmic oxidase A diacylglycerol kinase was detected Metabolomic: Three main lipid species could be observed	In terms of the number of induced genes: the oxidized forms of PE were more efficient than non-oxidized PE in inducing the mineralization. The most upregulated pathways in the presence of PE are alkane degradation and β-oxidation of fatty acids. The alkane degradation pathway is a central node in the degradation of the PE fragments generated by abiotic oxidation. Oxidases could well participate in the intracellular fragmentation of oxidized PE and extracellular oxidases to reduce the molecular mass of external PE. The redistribution of the phospholipid pattern and the presence of the enzyme diacylglycerol kinase suggests that PE fragments might serve as substrates for the β-oxidation pathway. Metabolic limiting steps were identified which could be fruitfully targeted for optimized PE consumption by *R. ruber*.	Gravouil *et al*., 2017

Heavy metals Plants were irrigated with 1000 mL of heavy metals contaminated water containing Cd, Pb, Cu and Ni at a concentration of 10 ppm. Reference plants were irrigated with tap water.	*Sorghum bicolor* (Sorghum) growing in an open glasshouse at Assiut University Experimental Farm, Assiut, Egypt	Transcriptomic: Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) in leaves of 12-weeks old plants irrigated either with tap-water or heavy metals contaminated water	Transcriptomic: The expression levels of all 15 genes were highly upregulated in response to heavy metals stress	The roles of the genes found are: SbZFP17, SbZFP346 and SbZFP6 genes are zinc finger proteins that are highly expressed in response to the stress imposed by heavy metals. SbPPR1 gene plays a substantial role in heavy metals stress tolerance too. SbLysMR1 plays a role in recognizing symbiotic bacteria, and it is induced in Cd, Cu, and Cr response. LAC9 (laccase gene) is expressed in response to the high concentration of Cu, Pb and Cd and is implicated in response to plant development and stresses. MAPKK gene plays a role in signal transduction of abiotic and biotic stress, and their expression is upregulated under higher concentrations of Cd and Cu. SbAVPL1 regulates solute transport across the vacuolar membrane of plant cells and plays a crucial role in accumulating heavy metals. The genes indicate that these clusters may provide a conserved evolutionary mechanism that might be implemented in common metabolic pathways, produce a protein complex through direct interaction, or serve as receptors in signaling cascades.	Abou-Elwafa *et al*., 2019
Pesticide Hexaconazole 98% (triazole fungicide) dissolved in acetone at a concentration of 50 mg L^{-1}.	*Sphingobacterium multivorum* from sewage activated sludge and soil from a pesticide factory producing hexaconazole	Genomic: NGS Transcriptomic: RT-PCR and NGS Metabolomic: Ultra performance liquid chromatography quadrupole-time of flight mass spectrometry (UPLC/Q-TOF MS)	Genomic: The genome contained 5523 genes Transcriptomic: It was detected the presence of 864 differential genes between the hexaconazole treatment and control, 337 upregulated genes and 527 down-regulated genes. Aldehyde dehydrogenase, monooxygenase, RND transporters and ABC transporters were upregulated Metabolomic: Three interesting metabolites were identified 2-(2,4-diclorophenyl-1-(1H-1,2,4-triazol-1-yl) hexane-2,5-diol; 2-(2,4-dichlorophenyl) hexane-1,2-diol and 1H-1,2,4-triazole	Differential genes are mainly related to metabolism; most of them are concerned with carbohydrate metabolism (39), energy metabolism (36) and amino acid metabolism (30). There are also six genes about xenobiotic biodegradation and metabolites. The reactions of oxidation, hydroxylation and substitution were involved during the degradation of hexaconazole. Hexaconazole can be oxidized, which may be due to the participation of monooxygenase. RND transporter may involve the exportation of toxic metabolites to maintain homeostasis of strain. ABC transporter may provide the essential phosphoric acid and amino acid to survive under a high concentration of hexaconazole. The results of these studies could provide a reference for in-situ remediation of hexaconazole.	An *et al.,* 2020

7 Remarks

Gene expression analysis using multi-omics is a valuable tool for the elucidation of unknown or hidden metabolic diversity of organisms, screening novel genes for a biodegradation response, identifying novel enzymes for biodegradation, and knowing the enzyme synergy (Lee *et al.,* 2021).

In the specific case of biodegradation processes, knowing what happens at the molecular level from the moment the biological system comes into contact with pollutants helps understand the whole process. After the entire process is known, the following optimization studies will be more straightforward. For example, all the identified enzymes involved in the biodegradation process may be cloned, isolated, and used as a biological tool in specific contamination situations.

Gene expression studies generate large amounts of information about the root of biological behavior, serving to apply to a specific industrial sector or other research groups with different objectives.

8 Conclusions

Environmental pollution is a serious and global problem. Hydrocarbons, plastics, heavy metals and pesticides are priority pollutants. They arrive at the environment by human activities first and negatively impact the environment and human health. Biodegradation is a process that could be helpful to eliminate or reduce pollutants in an economically and ecologically way. Several organisms with degradative capacity have been reported, but the efficiency is affected by different factors. For that, it is necessary a better understanding of the complex behavior of the organisms in detail.

The study of gene expression of degradative microorganisms using omics technologies provides relevant information that could be used to implement or improve the biodegradation process on a large scale.

These studies have offered crucial information about biodegradation that can be used to know or understand: genes transcribed, enzymatic activities, novel enzymes, survival mechanisms, secondary metabolites, hidden biodegradation pathways, exact metabolic pathways, and modifications of existing pathways under conditions of stress caused by pollutants. These aspects are important to optimize the success of the biodegradation approach.

The knowledge about the degradative organisms will help design or improve remediation strategies by manipulating the pathways adding or deleting one or more genes, incorporating new metabolic pathways into organisms, or modifying enzyme specificity and affinity. Also, the enzymes could be used to produce other high-value-added metabolites such as precursors for biotechnological or pharmaceutical products.

9 Acknowledgment

The authors are grateful to the National Council of Science and Technology, Mexico (CONACYT) for providing the financial support A1-S-47929.

The first author is grateful to CONACYT for the doctoral fellowship awarded (No. CVU: 858149).

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Chapter 4 Application of homeopathic preparations and biofungicides to prevent and control anthracnose (*Colletotrichum gloeosporioides*) in Haas avocado crops

Capítulo 4 Aplicación de preparados homeopáticos y biofungicidas para prevenir y controlar la antracnosis (*Colletotrichum gloeosporioides*) en los cultivos de aguacate Haas

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DOI: 10.35429/H.2021.3.67.86

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A. Marroquín, J. Olivares, D. Ventura and L. Cruz (Coord) Agricultural Sciences and Biotechnology. Handbooks-©ECORFAN-México, Querétaro, 2021.

Abstract

Avocado production in Mexican territory is considered the most important in the world because it contributes 45.95% of agri-food exports, various states contribute to position Mexico as the main producer, approximately 175 thousand hectares with fruits of different sizes are counted that are cultivated in the states of Michoacán, Jalisco and Nayarit to later be commercialized nationally and internationally to Guatemala, Canada, Japan and El Salvador.The import and export process is restricted to the phytosanity variable as a consequence of the appearance of pests and the lack of control for the elimination or reduction of the main sources that cause them, the consumer states / countries demand a continuous application of plant health measures in the field and in the shipping processes to ensure that the systems of prevention, growth, elimination of pests are supported and evidenced in a scientific and technical way; seeking to provide the Mexican countryside with the best techniques for the preservation, control and sale of the fruit. Within the implemented techniques, Agrohomeopathy manages the reduction of pests and diseases, considering economic and ecological effects, this research shows the application of homeopathic preparations and commercial biofungicides, to prevent and control the presence of anthracnose (*Collectotrichum gloeosporioides*) in the cultivation of hass avocado, the experimental process was established with 64 trees infected with anthracnose; Through a random sampling, 10 treatments and a control of homeopathic preparations and biofungicides were applied, placing the foliage by means of aspersion., The effect was observed by means of the decrease in the number of pustules in leaves and fruit, decrease in the length of the pustule (cm), pustule width (cm) both in fruits and leaves. The treatments used showed an effective control in the development of the *C. gloeosporioides* infection: the agrohomepathic doses of the *C. gloeosporioides* preparation at 10 CH showed an influence on the parameter of length by width in fruit and leaf; The microbiological product formulated from 5 strains of bacillus Fungizar 5B controlled the damage in the parameter of length by width in leaf, through the described implementation the effectiveness of Agrohomeopathy for the preservation of avocado and the reduction of the treated plague was verified.

Avocado, Agrohomeapatia, Homeopathic Preparations, Anthracnose

Resumen

La producción de aguacate en el territorio mexicano es considerada la más importante del mundo ya que aporta el 45.95% de las exportaciones agroalimentarias, diversos estados contribuyen a posicionar a México como el principal productor, se contabilizan aproximadamente 175 mil hectáreas con frutos de diferentes tamaños que se cultivan en los estados de Michoacán, Jalisco y Nayarit para posteriormente ser comercializados a nivel nacional e internacional a Guatemala, Canadá, Japón y El Salvador. El proceso de importación y exportación está restringido a la variable fitosanidad como consecuencia de la aparición de plagas y la falta de control para la eliminación o reducción de los principales focos que las provocan, los estados/países consumidores exigen una aplicación continua de medidas fitosanitarias en el campo y en los procesos de embarque para asegurar que los sistemas de prevención, crecimiento, eliminación de plagas estén sustentados y evidenciados de manera científica y técnica; buscando dotar al campo mexicano de las mejores técnicas para la conservación, control y venta de la fruta. Dentro de las técnicas implementadas, la Agrohomeopatía logra la reducción de plagas y enfermedades, considerando los efectos económicos y ecológicos, esta investigación muestra la aplicación de preparados homeopáticos y biofungicidas comerciales, para prevenir y controlar la presencia de antracnosis (*Collectotrichum gloeosporioides*) en el cultivo de aguacate hass, el proceso experimental se estableció con 64 árboles infectados con antracnosis; A través de un muestreo aleatorio, se aplicaron 10 tratamientos y un control de preparados homeopáticos y biofungicidas, colocando el follaje mediante aspersión, El efecto se observó mediante la disminución del número de pústulas en hojas y frutos, disminución de la longitud de la pústula (cm), ancho de la pústula (cm) tanto en frutos como en hojas. Los tratamientos utilizados mostraron un control efectivo en el desarrollo de la infección por *C. gloeosporioides*: las dosis agrohomeopáticas del preparado de *C. gloeosporioides* a 10 CH mostraron influencia en el parámetro de longitud por ancho en fruto y hoja; El producto microbiológico formulado a partir de 5 cepas de bacilo Fungizar 5B controló el daño en el parámetro de longitud por ancho en hoja, a través de la aplicación descrita se comprobó la efectividad de la Agrohomeopatía para la conservación del aguacate y la reducción de la plaga tratada.

Aguacate, Agrohomeapatia, Preparados Homeopáticos, Antracnosis

1 Introducción

The avocado harvest (*Persea americana Mill*) is an economic factor of great importance for the country, so its consumption and marketing has grown considerably, both in the national and international markets.

Londoño et al., (2007) comment that anthracnose (*Collectotrichum spp*.) Affects various foods for human consumption, pastures and crops, including fruit trees. Among the fruit trees most affected by anthracnose, the mango (*Mangifera indica L*.), the avocado (*Persea americana Mill.*), The tree tomato (*Cyphomandra betacea Cav*.) Stand out, indicating that the pathogen belongs to the domain; Phylum Eukaryota; Ascomycota, of class Ascomycetes (III) and the (NCBI, 2007) mentions that it is of the genus; *Colletotrichum* (in the amorphous state) and *Glomerella* (in the Telemorphic state). This infestation was observed by Zamora-Magdaleno, et al., (2001) in various plantations where C. *gloeosporioides* appeared in the growing fruits through round translucent spots in an interval of 0.5 - 1.0 mm; Later an elevation of orange color is formed that changes to dark brown (Figure 4.1 Interval of round translucent spots in fruit with anthracnose affectation), the lesions did not multiply, but they were numerous in the fleshy body of the avocado causing that during the phases of harvesting and packing will increase injuries due to the handling process**.**

The need to generate sustainable and economic means or alternatives that avoid or reduce the appearance of conditions such as anthracnose (*C. gloeosporioides*) in Hass avocado crops (*P.americana*), brings with it the development of the present research implemented an Agrohomeopathic process through the placement of various preparations by means of spraying to 64 trees in a plantation made up of 370 trees corresponding to the aforementioned fruit genus, the process was observed through the decrease in the number of pustules in leaves and fruit, decrease in the length of the pustule (cm), pustule width (cm) in both fruits and leaves.

In the first instance, a review of the existing literature regarding anthracnose (*C. gloeosporioides*) was carried out, considering the morphology and symptoms presented in avocado; in addition to establishing the existing advances with the use of biopreparations, organic and chemical preparations for the treatment of fungi. The methodology used is based on 2 phases: phase 1 is considered as the stage of identification and assurance of the work system detailing the environment and conditions of the area in which the experimental research will be applied; It is made up of 3 stages characterized by the application of the exploitative analysis; Phase 2 contemplates the development of the experimental process, describing in a specific way each one of the compounds used and the study of the different factors that intervene in the process of eradication of the condition. Subsequently, the results obtained are detailed, this for each study period, making a comparison of the results obtained from the analysis with those resulting from other investigations, finally, the conclusions obtained and the existence of present and future benefits with the application of the homeopathic preparations and biofungicides in the avocado plantation.

Figure 4.1 Interval of round translucent spots in fruit with anthracnose affectation

Consultation Source: Own Elaboration

1.1 Literature review
1.2 Generalities of anthracnose (*C. gloeosporioides*)
1.3 Taxonomy of *C. gloeosporioides*

(Agrios, 2005) It indicates that the pathogen belongs to the domain; *Eukaryota del Phylum*; Ascomycota, of class Ascomycetes (III) and the (NCBI, 2007) mentions that it is of Genus; *Colletotrichum* in (amorphous state) and *Glomerella* in (Telemorphic state).

(Villanueva-Arce,2006) They argue that the representative symptoms of anthracnose (*C. gloeosporioides*) of naturally infected fruits are characterized by sunken, circular or irregular necrotic lesions, with well-defined raised edges and masses of orange conidia. (Lopez, 2008) Indicates that the different stages of development of *Colletotrichum* species can be separated into: 1) deposition on the surface of the host, 2) subjection of the conidium on the surface, 3) germination of the conidia, 4) production of the appressorium, 5) penetration of the epidermis of the plant, 6) growth and colonization of the host tissue and 7) production of acérvalos and sporulation.

1.4 Symptoms of *C. gloeosporioides* in avocado fruits

(Garcia, 2009) Describes that avocado fruits with symptoms of anthracnose presented variation in the color of the colonies, which contrasts with the description of dark gray colonies for *C. gloeosporioides*, as well as the variation in size and shape of the conidia. that have served to separate species of Colletotrichum spp. in strawberry. (Rodriguez Lopez, 2013) He mentions that in the field, the fruits present symptoms called “smallpox” and “clove”. In reference to the first one, it begins with small light brown spots and, later, dark and sunken brown. Consequently, the lesion takes on a dry and brittle appearance, in the shape of a crater, becoming detached.

1.5 Use of biopreparations for the control of fungi

The way of administering medicines in homeopathy is directly related to the fundamental principles of this. After having taken that into account, the scale of dynamization of the remedies will be defined, then the potency and, later, the frequency of administration. Let us point out at this moment that we do not speak of dose itself, since this word traditionally refers to quantity or volume and in homeopathy, thanks to abundant clinical experience, we know that usually ten drops or globules do not have more effect than five (Eizayaga, 1991, p. 284 cited by Meneses, 2020). The most used dynamization scales are: decimal (X), centesimal (CH) and fifty-thousandth (LM). Each scale obeys defined pharmaceutical techniques and basically differs in the method of preparation.

Potency refers to the degree of dynamization (dilution + succussion). The higher the dilution and succussion, the higher the potency. Thus, 30 CH is more powerful than 24 CH since more dilutions and, therefore, more succussions have been involved in its preparation process. The frequency refers to the initial shot and its subsequent repetitions. The single, non-repeated dose, or single dose, is preferred when one is very sure of the remedy and when the state of the vital force does not make its frequent administration necessary (Meneses, 2020).

(Vargas Toledo, 2016) It demonstrated that the in vitro fungitoxicity against *Alternaria solani* by the homeopathic medicines *Propolis, Isotherapic* of *A. solani* and *Isotherapic* of ash, at 6, 12, 30 and 60 CH (centesimal hahnemaniana) dynamizations, and Sulfur , *Silicea Terra, Staphysagria, Phosphorus*, *Ferrum sulphuricum* and *Kali iodatum* at dynamizations 6, 12, 30 and 100CH, in which I use distilled water and a 30% hydroalcoholic solution as controls at 12, 30, 60 and 100CH dynamizations, in which the mycelial growth, sporulation and conidial germination of *A. solani* were evaluated. The results indicated that for mycelial growth only in *Sulfur* and *Staphysagria* 100CH it showed a suppressive effect in comparison with both controls. For sporulation, Propolis 6, 30 and 60CH and *Ferrum sulphuricum* 6 and 30CH caused inhibition and differed from both controls. Isotherapeutic of A. *solani* 6CH, *Isotherapeutic* of ash 6CH and *Ferrum sulphuricum* 30CH reduced the germination of the spores of the pathogen. (Hanif, Shahnaz, Tariq, & Imtiaz, 2015) They affirm that in the in vitro experiment, homeopathic granules of *Árnica montana* and *Thuja occidentalis* (100, 75 and 50%). v / w conc.) the result obtained was the inhibition of the fungi that infect the roots, such as *Fusarium oxysporum*, *Macrophomina phaesolina* and *Rhizoctonia solani*. The granules of *T. occidentalis* and *A. montana* (100% v / w conc.) However, the granules *T. occidentalis* (75% v / w conc.)

Transmitted a significant suppression of the mycelium of *R. solani* followed by *F. oxysporum* and *M. phaseolina* but *A. montana* (75% v / w conc.) It showed a greater zone of inhibition in the mycelial growth of *F. oxysporum* and maximum inhibition in *R. solani* and *M. phaseolina*. Both *A. montana* and *T. occidentalis* granules (50% v / w conc.) In which minimal inhibition occurs in the test fungi. The results show that *T. occidentalis* in all concentrations was considered effective for the inhibition of root rot fungi followed by *A. montana*. Therefore, the use of homeopathic granules has shown a positive effect in reducing the intensity of the root attacked by the pathogen in the field and improving the growth of the plant. Therefore, it is suggested that it should be applied on a large scale, as it is cheap, easily accessible, non-dangerous and environment-friendly.

1.6 Use of organic preparations for the control of fungi

(Eguívar, 2006) Describes that agroecological fungicides are normally preventive, so they must be applied before the onset of the disease. That is why agroecological fungicides function as "fungistatic" agents, since they primarily inhibit the germination of fungus spores. (Rodriguez, 2007) He comments that in response to the selection pressure due to the high doses and continuous applications of chemical products, it causes great economic losses. He adds that an economic and efficient dilemma for disease control is in this case the use of natural products emanating from plants.

1.7 Application of chemical products to reduce diseases caused by fungi

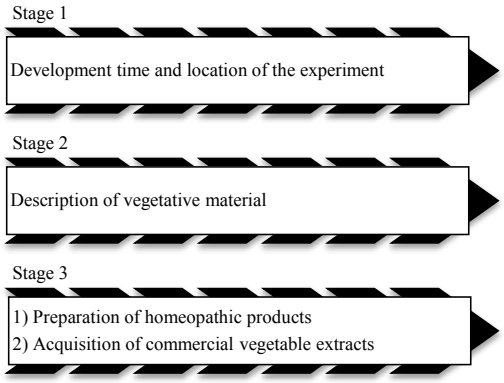
(Gutiérrez-Alonso, 2003). It evaluated the firmness to benomilo and thiabendazole in isolates of *Colletotrichum gloeosporioides* acquired from mango fruits cv. They have been affected by anthracnose in the producing regions of Veracruz, Guerrero, Michoacán, Sinaloa and Chiapas, Mexico. The fungicides were added to potato-dextrose-agar culture medium, at a rate of 0.1, 1, 5, 10, 50, 100, 200 and 400 ppm to evaluate their effect on the mycelial growth rate (TCM) and the germination of conidia, to estimate the mean lethal concentration (LC50) using a Pro bit analysis. Most of the isolates presented a TCM <0.5 mm / day at 50 ppm in both fungicides, except Mich; however, Ver-1, Sin and Mich presented an CL50> 20 ppm in benomilo and thiabendazole, being considered as resistant. Ver-2 and Gro did not exceed the 20 ppm threshold, except Gro with thiabendazole. Chia presented an CL50 <6 ppm in both fungicides, indicating sensitivity. Therefore, both fungicides did not deprive the germination of conidia *in vitro* and caused excessive deformation of germ tubes.

1.8 Methodology to be developed

The methodological process is made up of 2 phases; Phase 1 is called recognition and validation of the experimentation process and is analyzed through 3 stages (Figure 4.2 Stages that constitute Phase 1), in Phase 2 homeopathic preparations were applied through a series of experiments in the avocado plantation (application of experimental processes).

I. Phase 1: Recognition and validation of the experimentation process

Figure 4.2 Stages that constitute Phase 1



Consultation Source: Own Elaboration

1.9 Stage 1) Development time and location of the experiment

The experiment was developed in a period of 11 months in the community of Tlaxpanaloya, municipality of Naupan Puebla (Figure 4.3 Avocado fruit plantations). In this community, Hass Avocado plantations have been recently introduced, so far there are a total of 20 producers, with an area of 1.5 hectares planted. The experiment was established in the property called "Tantlahua" which is located between the coordinates 20.219353 ° North, -98.111944 ° West, at 1810 meters above sea level.

Figure 4.3 Avocado fruit plantations

Consultation Source: Own Elaboration

1.10 Stage 2) Description of vegetative material

The avocado trees that were used for the establishment of the experiment, had the following characteristics: average age 8 years, height 7 m, with 10 production cycles. Which had anthracnose damage with light brown spots on the leaves, and the presence of small circular lesions of black to brown color in the first stages of the fruit until its commercial maturity (Figure 4.4 Vegetative material).

Figure 4.4 Vegetative material

Consultation Source: Own Elaboration

1.11 Anthracnose (*C. gloeosporioides*)

The fungus *C. gloeosporioides* was obtained from the damaged leaves and fruits of avocado (Figure 4.5 Fruit with anthracnose affection), in which 0.05 g was taken to grind and 1/3 of the 5 g of sugar was added in a mortar, in which it was ground in a circular fashion for 6 min. At the end, the surface of the mortar was scraped with a stainless steel spoon for 4 min, the procedure was repeated, the second 2/3 was added and another 6 min was ground, then it was scraped again for 4 min, it was repeated again the procedure, the third third of sugar was added, ground for 6 min. It was scraped for 4 min. And the procedure was repeated. At the end, 1 C of the Triturado was obtained.

Figure 4.5 Fruit with anthracnose affection

Consultation Source: Own Elaboration

To obtain the 2 C of the crushed, 0.05 grams of the 1C of the crushed and 5 g of sugar were added to the mortar, to carry out the same procedure of obtaining the 1 C, obtaining the 2 C crushed. To obtain the 4 centesimal and the mother tincture of the anthracnose fungus, 0.05 g was added to a 30 ml amber glass bottle with 50 drops of distilled water and 50 drops of pure undenatured cane alcohol, it was succussed for two minutes and Two more were allowed to settle, resulting in 4 CH, likewise repeating the procedure until obtaining 10 CH and 30 CH. The rest of the homeopathic products were purchased at Dr. Arroyo's Homeopathic Pharmacy at the address of Aldama 110, Centro 56100, Texcoco de Mora, Mex. obtained at 6 CH.

1.12 Stage 3) Preparation of homeopathic products

The experiment consisted of the application of agronosodes such as:

1. *Árnica montana* 6CH and 20 CH
2. *Calcarea carbónica* 6CH, 20CH
3. *Chamomilla* at 6CH and 30CH
4. *Ferrum phosphoricum* at 6CH and 30C (Figure 4.6 Homeopathic products)
5. Anthracnose Agronosode (*C. gloeosporioides*) obtained from avocado fruit and leaves at 10 CH and 30 CH (Figure 4.7 Fruit with Anthracnose for Agronosode)

Figure 4.6 Homeopathic products

Consultation Source: Own Elaboration

Figure 4.7 Fruit with Anthracnose for Agronosode

Consultation Source: Own Elaboration

1.13 *Calcarea carbónica* 6CH

It contains no less than 85 percent of calcium carbonate calculated with reference to the dry substance, considered one of the most widely used polychrests in agro-homeopathy, it functions in the structure of the plant as a static factor, providing rigidity to the trunk and leaves as well it also generates the consistency of the epidermis of plants and fruits. From the mother tincture at 6 CH, 1 drop was added in a 30 ml amber glass bottle, 99 drops of pure undenatured cane alcohol were added, it was succussed for two minutes and left to rest for another two minutes. Obtaining the 7 CH. The same procedure was carried out until 20 CH was obtained (Figure 4.6: Homeopathic products).

1.14 *Árnica montana* 6CH

The substance is extracted directly from the fresh whole plant including the root, useful as a reestablishing agent in damage to the plant either by cuts, pruning or pest conditions; The preparation consisted of: from the mother tincture to 6 CH, 1 drop was added in a 30 ml amber glass bottle, 99 drops of pure undenatured cane alcohol were added, it was succussed for two minutes and left to rest for another two min. Obtaining the 7 CH. The same procedure was carried out until 20 CH was obtained (Figure 4.6: Homeopathic products).

1.15 *Chamomilla* 6CH

For the extraction of the substance, the entire plant is occupied when it is in bloom, the primary function is carried out by means of absorption in the root of the fruit, the preparation was carried out in the following way: from the mother tincture to 6 CH is 1 drop was added to a 30 ml amber glass bottle, 99 drops of pure undenatured cane alcohol were added, it was succussed for two minutes and left to rest for another two minutes. Obtaining the 7 CH. The same procedure was carried out until 30 CH was obtained (Figure 4.6: Homeopathic products).

1.16 *Ferrum phosphoricum* 6CH

It contains no less than 47 percent of ferrous salts expressed as ferrous sulfate octahydrate, it allows the improvement in the roots and the capillary system, optimizing the circulation of liquids in the extremities of the plant to avoid the generation of rotting processes, the preparation methodology was the following: from the mother tincture to 6 CH, 1 drop was added in a 30 ml amber glass bottle, 99 drops of pure undenatured cane alcohol were added, it was succussed for two minutes and left to rest for another two minutes. Obtaining the 7 CH. The same procedure was carried out until 30 CH was obtained (Figure 4.6: Homeopathic products).

1.17 Stage 3) Acquisition of vegetable extracts and commercial chemical products

1.18 Fungize 5B

It is a microbiological product formulated from 5 strains of *Bacillus*. (Complex of sporulated rhizobacteria, *Bacillus subtillis*, *B. amylillquefaciens*, *B. licheniformis*, *B. megaterium* and *B. mycoides*) Bacteria in Fungizar 5B compete for space and food with pathogenic fungi, in addition to producing compounds with fungicidal activity.

1.19 Clean Culture LT Extract of Gobernadora, (*Larrea tridentada*)

It is an organic bactericide - fungicide (Figure 4.8 Clean Crop LT) that inhibits or deactivates the enzymes of bacteria, mycelia and the fruiting bodies of fungi through the morphological denaturation caused by the components of this natural extract of Gobernadora (*L. tridentada*).

Figure 4.8 Clean Crop LT

Consultation Source: Own Elaboration

1.20 Commercial chemical

1.21 Benomilo

It is a commercial fungicidal product with radical and foliar absorption, with preventive and curative contact fungicidal activity for the control of fungal diseases.

Phase 2) Application of experimental process

1.22 Bioassay

Each concentration of the homeopathic product was succussed before its application, in a plastic container of 2 L capacity, 1.5 L of water was added and then a drop of the homeopathic preparation was added, it was succussed for a time of 2 min. After 2 min of rest, it was applied in a spray form and so on for the other treatments. For the application of Fungizar 5B (Figure 4.9 Application of Fungizar) it was every eight days at a rate of 3.7 ml in 1.5 liters of water, in the same way for the case of Clean Crop LT the applications were every eight days at a rate of 3.7 ml in 1.5 liters of water. In the combination of both products, 1.8 ml of each product were added in 1.5 liters of water. These products were applied in a conventional manner. In addition to homeopathic products, benomilo, organic products, water sucked as a control, and it was applied with pure water without succussion

Figure 4.9 Application of Fungizar

Consultation Source: Own Elaboration

The experiment was established in a 1.8 hectare plot, with a total of 370 trees of which 64 trees were selected by random sampling that showed damage caused by anthracnose. The applications were made to the foliage, throughout the tree, by spraying. (1.5L) (Figure 4.10 Bioassay applied by spraying system), every 8 days for *Árnica montana* 6CH, *Calcarea carbónica* 6 CH, *Chamomilla* 6 CH, *Ferrum phosphoricum* 6 CH, biopreparation of Anthracnose (*C. gloeosporioides*) obtained from fruit and leaves of avocado. 10 CH and every 15 days for *Árnica montana* 20 CH, *Calcarea carbonica*, 20 CH, *Chamomilla* 30 CH, *Ferrum phosphoricum* 30CH, biopreparation of Anthracnose (*C. gloeosporioides*) obtained from avocado fruit and leaves 30CH.

Figure 4.10 Bioassay applied by spraying system

Consultation Source: Own Elaboration

The experimental analysis was randomly completed with ten homeopathic products, two organic products and the combination of both, the benomilo fungicide applied in a conventional way pure water and succussed water that was considered as another treatment. Conforming 4 repetitions for each treatment and a control; succussed water, which was also considered as one more treatment. What make up 16 treatments. The parameters to be measured were the number of pustules, length, and width of the pustule in fruit as well as in leaf, for this one of the branches damaged by tree was selected.

A fruit and a leaf were selected which were monitored every eight, fifteen days for their analysis (Figure 4.11 Application of homeopathic preparations and biofungicides).

Figure 4.11 Application of homeopathic preparations and biofungicides

Consultation Source: Own Elaboration

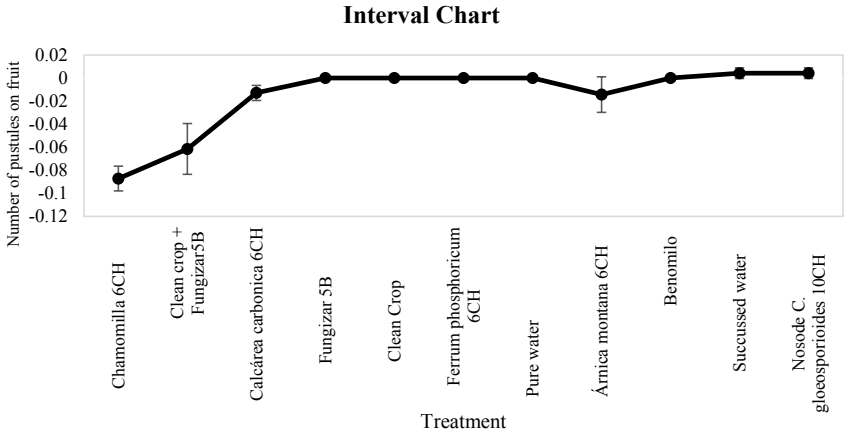
1.23 Results

Assumptions tests were performed on the data to find out if they met normality and homogeneity of variance, for which an analysis of variance was carried out. If no significance was found between the treatments, the Tukey test was used. If the assumptions were not met, a non-parametric analysis was performed.

1.24 Reduction of the fruit pustule implemented every 8 days

According to the data obtained from the reductions of the parameter of length by width in the fruit, in which 11 treatments including the control were evaluated in a period of eight days, where it was carried out that in all the reductions there are no significant differences between treatments compared to the control, in which statistically a minimal reduction of the pustule surface is appreciated in the last evaluation; and that *Chamomilla* 6CH in the reductions from 1 to 7 the growth was to a lesser extent compared to the control considering that different letters in the columns indicate significant differences between the means of the Kruskal Wallis ranges, $p \leq 0.05 \pm$ and of the Tukey treatments, $p \leq 0.05$ (Table 4.1 Abstract of significant LxA tests of the pustule in fruit, testing every eight days); *C. carbónica* 6CH increased the pustule to a lesser extent in reductions 2,4 and 7, on the other hand, both products in the first data collection showed that it did reduce, however, it increased for the other repetitions; and that the biopreparation of *C. gloeoporioides* 10CH did reduce the length by width of the fruit pustule in the last measurement in contrast to the witness (Graph 4.1 Tukey test for LxA of the pustule in fruit experienced every eight days).

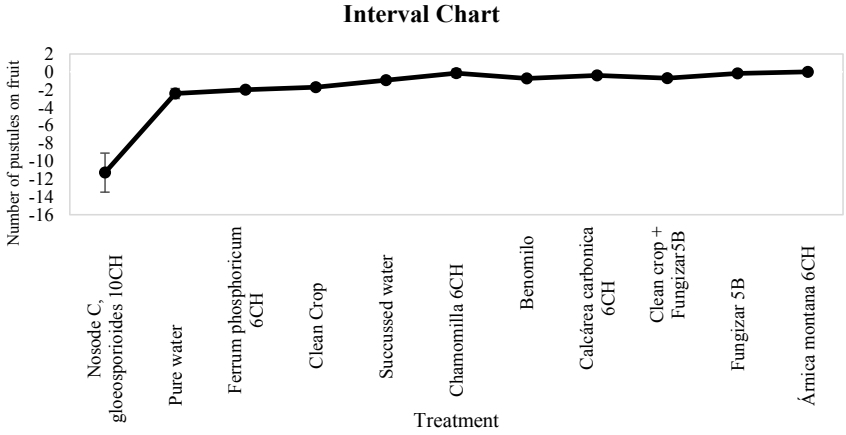
Graph 4.1 Tukey test for LxA of the pustule in fruit experienced every eight days



Consultation Source: Own Elaboration

The data shown in the parameter of the number of pustules in fruit evaluated every eight days, it can be seen that in reduction 2 there are no significant differences between treatments, and that in reductions 3,4, 5, 6 and 7 the nosode of *C. gloeoporioides* 10 CH presented significantly higher number of pustules with respect to the control of sucked water; in reduction 1 *Chamomilla* 6CH, *C. carbónica* 6CH, the combination of Clean crop + Fungizar 5B, Fungizar 5B did not reduce or increase the appearance of new pustules, on the other hand *Á. montana* 6CH in all measurements did not reduce or increase the appearance of new pustules in fruit (Graph 4.2 Tukey's test for Number of pustules in fruit).

Graph 4.2 Tukey's test for Number of pustules in fruit

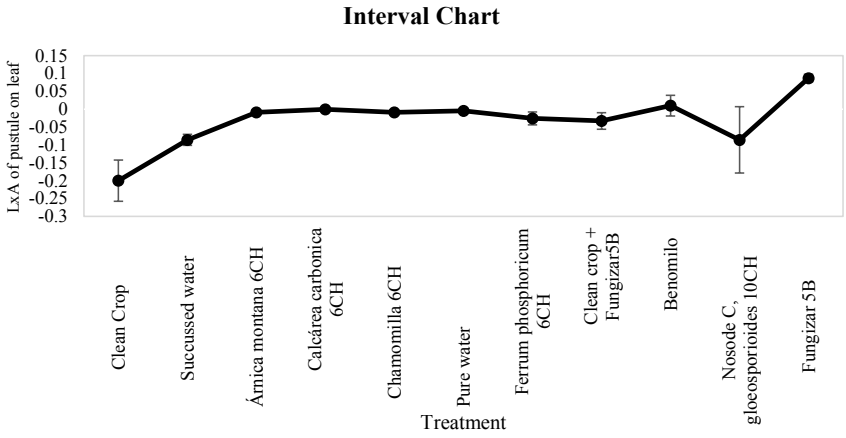


Consultation Source: Own Elaboration

1.25 Reduction of the leaf pustule evaluated every 8 days

The reductions measured every eight days in length by width of the leaf pustule are shown, where it is observed that in the reduction 1,2 and 7 considering that different letters in the columns indicate significant differences between the means of the ranges as shown In reduction 4, establishing a Kruskal Wallis test, $p \leq 0.05 \pm$ and of the treatments (Tukey, $p \leq 0.05$) (Table 1.2 Abstract of significant tests of the number of pustules in leaf evaluated every eight days) there are no differences between the treatments, and that benomilo in reductions 5 and 6 and that *C. gloeosporioides* 10CH 3, 5 and 6 and that Fungizar 5B in 3,4, 5 and 6 did not significantly reduce the pustule, on the contrary this parameter was higher in comparison to the control of sucked water (Graph 4.3 Tukey's test for LxA of the leaf pustule evaluated every eight days).

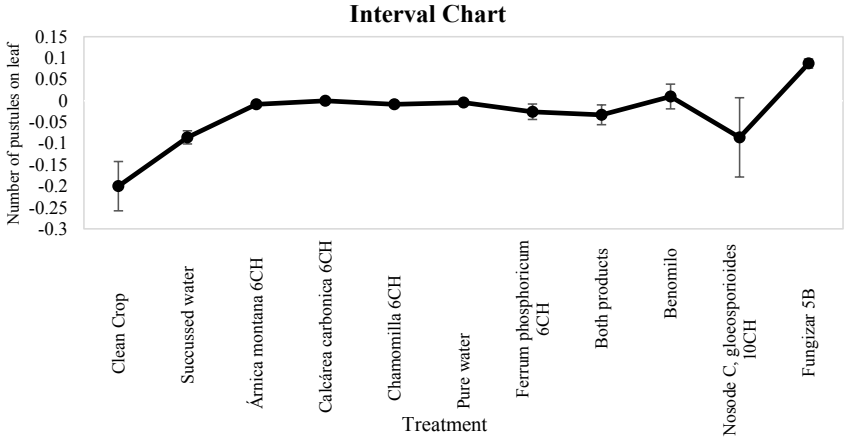
Graph 4.3 Tukey's test for LxA of the leaf pustule evaluated every eight days



Consultation Source: Own Elaboration

The results shown were evaluated every eight days, which shows the parameter of the number of pustules in the leaf, it is observed that in reduction 1 and 2 there were no differences between treatments, the nosode of *C. gloeosporioides* 10CH in reductions 3,4,5 , 6 and 7 increased the appearance of new pustules with respect to the control, *Chamomilla* 6CH, the combination of Clean crop + Fungizar 5B, fungizar 5B, F. *phosphoricum* 6CH, pure water, in reduction 3, the emergence of new pustules was with "less slowness", in reduction 4 with both products, Fungizar 5B and pure water, the appearance of new pustules was slow, on the other hand *C. carbónica* in reduction 7 did reduce compared to the witness (Graph 4.4 Tukey's test for number of leaf pustules evaluated every eight days).

Graph 4.4 Tukey's test for number of leaf pustules evaluated every eight days

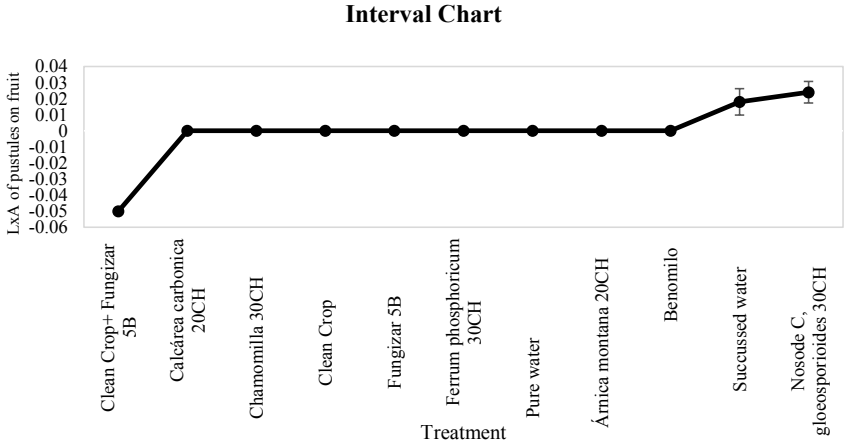


Consultation Source: Own Elaboration

1.25 Reduction of the fruit pustule implemented every 15 days

According to the data obtained in the reductions in the parameter of the number of pustules in fruit evaluated every 15 days, it is observed that in the reductions 1,2,3,4 (Table 1.3: LxA of the pustule in fruit evaluated every 15 days), there are no significant differences between treatments, however the combination of Clean crop + Fungizar 5B, Fungizar 5B, F. *phosphoricum* 30CH and *Chamomilla* 30CH in the reduction of one 1 without having significant differences between treatments did not decrease or increase the presence of new pustules in fruit, and that in the reduction 5 *Chamomilla* 30CH grew to a lesser extent compared to the witness (Graph 4.5 Tukey test for LxA of the pustule in fruit performed every 15 days).

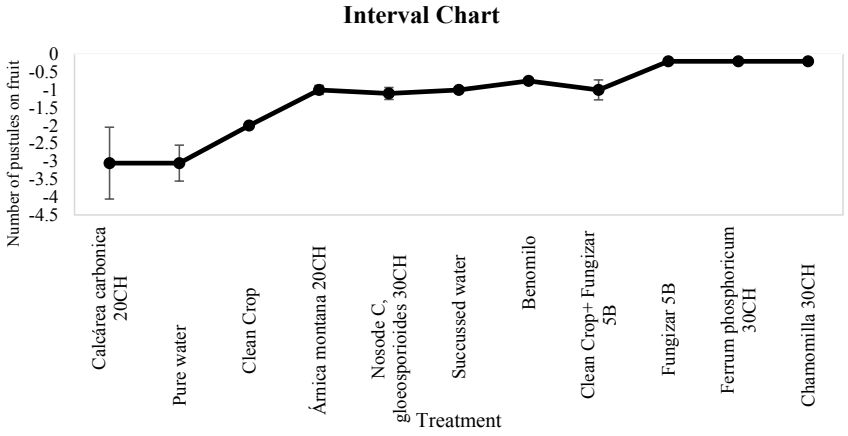
Graph 4.5 Tukey test for LxA of the pustule in fruit performed every 15 days



Consultation Source: Own Elaboration

The data shown on the reductions in length by width in fruit shows that there is no significant difference between treatments in the applications of every 15 days, so that the combination of Clean crop + Fungizar 5B did not stop the growth of the pustule in contrast with the values of the control of sucked water, and that the nosode of *C. gloeosporioides* 30CH in a similar way with the control of sucked water reduced the length by width of the fruit pustule less (Graph 4.6 Tukey's test for number of pustules in fruit evaluated every 15 days).

Graph 4.6 Tukey's test for number of pustules in fruit evaluated every 15 days

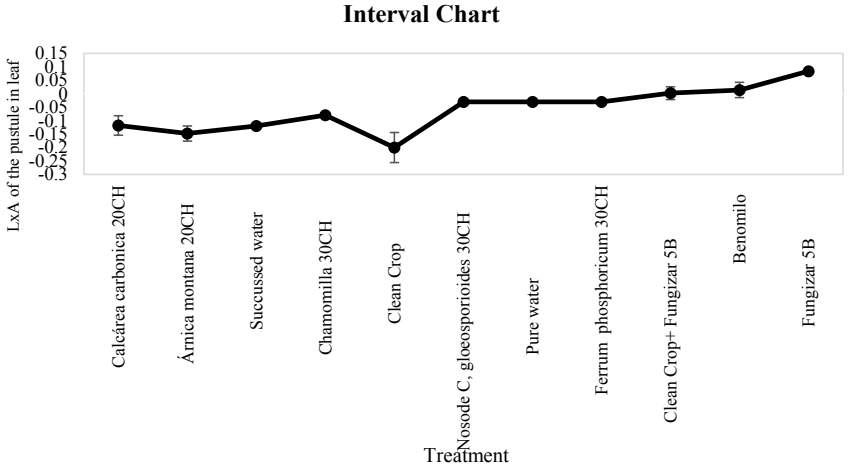


Consultation Source: Own Elaboration

1.26 Pustule reduction in update sheet every 15 days

In the reductions with the parameter number of pustules on the leaf, which were evaluated every 15 days, it is observed that there were no significant differences in the reductions 2, 3, 4 and 5 (Table 1.4: LxA of pustules on the check sheet every 15 days) ; However, in reduction 1, the treatments that slowly increased the appearance of new pustules were the combination of Clean crop + Fungizar 5B, Fungizar 5B, pure water and *F. phosphoricum* 30CH (Graph 4.7 Tukey's test for LxA of the pustule in leaf tested every 15 days).

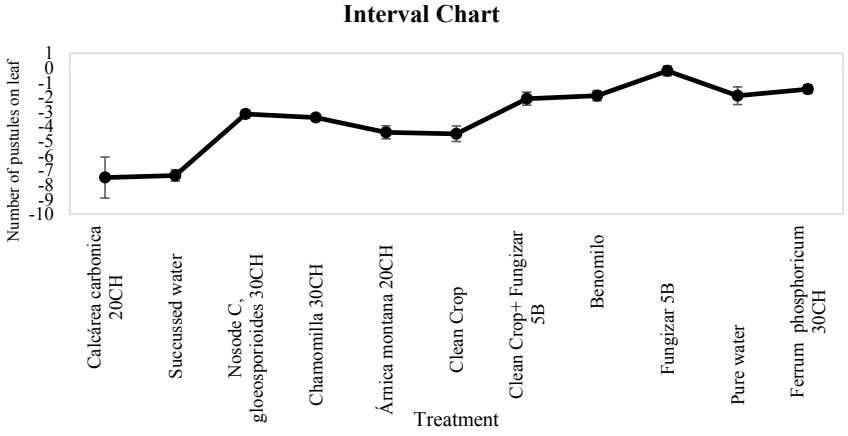
Graph 4.7 Tukey's test for LxA of the pustule in leaf tested every 15 days



Consultation Source: Own Elaboration

The results with parameter length by width in leaf evaluated every 15 days, it is observed that *C. carbonónica* 20 CH, *Á. montana* 20 CH in reduction 5 and 5 did not control the increase in the dimensions of the pustule with respect to the control of succussed water; Fungizar 5B considerably reduced the dimensions of the pustules compared to the control of sucked water (Graph 4.8 Tukey's test for the number of pustules in leaf evaluated every 15 days).

Graph 4.8 Tukey's test for the number of pustules in leaf evaluated every 15 days



Consultation Source: Own Elaboration

1.27 Annexes

Table 4.1 Abstract of significant LxA tests of the pustule in fruit, testing every eight days

* Kruskal Wallis test; * Tukey's test									
L x A of pustule on fruit									
Treatment	Reduction 1			Reduction 2			Reduction 7		
	Prom	R		Prom	R		Prom	R	
Chamomilla 6CH	-0.03	17.13	a	-0.08	18.13	a	-0.1	13.25	A
Clean crop + Fungizar5B	0.05	27.88	a	-0.05	18.5	a	-0.08	13.5	a
Calcárea carbónica 6CH	0	22.5	a	-0.03	18.88	a	-0.03	19	a
Fungizar 5B	0	22.5	a	0	24	a	0	24	a
Clean Crop	0	22.5	a	0	24	a	0	24	a
Ferrum phosphoricum 6CH	0	22.5	a	0	24	a	0	24	a
Pure water	0	22.5	a	0	24	a	0	24	a
Árnica montana 6CH	0	22.5	a	0	24	a	0	24	a
Benomilo	0	22.5	a	0	24	a	0	24	a
Succussed water	0	22.5	a	0	24	a	0.03	28.88	a
Nosode *C. gloeosporioides* 10CH	0	22.5	a	0	24	a	0.03	28.88	a

Consultation Source: Own Elaboration

Table 4.2 Abstract of significant tests of the number of pustules in leaf evaluated every eight days

* Kruskal Wallis test; * Tukey's test												
L x A of leaf pustule												
	Reduction 1			Reduction 2			Reduction 3			Reduction 7		
Treatment	Prom	R		Prom	R		Prom	R		Prom	R	
Clean Crop	0	23.5	a	0.0	27.5	a	-0.2	20.5	ab	-0.3	15	a
Succussed water	0	23.5	a	-0.1	16.63	a	-0.1	12.25	a	-0.1	15.5	a
Árnica montana 6CH	-0.03	18.38	a	0.0	22.75	a	-0.03	22.5	ab	0.0	21.13	a
Calcárea carbónica 6CH	0	23.5	a	0.0	22.75	a	0	27	ab	0.0	21.13	a
Chamomilla 6CH	-0.03	18.38	a	0.0	22.75	a	-0.03	22.5	ab	0.0	21.13	a
Pure water	0	23.5	a	0.0	22.75	a	-0.03	22.5	ab	0.0	21.13	a
Ferrum phosphoricum 6CH	0	23.5	a	-0.1	16.63	a	-0.08	16.75	ab	0.0	23.63	a
Clean crop + Fungizar5B	0	23.5	a	-0.1	20.88	a	-0.13	15	ab	0.0	24.38	a
Benomilo	-0.03	22.88	a	-0.1	16.63	a	0	26.38	ab	0.0	25.25	a
Nosode *C. gloeosporioides* 10CH	-0.03	18.38	a	0.0	26.63	a	0.03	30.88	b	0.0	29.25	a
Fungizar 5B	0.03	28.5	a	0.1	31.63	a	0.08	31.25	b	0.1	30	a

Consultation Source: Own Elaboration

Table 4.3 LxA of the pustule in fruit evaluated every 15 days

* Kruskal Wallis test; * Tukey's test															
L x A of pustule on fruit															
	Reduction 1			Reduction 2			Reduction 3			Reduction 4			Reduction 5		
Treatment	Prom	*R		Prom	*R		Prom	*R		Prom	*R		Prom	*R	
Clean Crop+ Fungizar 5B	-0.05	17.5	a	-0.05	17.13	A	-0.05	16.75	a	-0.05	16.75	a	-0.05	16.75	a
Calcárea carbónica 20CH	0	23	a	0	22.5	A	0	22	a	0	22	a	0	22	a
Chamomilla 30CH	0	23	a	0	22.5	A	0	22	a	0	22	a	0	22	a
Clean Crop	0	23	a	0	22.5	A	0	22	a	0	22	a	0	22	a
Fungizar 5B	0	23	a	0	22.5	A	0	22	a	0	22	a	0	22	a
Ferrum phosphoricum 30CH	0	23	a	0	22.5	A	0	22	a	0	22	a	0	22	a
Pure water	0	23	a	0	22.5	A	0	22	a	0	22	a	0	22	a
Árnica montana 20CH	0	23	a	0	22.5	A	0	22	a	0	22	a	0	22	a
Benomilo	0	23	a	0	22.5	A	0	22	a	0	22	a	0	22	a
Succussed water	0	23	a	0	22.5	A	0.03	27.38	a	0.03	27.38	a	0.03	27.38	a
Nosode *C. gloeosporioides* 30CH	0	23	a	0.03	27.88	A	0.03	27.38	a	0.03	27.38	a	0.03	27.38	a

Consultation Source: Own Elaboration

Table 4.4 LxA of pustules on the check sheet every 15 days

* Kruskal Wallis test; * Tukey's test															
L x A of pustule on fruit															
	Reduction 1			Reduction 2			Reduction 3			Reduction 4			Reduction 5		
Treatment	Prom	*R		Prom	*R		Prom	*R		Prom	*R		Prom	*R	
Calcárea carbónica 20CH	-0.03	23.63	ab	-0.05	21	ab	-0.15	15.13	ab	-0.18	13.25	a	-0.18	13.25	a
Árnica montana 20CH	-0.05	22.13	ab	-0.15	16.5	ab	-0.18	13	a	-0.18	13.25	a	-0.18	13.25	a
Succussed water	-0.08	17.25	ab	-0.13	13	a	-0.13	13.75	a	-0.13	14.13	ab	-0.13	14.13	ab
Chamomilla 30CH	-0.08	13.88	a	-0.08	16.75	ab	-0.08	18	abc	-0.08	18.38	abc	-0.08	18.38	abc
Clean Crop	0	28.5	ab	-0.25	18.13	ab	-0.25	18.75	abc	-0.25	18.88	abc	-0.25	18.88	abc
Nosode *C. gloeosporioides* 30CH	-0.03	23.63	ab	-0. 03	24	ab	-0.03	24.63	abc	-0.03	24.88	abc	-0.03	24.88	abc
Pure water	-0.03	23.63	ab	-0.03	25.25	ab	-0.03	26	abc	-0.03	26.13	abc	-0.03	26.13	abc
Ferrum phosphoricum 30CH	-0.03	23.63	ab	-0.03	25.25	ab	-0.03	26	abc	-0.03	26.13	abc	-0.03	26.13	abc
Clean Crop+ Fungizar 5B	-0.08	21.63	ab	0	26.38	ab	0.03	27.25	abc	0.03	27.38	abc	0.03	27.38	abc
Benomilo	-0.08	17.25	ab	0	28.25	ab	0.05	31.63	bc	0.05	31.75	bc	0.05	31.75	bc
Fungizar 5B	0.1	32.38	b	0.08	33	b	0.08	33.38	c	0.08	33.38	c	0.08	33.38	c

Consultation Source: Own Elaboration

1.28 Discussion

Farmers' limited knowledge of the identity of plant diseases, routes of transmission, and appropriate management methods is a key but little-exposed problem. Islam et al., (2020) conducted a sample of 260 farmers who produce chili in Bangladesh to explain their knowledge, perceptions and working methods in relation to chili anthracnose, a fungal disease caused by *Colletotrichum*.Total crop yield was reduced by 4% for the average farmer. However, only 22% of farmers knew it was a fungal disease and only 25% could tell how the disease spreads in the field. In a study of students in general, students show interest in learning about Homeopathy, however they expressed difficulty in understanding its philosophy. Some of the factors that influence this ignorance of homeopathy is the scarcity of reliable and updated information, reliable and updated materials. This contributes to the spread of misleading information, generating disbelief and low demand for this type of treatment. Therefore, it is necessary to reflect on the importance of explaining about homeopathy within the University (Zen *et al*., 2021).

The works that have been carried out in the last decades in Mexican lands to use Homeopathy in favor of the health of animals and crop plants deserve a special mention (Rodríguez and Pérez, 2020). With regard to agrohomeopathy, the most notable efforts have come from the Chapingo Autonomous University, where several researchers have conducted studies on the effects of homeopathic medicines on crops, with very positive results. Dr. Felipe Ruiz Espinoza explained that a central part of agro-homeopathy is safety, and its non-toxicity (Ponce, 2015).

As Paracelsus stated: "any substance can be a poison or a medicine, the difference is the dose." In the same way, it can also be said that every substance is a medicine until proven otherwise. Homeopathy is a therapeutic method that is based on principles such as "the similar cures the similar", experimentation in the healthy man and unique medicine, in addition to dilution and dynamization (Zen et al., 2021). Homeopathy was developed on the principle of individuality and firmly believes that only one remedy at a time, sourced from our peers in nature, has the essential energy to be able to heal us. By applying only one remedy at a time, it is easy to see if and what a reaction occurs. In the same way, it can be verified if an improvement occurs, something other than applying several remedies together or at the same time because the common thread is lost many times and it is not known which remedy has produced what (Meneses, 2020).

The use of homeopathic preparations in horticultural crops allows to restore their homeostasis and reduce production losses caused by biotic and abiotic factors (Lösch, *et al*., 2021).

In this investigation for the applications that were evaluated every eight days in a curative way, in the parameter length by width in fruit, Chamomilla 6CH and C. carbonónica 6CH during 48 days, constants were maintained regarding the damage by the fungus, not registering the increase of the pustule. In the parameter number of pustules in fruit, the nosode of *C. gloeosporioides* at 10CH at 24 days showed a lower presence of pustules. For the parameter of number of pustules on the leaf, the nosode of *C. gloeosporioides* at 40 days reduced the damage. In the record of measurements, length by width of the leaf, Clean crop at 32 days registered less control compared to the other treatments.

Ishwarya et al., (2018) used a combination of lemongrass oil, cinnamon oil and thyme oil for the management of postharvest anthracnose of pomegranate caused by *Colletotrichum gloeosporioides* and tested at a concentration of 0.1% *in vitro*. The new formulation THYCILEM 30 EC based on essential oils completely (100%) inhibited the growth of the pathogen at a concentration of 0.1%. In vivo results revealed that pre-harvest spraying with the new THYCILEM 30 EC (0.1%) followed by a subsequent immersion with the new THYCILEM 30 EC (0.1%) for 5 minutes was more effective in reducing anthracnose. of pomegranate, which had the minimum PDI of 13.43 after 24 days of treatment under harvest conditions, compared with 87.50 PDI in the untreated control.

Taking into account the data obtained in the parameter of length by width in fruit, the nosode of *C. gloeosporioides* 10CH 56 days reduced the damage as did *Á. montana* 6CH at 32 days, but the number of pustules in fruit remained constant. The length per width parameter in the leaf, the treatments that reduced the damage caused by the fungus were, benomilo® at 40 days, the nosode of *C. gloeosporioides* 10 CH at 40 days and Fungizar 5B at 24 days began to control damage by the fungus. Situation that may coincide with Rodrigues, et al. (2020) who carried out an investigation to evaluate the fungitoxicity of high dilutions of tectone extract (*Tectona grandis*) in the mycelial growth, sporulation and germination of the fungus *Colletotrichum gloeosporioides* and concluded that the variables relative percentage of mycelial development (PRD) and sporulation varied according to dynamization, with a maximum reduction of 7% in PRD (33 CH) and a 70% increase in sporulation (3 and 21 CH).

Likewise, Rissato et al., (2018) demonstrated the control of white mold (*Sclerotinia sclerotiorum*) in common beans (*Phaseolus vulgaris L.*) using extremely dilute aqueous solutions of Phosphorus and *Calcárea carbónica*, at 6CH, 12CH, 24CH, 36CH and 48CH dynamizations. These results indicate the potential of *Phosphorus* 12CH, *Phosphorus* 48CH, *Calcárea carbónica* 12CH, and *Calcárea carbónica* 48CH to control *S. sclerotiorum* in common beans. In addition to slowing the progression of the disease up to 83%, reducing the number of dead plants up to 90%. While, for this research, in the parameter of number of pustules in leaf, the treatment that began to reduce the damage of the fungus was *C. carbónica* 6 CH at 56 days.

Lösch, et al., (2021) found that in sweet pepper the homeopathic preparation Sulphur allowed positive increases in the development of plants and production and diameter of fruits under field cultivation. *Calcárea carbónica* showed significant results at the height of plants grown in greenhouse. During the cultivation the presence of caterpillars, ants, aphids, mites, fungi and bacteria was found. Homeopathic preparations did not show evident effects in reducing the populations of these pathogens in attacked plants and fruits, but they can favor the resilience of plants affected by these parasites, helping in growth after damage. *Calcárea carbónica* showed a tendency to lower amounts of fruits with signs of anthracnose.

Analyzing the results of the evaluations applied preventively every 15 days, the treatments that reflected a greater control of the fungus is the nosode of C. gloeosporioides 30CH at 30 days after the application in the parameter of length by width in fruit and Fungizar 5B. Which contrasts with the evaluation of the effectiveness of Bacillus subtilis, Rhodotorula minuta and its combination compared to benomilo with pre-harvest applications, for post-harvest control of anthracnose, coinciding with Martínez et al., (2019) when evaluating in the Municipality of Zimatlán de Álvarez Oaxaca 14 homeopathic treatments and four controls, to obtain Capsicum annuum fruits without Anthonomus eugenii. They found that the preparations of Lachesis trigonocephalus T (Crushing) 7 CH (Centesimal Hannemaniana) and Allium cepa Ø (Tincture) 6 CH effectively preserved the weight of the fruits, presenting 40% less chopped areas per fruit, while the Weevil field treatments T 6CH, Strychninum T 6CH, and T 200CH greenhouse weevil moderately protected the fruit weight of C. annuum plants against this condition, 50% fewer weevils per fruit. Therefore, it is confirmed that the use of different homeopathic preparations are an effective alternative to protect plants and crops, which will contribute to reducing the use of chemical elements and protecting the environment.

1.29 Appreciation

To the Universidad Xicotepetl A.C. for financing this research project.

1.30 Conclusions

In the evaluation of 10 homeopathic products, *Á. montana* 6CH and 20CH, *C. carbónica* 6CH, 20CH., *Chamomilla* at 6CH and 30CH, *F. phosphoricum* at 6CH and 30CH, and a biopreparation of Anthracnose (*C. gloeosporioides*) obtained from fruit and avocado leaves at 10 CH Y 30 CH, 2 organic products Fungizar 5B, Clean Crop and the combination of both products as well as a commercial chemical Benomilo®, suctioned water and pure water as a witness. The treatments that showed effective control over the infection of *C. gloeosporioides* were the agrohomepathic doses of the *C. gloeosporioides* preparation at 10 CH, in which the parameter length by width in fruit stood out at 56 days for its control applying it every eight days, as well as in the parameter of length by width in leaf at 40 days, in the same way for the applications evaluated every 15 days at 30CH at 30 days after application.

The microbiological product formulated from 5 strains of Bacillus Fungizar 5B being applied every eight days demonstrated to have a control of the damage in the parameter of length by width in leaf at 24 days at a dose of 3.7 ml of the product per 1.5 liters in water. The parameter length by width in leaf evaluated every 15 days, controlled the damage 30 days after its application in doses of 3.7 ml of the product per 1.5 liters of water.

It should be noted that homeopathic products offer longevity, this aspect is important because in an unsystematic way to foods of plant origin, when applying these products, they have a longer shelf life. At the end of the experiment, it is experienced that the treatment of *Á. montana* 6CH lengthened the days to maturity with an advantage of 8 days compared to the fruits where the treatment was not applied, in addition to observing that it statistically stopped the damage of the fungus in the parameter of the number of spots on the fruit at 32 days. However, it was found that for the fruits that were not treated with any product, the damage of the fungus was just during the ripening of the fruit.

This is why agrohomeopathy and particularly with the use of low or high dynamisations of agronosodes will depend on the degree of progression of the disease or pest damage, in addition to the timely choice of the centesimal dose and application frequency, the recovery of the plant's health in a tangible and permanent way, since in the case of diseases, agronosodes and high homeopathic dynamisations eliminate the damage caused by pathogenic organisms. Ultimately, this represents a real economic possibility for producers and the environment, being able to show its specific incidence on pests and independent diseases that affect the agricultural sector.

In other words, Agrohomeopathy is scientific knowledge that complements traditional agriculture, considering itself as an alternative for agricultural production, which is currently in constant evolution, with great scientific advances and at the same time taking advantage of ancestral knowledge, to mitigate the high levels of contamination, the irrational use of pesticides, making alternatives a reality with natural, simple, viable and economic means.

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Chapter 5 Biosynthesis of Metallic Nanoparticles and their Applications

Capítulo 5 Biosíntesis de Nanopartículas Metálicas y sus Aplicaciones

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DOI: 10.35429/H.2021.3.87.121

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A. Marroquín, J. Olivares, D. Ventura and L. Cruz (Coord) Agricultural Sciences and Biotechnology. Handbooks-©ECORFAN-México, Querétaro, 2021.

Abstract

The development of nano-sized materials increasingly requires the implementation of synthesis methods that are friendly to the environment and that have the ability to implement them in medical, therapeutic, pharmacological, food and environmental areas. As such, green methods have been gaining ground in recent years. Within these processes, there is a huge range of species belonging to different groups (such as bacteria, algae, yeasts, fungi, and plants) with the necessary qualities to generate metallic NPs with particular size and shape characteristics, within which plants stand out. This is due to the simplicity of the process as well as their easy scaling. Additionally, the studies carried out indicate the parameters to be considered in order to carry out a good bioreduction process and obtain both monometallic and highly functional bimetallic nanoparticles. It should be noted that in addition to the economic and ecological advantages of the nature of these methods, the biological molecules that participate as reducing agents also provide stability to the NPs, in some cases conferring superior qualities in catalytic and clinical applications.

Bioreduction, Vegetal extract, Phytochemicals, Catalyst

Resumen

El desarrollo de materiales nanométricos requiere cada vez más la implementación de métodos de síntesis amigables con el ambiente y que tengan la capacidad de implementarlos en los ámbitos médico, terapéutico, farmacológico, alimentario y medioambiental. Como tal, los métodos ecológicos han ido ganando terreno en los últimos años. Dentro de estos procesos, existe una amplia gama de especies pertenecientes a diferentes grupos (como bacterias, algas, levaduras, hongos y plantas) con las cualidades necesarias para generar NP metálicas con características particulares de tamaño y forma, dentro de las cuales destacan las plantas. Esto se debe a la simplicidad del proceso, así como a su fácil escalado. Además, los estudios realizados indican los parámetros a considerar para llevar a cabo un buen proceso de biorreducción y obtener nanopartículas tanto monometálicas como bimetálicas altamente funcionales. Cabe señalar que además de las ventajas económicas y ecológicas de la naturaleza de estos métodos, las moléculas biológicas que participan como agentes reductores también brindan estabilidad a las NP, en algunos casos confiriendo cualidades superiores en aplicaciones catalíticas y clínicas.

Biorreducción, Extracto vegetal, Fitoquímicos, Catalizador

1. Introduction

Nanotechnology is a fascinating multi-disciplined field that involves the design and engineering of functional systems on the molecular scale. It can be defined as the art and the science of the manipulation of matter at the nanoscale to create new and unique materials. Its main characteristic is a dimensional structure smaller than 100 nm, which is important in the fields of materials science, medical and life sciences, and physical and chemical sciences (Nayantara & Kaur, 2018; Vijayaraghavan & Ashokkumar, 2017). They mainly present themselves in characteristics such as catalytic reactivity, thermal conductivity, nonlinear optical behavior, and chemical stability, owing to their high surface-area-to-volume (Agarwal et al., 2017).

The development of metallic nanoparticles has been integrated in diverse areas such as medicine, chemistry, engineering, and biology, as well as participating in industries such as cosmetics, food, pharmaceuticals. It is equally involved in the development of new technologies for the detection, cleaning, and prevention of contamination. It is estimated that the global market of nanoparticles in biotechnology and pharmaceuticals grew by up to $79.8 billion in 2019, and that it will continue growing by around 22% annually (Nayantara & Kaur, 2018; Scaria et al., 2020). The synthesis and assembly of nanoparticles through biological routes allows the development of clean, non-toxic, and environmentally acceptable procedures involving organisms that vary from bacterias to bigger plants. The three main aspects of bioreduction related with the principles of green chemistry are: 1) the choice of non-toxic solvents for the reduction reaction, as distilled water is generally used in green synthesis. This is done to avoid the use of toxic organic solvents which are usual in chemical synthesis such as ethanol, dimethylformamide, ethylene glycol, toluene, and chloroform, owing to the use of hydrophobic stabilizing agents. 2) environmentally benign reducing agents and non-harmful stabilizing agents which form part of the biomolecules of the used organisms.

Many of these have the capacity to act as reducers and stabilizers in a simultaneous manner, substituting the use of chemical compounds such as sodium borohydride, hydrazine, and elemental hydrogen as reducing agents and the use of natural or synthetic polymers such as rubber, chitosan, cellulose, and copolymer micelles as stabilizers. In this sense the recovery that the biomolecules provide on the surface of the NPs makes them biocompatible, opening the possibility of applications in biomedicine and related fields (Gan & Li, 2012; Nayantara & Kaur, 2018; D. Sharma et al., 2019).

1.1 Basic Approaches

The synthesis methods for nanoparticles are commonly classified by two main categories: 1) from top to bottom or 'Top down'. 2) from bottom to top or 'Bottom up' (Carrillo-Inungaray et al., 2018).

The 'Top down' approach is based on mechanical size reduction methods, gradually breaking down the volume of the material into structures on the nanoscale, using lithographic techniques such as: grinding, milling, sputtering, and thermic or laser ablation (Agarwal et al., 2017; Chinnasamy et al., 2018; Gan & Li, 2012; Vijayaraghavan & Ashokkumar, 2017).

The 'Bottom up' approach is based on assembly through smaller entities in the nanoscale range (10-100 nm) such as atoms or molecules. Based mainly on chemical and biological methods, this approach increases the possibility of producing more chemically homogenous metallic particles with less defects (Agarwal et al., 2017; Chinnasamy et al., 2018; Gan & Li, 2012; Narayanan & Sakthivel, 2010b; Vijayaraghavan & Ashokkumar, 2017).

Nanoparticles have physical, chemical, electronic, electric, mechanical, magnetic, thermic, dielectric, optic and biological properties and characteristics. These are generated by the reduction of their dimension regarding their atomic surface, surface energy, the reduction of imperfections and spatial confinement, which are in summary size, shape, and crystalline structure (Anu et al., 2020; Narayanan & Sakthivel, 2010).

2. Methods to obtain nanoparticles

In accordance with the approach that is chosen for obtaining metallic nanoparticles, physical or chemical methods were used. As was previously mentioned, the 'Top down' approach is mostly related to physical methods, whilst the 'Bottom up' approach is mainly related to distinct chemical methods.

2.1 Physical methods

Physical methods include laser ablation, evaporation-condensation, ball milling, plasma arcs, and mechano-chemical synthesis, among others (Jamkhande et al., 2019).

Laser ablation

This is a technique that produces colloidal nanoparticles in a variety of solvents, taking place in a vacuum chamber in the presence of certain inert gases. The solid material sits under a thin layer and is exposed to irradiation with a pulsed laser, mainly the Nd: YAG laser (yttrium-aluminium-garnet doped with neodymium) to an output of 106 μm, and their Ti: Sapphire harmony laser (sapphire doped with titanium). The irradiation of material through lasers leads to the fragmentation of solid material in the shape of nanoparticles, which stay in the liquid that surrounds the target and produce a colloidal solution. The duration and energy of the pulse laser determine the relative quantity of atoms and particles that form. Parameters, such as the duration of the laser pulse, the wavelength, the ablation time, the laser fluidity, and the average effective surrounding liquid with or without surfactant, influence the efficiency of the ablation and the characteristics of the formed particles (Jamkhande et al., 2019; Vijayaraghavan & Ashokkumar, 2017).

It is a relatively simple and efficient technique for obtaining large quantities of nanometric particles in the form of a suspension. Their properties can change in accordance with the used laser and the nature of the suspension. Another important advantage is the absence of chemical reagents in the solutions (Jamkhande et al., 2019; Vijayaraghavan & Ashokkumar, 2017).

Evaporation-condensation

This is generally performed using a tubular oven at atmospheric pressure. The source or original material collects in a container centered in the oven which evaporates in a carrier gas. However, the synthesis of nanoparticles using a tube oven at atmospheric pressure has some disadvantages: for example, the tube oven occupies a large space, consumes a large amount of energy whilst elevating the environmental temperature around the original material and requires a lot of time to achieve thermal stability (Vijayaraghavan & Ashokkumar, 2017).

High-energy ball milling

This is a mechanical technique that can be affected by certain variables in the process. It is classified between low energy and high energy milling, depending on the induced mechanical energy to the powder mix. The nanometric particles are produced through a process of high-energy ball milling, and this method is mainly used for the synthesis of intermetallic nanoparticles. The procedure consists of placing a large amount of the powder of the material you wish to reduce into a container together with various heavy balls. A high mechanical energy is applied to the powdered material with the help of a high-speed rotating ball (Jamkhande et al., 2019). The reduction of the particle size can be carried out using different high energy mills: those with attrition balls, planetary balls, vibrating balls, and low energy rotation. In each of these methods, the high energy heavy balls move freely, and can roll above the surface of the chamber that contains the ground material in a series of parallel layers. Alternatively, they can also fall freely and impact the powder. These high impact collisions reduce the material without generating chemical changes (Jamkhande et al., 2019; Vijayaraghavan & Ashokkumar, 2017).

Plasma arc technique

The high temperatures associated with the formation of the arc or plasma are used to effectively separate the atomic species of the prime matter. These are quickly recombined outside of the plasma to form nanometric sized particles (Vijayaraghavan & Ashokkumar, 2017).

2.2 Chemical methods

Chemical methods include chemical reduction, microemulsion, thermal decomposition, sol-gel, solvothermal, and electrochemical deposition.

Chemical reduction

Ionic salt is reduced in an appropriate way in the presence of a surfactant using different reducing agents. The formed metal nanoparticles are stabilized using trisodium citrate (TSC) or sodium lauryl sulfate (SLS). On occasion a stabilizing agent is used together with a reducing agent. The most common reducing agents are Sodium borohydride ($NaBH_4$), Potassium bitartrate ($KC_4H_5O_6$), glucose, ethylene glycol, ethanol, sodium citrate, hydrazine hydrate, ascorbate, and elemental hydrogen (Jamkhande et al., 2019; Vijayaraghavan & Ashokkumar, 2017).

Microemulsion process

This takes place in the aqueous nuclei of inverse micelles that are dispersed in an organic solvent and stabilized with a surfactant. The dimensions of these aqueous nuclei are in the nano regime and, as such, are referred to as nanoreactors. The product obtained through the reaction is homogeneous. This is one of the versatile and reproducible methods that allow the control of particle properties such as: size, morphology, geometry, homogeneity, and surface area (Vijayaraghavan & Ashokkumar, 2017).

Thermal decomposition

This is one of the most common chemical techniques for producing stable monodisperse suspensions with the ability of self-assembly. The nucleation occurs when the metal precursor is added to a heated solution in the presence of a surfactant. Meanwhile the growth phase takes place at a higher reaction temperature. The composition and size of the formed particles depend on parameters such as the reaction time, temperature, and the length of the surfactant molecule (Vijayaraghavan & Ashokkumar, 2017).

Sol-gel technique

This technique involves one of the following: 1) a mixture of preformed metallic colloids (oxides) in a sol which contains the shaping type of the matrix followed by the formation of gel; 2) the direct mix of metal/metal oxide or nanoparticles inside a sol made of pre-hydrolysed silica or 3) the complexation of metal with silone and the reduction of the metal before hydrolysis. In this method, a network formation is introduced using colloidal suspension (sol) and gelatin to form a network in a continuous liquid phase (gel). Initially, a homogenous solution of one or more selected alkoxide is prepared. A catalyst is added in order to start a reaction at a controlled pH. The formation of sol-gel involves four main steps: hydrolysis, condensation, particle growth, and particle build-up (Jamkhande et al., 2019).

Solvothermal method

This is used for the preparation of nano-phases in the presence of water or other organic chemicals such as methanol, ethanol, and polyol and solvents. The reaction is produced in a container at a pressure that allows the solvent (water and alcohol) to heat up above their boiling temperature. The crystallisation kinetic (formation of crystals) can be increased by one or two orders of magnitude through the employment of reactions assisted by microwaves (solvothermy by microwaves) (Jamkhande et al., 2019).

Electrochemical deposition

Electricity is used as a controlling force. The method consists of passing an electrical current between two electrodes separated by an electrolyte, and the synthesis of nanoparticles is produced in the electrode / electrolyte interface. The size of the particles can be controlled by changing the current density (Vijayaraghavan & Ashokkumar, 2017).

When done well, the chemical and physical processes offer greater control over the size and shape of the obtained nanoparticles. Generally, their disadvantages lie in the use of toxic chemicals on their surfaces, the use of non-polar solvents, expensive equipment that requires a high consumption of energy, and the low capacity for scaling in production.

3. Bioreduction of metallic ions and approach on green chemistry

Owing to the impact of nanomaterial synthesis on the environment generated by the use of physical and chemical methods, it has been sought to develop more environmentally friendly means of synthesis that are also consistent with the principles of green chemistry. This is why biological methods involving the reduction of metallic ions using extracts or live biological mass have been investigated, such as sources of reducers, equally so with those that are intracellular and extracellular (Agarwal et al., 2017; Carrillo-Inungaray et al., 2018; Kharissova et al., 2013; Nasrollahzadeh et al., 2020).

The intracellular processes take place within the cell. No previous treatment is required because the process is based on metabolic pathways that are probably responsible for synthesis, such as photosynthesis, respiration, and nitrogen fixation (A. Sharma et al., 2015). Diatom algae from the genera *Chaetoceros sp*., *Skeletonema sp.*, and *Thalassiosira sp.* are among the most used microorganisms (Mishra et al., 2020).

Extracellular processes are referred to as the processes that take place outside the cells, mainly supported by the exudates of cellular metabolism that comprise metabolites, ions, pigments, many proteins (enzymes) and non-protein entities such as DNA, RNA, microbial subproducts (hormones, antioxidants) and lipids (Khanna et al., 2019).

Biosynthesis methods surpass other classic procedures thanks to their advantages - the wide availability of biological entities, ecological procedures, cost-effectiveness and easy scaling (Jamkhande et al., 2019; Kuppusamy et al., 2016). Bacteria, fungi, yeasts, algae, and plants are among the different organisms that are used.

3.1 Bacteria

Prokaryotes have gained attention as a means of synthesis for metallic NPs owing to their abundance in the atmosphere and their capacity to adopt extreme conditions. Their advantages relate to their rapid multiplication - some well-known species are easy to grow and manipulate. However, regarding their disadvantages, it stands out that the detection of suitable microbes is a process that requires a lot of time. A careful control of both the cultivation method and the whole process is necessary in order to avoid contamination. This is combined with the lack of control regarding the size and shape of the NPs and the associated cost of the means used to grow bacteria (Agarwal et al., 2017; Jamkhande et al., 2019). Saravanan et al. (2021) report investigations with the genera *Pseudomonas*, *Bacillus*, *Streptomyces*, *Escherichia*, *Aeromonas*, *Enterobacter*, and *Klebsiella* used for the reduction of Ag, Au, ZnO, Cu and Cd NPs. On the other hand, Yusof et al. (2020) used cellular biomass and supernatant from *Lactobacillus plantarum* in order to obtain ZnO NPs ranging between 191.8-291.1 nm in size. Joshi et al. (2018) used *Geobacter sulfurreducens* with the goal of obtaining Fe NPs with magnetic properties. The process produced a mineral phase of Fe(II) with similar characteristics to magnetite, due to which the authors suggested that these nanoparticles could be used for the cleaning of soils contaminated with other metals. For obtaining Au NPs, *Rhodopseudomonas capsulata* (He et al., 2007), *Deinococcus radiodurans* (Li et al., 2016), *Shewanella oneidensis* y *Shewanella xiamenensis* (Wu & Ng, 2017) have been tried.

3.2 Fungi

The extracellular synthesis of NPs through fungi is very useful owing to the great scale of production and the economic viability. In this case, the enzymes and proteins secreted by the fungi work as reducing agents, allowing the synthesis through metallic salts. The fungi variants are chosen over bacteria owing to their greater tolerance and bioaccumulation of metals, as well as their capacity to reduce great quantities of NPs (Agarwal et al., 2017; Jamkhande et al., 2019; Singh et al., 2018). *Aspergillus* is among the commonly implemented genera, Ninganagouda et al. (2013) used the species *A. flavus* for the extracellular reduction of Ag, from which they evaluated its antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumonia*, with effective results. *A. niger* has also been used for the reduction ZnO (Kalpana et al., 2018), as well as *A. fumigatus* for the biosynthesis of CuO (Ghareib et al., 2019). In both cases, the objective was to use the obtained NPs for photocatalytic processes in the removal of ZnO, as well as evaluating its catalytic activity in organic synthesis (Shamsuzzaman et al., 2017). In this sense, through their study, Ovais et al. (2018) emphasize some works in which various genera have been used for the reduction of NPs. In the case of intracellular biosynthesis, *Aureobasidium*, *Fusarium*, and *Rhizopus* have been used for gold and silver NPs; for the extracellular processes, the *Candida* and *Aspergillus* genera have been worked with for the reduction of Ag, ZnO, and Co.

3.3 Algae

Algae are photosynthetic organisms that vary from unicellular types (*Chlorella*) to multicellular types (kelps). They lack basic vegetal structures like roots and leaves and are classified according to the pigment present inside them, such as the red pigment in Rhodophyta, the brown pigment in Phaeophyta, and the green pigment in Chlorophyta (Agarwal et al., 2017). They are considered to be potential sources of a broad group of secondary metabolites, proteins, and pigments with which they can serve as nano factories of metallic nanoparticles (Khanna et al., 2019). Some implemented species are *Macrocystis pyrifera* for the reduction of CuO NPs (Araya-Castro et al., 2021), *Botryococcus braunii* in obtaining Cu, Ag, Pt and Pd NPs (Arya et al., 2018, 2020), *Chlamydomonas reinhardtii* for Cu NPs (Žvab et al., 2021), whilst Salem & Funda (2020) report in their evaluation that species from the genera *Chlorella*, *Phaeodactulum*, *Sargassum* and *Shewanella* have been used for the reduction of Au, Cd, and Pt NPs.

4. Biosynthesis of nanoparticles of metallic ions using plants

Compared with the synthesis of nanoparticles mediated by microorganisms, the use of plants presents diverse advantages owing to the rich biodiversity and easy availability of vegetative organisms that have been explored for the synthesis of nanomaterials (Kuppusamy et al., 2016).

They also represent less biological risks during production and eliminate the laborious process of cell cultivation, which has led to plants being considered the better option to reduce metallic ions, as well as being ideal candidates for the production on a great scale. This is in addition to producing stable NPs which are variable in both size and shape (Agarwal et al., 2017; Gan & Li, 2012; Nasrollahzadeh et al., 2020).

The metallic ions are biologically reduced to zero-valent metals or particles of metal oxide due to the phytochemicals present in the plant extracts constituted by their primary and secondary metabolites. They participate in a constant way in the redox reaction of their metabolic pathways, and act as reducing agents and stabilizers, minimizing the agglomeration and oxidation of nanoparticles. In addition, the process is generally carried out aqueously, thus dispensing with other organic solvents, and as such reducing the generation of toxic waste (Agarwal et al., 2017; Gan & Li, 2012; Jamkhande et al., 2019; Nasrollahzadeh et al., 2020). In accordance with Agarwal et al. (2017), pigments, terpenoids, flavonoids, alkaloids, carbonyls, amide groups, amines, phenols, vitamins, and amino acids are among the main reducing groups, whilst the groups that work as stabilizers are carboxylic, phenolic acid, and ascorbic acid.

4.1 Phytochemicals present in vegetal extract

Flavonoids, terpene, sugars, and secondary metabolites are among the main phytochemicals responsible for the reduction of metallic ions. Some examples are shown in figure 5.1 (Singh et al., 2018).

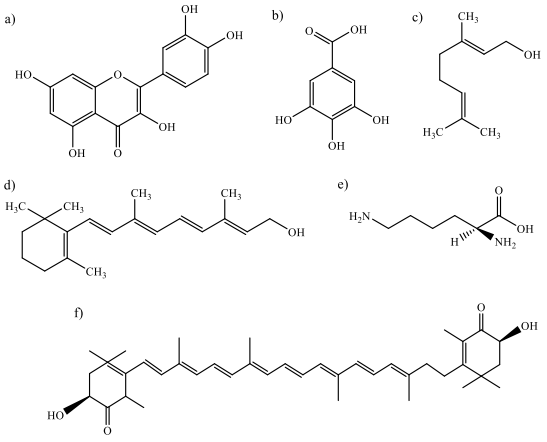
Flavonoids are a wide group of polyphenolic compounds that are soluble in water. They comprise various classes such as flavonols, isoflavonols, anthocyanins, chalcones, flavones and flavonoids, with the capacity to actively chelate metallic ions and reduce them to nanoparticles. It has been proposed that they are the main component of the aqueous extract in most plants (Gan & Li, 2012). Flavonoids contain various functional groups capable of forming NPs. It has been postulated that the tautomeric transformation of flavonoids, from the -enol shape to the -ceto shape can release an atom of reactive hydrogen capable of reducing to metallic iron. For example, quercetin is a flavonoid with very strong chelating activity, owing to its ability to chelate in three different positions involving carbonyl and hydroxyl group in the C3 and C5 positions, and the catechol group in the C3' and C4' locations (Makarov et al., 2014; Singh et al., 2018).

Terpenoids are a group of diverse organic polymers, made up of units of five carbon isoprenes with strong antioxidant activity. They are commonly found in essential oils from diverse medicinal plants (Makarov et al., 2014). The sugars present in the vegetal extract have also been shown to be responsible for the formation of NPs. Monosaccharides such as glucose and fructose can act like antioxidants when they have suffered a tautomeric transformation from ketone to aldehyde. The reducing capacity of disaccharides and polysaccharides depends on the capacity on whichever of their individual components in order to adopt a form of open chain within an oligomer and providing the metallic ion access to an aldehyde group (Makarov et al., 2014; Singh et al., 2018).

In addition, amino acids and proteins have also shown the capacity to reduce. Firstly, it has been observed that this varies between different amino acids. For example, in tyrosine, the hydroxyl groups are responsible for the reduction, whilst for glutamine and asparagine, it's the carbonyl groups. In the case of proteins, their reducing capacity and even their size, shape, and quantity of NPs will depend on the sequence of amino acids that composes them, and the access that the metallic ion has to the molecule's reducing location (Makarov et al., 2014).

All these phytochemicals are found distributed throughout the body of the plant, which is to say in roots, stems, leaves, flowers, fruits, and seeds. This has allowed all organs to be used for the bioreduction of many metals, as can be seen in table 5.1 (Agarwal et al., 2017).

Figure 5.1 Examples of phytochemicals present in plants: a) Quercithin (flavonium); b) Gallic acid (phenolic compound); c) Geraniol (terpenoid); d) Retinol (vitamins); e) L-lysine (amino acid); f) Carotenoid (pigment).



4.2 Proposed mechanisms

The elucidation of the exact mechanisms associated with the reduction of nanoparticles can be complicated - it is still not fully understood. However, some authors present certain proposals that can shed light on these mechanisms (Asghar et al., 2018).

Asghar et al. (2018) cite a possible general reaction mechanism for metallic ions in contact with phytochemicals present in vegetal extracts which could be considered for the reduction of a metal determined in equation 1:

$$nM + n(Ar - OH)n \rightarrow nM^{0}NPs + n(Ar = O) + 3nH^{+} \quad (1)$$

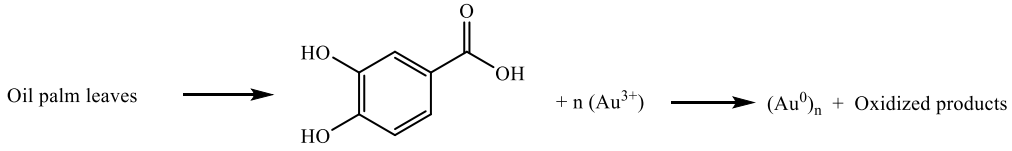
…where n is the number of oxidized groups for the metallic ion, M represents the metallic ion, and Ar represents the aromatic groups. The formation of NPs is confirmed by a change in pH in the solution, which decreases at the end of the reaction.

For example, Devatha et al. (2016) propose a reduction mechanism for Fe^{2+}, where they indicate that the presence of polyphenolic groups directly reduces the iron ions to zerovalent iron. This is explained through stoichiometric equation 2, where Ar represents the phenyl groups:

$$nFe^{2+} + 2Ar\text{-}(OH)n \rightarrow nFe^{0} + 2nAr = O + 2nH^{+} \quad (2)$$

Ahmad et al. (2018) report that they identified the presence of protocatechuic acid (polyphenolic compound) in the palm oil that they used for the reduction of trivalent gold in their analysis of FTIR. They mention that owing to the fact this compound was reported previously as one of the most abundant of this material, it was taken as a model compound that reacts with trivalent gold ions to oxidize by giving electrons to Au^{3+} in order to reduce them to gold atoms, as is shown in figure 5.2.

Figure 5.2 Reduction mechanism for Au^{3+} with oil palm leaves *(modified from Ahmad et al., 2018)*.



In the same way, possible mechanisms for bimetallic nanoparticles that intervene in the reduction process have been suggested. Such is the case for Olajire & Mohammed (2020), who carried out a green synthesis of Pd/Au using *Ananas comosus* leaves as a reducing agent.

They indicate that the analysis of the FTIR spectrum evidence that reduction of the ion mix is predominantly carried out by O-H groups of polyphenolic compounds, one of the isolated bioactive compounds of the leaf extract. For this reason, they propose a reaction mechanism between the polyphenolic compound and the mixture of palladium/gold ions (Pd^{2+}-Au^{3+}), where the electrons are given by the polyphenolic compound to the vacant d orbital of the mixture of palladium/gold ions, and later they are converted into a palladium atom (Pd^0) - gold atom (Au^0) (Fig 5.3). It is observed that through the mechanism, the π electrons of the polyphenol aromatic ring can transfer electrons to the vacant d orbital of the palladium-gold ions and convert them into free atoms.

Figure 5.3 Proposed mechanism for bioreduction of the ions mixture Pd^{2+} y Au^{3+} a Pd y Au with polyphenolic compounds in *A. comosus (modified from Olajire & Mohammed, 2020)*

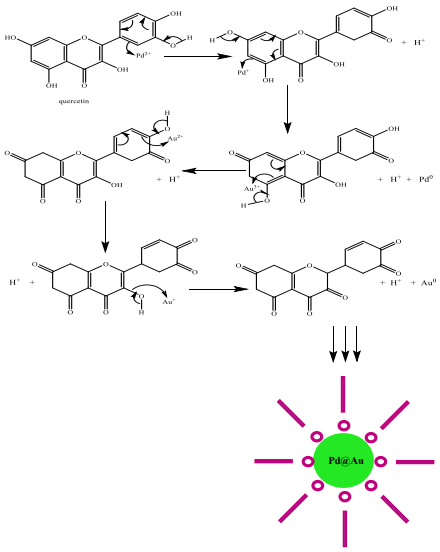


Table 5.1 Bioreduction of metallic particles using plants

Metal	Reducing agent	Plant organ	Chemical species obtained	Form	Average diameter (nm)	UV-Vis (λ_{max}) nm	Functional groups	Applications	Author, year.
Ag	*Artemisia annua* L.	Leaves	Ag^0	Spherical	49.4 - 88	400- 500	ND	Antibacterial activity	(Aghajanyan et al., 2020)
	Azadirachta indica	Leaves	Ag^0	Spherical	34	436- 446	N-H amines C=O alkyl groups C-O, C-OC- flavonoids and terpenoids	Antibacterial activity	(Ahmed et al., 2016)
	Salvia officinalis	Leaves	Ag^0	Spherical	40	439- 446	C=O, O-H, N-H phenolic compounds, flavonoids, proteins and saponins.	Dye removal	(Albeladi et al., 2020)
	Commiphora myrrh	Resin	Ag^0	Spherical	22	445	O-H, C-H phenolic compounds.	Antibacterial activity	(Alwhibi et al., 2020)
	Green tea Black tea	Leaves	Ag^0	Spherical	10 -20	ND	O-H, C-H y C-O-H phenolic compounds.	Antibacterial activity	(Asghar et al., 2018)
	Lippia citriodora	Leaves	Ag^0	Spherical	14	430	C-O, O-H, C=C phenolic compounds.	Medical activity	(Cruz et al., 2010)
	Acalypha wilkesiana	Leaves	Ag^0	Spherical	10 -26	450	O-H, C-H, C=C phenols, terpenoids, saponins and flavonoids.	Antibacterial activity	(Dada et al., 2019)
	Vitex sp.	Leaves	Ag^0	Spherical	86	ND	C-H, O-H, C-O-C polyphenols	Antibacterial activity	(Deeksha et al., 2021)
	Clitorea ternatea	Flowers	Ag^0	Cubical	18 -50	400- 450	C-CH phenols and anthocyanins C=O y OH phenolic compounds.	Antibacterial activity	(Fatimah et al., 2020)
	Acanthe phylum bracteatum Persian manna	ND	Ag^0	Spherical	40	425	ND	ND	(Forough & Farhadi, 2010)
	Nelumbo nucifera	Flowers	Ag^0	ND	90	450	N-H, C-C, C-O aromatic groups, alcohol	ND	(Hitesh & Lata, 2018)
	Anthurium andraeanum	Leaves	Ag^0	Spherical	12- 46	419	ND	Antibacterial activity	(Korkmaz, 2020)
	Ocimum sanctum	Leaves	Ag^0	Spherical	3- 20	436	O-H alcohols, polyphenols, carboxylic acids. C-N aromatic amines N-H primary amines	ND	(Mallikarjuna et al., 2011)
	Prunus domestica	Fruit	Ag^0	Spherical	50	380- 450	C-N, N-H amines C-H, O-H carboxylic acids and aldehydes	ND	(Mohaghegh et al., 2020)
	Catharanthus roseus	Leaves	Ag^0	Spherical	48- 67	440	ND	Antibacterial activity	(Mukunthan et al., 2011)
	Allium sativum L. *Camellia sinensis* L. *Curcuma longa* L.	Slice Leaves Rhizome	Ag^0	Spherical	8.19 8.37 6.13	447- 451	OH, C-H, C-N, N-H phenolic compounds, flavonoids, proteins, sugars.	Antioxidant and cytotoxic activity.	(Selvan et al., 2018)
	Terminalia bellerica	Grain	Ag^0	Spherical	29.6	350- 450	$-NH_2$, -OH proteins, C=O terpenoids or other aromatic groups. C-O-C proteins or saccharides.	Catalytic activity	(Sherin et al., 2020)
	Olea europaea	Fruit	Ag^0	Spherical	11.6- 20.7	436	O-H, N-H, CO-O-CO alcohols, phenols and proteins.	Antimicrobial activity	(Umai et al., 2020)
	Phyllostachys aurea	Leaves	Ag^0	Spherical	13 ± 3.5	420- 450	ND	Antibacterial activity	(Yasin et al., 2013)
Au	*Elaeis guineensis*	Leaves	Au^0	Spherical	27.89 ± 14.59	500- 550	O-H polyphenolic compounds,	ND	(Ahmad et al., 2018)

Metal	Reducing agent	Plant organ	Chemical species obtained	Form	Average diameter (nm)	UV-Vis (λ_{max}) nm	Functional groups	Applications	Author, year.
							C=O y N-H proteins and carboxylic acids C-N, C-O phenolic compounds and amines.		
	Jasminum auriculatum	Leaves	Au^0	Spherical	8 - 37	547	O-H, C=O, N-H flavonoids, terpenoids, alkaloids and polyalcohols.	Catalytic and antibacterial activity	(Balasubramanian et al., 2020)
	Citrullis lanatus	Fruit	Au^0	Spherical, hexagonal, and triangular	100- 200	560	ND	Antibacterial activity	(Chamsa-ard et al., 2019)
	Vitis vinifera L.	Fruit	Au^0	Spherical	57.1 ± 16.4	550.6	C=O, C=C, O-H phenolic compounds, carboxylic acids.	ND	(Dzimitrowicz et al., 2018)
	Gnidia glauca	Flowers	Au^0	Spherical, triangular and prism	5- 20	540	O-H, C=C, N-H, phenols, alcohols	Catalytic activity	(Ghosh et al., 2012)
	Coleus amboinicus	Leaves	Au^0	Spherical, triangular, and hexagonal	20.5 ± 11.45	536	C-N Aromatic amines, C-OH secondary alcohols	ND	(Narayanan & Sakthivel, 2010a)
	Camellia sinensis	Leaves	Au^0	Spherical, triangular y hexagonal	18	536 529 (24 h)	Confirm the presence of polyphenols	Metal ion sensor	(Silva-De Hoyos et al., 2019)
	Citrus paradisi	Fruit	Au^0	Spherical Triangular and hexagonal	10- 90 75- 325	540- 568	O-H, C-H, C=O, C-O-C polysaccharides	Metal ion sensor	(Silva-De Hoyos et al., 2020)
	Gymnocladus assamicus	Scabbard	Au^0	Hexagonal, pentagonal and triangular	13.31 ± 2.5	526	ND	Catalytic activity	(Tamuly et al., 2013)
	Aegle marmelos, *Eugenia jambolan* *Annona muricata*	Fruits	Au^0	Cubical	18 16 28	519 523 526	C=O, N-H, O-H, aromatic compounds.	Anticancer activity	(Vijayakumar, 2019)
	Muntingia calabura L.	Leaves	Au^0	ND	36.93	543.5	O-H, C=O flavonoids, tannins, terpenoids, C-N, C-H proteins.	ND	(Wahab et al., 2018)
	Phoenix dactylifera L.	Leaves	Au^0	Spherical	32- 45	552	C=O, O-H carbohydrates, tannins, flavonoids, phenolic acids.	Catalytic activity	(Zayed & Eisa, 2014)
Fe	Neem Mango Rose Clove Carom	Leaves Leaves Leaves Buds Seeds	Fe_3O_4 y α-Fe Fe_3O_4 y α-Fe Fe_2O_3, Fe_3O_4 y α-Fe Fe_2O_3, Fe_3O_4 y α-Fe α-Fe	Spherical Spherical Spherical Spherical Spherical	Micro/nano	320- 325 470- 475	C=C alkene groups -PH phosphines. N-H secondary amines. C-O-C, C=O polysaccharides, nucleic acids, and proteins	ND	(Afsheen et al., 2018)
	Green tea	Leaves	α-Fe_2O_3	Spherical	40 -80	570	ND	Photocatalytic activity	(Ahmmad et al., 2013)
	Green tea Black tea	Leaves	Fe^0	Spherical	42- 60	ND	C-OH, C=C, C-O-C flavonoids.	Antibacterial activity	(Asghar et al., 2018)
	Green tea	Leaves	FeO	Quasi-spherical	25.5 ± 0.6	270	ND	Dye removal	(De León-Condés et al., 2019)
	Magnifera indica *Murraya koenigii* *Azadiracta indica* *Magnolia champaca*	Leaves Leaves	Fe^0 Fe^0 Fe^0 Fe^0	Spherical Spherical Spherical Spherical	100- 150 100- 150 96- 110 99- 129	216- 256 256- 277 296- 325 259- 282	O-H, C-H, C-H_2 phenolic and aliphatic compounds	Domestic wastewater treatment	(Devatha et al., 2016)
	Green tea Oolong tea Black tea	Leaves	α-Fe, Fe_2O_3, Fe_3O_4 y FeOOH	Spherical	40- 50	500- 700	ND	Dye removal	(Huang et al., 2014)
	Moringa oleifera (MOS) (MOL)	Seeds Leaves	Fe^0	Spherical	2.6- 6.2 3.4- 7.4	210- 240	O-H, N-H, C-O, C-H, C= proteins and fatty acids O-H, C-H, C=O amino acids, flavonoids, phenolic compounds.	Nitrate removal and antibacterial activity	(Katata-Seru et al., 2018)
	Eucalyptus sp.	Leaves	FeNPs RGO/FeNPs	Spherical	4- 7	330- 450 270 y 330- 450	C-O, C-C, C=O graphene groups, characteristic groups of eucalyptus were identified.	Adsorbent activity	(X. Weng et al., 2018)
Cu	Green tea Black tea	Leaves	Cu^0	Spherical	26- 40	ND	C-H, O-H flavonoids, and phenolic compounds -OH, C-O-C phenolic groups	Antibacterial activity	(Asghar et al., 2018)
	Punica granatum	Fruit shell	CuO	Spherical	10- 100	282	O-H, N-H phenolic groups and alcohols, C=C, C=O, C-OH, C-N proteins.	Insecticide	(Ghidan et al., 2016)
	Azadirachta indica	Flowers	Cu^0	Spherical	44.9	560	N-H, C=O, C-OH terpenes, proteins.	Antibacterial activity	(Gopalakrishnan & Muniraj, 2019)
	Eucalyptus globulus L. *Mentha piperita*	Leaves Leaves	Cu^0 Cu^0	Triangular Cluster	60- 75 36- 50	ND	ND	Antifungal activity (Phytopathogens)	(Iliger et al., 2021)
	Ziziphus spina-christi	Fruit	Cu^{2+} y Cu^+	Spherical	5- 20	551	C-H, -NH, -OH, C=O phenols, alcohols, carboxylic groups.	Dye removal and antibacterial activity	(Khani et al., 2018)
	Abies spectabilis	Aerial parts	CuO	Spherical	50	403	C-O-C, C-O, C=O y OH	Anti-inflammatory and contraceptive activity	(Liu et al., 2020)
	Azadirachta indica	Leaves	Cu^0	Cubical	48	560	O-H phenolic groups C-N aromatic amines C-H Aldehydes	ND	(Nagar & Devra, 2018)
	Syzygium aromaticum	Buds	Cu^0	Spherical	15	580	C-H, C-N secondary amines, -C=C-, C=O proteins.	Antimicrobial activity	(Rajesh et al., 2018)
	Calotropis procera	Leaves	CuO	Cylindrical	40	292 y 355	O-H adsorbed on the NPs	ND	(Reddy, 2017)
	Bauhinia tomentosa	Leaves	CuO	Spherical	22- 40	384	C-H, C=O, N-O, C-O phenolic groups, tannins, and proteins.	Antibacterial activity	(Sharmila et al., 2018)
	Achras sapota Linn.	Fruit	Cu^0	Spherical	20 -40	603	OH, C-H, -C=C aromatic groups.	Cytotoxic activity	(Thakore et al., 2019)
Zn	*Delonix regia*	Leaves	ZnO	Hexagonal and different superstructures	20	312	C=C, C-N, C-O amino acids, heterocyclic compounds.	Antibacterial activity, with anticancer potential.	(Begum et al., 2020)
	Medicago sativa	Leaves	Zn^0	Hexagonal	2- 5.6	335	ND	ND	(Canizal et al., 2006)
	Costus Igneus	Leaves	ZnO	Spherical	31	210 -230	ND	ND	(Chinnasamy et al., 2018)
	Jatropha sp.	Latex	ZnO	Hexagonal	100	310- 365	ND	Semiconductor	(Geetha et al., 2016)
	Nyctanthes arbor-tristis	Flowers	ZnO	Hexagonal	12-32	365- 369	N-H, C=O, C-N, C-H aromatic groups, amines and alkynes.	Antifungal activity	(Jamdagni et al., 2018)
	Moringa oleifera	Leaves	ZnO	Spherical	6- 10	350- 380	O-H, NH_2, H-C=O, C-H, OH-C=O bioactive compounds.	Electrochemical activity	(Matinise et al., 2017)
	Lycopersicon esculentum *Citrus sinensis* *Citrus paradisi* *Citrus aurantifolia*	Fruits shell	ZnO	Polyhedral	9.7 ± 3	ND	Aromatic rings.	Photocatalytic activity	(Nava et al., 2017)
	Passiflora caerulea	Leaves	ZnO	Spherical	70	380	OH, C=C, C=O, C-N polyphenols, proteins, C-O, C-N, C-H amino acids, N-H amide groups.	Antimicrobial activity	(Santhoshkumar et al., 2017)
	Eucalyptus globulus	Leaves	ZnO	Spherical	11.6	361	O-H polyphenols, C-O carboxylic acids.	Photocatalytic activity	(Siripireddy & Mandal, 2017)
	Scutellaria baicalensis	Roots	ZnO	Spherical and irregular	33.14- 99.03	316	C-H, N-H aliphatic amines and amides, -CH aromatic groups.	Antioxidant activity	(Tettey & Shin, 2019)
	Atalantia monophylla	Leaves	ZnO	Spherical	30	352	C-O, C-H, -OH proteins, N=O, N-O, C-N aromatic amines.	Antimicrobial activity	(Vijayakumar et al., 2018)

Own Elaboration

5. Influential parameters for obtaining nanoparticles

Different factors such as pH, temperature, concentration of metal salts, or the quantity of vegetal extract, play an important role in the control of nucleation, formation, and stabilization of NPs. Changes in these parameters can induce changes in the size and shape, as well as preventing the agglomeration of NPs (Khanna et al., 2019; Rai & Yadav, 2013).

5.1 Concentration of metallic ions

In the optimization studies of Ghosh et al. (2012), for the reduction of Au with *Gnidia glauca* flowers, they found that a concentration of 0.7 mM facilitates the synthesis best in comparison to other concentrations. They tried variations from 0.1 mM to 5 mM and found that although the synthesis speed increases with the concentration up to a value of 1 mM, in higher concentrations a reduction reaction is no longer observed. As such they conclude that this parameter plays an important role in the process. Ahmed et al. (2016) carried out green synthesis of silver nanoparticles through silver nitrate. For its optimization, they evaluated the concentration of the metallic salt from 1mM to 5mM, in accordance with the absorption spectrums obtained through UV-vis. As the concentration increases, so too does the absorption of the band locate at 445 nm. Jamdagni et al. (2018) used different concentrations of zinc acetate in order to optimize the synthesis of ZnO NPs with *Nyctanthes arbor-tristis*. They observed that an increase in the concentration of 0.0025 M to 0.01 M generates an increase in the absorption accompanied by a constriction of the band. Upon increasing to 0.02 M, a decrease in and broadening of the band absorption are generated. The investigators concluded that the increase in ion concentration beyond the threshold value leads to a decrease in nanoparticle synthesis.

In accordance with that reported by Chinnasamy et al. (2018), for the synthesis of ZnO nanoparticles with insulin plants. Through an ANOVA analysis, they found that the concentration of the metallic salt, in the case of zinc nitrate, has a greater contribution in determining the size of NPs compared to other considered parameters, such as reaction time, temperature, and quantity of vegetal extract. In this sense the studies carried out by Nagar & Devra (2018) on the optimum concentration of Cu salts indicate that upon increasing the concentration from 6mM to 7.5mM, the size of the NPs decreases. This is, according to the authors, due to the fact that the generation of NPs occurs in two steps - firstly, the nuclei are generated, and then the NPs grow. Increasing the concentration allowed the fast generation of nuclei that grew slowly, obtaining smaller nanoparticles. However, upon adding an excessive concentration of precursor salt, the high generation of nuclei will, as a result, give a greater agglomeration, increasing the final size of the NPs.

Dada et al. (2019) carried out the optimization of the synthesis process for obtaining Ag NPs with *Acalypha wilkesiana*, varying the molar concentration of the metallic salt. They found that in higher concentrations, the increase in size of the particles increases the intensity of the spectrum of the plasmon. Gopalakrishnan & Muniraj (2019) did something similar with the green reduction of copper with *Azadirachta indica* (Neem flower) trying concentrations of metal salts from 1mM to 10 mM. The optimal condition, determined by the maximum absorbance intensity of the SPR was 2mM - with higher concentrations the absorbance band begins to decrease.

5.2 Vegetal Extract

The formation of nanoparticles occurs through ionic or electrostatic interactions between the complex metals and functional groups at the surface of the biomass. It has been observed that many phytochemicals in plants are involved as reducing or protective agents during the formation of nanoparticles, and that their concentrations are critical to the way in which the process is directed (Gan & Li, 2012).

The surface chemical is one of the main factors that not only determines the activity of NPs but also plays an important role in its stability. Phenolic compounds that cover biogenic metallic nanoparticles confer them greater stability in comparison with other used reducing agents. In general, phytochemicals are adsorbed on the surface of the particles through various mechanisms. The assemblage pattern on the surface of the metal depends on various intermolecular interactions between the adsorbed molecules and the surface of the metal (Amini, 2019). It is believed that one of the adsorption mechanisms is attributable to the presence of π electrons and carbonyl groups in the molecular structures of phytochemicals (Gan & Li, 2012).

It is the availability of reducing and protective agents that determines if the metal precursors are able to reduce and eventually lead to the formation of nanoparticles. Thus, the concentration of vegetal biomass used during biosynthesis should not be overlooked, as it determines the grade of reduction and stabilization exercised by the biomolecules, which could affect the resulting size and shape of the nanoparticles (Gan & Li, 2012).

Cruz et al. (2010) evaluated the quantity of added vegetal extract for the reduction of Ag with *L. citriodora* and observed that this influences the formation of NPs. Upon analysing the absorbance spectrums in UV-vis of the SPR, they observed an increase when they increased the quantity of used vegetal extract. However, when carrying out the analysis of the size and shape of the nanoparticles through TEM, they conclude that there is not a change in these characteristics related to the quantity of vegetal extract. Gan & Li (2012) propose in their revision that when the concentration of the vegetal extract increases, so too does the electron density such as charged groups in the reducers. This would limit the free electrons of the metal cluster within a small volume and would increase the surface charges of the metal clusters in a way that makes the resulting surface charges exercise a repulsive force that could lead to a reduction in the size of the particles.

In the green synthesis of silver carried out by Ahmed et al. (2016), for the evaluation of the vegetal extract as a reducing agent, they tried quantities from 1 to 5 mL. They observed through monitoring with UV-vis that upon increasing it to 4 mL, a slight movement from 445 to 448 nm is observed, which in accordance with the authors means there is a change in the size of the particles. In a similar way, Agarwal et al. (2017) relayed that in accordance with the revised documents, when the quantity of vegetal extract is increased, the size of the NPs decreases.

In 2018, Nagar & Devra carried out the green reduction of copper with *A. indica* leaves. In order to obtain the optimal conditions for reduction, they evaluated distinct percentages of vegetal extract from 5% to 25%. They observed that with the lowest percentage, they obtained a weak absorption band of the SPR through UV-vis, whilst at 20%, the band intensity increased, which they suggest is related to the reaction speed. They corroborated this through a graph of the conversion rate versus the vegetal extract percentage - the results indicate that the conversion rate increases with the increase of the extract percentage up to 20%. With higher percentages than this, the speed remains constant, which indicates agglomeration of the NPs. This is due to the excess of present biomolecules that promote a secondary reduction process, which begins on the surface of the performed nuclei, which increases the size of the NPs. Jamdagni et al. (2018) evaluated the influence of the quantity of flower extract from *Nyctanthes arbor-tristis* added to the synthesis of ZnO. They determined that an increase from 0.25 to 1 ml of extract in 50 mL of metal solution improves the absorption and bandwidth, whilst any increase or decrease of this volume generates a decrease in the absorption and bandwidth, and thus decreases the synthesis of NPs. On the other hand, Gopalakrishnan & Muniraj (2019) report in their optimization of the process that they evaluated different volumes of vegetal extract for the reduction of copper through the Neem flower, from 8 to 12 mL. They found the optimum volume was achieved at 10 mL, which signals that the results were based on the maximum absorption intensity of the band of the SPR found at a wavelength of 560 nm.

5.3 pH

Various investigations have shown that the size and shape of biosynthesized nanoparticles can be manipulated by varying the pH of the reaction mixtures (Rai & Yadav, 2013). An important influence of the pH of the reaction is its capacity to change the electric charge of the biomolecules, which could affect its stabilizing capacity and, subsequently, the growth of NPs. This could result in the favorable formation of certain shapes in a particular pH range in a way that could achieve a greater stability (Gan & Y 2012).

Nagar y Devra (2018) evaluated the synthesis of copper through *A. indica*. The authors considered the optimization of different factors to be important for their reduction process, among which was pH. They observed that for acidic values (4.5) the reduction reaction didn't occur, probably due to the inactivation of the involved biomolecules. For a high pH (6.6), small NPs were produced, whilst at higher pHs, the generation of bigger sized NPs was observed, which suggests an agglomeration process. The authors also report that during the synthesis process, the pH decreases owing to the release of H^+ ions on the part of the species in the extract due to their oxidation in the presence of Cu^{2+} ions. Dada et al. (2019) investigated the effect of the pH on the formation speed of Au NPs and found that it increases when the pH is increased to 9.0. They propose that this is due to the responsible OH groups of the reduction, and that the formation of silver nanoparticles is more favorable in basic means corroborated by the SPR obtained through basic means.

Aghajanyan et al. (2020) evaluated the effect of pH on the synthesis of Au using *Artemisia annua*. They observed that the formation of smaller spherical NPs is favored in neutral and alkaline pH (7.0, 9.0). They evaluated the values of acidic pH (3.0, 5.0) in the same way, and did not observe the formation of NPs. In a similar way, Gopalakrishnan & Muniraj (2019), in optimizing the pH value for obtaining copper nanoparticles, signal that the most favorable pH was 9.0, having evaluated pH values from 8.0 to 12.0. Jamdagni et al. (2018) also evaluated the optimum pH for the reduction to ZnO. The evaluated values varied from 9.0 to 13.0 - the lowest values did not show the formation of NPs, whilst with 12.0 and 13.0, the characteristic absorption band was observed. However, the band obtained at pH 12.0 was the clearest, and as such this value was considered the most suitable for the reduction.

5.4 Temperature

The temperature is considered to be a crucial factor in the formation of NPs, affecting the process of nucleation and their size. Cruz et al. (2010) report the evaluation of two temperatures used in the reduction of Ag with *L. citriodora* of 25 and 95°C. The results through UV-vis showed that upon increasing the temperature, a displacement in the absorption band of 440 to 420 is given. They also observed that the band of 440 nm obtained at 25°C modifies itself 24 h after the reaction, reaching 420 nm, whilst with the temperature at 95°C, both the intensity and the maximum length of the absorption band remain constant. The analyses with a transmission electron microscope suggest that this decrease in wavelength is related to the distribution of the size of the NPs. For Ghosh et al. (2012), the study of temperature optimization revealed a direct effect on the speed of the reaction kinetic for gold NPs. They observed that low temperatures (0 to 20°C) do not allow synthesis, whilst at average temperatures (30 to 40°C) a moderate synthesis was observed, and at 50°C a maximum speed was found, which supports what was previously reported, which indicates that high temperatures play a key role in improving the speed of the reaction. Nagar & Devra (2018) studied the effect of temperature on the formation of copper NPs and observed that upon increasing the temperature from 60 to 85°C, the rate of conversion increases in a way in which the effect of the temperature on the nucleation speed is greater than that on the speed of growth. However, when the temperature is too high, it promotes the nuclei to be colloidal, and they agglomerate, which is why the optimum temperature is 85°C.

On the other hand, Dada et al. (2019) studied the effect of temperature on the synthesis of silver nanoparticles, for which they varied the temperature from ambient temperature to 100°C. They observed that the increase in temperature generates an increase in the intensity of the band of the plasmon as a result of the bathochromic change, which results in a decrease in average diameter of the NPs. In order to ascertain the effect that was generated in green synthesis, Aghajanyan et al. (2020) used UV-vis spectrophotometry in order to monitor the formation of the surface plasmon resonance (SPR). The spectra evidenced the formation of Ag NPs in a short time between 40 and 60°C, and although they observed the formation of NPs at ambient temperature, this was slower, and the absorption band generated by SPR was not as pronounced as at the higher temperatures. Moreover, Gopalakrishnan & Muniraj (2019) also tested temperatures of 35, 80, and 90°C during the optimization process for obtaining Cu NPs, reporting that 80°C was the best. In the same way, Jamdagni et al. (2018) signaled that the most suitable for the reduction of ZnO with *Nyctanthes arbor-tristis* is 90°C, as they obtained the most prominent and narrow absorption band with only this temperature.

As observed, there are no unique conditions that can apply to each metallic salt, and which generate NPs with specific characteristics. As such, carrying out an optimization process that considers the following ranges as distinct parameters is recommended. Regarding the concentration of metallic salts, it is suggested that the working concentrations be considered to be in the range of 1 to 10 mM mainly in the case of noble metals. In accordance with that revised for other metallic ions, it is possible to increase the range to 0.1 M. It is important to remember that excessive concentrations can limit the reduction, and thus generate agglomeration that can affect the characteristics that are sought in NPs. The salt precursor also proves to be important given that when dealing with a nitrogenous salt, the concentrations can be smaller. In the case of vegetal extract volume, as reported previously, upon increasing this factor, the size of the NPs decreases. As such, the use of percentages higher than 5% is recommended. For the pH, it is recommendable to work between the values of 5.0 and 12.0, given that lower values will not have a reduction process. For the temperature, it is better to work with high temperatures, or those that are at least higher than 50°C and lower than 100°C.

6. Characterization techniques

Once prepared, the metallic nanoparticles conform to diverse characterization techniques in order to determine their size, shape, distribution, morphology, and surface area. The spectroscopic and diffractographic techniques involved include UV-visible spectroscopy (UV-vis), energy dispersion spectroscopy (EDS), x-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), x-ray photoelectron spectroscopy (XPS), and they are used to analyses the chemical composition, structure, and crystalline phase of NPs. On another note, microscopic techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), high resolution transmission electron microscopy (HR-TEM), and atomic force microscopy (AFM), are employed in order to determine the size and morphological characteristics of the NPs (Khanna et al., 2019).

Table 5.2 Characterization techniques implemented in the study of bioreduced metallic nanoparticles

NPs	UV-Vis	DRX	EDS	DLS	XPS	IR-TF	SEM	TEM	AFM	TGA	BET	Author, year
Ag	•			•				•				(Aghajanyan et al., 2020)
	•			•		•		•				(Ahmed et al., 2016)
	•	•				•		•		•		(Albeladi et al., 2020)
	•					•		•				(Alwhibi et al., 2020)
	•		•			•	•					(Asghar et al., 2018)
	•	•	•			•		•				(Cruz et al., 2010)
	•		•			•	•	•				(Dada et al., 2019)
		•				•	•					(Deeksha et al., 2021)
	•	•			•	•	•	•				(Fatimah et al., 2020)
	•	•	•				•					(Forough & Farhadi, 2010)
	•	•				•						(Hitesh & Lata, 2018)
	•	•					•	•				(Korkmaz 2020)
	•	•				•		•				(Mallikarjuna et al., 2011)
	•	•			•	•	•	•				(Mohaghegh et al., 2020)
	•		•				•					(Mukunthan et al., 2011)
	•	•	•			•	•	•				(Selvan et al., 2018)
	•	•	•			•	•	•				(Sherin et al., 2020)
	•	•	•			•	•					(Umai et al., 2020)
	•	•	•					•				(Yasin et al., 2013)
Au	•	•			•	•	•	•		•		(Ahmad et al., 20189
	•		•			•		•				(Dzimitrowicz et al., 2018)
	•	•		•		•		•				(Ghosh et al., 2012)
	•	•	•			•	•	•				(Narayanan & Sakthivel, 2010a)
	•	•	•					•				(Tamuly et al., 2013)
	•		•			•		•				(Vijayakumar, 2019)
	•	•				•	•					(Wahab et al., 2018)
Fe	•	•		•		•	•					(Afsheen et al., 2018)
	•	•			•		•	•				(Ahmmad et al., 2013)
	•		•			•	•					(Devatha et al., 2016)
	•	•	•				•				•	(Huang et al., 2014)
	•	•				•		•				(Katata-Seru et al., 2018)
	•		•		•			•				(Weng et al., 2018)
Cu	•	•				•	•					(Ghidan et al., 2016)
	•	•				•	•	•				(Gopalakrishnan & Muniraj, 2020)
		•					•	•				(Iliger et al., 2021)
	•	•				•	•	•				(Khani et al., 2018)
	•					•		•				(Liu et al., 2020)
	•	•	•			•	•	•				(Nagar & Devra, 2018)
	•	•				•		•				(Rajesh et al., 2018)
	•	•				•	•			•		(Reddy, 2017)
	•	•	•			•		•				(Sharmila et al., 2018)
Zn	•	•				•	•	•				(Begum et al., 2020)
	•	•						•				(Canizal et al., 2006)
	•	•	•			•	•					(Chinnasamy et al., 2018)
	•	•	•			•	•	•				(Geetha et al., 2016)
	•	•		•		•		•				(Jamdagni et al., 2018)
	•	•	•			•		•				(Matinise et al., 2017)
	•	•	•			•	•		•			(Santhoshkumar et al., 2017)
	•	•	•	•			•	•				(Siripireddy & Mandal, 2017)
	•		•			•	•	•				(Tettey & Shin, 2019)
	•	•	•			•	•	•				(Vijayakumar et al., 2018)

Own Elaboration

6.1 UV-vis

UV-vis spectrophotometry is employed to confirm the synthesis of NPs (Agarwal et al., 2017). Various investigators agree that one of the first signs of the reduction of metals is the change in color between the solutions before and after the reduction reaction. They mention that this change indicates the formation of nanoparticles, confirmed by the visible surface plasmon resonance (SPR) in the UV-vis absorption spectrum (Asghar et al., 2018; Kuppusamy et al., 2016). As observed in table 2, it is one the first pieces of evidence that the reaction reduction has taken place. The surface plasmon resonance for each type of particle can be found in the form of characteristics in a specific region of the absorption spectrum in a way that the SPR of silver NPs can be found between 400 to 500 nm, whilst for gold NPs, they are generally found between 500 to 550 nm, as shown in table 5.1.

SPR gives a spontaneous spectroscopic signal generated by the formation of nanostructures, which is due to the free electrons which emerge due to the conduction and valence bands being very close to each-other. When the oscillating electromagnetic field (in the light) generates a coherent collective oscillation of electrons from the conduction band of the metals during their exposure to the light, it provokes a separation of charge around the surface of the metals (Ahmad et al., 2018; Dada et al., 2019).

6.2 FTIR

Analyses through Fourier-transform infrared spectroscopy (FTIR) are relevant for determining the functional groups present in vegetal extracts, which allows the investigation of the underlying synthesis mechanism, and the surface chemical. Usually the ranges used are found between 4000 and 400 cm^{-1}, with a resolution of 4 cm^{-1}, which gives a clear idea of the reducing agents responsible for the covering, reduction, and stabilization of the NPs. The main limiting factor of this technique is found in the superposition grade of the IR absorption bands in the complex biological matrix (Dada et al., 2019; Khanna et al., 2019).

The comparison between the transmittance spectra of the aqueous vegetal extract and the means of reaction offers information on the biomolecules involved in the process (Dada et al., 2019; Khanna et al., 2019). The extracts contain numerous functional groups like C=C (alkenes), C=N (amide) O=H (phenolics and alcohol), N- H (amine), C - H and COO- (carboxylic groups).

6.3 XRD

The purity, crystalline size, geometry, orientation, and phases can be determined through XRD data. Generally, the diffraction patterns are compared with a standard crystallographic database such as JCPDS in order to have the structural information. Using the Debye Scherrer formula (3), an approximation of the particle size is obtained. XRD functions well with the identification of NPs in one or various phases. Additionally, the diffractogram is influenced by amorphous NPs that have varying interatomic longitudes (Khanna et al., 2019).

$$D = \frac{K\lambda}{\beta coscos\,\theta} \tag{3}$$

...where K is the Scherrer constant (K = 0.94), D is the average size of the crystal, β is the total width at half the maximum of the spikes (FWHM) for a Gaussian adjustment, λ corresponds to the wavelength of the used radiation Cu Kα (λ= 0.1546 nm), and θ is half of the diffraction angle of the Bragg peak (Rajesh et al., 2018).

6.4 XPS

Typically for the determination of chemical types present in a sample, an analysis through x-ray photoelectron spectroscopy (XPS) is turned to. It can also shed light on the interaction between NPs and their adjacent biomolecules, as well as signaling the presence of secondary or undesired elements that could reduce its efficiency or lead to a secondary reaction and process contamination (Ealia & Saravanakumar, 2017; Khanna et al., 2019).

6.5 SEM/TEM

Scanning electron microscopy (SEM) offers information on particles at the nanoscale and helps to determine the surface morphology and the dispersion of free NPs or those in the matrix. Transmission electron microscopy (TEM) is more commonly used for the size and shape and can also offer information on the number of layers of the material, given that it varies from a low or high increase. However, when both are combined with EDAX or EDS, information is provided on the elements present. When the precise shape, size, and crystalline structure need to be known, HR-TEM is used (Khanna et al., 2019). As has been observed, all the implemented characterization techniques provide important and necessary information regarding the characteristics of bioreduced NPs. Among these techniques, a conclusive tool for said characteristics is transmission electron microscopy. However, it is appropriate to highlight that UV-vis spectrophotometry is the first technique chosen to confirm the presence of NPs in the bioreduction solution (Powar & Patel, 2019; Vijayaraghavan & Ashokkumar, 2017).

7. Applications

Metal nanoparticles are of great interest to several disciplines including biotechnology / biomedicine, bioremediation, agriculture, catalysis, biosensors, among others (Rai & Yadav, 2013).

7.1 Monometallic Particles

Silver

According to Dada et al. (2019), of all metal nanoparticles, those of silver have been largely explored by researchers around the world due to their versatility, simplicity of synthesis, adaptability, morphology, and high surface area. One of its main applications resides in the evaluation of their antibacterial activity. In this sense, Yasin et al. (2013) obtained AgNPs with bamboo leaves through bioreduction, tested against the pathogenic bacteria *E. coli* and *Staphylococcus aureus*; the results indicate that the effectiveness of the NPs applied increases with the concentration used, however, they reported that 20 μg / mL is the minimum concentration that reduces bacterial proliferation.

Korkmaz (2020) evaluated the bactericidal activity of AgNPs obtained by green reduction with *Anthurium andraeanum* against the bacteria: *Enterobacter aerogenes*, *Salmonella infantis*, *Salmonella typhimurium*, *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Bacillus epidermidis*. MIC and MBC tests indicated that the minimum inhibitory concentration against all species was found at 0.125 mg / mL, while the bactericidal effect for all strains was 1 mg / mL except for *S. aureus*. Alwhibi et al. (2020), synthesized AgNPs with *Commiphora myrrha* and tested its antibacterial activity against several gram-negative strains. According with the authors, the antibacterial effectiveness is explained by two pathways, the first is membrane damage through an association / interaction of NPs with biomolecules and DNA, causing the inhibition of cell multiplication, and second by formation of reactive oxygen species through their interaction with enzymes and / or biomolecules generating cell damage or destruction. This same study concerned the anticancer activity of NPs and found that cell viability is reduced by increasing the concentration of nanoparticles used, reaching 30% cell viability with 100 μL of AgNPs.

Silver NPs have also been applied as catalysts, Albeladi et al. (2020), for example carried out the green reduction of Ag with *Salvia officinalis* and evaluated their ability to degrade the Congo red dye (CR) through a catalytic reduction with sodium borohydride ($NaBH_4$). It was found that the addition of Ag NPs significantly increase the degradation of the dye up to 82.22 % due to the high availability of active sites on their surface where electron transfer is mediated. Sherin et al. (2020), also tested the catalytic capacity of AgNPs synthesized with *Terminalia bellerica* for the degradation of four organic pollutants (4 nitrophenol, methyl blue, eosin yellow and methyl orange), the results showed good catalytic activity with the dose dependent on ambient conditions.

Gold

Gold nanoparticles are among the most used for medical applications as reported by Amini et al. (2019); their antibacterial activity was tested by Chamsa-ard et al. (2019), against *Escherichia coli* and *Staphylococcus epidermidis*, with NPs obtained by bioreduction with *Citrullis lanatus*; the fruit was split in two parts, the inner red and the outer green; each portion allowed obtaining AuNPs with outstanding antibacterial properties, which is why the authors consider that the AuNPs are potential candidates for incorporation in new types of antibiotics. Also, the authors highlighted the effectiveness of the production technique based on green chemistry and its low cost.

Balasubramanian et al. (2020), obtained AuNPs by green reduction with *Jasminum auriculatum* leaf extract and evaluated their antimicrobial activity against various species of pathogenic bacteria and fungi, the results obtained by the disk diffusion method showed that their efficiency depends on the size and amount of NPs; the authors referred that the probable mechanisms consist of: 1) the damage caused to the cell membrane and the sulfide and phosphate groups of DNA and protein by the interaction with the NPs, and 2) the induction of Reactive oxygen species that can damage the cell membrane, DNA, and lead to cell death.

Balasubramanian et al. (2020), also evaluated the anticancer activity of the AuNPs produced and the results of cytotoxicity against HeLa cancer cells indicate that the inhibition capacity depends on the time and dose administered: the gold nanoparticles suppress the cell viability by 69.28 % with a dose of 200 µg / mL. In this same line, Vijayakumar (2019) prepared AuNPs with extracts of the fruits of *Aegle marmelos*, *Eugenia jambolana* and *Annona muricata*. The results indicate that the inhibition of cell viability depends on the concentration of the administered NPs, but it was also evidenced that the phytochemicals involved in stabilizing these also influence their cytotoxicity, the NPs reduced with Annona were 30% more effective than the others with concentrations of 120 µg / mL.

Iron

Iron nanoparticles, FexOy and FeOOH (a total of 16 polymorphic structures) attract increasing interest due to the rapid development of their applications (Kharissova et al., 2013), among these Katata-Seru and collaborators (2018) evaluated the antibacterial effectiveness of FeNPs obtained by green reduction with *Moringa oleifera* leaves and seeds (MOL-FeNPS and MOS-FeNPs) against antibiotics such as ampicillin, gentamicin, erythromycin and vancomycin on gram negative strains; the results show that MOS-FeNPs are more effective than MOL-FeNPs against *E. coli* because they are the smallest NPs. The authors considered that the positive charge of the Fe ions may be responsible for the antibacterial activity through the attraction between the negative charge of the cell membrane of microorganisms.

Huang et al. (2014), obtained FeNPs by bioreduction using green tea, black tea and oolong tea (GT-FeNPs, BT-FeNPs and OT-FeNPS respectively) to apply them in a comparative study aiming to remove malachite green dye (GM); the results indicate that the highest efficiency was obtained with GT-FeNPs (81.6%). The authors suggested that the polyphenol molecules associated with the NPs of Fe which, by inducing its corrosion, allows the release of electrons capable of breaking the –C = C- and –C = N- bonds in the benzene rings. On the other hand, Devatha and collaborators (2016) evaluated the FeNPs obtained by green reduction of with *Mangifera indica* extracts (MI-FeNPs), *Murraya koenigii* (MK-FeNPs), *Azadirachta indica* (AI-FeNPs) and *Magnolia champaca* (MC-FeNPs).

For the treatment of domestic wastewater, the results indicate that the best treatment is obtained with AI -FeNPs with a removal efficiency of 98.1%, 84.3% and 82.4% for phosphates, ammoniacal nitrogen and COD respectively, using 1 g / L of NPs. Ouyang et al. (2019), carried out the reduction of Fe with a pure extract of polyphenols, to evaluate its catalytic capacity in the removal of Lincomycin (LCM), for which various systems were tested with NPs (GFe0.25, GFe0.5 and GFe1.0), it was found that with the GFe0.5 the degradation rate of the MCL after 90 min was 93.85% using a dose of 0.01 g / L.

Copper

Copper oxide (CuO) is an important semiconductor metal oxide with a band gap of 1.7 eV that can be obtained by green reduction and be used in the formulation of pesticides, fungicides and antibacterials, such as Ghidan et al. (2016) who evaluated CuO nanoparticles obtained by bioreduction with *Punica granatum* to eliminate the green peach aphid with an 86% efficiency in percentage of mortality, using a 8000 µg / mL concentration of NPs. Ilger et al. (2021), on the other hand, evaluated the effect of Cu NPs obtained with *Eucalyptus globulus* L. against the fungus *Colletotrichum capsici*, which causes rotting of chili fruit that affects farmers in India, the results obtained showed that concentrations of 500 ppm and 1000 ppm of NPs inhibit micellar growth completely. In addition they prolong the incubation period by reducing the number and length of the lesions caused by this fungus. Gopalakrishnan and Muniraj (2019) obtained CuNPs with *Azadirachta indica*, tested its antibacterial activity against *Enterococcus faecalis*, *Proteus mirabilis*, *Klebsiella pneumonia* and *Staphylococcus aureus* and obtained a high efficiency against *P. mirabilis* using 40 µg / mL of NPs.

In the medical area CuONPs have been evaluated as anti-inflammatory and anti-nociceptive in mice as reported by Liu et al. (2020), who obtained CuONPs by bioreduction with *Abies spectabilis*, their results indicate that CuONPs are effective by inhibiting noniceptive and inflammatory responses in mice with administered doses of 15 µg / Kg without producing behavioral changes in them, however, more studies are required to understand the exact therapeutic mechanisms. CuNPs have also been evaluated for wastewater treatment: Khani et al. (2018), reduced copper using *Ziziphus spina-christi* (L.) Willd. To apply the NPs obtained in the adsorption of the crystal violet dye, after optimizing the process, it was found that with the conditions of pH 9.0, dye concentration of 35 µg / mL, stirring time 7.5 min and 80 mg amount of sorbent, the removal efficiency reached 95%.

Zinc

Zinc oxide (ZnO) exists within the earth's crust as a mineral, zincite, however, most of it that is used commercially is obtained from synthetic methods. ZnO is not toxic and is compatible with human skin, making it an acceptable additive for textiles and surfaces that are in direct contact with the skin (Mirzaei & Darroudi, 2017). ZnO NPs have been tested as antimicrobial, Gunalan et al. (2012), performed the bioreduction of ZnO with aloe extract to evaluate its antimicrobial activity compared with NPs prepared by a chemical method, the results obtained for the minimum inhibitory concentration (MIC) suggest that the small NPs prepared by the green method show an improved microbicidal activity due to the greater surface area in relation to volume. The authors observed that ZnO NPs have a selective antimicrobial activity for both the bacteria and fungi evaluated; therefore, they considered that these constitute an effective agent against pathogenic microorganisms. Later, Santhoshkumar et al. (2017) obtained ZnO from green reduction with *Passiflora caerulea* to evaluate its antimicrobial activity against urinary tract pathogens such as *E. coli*, *Streptococcus sp*., *Enterococcus sp*., *Klebsiella sp*. The results showed that NPs have a dose-dependent effect, having a greater effectiveness for gram- than for gram + bacteria.The authors pointed out that NPs have different mechanisms of action against bacteria due to their structural differences.

In the medical area, Tettey and Shin (2019) synthesized ZnO NPs with *Scutellaria baicalensis* root extract, to evaluate its antioxidant and cytotoxic activity, in terms of antioxidant activity it was observed that it is dependent on the applied dose, namely it increases with increasing dose, in comparison with a standard antioxidant, the effectiveness was low, which the authors explained was not conclusive, thus they recommended more explorations in this regard. Regarding the cytotoxic activity, it was observed that the antiproliferative activity is also dose-dependent when tested against the growth of HeLa cells, the main possible mechanism according to the authors is the generation of free radicals that induce apoptosis in these cells, though they were tested with cells of the immune system and did not show toxicity at concentrations up to 1 mg / mL, which suggests that these NPs generated by green pathways could be promising candidates for the design of agents with a combined antioxidant and anticancer effect.

7.2 Bimetallic particles

Bimetallic nanoparticles differ from monometallic ones in that they contain two metallic ingredients, for their preparation from two precursor salts, two methods can be adopted:

1) co-reduction, which is the simultaneous reduction of two metals and 2) successive reduction in the that one metal is reduced over the nuclei of the other. Depending on the method used, nanoparticles will be produced in alloy or in layers (core-shell). Alloys have received increased attention due to the possibility of setting their properties over a wide range, simply by varying the composition of the alloy (Sumbal et al., 2019; Thakore et al., 2019). Bimetallic nanoparticles are of great interest for their applications in catalysis, electronics, as optical materials and coatings, and various methods have been reported to obtain them, however, reports on green routes to obtain bimetallic NPs are still few (Lagashetty et al., 2019; Schabes-Retchkiman et al., 2006).

Regarding the applications in the medical area, bimetallic NPs have been tested in antibacterial studies such as the one carried out by Al-Haddad et al. (2019), where they implemented the green reduction of bimetallic Cu-Ag particles with extract of *Phoenix dactylifera* leaves, evaluating the antibacterial activity of NPs against gram-positive and gram-negative results indicate that the bimetallic system exhibited good bacterial resistance at very low concentrations with an inhibition zone greater than 20 mm for both strains. Lagashetty et al. (2019), obtained Ag-Au bimetallic NPs by green reduction with Piper betle against *Bacillus subtils* and *Klebsiella planticola* strains: the results indicate that NPs are more effective for *B. subtils*. Merugu et al. (2020), used *Borassus flabellifer* for the reduction of bimetallic NPs Ag / Cu and Cu / Zn, evaluating its antibacterial activity against *Alcaligenes faecalis*, *Staphylococcus aureus*, *Citrobacter freundii*, *Klebsiella pneumonia* and *Clostridium perfringens*, the results indicate that NPs Ag / Cu and Cu / Zn are more effective than the tested control drugs (ciprofloxacin and amoxicillin), except for *Staphylococcus aureus* where amoxicillin showed better results.

In studies on anticancer and antioxidant activity, Elemike et al. (2019), developed a Ag-Au bimetallic system using *Stigmaphyllon ovatum* to test its cytotoxic capacity with HeLa cell lines; the results showed that, compared to bimetallic NPs the system showed greater activity due to possibly the synergistic effect of the two metals. According with the authors, the ability to inhibit cell viability is related to the ability of metals to generate Fenton-like reactions by means of which reactive oxygen species are produced that damage DNA and lead to cell death. In this same sense, Thakore et al. (2019), evaluated the cytotoxic effect of the Cu / Ag alloy bioreduced with sapota fruit latex, the tests with different proportions of the metals did not show cytotoxic effects, so its therapeutic application could lead to products biocompatible with the latex-coated alloy. Merugu et al. (2020), tested their bimetallic NPs against HeLa cancer cells and their antioxidant activity, with respect to the first test, the results indicate that the inhibition of cell viability increases with increasing dosage of NPs for both combinations Ag / Cu and Cu / Zn.

7.3 Biosynthesis of Fe / Cu bimetallic nanoparticles and their application in the removal of indigo carmine

One other area to use of NPs is in environmental remediation, specifically the treatment of water and wastewater through Advanced Oxidation Processes (AOP); this is a group of techniques that consider the environment the subject for the elimination of persistent organic pollutants, pathogens and by-products of water disinfection through the *in situ* formation of powerful oxidizing agents such as the hydroxyl radical ($HO^{\bullet}$). One of these processes is the Fenton reaction, which is based on the decomposition of hydrogen peroxide catalyzed by iron ions in an acid medium, among its main advantages are its low cost, ease of application at room temperature and ambient pressure. However, one of its main disadvantages lies in the high amounts of iron necessary to carry out the process (50-80 mg / mL) (Oruç et al. 2019; Scaria et al. 2020). Recently, the use of bimetallic catalysts (Elias E. Elemike et al. 2019; Xiulan Weng et al. 2017; Zhu et al. 2018) has shown that they have a better catalytic activity as result of the surface area increase and the synergistic effect between the two metals, which not only improves the degradation of pollutants complexes, but it is also useful to achieve the complete mineralization of their by-products. In this sense the Fe-Cu bimetallic catalysts have attracted attention because, as has been suggested, there is the interaction between the redox couple of the two Fe-Cu metals that could accelerate electronic transfer at the interface and thus improve the activation of H_2O_2 by the catalyst (Scaria et al. 2020; Sun et al. 2019; Tang & Wang, 2020; Wang et al. 2018).

Eucalyptus globulus belongs to the Myrtaceae family; due to its rapid growth which increases its timber biomass, the Eucalyptus genus is widely cultivated in the Mediterranean, its leaves are used for traditional remedies of several respiratory conditions (Boulekbache-Makhlouf et al. 2013).

Among its main components are holocellulose (83.2%), lignin (34.1%), and extractives (6.5%), where the latter are made up of lipophilic compounds and polyphenolic compounds responsible of its anti-inflammatory, antibacterial and antioxidant properties (González et al. 2017), the use of *Eucalyptus globulus* has already been reported for the synthesis of metallic nanoparticles (Iliger et al. 2021; Siripireddy & Mandal, 2017), its biomolecules play a key role in the reduction of ions at the nano scale and stabilization of nanoparticles (Iliger et al. 2021), in addition to being an economical and environmentally friendly method.

7.3.1 Materials and Methods

Preparation of the vegetal extract

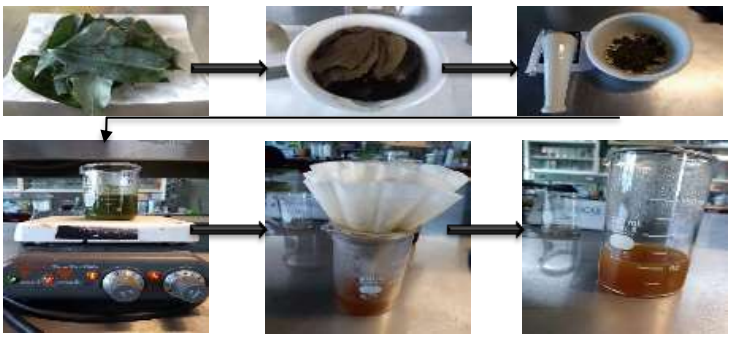
Generally, the biosynthesis of metallic nanoparticles is carried out in aqueous solution (Amini et al. 2019), various authors agree on the procedure to obtain the plant extract, considering small variations such as plant organ used, amount of biomass per volume, boiling time (Agarwal et al. 2017; Ahmad et al. 2018; Rajesh et al. 2018; Siripireddy & Mandal, 2017).

In this research, eucalyptus leaves, moringa leaves and hibiscus flower were used to obtain the plant extract with which the Fe / Cu bimetallic particles were reduced. The leaves were acquired in the municipal market of Metepec, State of Mexico, transported to the laboratory and washed thoroughly to remove dirt and dust, then were placed in a paper bag in an oven at 100 °C for 24 hours, until they look completely dry. They were macerated to obtain a fine powder that was kept in an airtight container until use.

The infusions were prepared following the process reported by Siripireddy and Mandal (2017), namely: 10 g of eucalyptus, moringa and hibiscus leaf powder were weighed separately (Fig. 5.4); each sample was placed in a beaker and 70 mL distilled water were added, then the obtained suspensions were placed on a heating plate at constant stirring until boiling, the infusions were allowed to boil for 10-15 min, after which were removed and the supernatant was filtered, obtaining 50 mL of infusion. It was allowed to cool to room temperature and used for reduction reaction.

The three infusions prepared were tested to obtain Cu particles to evaluate which reducer was more efficient to be used to carry out the reduction reaction of the Fe / Cu bimetallic catalyst.

Figure 5.4 Procedure for the elaboration of the vegetal extract from eucalyptus leaves



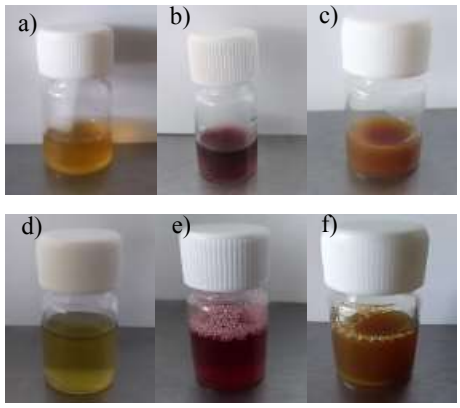
Own Elaboration

Reduction reaction

For the reduction reaction, 150 mL of $CuSO_4 \cdot 5H_2O$ 0.05 M were prepared with distilled water in a volumetric flask. The reduction reaction was carried out with the moringa extract plus the copper solution in a 1:1 volume ratio, at room temperature under constant stirring for one hour, but no color change was observed in the solution that remained a light green color, which suggested that the reduction process was not taking place (Fig. 5.5d). Subsequently, the hibiscus solution and the copper solution were tested under the same conditions; although the color change was not very evident, the resulting solution was filtered because a little turbidity was observed, obtaining very few particles (Fig. 5.5e).

Finally, the test with the eucalyptus infusion resulted in the color change from light brown of the infusion to dark brown that was due combination to reaction with the copper solution after the first 5 minutes (Fig. 5.5f), so this infusion was used as a reducing agent for the Fe / Cu bimetallic particles.

Figure 5.5. a), b) and c) infusions of moringa, hibiscus and eucalyptus before the reduction reaction; d), e) and f) solutions after one hour of reduction reaction with copper



For the reduction of Fe / Cu bimetallic particles, 50 mL were prepared with $CuSO_4 \cdot 5H_2O$ and $FeSO_4 \cdot 7H_2O$ at a concentration of 0.05 M in distilled water, once the bimetallic solution had been prepared, the infusion of eucalyptus leaves (IEH) was added in a 1: 1 volume ratio, the color change from light brown to dark was observed immediately, the reaction was kept under constant stirring for 2 h, then the solution was left to rest for 24 h, after which it was filtered on a Büchner funnel; the particles obtained were dried at 70 °C for 48 hours and stored in 3 mL hermetic vials

Characterization of the Fe/Cu NPs

Determination of the SPR of the IEH-Sol containing Fe / Cu solution by means of UV-visible with a Perkin-Elmer Lambda 25 spectrophotometer.

Indigo carmine removal

Indigo carmine reagent grade was added with distilled water to set the pH with 4 M sulfuric acid and hydrogen peroxide at 30% analytical grade, the removal of the dye was monitored by UV-visible spectrophotometry with the Perkin-Elmer Lambda spectrophotometer 25, applying equation 4, the results obtained were fed into the statistical software Minitab Ver. 18.0 to design and carry out a factorial experimental 2^3; the factors evaluated were: dye concentration (200 - 400 mg / L), dose of 30% hydrogen peroxide (5 and 10 µL) and catalyst dose (1 and 5 mg). The volume used for each test was 10 mL at a pH of 3.0.

$$\% \text{ Remoción de IC} = \frac{C_0 - C_t}{C_0} * 100 \quad (4)$$

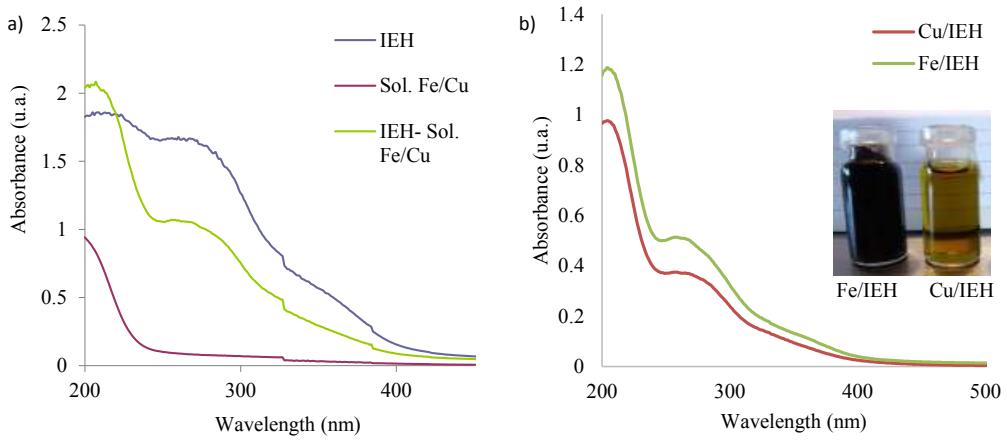
7.3.2 Results

Characterization of the Fe/Cu NPs

UV-vis spectrophotometry is a technique that allows establishing the formation of NPs (Katata-Seru et al. 2018), after the change in color of the infusion of eucalyptus leaves (IEH) from light brown to black after it enters into contact with the bimetallic solution; the reduction process is evidenced and the formation of nanoparticles due to surface plasmon resonance (SPR), as corroborated by the UV-vis absorption spectra that were obtained from the IEH, for the Fe / Cu bimetallic solution and for the reduction solution (IEH-Sol, Fe / Cu).

As seen in graph 1a) the IEH spectrum shows two absorption maxima at 270 and 220 nm, when added the bimetallic solution a decrease is observed in the maximum of 270 nm while that close to 200 nm increases reaching a maximum at 208 nm that corresponds to the bimetallic nanoparticles obtained. Graph 5.1b) shows the spectra corresponding to the individual reduction solutions of Fe / IEH and Cu / IEH; the spectrum of Fe / IEH agrees with that reported by Katata-Seru et al. (2018).

Graph 5.1 UV-Vis spectra: a) spectrum of the reduction reaction solutions in the green infusion of eucalyptus leaves plus iron and in the red infusion of eucalyptus leaves plus copper; b) spectra in the blue infusion of eucalyptus leaves (IEH), red bimetallic solution (Fe / Cu) and in green reduction solution (IEH-Sol. Fe / Cu)



Proposed reduction mechanism

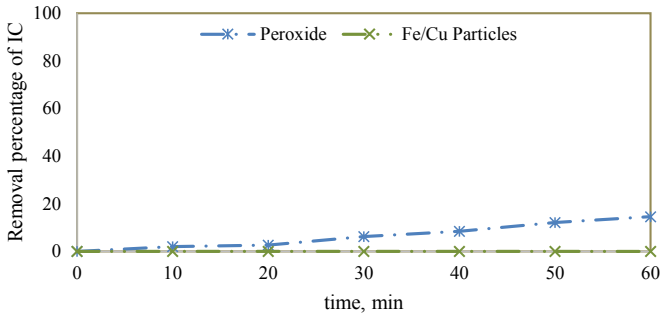
Eucalyptus (*Eucalyptus globulus*) is commonly used to treat respiratory diseases such as bronchitis, asthma, and influenza, among others; its antioxidant and antimicrobial capacity has been recognized by studies such as that of Boulekbache-Makhlouf et al. (2013), who found among its components the polyphenols, mainly hydrolysable tannins, responsible for said activity. Furthermore, Gonzales-Burgos et al. (2018), reported that among the flavonoids present in eucalyptus extracts the most abundant compound is chlorogenic acid and the group of quercetin glucosides which dispaly multiple hydroxyl groups that can act as reducing metal ions, as shown in the mechanism proposed in equation 5. Due to the redox potentials of the two metals (E^0 ($Fe_{(III)}$ / $Fe_{(II)}$) = 0.77 V) and Cu (E^0 ($Cu_{(II)}$ / $Cu_{(III)}$) = 0.17 V) it is proposed that iron reacts faster so that the Fe nuclei are the first to form to begin growth process, where the copper nanoparticles aggregate over the surface.

$$nCu^{2+} + nFe^{2+} + 4nAr\text{-}(OH)_n \rightarrow nFe(Cu)_{(s)} + 4n\ Ar{=}(O)_n + 4nH^{+} \quad (5)$$

Indigo carmine removal

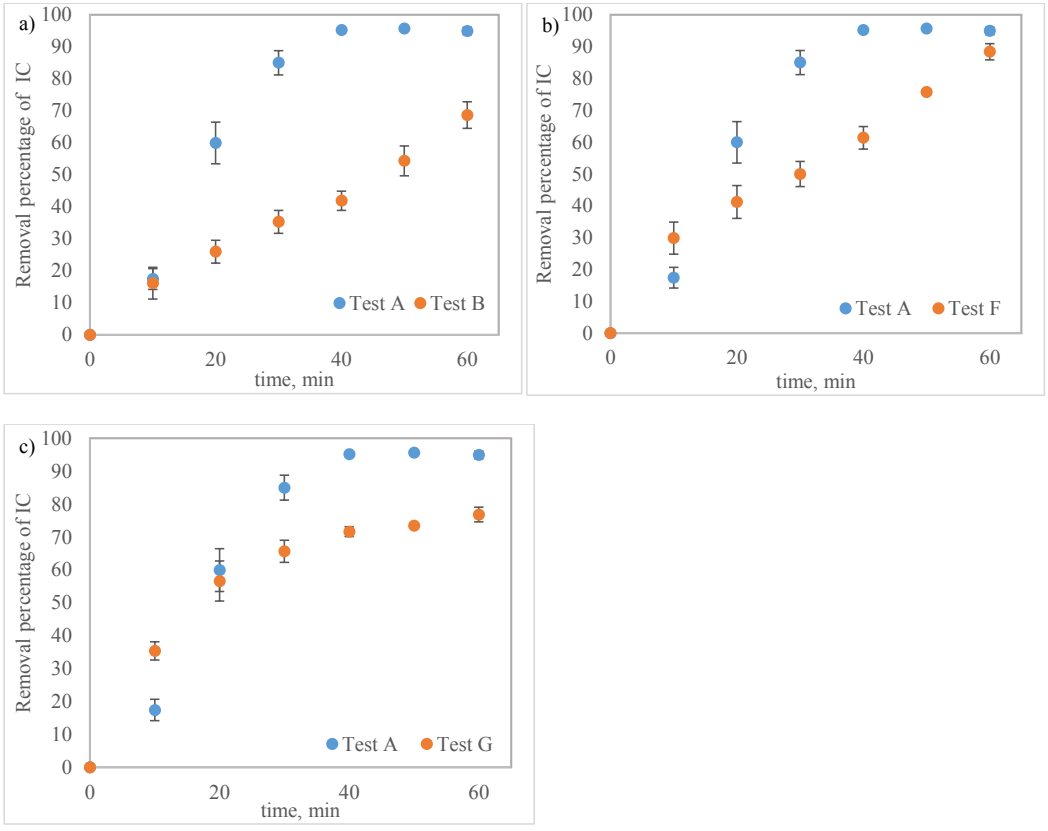
Preliminary calibration plots were made for the IC (Abs_{610} = 0.0447 (C_{IC}) - 0.0021) and the Isatin (Abs_{245} = 0.1655 (C_{Is}) - 0.0383), their main degradation by-product, obtaining a coefficient of determination of 0.9966 and 0.9981 respectively. Additionally, peroxide and Fe / Cu bimetallic particles were tested individually to observe their effect on color, as can be seen in graph 2, both peroxide and bimetallic particles remove color in very low percentage during the treatment time, 0.002 and 14.613% respectively.

Graph 5.2 Evaluation of the individual reagents for removal of the IC dye. Working conditions, volume: 10mL; dye concentration: 200 mg / L; pH: 3.0; amount of particles: 1mg; amount of H_2O_2: 10 µL



From the developed experimental design, a total of eight experiments were obtained with all possible combinations of the levels for each factor, each experiment was assigned the letters in alphabetical order A, B, C, D, E, F, G and H, all the experiments were carried out in triplicate, working with a set volume of 10 mL and with a set pH = 3.0.

Graph 5.3 Indigo carmine removal with Fenton-like reactions with Fe / Cu bimetallic nanoparticles: a) evaluation of the influence of the IC dye concentration (200 - 400 mg / L); b) evaluation of the influence of the amount of NPs Fe / Cu (1 - 5 mg); and c) evaluation of the influence of the H_2O_2 dose (5 - 10 µL). Working conditions: volume of 10 mL, pH 3.0, constant stirring (ANOVA analysis $p < 0.05$)



Regarding the effect of the concentration of the dye, as can be seen in graph 5.3a), when the dose of H_2O_2 (10 µL) and Fe / Cu bimetallic particles (5 mg) are kept constant, increasing the concentration of dye from 200 mg / L (test A) to 400 mg / L (test B).

The removal percentage is significantly decreased (ANOVA $p < 0.05$) from 94.92 to 68.66%, it is also important to note that after 40 minutes of test A maximum removal was reached. When analyzing the effect of the catalyst dose in graph 3b), it is apparent that by reducing the amount of catalyst (from 5 to 1 mg), keeping the dye concentration (200 mg) and the H_2O_2 dose (10 µL) constant, the removal percentage decreases from 94.92 to 88.37%, the effectiveness of the catalyst may be related to the standard reduction potentials of Fe (E^0 ($Fe_{(III)}$ / $Fe_{(II)}$) = 0.77 V) and Cu (E^0 ($Cu_{(II)}$ / $Cu_{(III)}$) = 0.17 V) where part of the Cu (I) generated on the Fe surface could promote Fe (II) regeneration through a thermodynamically favorable electron transfer process (6) (Tang & Wang, 2020).

$$Cu^{+} + Fe^{3+} \rightarrow Cu^{2+} + Fe^{2+} \qquad \Delta E^0 = 0.60 \text{ V} \tag{6}$$

The effect of H_2O_2 dosage (from 10 to 5 µL) can be observed in graph 3 c) also decreases the removal percentage from 94.92 to 76.83%; however, it is important to note that at 30 min in the G test, it exceeds 60% removal so that it is possible to reduce the H_2O_2 dose to an intermediate value and that this would not significantly affect the removal process and would also reduce the operating cost. It should be noted that since H_2O_2 is the main source of the $HO^{\bullet}$ radical, it is important that it is not insufficient since this generated a decrease in the degradation rate of the dye due to lower generation of $HO^{\bullet}$. Nonetheless, higher concentrations of H_2O_2 can be generated, more $HO^{\bullet}$ that may accelerate the decomposition of organic compounds, excess peroxide will have a predatory effect on $HO^{\bullet}$ radicals through the self-decomposition of H_2O_2 to O_2 and H_2O (equations 7-9) limiting the degradation process (Oruç et al. 2019).

$$H_2O_2 + HO^{\bullet} \rightarrow HO_2^{\bullet} + H_2O \tag{7}$$

$$HO_2^{\bullet} + HO^{\bullet} \rightarrow H_2O + O_2 \tag{8}$$

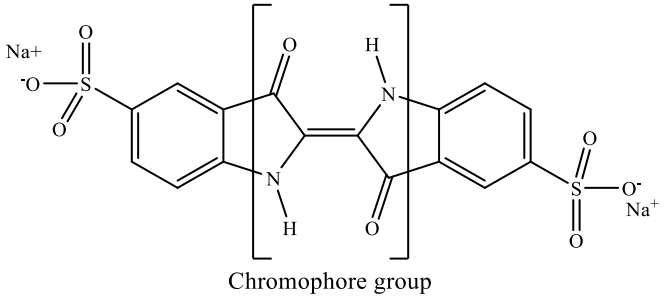
$$2H_2O_2 \rightarrow 2H_2O + O_2 \tag{9}$$

The analysis of variance (ANOVA) carried out in the statistical software Minitab Ver. 18.0, with a confidence level of 95%, using the IC percentage removal as the response variable in the analysis, shows that there are statistically significant differences ($p < 0.05$) between the different treatments, this suggests that the factors that have a greater effect on the response variable are the concentration of color and bimetallic particles. Since the value of R2 (94.31%) and fitted R2 (91.83%) are very close to 1.0, this indicates a good fit between the observations and that predicted by the model. The optimization of the experimental designates test A as the best.

UV-vis analysis of the Indigo Carmine removal

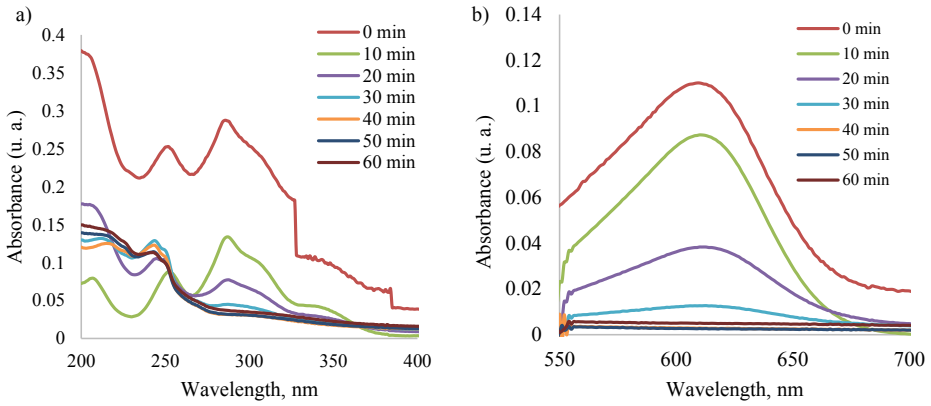
In the UV-vis spectra obtained from the indigo carmine dye (Fig. 5.6), two main absorption maxima can be identified as: the first in the visible region at 610 nm, typical of the IC dye, can be attributed to the orbital of the $n \rightarrow \pi$ group of the double bond system (transition of the non-binding electrons to the antiband π) and the second in the ultraviolet region at 287 nm characteristic of the benzene rings present in the molecule (aromatic compounds present absorption maxima between 210-320 nm).

Figure 5.6 Chemical structure of the indigo carmine *(modified from De León Condés et al., 2019)*



Chromophore group

Graph 5.4 Indigo carmine UV-visible spectra: a) segment of the 200-400 nm range; b) segment of 550-700 nm characteristic absorption of the blue of the indigo carmine



The graph 5.4b) shows the absorption band associated with the chromophore group of the dye molecule decreasing with time, which suggests an oxidation process by which the IC degrades during the first 30 minutes, while plot 4a) depicts the emergence of a maximum around 245 nm characteristic of isatin, that begins to be appear after 20 min reaction, reaching a maximum at 30 min; this decreases at the end of the experiment, therefore, in addition to the degradation of the IC , the main by-product generated from this process is also degraded, which is consistent with that reported by Torres-Blancas et al. (2017), who applied a Fe-Cu bimetallic catalyst in the ozonation process to remove the indigo carmine dye: this does not only improves the efficiency of the process, but also impacts the mineralization (90%) of its by-products in a short time through the generation of hydroxyl radicals. For the synergy of the ozonation, peroxidation, Fenton and Fenton-like concurrent reactions, much like the Fenton and Fenton-like processes evaluated with the Fe / Cu bimetallic particles, the reaction mechanism is very similar to that proposed by Torres-Blancas, shown in equation 10, where the generation of the $HO^{\bullet}$ radical can be seen responsible for the degradation of the indigo carmine dye.

$$Fe^{2+} + Cu^{2+} + 3H_2O_2 \rightarrow 3HO^{\bullet} + HO^{\bullet}_2 + H_2O + Fe^{3+} + Cu^{2+} \quad (10)$$

Bioreduction with eucalyptus is efficient to obtain monometallic and bimetallic Fe / Cu nanoparticles, which is verified through UV-Vis spectra of surface plasmon resonance (SPR) characteristic of nano-sized particles. The spectrum Fe / IEH is in close agreement with that reported in the literature at a wavelength of 208 nm. Thus, it is important to point out that the phytochemicals present in the eucalyptus extract are very important for the particles stability, to prevent their agglomeration, that would occur with other chemical reducers. The proposed mechanism suggests that iron ions are first reduced so that copper NPs deposit over their surface. The catalytic activity of the elaborated Fe / Cu bimetallic particles was tested for the removal of the indigo carmine dye in standard solutions through Fenton-like reactions, reaching an efficiency of 94.92% with optimal conditions, in addition to the removal of the Isatin by-product. Statistical analysis indicates that the concentration of the dye and the amount of bimetallic particles used have a significant effect on the removal of the dye.

8. Toxicity

The toxicity of nanomaterials depends on several factors such as: size, shape, size and shape of their distribution, surface charge and surface chemistry. To make a valid comparison in addition to the type of associated phytochemical, it is necessary to consider other factors such as reactivity, mobility, solubility and aggregation. (Amini, 2019; Sajid et al., 2015). Based on the review by Ajdary et al. (2018), NPs can enter the body through various means, like inhalation, skin and digestion; depending on their physicochemical characteristics and their method of preparation, NPs can access vital organs through blood flow and induce tissue and cell damage. Cell penetration occurs through diffusion intrusion, endocytosis, and membrane proteins such as the phospholipid layer; several investigations indicate that NPs activate oxidative stress by increasing the generation of reactive oxygen species (ROS), intervening in the expression of genes involved in inflammation, consequently generating damage to proteins, cell membrane and DNA (Fard et al., 2015).

Further, the presence of NPs in the environment gives rise to transformations capable of modifying their degree of toxicity, which still requires studies and test methods to single out the processes underway in different environmental spheres that allow identifying risk levels. (Zhang et al., 2018). Considering the above and the rapid growth, development, and applications of NPs, regulation via legislations, laws and rules are being implemented by various governmental organizations to minimize or avoid the risks associated with these materials. However, there are neither international regulations, nor protocols and legal terminology for management and production, nor toxicity tests or environmental impact assessments of NPs (Jeevanandam et al., 2018), so that management and production in research centers acquires relevant importance.

9. Conclusions

Bioreduction processes using plants show broad advantages over other methods related to the availability of plant species with antioxidant capacity, the complex matrix of the extract that provides not only chemical species with reducing capacities, but also provides stabilizing species that when associated with the surface of the NPs, can influence positively the application to be evaluated, mainly generating high expectations in the medical and catalytic area.

The manufacturing processes must consider the aforementioned aspects like pH, temperature, metal ion concentration, as well as the amount of plant extract, so it is important to carry out an optimization process to synthesize nanoparticles displaying the best qualities, which can be corroborated with the fundamental characterization studies such as UV-vis Spectrophotometry, X-ray Diffraction (XRD) and microscopy, either scanning electron microscopy (SEM) or transmission electron microscopy (TEM) to ensure the success of the tests to be carried out.

The application of bimetallic NPs obtained by bioreduction, as catalysts for advanced oxidation processes such as Fenton-like, is a viable field in development where high efficiencies can be found in the removal of organic pollutants; subsequent and complementary studies are required to shed light on the degree of mineralization of the pollutants and the toxicity of the particles used in the process.

Acknowledgements

The first author thanks CONACyT for the national scholarship granted for postgraduate studies, as well as the UAEMex 4986/2020 CIB Project for the finance provided; special thanks are given to the LIA María Citlali Martínez Soto for the support of the documentary film material, to the Dr. Mario Alberto Romero Romo for his support in the translation of this document.

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Chapter 6 Application of beneficial microorganisms rhizobacteria to improve plant production in protected natural areas

Capítulo 6 Aplicación de microorganismos benéficos rizobacterias para mejorar la producción vegetal en espacios naturales protegidos

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DOI: 10.35429/H.2021.3.122.130

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A. Marroquín, J. Olivares, D. Ventura and L. Cruz (Coord) Agricultural Sciences and Biotechnology. Handbooks-©ECORFAN-México, Querétaro, 2021.

Abstract

Protected Natural Areas generate environmental services, form soil, are habitats of wide biodiversity of plants, animals, insects and microorganisms, recharge of aquifers, capture of CO_2, buffer the effects of global climate change. They provide economic resources for the communities that live in the areas. Numerous actions have been generated to maintain the conservation of biological diversity. Through comprehensive strategies and actions, the impact of damage caused by anthropocentric activities can be reduced. Forest soils have an enormous variety of living forms that obtain their energy mainly from forms of organic matter derived from plants and animals. The major biological component of forest soils are the roots of plants, microorganisms and animals in the soil. A group of beneficial bacteria that inhabit the rhizosphere region receive organic acids from plants, and the bacteria provide plant protection and better nutrient uptake. Among the benefits that bacteria provide directly are: nitrogen fixation, phosphorus solubilization, production of phytohormones such as: auxins, gibberellins, indole acetic acid. In the state of Guanajuato, the works that have been carried out with isolated rhizobacteria from the soil are from several Protected Natural Areas and guava orchards. Rhizobacteria isolates have been tested on fruit, food and ornamental plants.

Soil, Forest, Conservation, Bacteria

Resumen

Las Áreas Naturales Protegidas generan servicios ambientales, forman suelo, son hábitats de amplia biodiversidad de plantas, animales, insectos y microorganismos, recarga de acuíferos, captura de CO2, amortiguan los efectos del cambio climático global. Proporcionan recursos económicos a las comunidades que viven en las zonas. Se han generado numerosas acciones para mantener la conservación de la diversidad biológica. Mediante estrategias y acciones integrales, se puede reducir el impacto de los daños causados por las actividades antropocéntricas. Los suelos forestales tienen una enorme variedad de formas vivas que obtienen su energía principalmente de formas de materia orgánica derivadas de plantas y animales. El principal componente biológico de los suelos forestales son las raíces de las plantas, los microorganismos y los animales del suelo. Un grupo de bacterias beneficiosas que habitan en la región de la rizosfera reciben los ácidos orgánicos de las plantas, y las bacterias proporcionan protección a las plantas y una mejor absorción de nutrientes. Entre los beneficios que las bacterias proporcionan directamente están: fijación de nitrógeno, solubilización de fósforo, producción de fitohormonas como: auxinas, giberelinas, ácido indol acético. En el estado de Guanajuato, los trabajos que se han realizado con rizobacterias aisladas del suelo son de varias Áreas Naturales Protegidas y huertas de guayaba. Los aislamientos de rizobacterias se han probado en plantas frutales, alimenticias y ornamentales.

Suelo, Bosque, Conservación, Bacterias

1 Introduction

Forests and Protected Natural Areas are essential ecosystems for life. Many cultures have relied on the products obtained from the forest: wood as fuel or in construction, charcoal, hunting animals, resins, fruits, medicinal plants, etc. In the Mexican territory, at the federal and state level, territorial and aquatic spaces have been designated for conservation, the Protected Natural Areas (PNA) are defined as the areas of the territory where the original environments have not been significantly altered by the activity of the being. human beings either require preservation or restoration. The aim is to conserve the biodiversity of the different ecosystems. The PNAs are regulated by the General Law of Ecological Balance and Environmental Protection with protection, conservation, restoration and development regimes. PNAs generate environmental services, form soil, are habitats of wide biodiversity of plants, animals, insects and microorganisms, recharge of aquifers, capture of CO_2, buffer the effects of global climate change.

They provide economic resources for the communities that live in the areas. There is concern about the proper use of natural resources and to achieve this, numerous actions have been generated to maintain the conservation of biological diversity. Through comprehensive strategies and actions, the impact of damage caused by anthropocentric activities such as deforestation, fires, charcoal production, livestock, agriculture, wildlife hunting, expansion of cities, extraction of soil and litter for sale as potting soil, extraction of plant and animal specimens in endangered cases, among others.

A natural resource within the PNA is the soil, a system of inorganic and organic components and living organisms and microorganisms. In the soil, all the vegetation of the natural areas develops, in which the plants obtain mechanical support, water and nutrients. The soil is a very dynamic system, in which a great variety of biochemical reactions are carried out, facilitated by the microbial communities that inhabit it, the formation of the soil itself and its organic matter, the recycling of nutrients, etc.

A group of beneficial bacteria that inhabit the rhizosphere region receive organic acids from plants and the bacteria provide plant protection and better nutrient uptake. Among the benefits that bacteria provide directly are: nitrogen fixation, phosphorus solubilization, production of phytohormones such as: auxins, gibberellins, indole acetic acid. Other benefits of the plant-rhizobacteria interaction is protection against phytopathogenic microorganisms by the production of toxic compounds that stop the advance of the phytopathogen.

Importance of protected natural areas

The Protected Natural Areas are the areas of the state territory where the original environments have not been significantly altered by human activity or need to be preserved or restored, and in the specific case of those classified as areas of sustainable use, it has for The objective of producing goods and services that respond to the economic, social and cultural needs of the population, based on the sustainable use of compatible uses, being located in areas that include hydrological basins, forest resources and elements of wild flora and fauna, in the that there are agricultural developments, recreational potential and rural populations (Institute of Ecology of the State of Guanajuato, 2015).

The geological characteristics of the area are basalt for the most part, combined with zones of volcanic breccia, both extrusive igneous rocks. The highest parts of the hills present Haplic Faeozem-type soils, easy to erode when found on slopes and slopes, and the remaining surface is dominated by vertisol pelic soils with a stony phase (Instituto de Ecología del Estado de Guanajuato, 2004).

The Guanajuato, Mexico state is located between the parallels 19º 55 '08' 'and 21º 52' 09 " of north latitude and the meridians 99º 41 '06' 'and 102º 09' 07 " of west longitude. The total surface of the state is 30,589 km, see in figure 6.1. The predominant vegetation of the PNA is oak forest (*Quercus deserticola*), tropical deciduous forest and crasicaule scrub. In The Gavia it is represented mainly by trees of the Bursera genus and other species such as *Ipomoea spp.* and *Acacia spp*. The crasicaule scrub in The Culiacán hill is represented by species with succulent stems, associated with spiny-type species, herbaceous elements and some kinds of grasses, while in Gavia there are *Opuntia*, *Myrtillocactus*, *Mimosa spp*., *Acacia spp*., and *Ipomoea spp.* (Institute of Ecology of the State of Guanajuato, 2004) see in figure 6.2.

Figure 6.1 Map of Protected Natural Areas in the state of Guanajuato, Institute of Ecology of the State of Guanajuato, Mexico

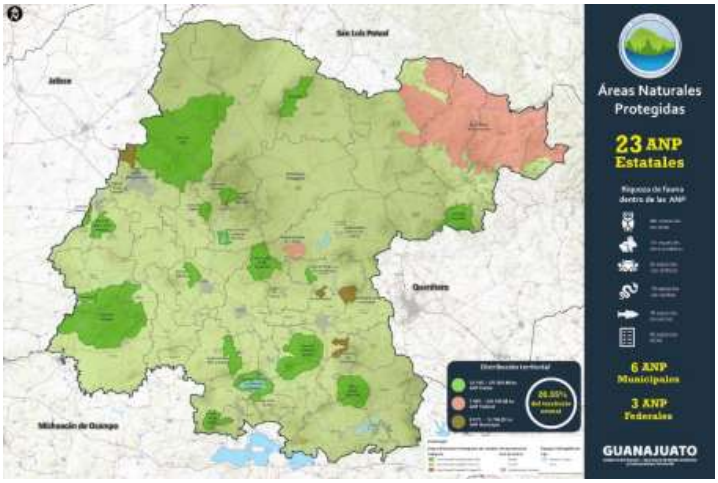
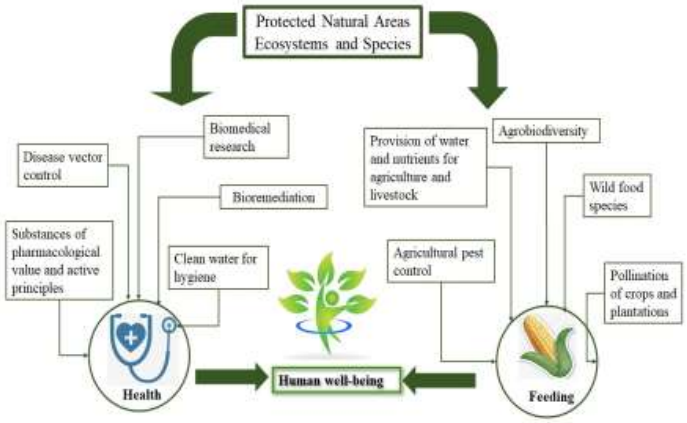


Figure 6.2 Oak forest (*Quercus spp.*) in Protected natural areas in Guanajuato, Mexico

The importance of preserving protected natural areas also lies in the services that these areas provide to humanity, which are observed in the figure 6.3.

Figure 6.3 Services provided by protected natural areas, Secretary of the Environment and Natural Resources and National Commission for Protected Areas (CONANP)



Threats to protected natural áreas

One of the main problems of Cerro del Culiacán and La Gavia is deforestation, caused mainly by changes in land use, use of trees for firewood and handicraft production, extensive cattle ranching, effects by pests and forest fires.

The aquifer resource is extremely scarce and combined with deforestation and loss of soil, it causes an increase in the speed of runoff and a decrease in infiltration and water retention rates; in addition to presenting uncontrolled exploitation of existing material banks in the area (IEE, 2015).

The accelerated destruction of forests by the change of land use for agriculture, livestock, expansion of cities, roads, excessive logging, charcoal production, pests among other forms of soil disturbance. Agrochemical compounds (pesticides, herbicides and fertilizers) can have an adverse effect if they are used in excess, since they can affect the physiology and biochemistry of the soil, reflecting in reduced respiration, enzymatic activity, microbial biomass, nitrogen content and changes in soil microbial diversity (Yang et al., 2000).

Excessive logging is a very common practice in forests and in some it is selective towards some tree species. Deforestation can lead to soil erosion and destatization of the water table, which in turn favors floods or droughts and the loss of biodiversity. The opening of roads and the extraction processes cause the compaction of the soil, making it more susceptible to the loss of nutrients (FAO, 2000, WRI, 2001). Extraction of soil and litter that is sold as potting soil, the sale of these elements leads to more vulnerability to erosion, less water retention, loss of soil organic matter and nutrients, in addition to requiring hundreds to thousands of years for soil formation depending on geochemical, climatic and geographic conditions.

Soil and microbial communities

Forest soils have an enormous variety of living forms that obtain their energy mainly from forms of organic matter derived from plants and animals. The major biological component of forest soils are the roots of plants, microorganisms and animals in the soil. Together these organisms play an important role in the function of forest ecosystems through their participation in the degradation of organic matter. Through these processes there is a positive effect on the availability of nutrients for plant growth and on the structure of the soil.

Soil as an ecosystem is made up of five main components: mineral matter, water, air, organic matter, and living organisms. Soil contains five main groups of microorganisms: bacteria (archaea), actinomycetes, fungi, algae, and protozoa (Alexander, 1980). Bacteria are particularly prominent because there are many populations in a soil and because they are the most abundant group. The role of bacteria in the soil is very varied: they may be participating in the regulation of biogeochemical cycles, in association or interaction with plants, nitrogen fixation, growth promotion, degradation of xenobiotic compounds, recirculation of nutrients, degradation of cellulose, hemicellulose, lignin, polysaccharides, hydrocarbons, proteins, urea, immobilization of organic phosphorus, oxidation of sulfur, formation of organic iron compounds, mobilization of potassium, etc., (León, 1991).

Rhizobacteria

Plant growth-promoting rhizobacteria are a group of bacteria that inhabit the root of plants and the soil attached to it, this space is known as the rhizosphere (Cassán et al., 2009). A variety of organic acids are produced in the rhizosphere that can be metabolized by rhizobacteria. The rhizobacteria in turn provide nutrients from the soil to the plant (Marschner, et al, 2004; Lugtenberg and Kamilova, 2009). This group of bacteria provide benefits to plants through various mechanisms: N_2 fixation, phytohormone production, phosphate solubilization, synthesis of enzymes such as ACC deaminase that reduces ethylene levels, biological control, siderophore production, antibiotics, activation of the induced systemic response and production of lytic enzymes (Glick, 1995; Dobbelaere, et al, 2003; Esquivel et al., 2013). The products generated by the various mechanisms have direct and indirect effects on the plant's development and growth, such as: improvement in germination, greater development of the root, stems, leaves and fruits or defense against phytopathogenic organisms (Glick, 1995; Dobbelaere et al., 2003; Esquivel et al., 2013). Bacteria with ACC deaminase activity are of special interest since these can decrease the level of ethylene in the plant root, due to the degradation of the ethylene precursor, 1-aminocyclopropane -1-carboxylic acid (ACC). Bacteria carry out this process by means of the ACC deaminase enzyme, which by degrading ACC generates ammonium and □-ketobutyrate products, thus the bacteria attached to the root of the plant consume ACC and lower the level of ethylene associated with stress signals and favor root elongation (Glick, et al, 1999, Holgin et al., 2003, Esquivel et al., 2013).

The use of rhizobacteria in plants of agronomic importance has resulted in an important alternative to production systems with a high consumption of fertilizers and agrochemicals (Grageda, et al., 2012; Martínez, et al., 2013), however, research and monitoring of use for plants of forest importance or for recovery and conservation of PNA is much less. In some of these works they found that the benefits of rhizobacteria have been used for reforestation of desert areas (Berreto, et al, 2007; Ogata, et al, 2008). Due to its ability to fix nitrogen, *Azospirillum brasilence* has been used in cardón plants where they achieved improvement in the development of the plant and in the regeneration of the soil (Holguin, et al., 2003). The genera of *Pseudomonas*, *Rhizobium*, *Bradyrhizobium*, *Azotobacter*, isolated from the rhizosphere of *Caesalpinia spinosa* "tara" trees were tested on alfalfa (*Medicago sativa*), tara (*Caesalpinia spinosa*), pallar (*Phaseolus lunatus*) and bean (*Phaseolus vulgaris*) seeds and Ogata, et al (2008) observed that the tested strains increase the germination of the mentioned crops.

In another work, strains were isolated from the rhizosphere of *Anacardium excelsum*, which is an arboreal species native to dry forests in Central and South America. The isolates were from *Pseudomonas fluorescens*, *P. putida* and *Bacillus licheniformis* and were used to evaluate germination and growth of *Anacardium excelsum* seedlings, finding positive effects in both processes (Barreto, et al 2007).

Other authors reported the effectiveness in forest restoration, testing growth-promoting rhizobacteria in 11 native species (*Tipuana tipu* (Benth O.) Kuntze (white tipa), *Pterogyne nitens* Tul (*Tipa colorada*), *Aspidosperma quebracho* blanco Schlecht (Quebracho blanco), *Schinopsis lorentzii* (Griseb) Engler (Quebracho colorado santiagueño), *Enterolobium contortisiliquum* (Well) Morong (Pacará, timbó), *Jacaranda mimosifolia* D. Don (Jacarandá or tarco), *Cedrela lilloi* C. DC. (Cedro coya or hairy cedar), Caesalpinia paraguariensis (D. Parodi) Burkart (Guayacán), *Prosopis chilensis* (Mol) Stuntz Chilean Algarrobo, Juglans australis griseb. (Nogal criollo), *Anadenanthera colubrina* Griseb (Cebil Colorado) from the forests of Jujuy, Argentina. Here they observed improvements such as the germination, development, establishment in the site to be recovered and plant health, proving that the isolated strains are effective for the purposes (Lázzaro, et al., 2011).

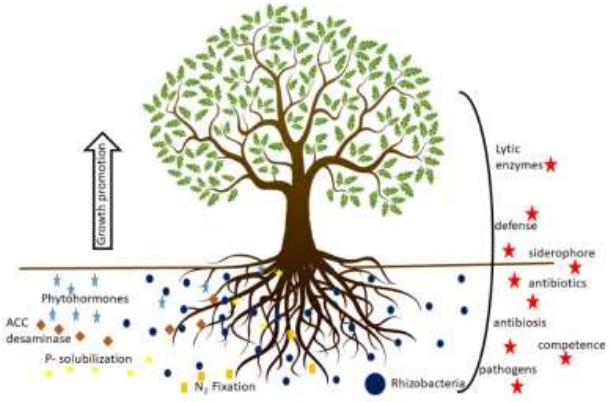
With other purposes, it has begun to use plant growth promoting bacteria as components of environmental improvement for the recovery of soils in degraded arid zones, reintegrating native vegetation with plant growth promoting microorganisms (García, et al., 2013).

Biofertilizers made from bacteria

The soil is inhabited by many microorganisms, among them are bacteria that can be free-living in the soil, in soil attached to the roots of plants and in the cells of the roots of plants, forming a symbiosis (association between two organisms with benefits for both). Of all the groups of bacteria that inhabit the soil, the so-called PGPR stands out for its acronym in English (Promoting Grow Plant Rhizobacteria), in Spanish rhizobacteria that promote plant growth, rhizobacteria refers to that inhabit the space called rhizosphere (Cassán et al., 2009), soil space in close contact with plant roots. This type of plant-bacteria interaction occurs in a special way in the rhizosphere because the roots of the plants produce a variety of organic acids that can be metabolized by bacteria and bacteria in turn provide nutrients from the medium to the plant (Marschner, et al., 2004; Lugtenberg, and Kamilova, 2009).

Two mechanisms have been described by which plant growth-promoting bacteria provide a benefit to the plant, which are known as: direct and indirect mechanisms. Among the benefits that bacteria provide directly are: nitrogen fixation, phosphorus solubilization, production of phytohormones such as: auxins, gibberellins, indole acetic acid (Glick 1995; Dobbelaere et al., 2003; Esquivel et al., 2013). On the other hand, the benefits that this group of bacteria provides to plants indirectly through the production of compounds that can be antibiotics, siderophores (a molecule that traps iron), among others, that act on other microorganisms that can cause diseases in plants. plants, these mechanisms are seen in figure 6.4 (Glick 1995; Dobbelaere et al., 2003; Esquivel et al., 2013).

Figure 6.4 Mechanisms of the beneficial effects of rhizobacteria on plants



Applications of rhizobacteria with plants from natural áreas

It is well known that there is a continuous deterioration in environmental quality throughout the planet due to human activities on natural areas, mainly deforestation, burning of waste with excessive release of pollutants, such as heavy metals, extraction of forest soil (potting soil), change in the use of land for agricultural production with excess of agrochemicals and pesticides and grazing areas and animal production. Some of the emerging strategies are the use of technologies that plants use as extractors, mitigators and stabilizers of pollutants (phytoremediation) or that stabilize and recover soil productivity (reforestation) in conjunction with beneficial rhizobacteria and other organisms such as mycorrhizal fungi.

Some plants cannot establish in these degraded systems, and even if they do, they are generally affected by adverse environmental conditions, such as excessive concentration of pollutants, extreme pH (high or low), shortages. of nutrients, poor soil structure (organic matter and others) and a severely affected or even eliminated microbial community; As a way to help in its establishment, its inoculation with beneficial rhizobacteria and arbuscular mycorrhizal fungi has been proposed. These microorganisms are currently under experimentation in programs of revegetation, reforestation of eroded soils, biological wastewater treatment, phytoremediation, phytostabilization and restoration of ecosystems (De-Basch, 2013).

The use of rhizobacteria in plants of agronomic importance has resulted in an important alternative to production systems with a high consumption of fertilizers and agrochemicals, thus working towards sustainable agriculture (Orona and Leos, 2020), however, research and monitoring of use for plants of forest importance or for recovery and conservation of PNA is much less. In some of these works they found that the benefits of rhizobacteria have been used for reforestation of desert areas (Berreto et al., 2007). In another work, strains were isolated from the rhizosphere of *Anacardium excelsum*, which is an arboreal species native to dry forests in Central and South America. The quantity and diversity of growth-promoting rhizobacteria has been considered as a biological marker of soil health and quality, comparing a forest and an agricultural soil, taking into account the physicochemical properties of each soil (Flores et al., 2018). In some works, efficient microorganisms have been used to improve plant production, as indicated by Sucapuca (2021) by applying efficient microorganisms in wheat crops in winter season (*Triticum aestivum L.*) and found improvements in forage yield and nutrient content, in addition to continuing to use it, it could reduce costs of production. In another proposal, efficient microorganisms were used in urban orchards for tomato (*Solanum lycopersicum*) production, finding improvement in production by using efficient microorganisms, with the possibility of reducing the application of agrochemicals (Frías, 2021).

In the state of Guanajuato, the works that have been carried out with isolated rhizobacteria from the soil are from various PNAs and from guava orchards. The rhizobacteria isolates were characterized and a collection was generated with gram positive and gram negative bacteria, the majority of the genus Bacillus spp. To verify their capacity for growth-promoting rhizobacteria, they were tested with plants of importance for the PNA such as Huizache (*Acacia farneciana*), which is a vegetation of the region and of economic and ethnobotanical importance; Furthermore, guava (*Psidium guajava*), lentil (*Lens culinaris*), cucumber (*Cucumis sativus*), radish (*Raphanus sativus*) have also been tested with plants of nutritional importance; in ornamentals such as marigolds (*Tagetes erecta*) and sunflowers (*Helianthus annuus*). In all cases there have been beneficial effects from the germination of the seeds and their development. Additionally, the rhizobacteria isolated from the PNAs also presented plant protection and health capabilities by producing compounds that reduce or stop the growth of phytopathogenic fungi, that is, they have potential for biological control, the benefits in seed germination are indicated (Gómez-Luna, et al 2012; Gómez-Luna, et al 2018; Gómez-Luna, et al 2020).

Conclusions

- Protected Natural Areas provide diverse services and resources for human beings and it is their responsibility to make a sustainable use of resources.

- A biotechnological and eco-friendly strategy is the use of beneficial microorganisms such as rhizobacteria, isolated from the soils of the PNA and applied to improve the plant production of the area and also plants of nutritional importance.

– With the application of rhizobacteria, the use of agrochemicals in plant production could be reduced.

– Rhizobacteria have the ability to be used as biofertilizer, biocontrol and biostimulant of the soil and with this regenerate or conserve the PNA and thus all its resources and services.

– These rhizobacteria that already exist naturally in the soils of the PNA.

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Chapter 7 Adaptability and rusticity of zebu breeds over pure European breeds in the climates of the Mexican tropics

Capítulo 7 Adaptabilidad y rusticidad de las razas cebú sobre las razas Europeas puras en los climas del trópico Mexicano

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DOI: 10.35429/H.2021.3.131.140

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Abstract

This investigation was carried out in a production unit in the north of the state of Puebla, Mexico, located between the parallels 19º 47 '06 "and 19º 58 '12" north latitude and 97º 18 '54 "and 97º 23 '18" in western longitude. With the aim of carrying out the phenotypic differences (coat color, ear morphology, horns, profile and body condition) between the zebu breeds (Brahman, Guzerat, Indubrasil) with the highest demand in the livestock region. Likewise, describe its adaptability to tropical climates that exceed 30 °C and its superiority over synthetic and pure European breeds destined for meat production (Beefmaster, Charbray, Brangus, European Swiss, Angus and Hertford). Phenotypic characteristics were recorded individually for each of the animals using descriptive statistics to establish the breed patterns. A total of 37 stallions with variable age and weights were evaluated. The main breeds evaluated were Brahman (2.70%), Indubrasil (54.05%), Guzerat (2.70%) crossbreeds European Swiss (13.51%) and Zebu x European Swiss (27.02%), which presented very specific phenotypic characteristics among these breeds. Despite the data collected from the zebu breeder associations, there is very little information on the phenotypic differences of the zebu breeds that have been present for centuries in the systems under which it survives, demonstrating at the same time its greater adaptability and superiority. to tropical soils and pastures, when compared with synthetic and pure European breeds intended for meat production.

Cattle, Zebu, Adaptability, Breeds, Tropics

Resumen

Esta investigación se llevó a cabo en una unidad de producción en el norte del estado de Puebla, México, ubicada entre los paralelos 19º 47 '06 "y 19º 58 '12" de latitud norte y 97º 18 '54 "y 97º 23 '18" en longitud occidental, con el objetivo de realizar las diferencias fenotípicas (color de pelaje, morfología de las orejas, cuernos, perfil y condición corporal) entre las razas indias (Brahman, Guzerat, Indubrasil) de mayor demanda en la región ganadera. Asimismo, describe su adaptabilidad a climas tropicales que superan los 30 °C y la superioridad sobre las razas europeas puras destinadas a la producción de carne y leche (Suizo Europeo, Brahman x Suizo Europeo). Las características fenotípicas se registraron individualmente para cada uno de los animales utilizando estadísticas descriptivas para establecer los patrones de raza. Se evaluaron un total de 37 sementales con edad y peso variables. Las principales razas evaluadas fueron Brahman (2.70%), Indubrasil (54.05%), Guzerat (2.70%) Suizo Europeo (13.51%) y cruza Cebú x Suizo Europeo (27.02%), que presentaron características fenotípicas muy específicas entre estas razas. A pesar de los datos recogidos de las asociaciones de criadores de cebú, existe muy poca información sobre las diferencias fenotípicas de las razas indias que han estado presentes durante siglos en los sistemas bajo los que sobreviven, demostrando al mismo tiempo su mayor adaptabilidad y superioridad a los suelos y pastos tropicales, en comparación con las razas europeas puras destinadas a la producción de carne y leche.

Bovinos, Cebú, Adaptabilidad, Razas, Trópico

Introduction

Zebu breeds originated in 1900, with the importation of cattle from countries such as India and France, in order to develop new breeds that could adapt to certain tropical zones (Cañas *et al*., 2008). Cattle raising in almost all Latin American countries began when the colonizers brought cattle from temperate countries, which did not find favorable conditions for expansion in their new environment. In general, the efforts of technical services to introduce and adapt improved European breeds were unsuccessful. Imports by thousands of breeders, carried out at different times, yielded poor results and European thoroughbreds did not survive, perished due to lack of adaptation or were absorbed when crossed with native cattle (Montes *et al*., 2009).

The establishment of European breeds in the tropics has been done with obvious difficulty. Practice has shown the difficulties of raising European cattle in tropical regions. When encountering adverse conditions, cattle rot rapidly and after a few generations are no longer the size of those that preceded them; meat and milk production is reduced, the birth rate decreases and the mortality rate increases (BIF, 2002).

The inability of the European bull to eliminate excess body heat hinders adaptation to regions with hot climates and low nutritional quality of forages. Zootechnical climatology studies demonstrate the difficulties of raising fine, highly specialized animals in tropical and subtropical areas. Under these conditions, livestock production in the tropics is limited to raising native breeds, some already in the process of improvement, or imported livestock of Asian origin. Therefore, it was necessary to resort to a bovine type natural to the tropics (ASOCEBU, 2007).

The Zebu breeds emerged as a cattle saving breed, revitalizing the blood of exhausted herds of Creole cattle, with low economic performance or giving conditions of resistance to improved cattle of European origin. Brazil was the first importer of Asian breeds, multiplying the efforts for their introduction, extending them throughout the national territory and beginning to take care of their improvement (BIF, 2002).

Cattle of Zebu or Hindustani origin are breeds that have attracted attention for their great adaptation to tropical climates, hardiness and resistance to the adverse conditions of the tropical environment. Brazil and India are tropical countries. The cattle that live and prosper in that Asian nation, in spite of all adverse factors, were the cattle naturally indicated to populate Brazil, thus understanding the value of Bos indicus for its cattle (Domínguez *et al*., 2003). The objective of this study was to determine the phenotypic differences (coat color, morphology of the ears, horns, profile and body condition) between the zebu breeds (Brahman, Guzerat, Indubrasil) most in demand in a livestock production unit in the northern part of the State of Puebla. Likewise, to describe their adaptability to tropical climates that exceed 30 °C and their superiority over synthetic and pure European breeds for meat production (Beefmaster, Charbray, Brangus, Swiss European, Angus and Hereford.

Material and Methods

This research was carried out in a production unit in the north of the state of Puebla, Mexico, located between parallels 19º 47 '06" and 19º 58 '12" north latitude and 97º 18 '54" and 97º 23 '18" west longitude. The objective was to carry out the phenotypic differences between the Indica breeds most in demand in the cattle region. For this purpose, a form was developed with a capacity for 37 animals, which was used to record the number and breeds encountered during the tours. To better locate the cattle herds, we had the valuable collaboration of the manager of the livestock production unit, who was a key informant on the breeds evaluated. On several occasions it was necessary to enter the production unit through long roads that can only be accessed on foot or on horseback, sometimes even crossing rivers that are quite low due to the dry season.

In order to carry out the phenotypic differences between the Indica breeds, a format was developed, which was used to record the phenotypic characteristics individually for each of the animals, such as: breed type, sex, age, coat color, type of horn, direction of the ears and body condition. Some important aspects of the management given to the animals and the particular environment in which they were found were also recorded, such as the type of predominant vegetation and the degree of utilization of the paddocks or grazing areas. The body condition of the cattle evaluated was also recorded by direct observation of the animal, according to a subjective scale ranging from 1 (skinny) to 5 (fat). The data were captured in a general information bank (Microsoft Office Excel package), from which descriptive statistics were performed taking into account the database of the evaluated breeds. (paquete Minitab, versión 10.1).

Results and Discussion

This study determined that, in a single production unit of 400 hectares, there are Indica breeds and their crosses (n=37 bulls), whose existence is officially recognized at the international level, among them: Brahman, Guzerat, Indubrasil, Swiss European and the F1 cross (Table 7.1). These breeds have undergone significant improvements, to the point of constituting, in certain aspects, different and improved types of the equivalent Hindustani varieties, even with new characteristics acquired by virtue of the action and especially of a great genetic selection of these breeds. So that the development of livestock does not constitute, from the socioeconomic point of view, a delay in agriculture, it is necessary to introduce new and better methods of breeding and fattening, animal feeding and sanitary defense, pasture improvement, fodder preparation, selection and improvement of Indian breeds, using breeds such as the European Swiss and the Brahman x European Swiss crosses (F1) (De Lira, 2008).

Table 7.1 Breeds and crossbreeds of cattle evaluated at the production unit

Total animals	Brahman	Indubrasil	Guzerat	Swiss European	Cross (F1) Brahman x European Swiss
37	1	20	20	5	10
%	2.70	54.05	2.70	13.51	27.02
Body Condition	3.5	4.0	3.2	3.0	3.0

Source: Own Elaboration/2021

As can be seen in Table 7.2, three Indica breeds, one European breed and an F1 cross were located in the production unit evaluated. It was observed that there are very notable phenotypic differences between the Indica breeds, European Swiss and the Brahman x European Swiss cross (F1). According to the statistical and descriptive analysis, the predominant breed was the Indubrasil (54.05%) with a body condition of 4.0, which since its creation has shown great hardiness, masculinity, maternal ability, meat and milk production, excellent feed conversion in fattening bulls and great adaptability to climate, pastures and soil in a tropical climate.

Table 7.2 Phenotypic differences between the breeds and crossbreeds of cattle evaluated in the production unit

Race	Coat color	Ear morphology	Horn morphology	Head profile	Body condition range
Brahman	White Gray Red	Short and slightly dangling	Tilted upwards instead of downwards and outwards	Straight	2.0 - 4.0
Guzerat	Light gray Dark gray	Elongated and oblique broad ears	Rather thick long horns of circular section; implanted vertically and projected upward symmetrically in the form of an arc like a half lyre and ending in a backward direction	Subconcave	2.0 - 4.0
Indubrasil	White Gray Red	They are large pendants, long up to the middle of the neck and the tip is directed forward.	The horns are black, medium-sized and laterally set, directed backwards	Subconvex	2.0 - 4.0
Swiss European	Light brown Dark brown	They are short and outward facing, giving the appearance of a tree leaf.	The horns are white with black tips, medium or small, some of them do not present	Straight	2.0 - 4.0
Brahman x European Swiss (F1)	Light gray Dark gray Light brown Dark brown	They are short, slightly bent at their extremities, directed outwards	The horns are black, short and laterally set.	Straight	2.0 - 4.0

Source: Elzo et al. (2003)

Brahman breed

Noting that Brahman cattle (Fig. 7.1 and 7.2) emerged from four *Bos indicus* breeds that contributed to the foundation of the American Brahman; Guzerat, Nelore, Gyr and Krishna Valley, which arrived in the United States in different shipments between 1854 and 1946. These animals were carefully crossbred, strictly selected and rigorously culled to form a new beef breed that would adapt to the world's most hostile tropical and subtropical climates. Now established in 60 countries around the world, it has improved meat production around the world, making meat production more efficient in the tropics (Elzo *et al.,* 2003). Phenotypically they are large in size, the head is long compared to other meat producing breeds. The horns generally appear tilted upward instead of downward and outward, as in the European horned breeds. Short, slightly pendulous ears, voluminous belly, straight profile, short thick neck, well defined hump centered on the shoulders, loose and mobile skin, large dewlap, all combined with excellent meat characteristics and muscular expression. The color varied from light red to black, with gray being the predominant color (Montes *et al.,* 2008).

Figure 7.1 Gray Brahman

Figure 7.2 Red Brahman

Source: Montes et al. (2008)

Guzerat breed

The Guzerat breed (Fig. 7.3 and 7.4) originated in the northern and southwestern region of India. The animals of this breed show in both sexes a majestic appearance and great presence when walking, as they carry their heads erect, with voluminous and striking horns (Castaño, 2003). In short, their appearance denotes physical strength. The head is moderately wide and short in the male, and longer and narrower in the female; subconcave profile, straight face and wide muzzle, pigmented in black; large black eyes with meek expression; long horns quite thick of circular section; implanted vertically and projected upwards symmetrically in the form of an arc like a half lyre and ending in a backward direction; wide, elongated and oblique ears; its short neck is relatively thick.

The body well developed, with a deeper and longer thorax, hump of good size and shape; limbs of medium length, strong bones. Black skin colors and dark and light gray coat in both males and females, large and pendulous foreskin and both birth weight and growth rate are similar in both sexes (Arboleda *et al*., 2008).

Figure 7.3 Guzerat dark gray

Figure 7.4 Guzerat light gray

Source: Arboleda et al. (2008)

The selection of this breed led to produce animals with good finishing aptitudes for butchering, demonstrating in practice and in weight gain tests. The same result for milk production, instituting selective programs and individual cow productivity controls with the purpose of founding the dairy Guzerat. Specimens of this breed were transported to the United States at the beginning of the 9th century and then had a preponderant participation in the integration of the Brahman breed and even some specimens (females and males) were taken to Central and South American countries, achieving significant progress. The average birth weight is 28 kg and at weaning (adjusted to 210 days) is 1184 kg. Bulls in good condition can weigh 730 kg (478 kg at 4 years of age) and adult cows 460 kg. However, males have been recorded reaching 110 kg and females 780 kg (Cañas *et al.,* 2008).

Indubrasil breed

It should be noted that the Indubrasil breed originated in the Triangulo Mineiro Brazil, located in the west of the state of Minas Gerais and particularly in the surroundings of Uberaba, at the beginning of the 20th century. The Indubrasil arrives through crossbreeding of Asian breeds such as Gyr, Guzarat and Nelore. And initially it was named as Brazil induced cattle for having in a single breed the best characteristics of the three breeds introduced in Brazil (Montes et al., 2009). A very important characteristic of the breed is its typical ears, which are hanging ears that can range from medium to large, the shape of the ear varies, but they are directed with the tip forward (Fig. 7.5 and 7.6).

Figure 7.5 Ears on male Indubrasil

Figure 7.6 Ears on female and calf Indubrasil

Source: Teyer et al. (2003)

It is an animal of large body volume, the color of the coat ranges from light gray, dark gray and red, it is a vigorous breed like the Nelore, with a somewhat nervous temperament (Fig. 7.7 and 7.8). Gyr cattle maintain some characteristics of the subconvex head profile. The horns are medium-sized and laterally implanted, directed backwards, reports of adult males of the Indubrasil breed are that they are animals that can exceed 1200 kg in weight, and cows can weigh up to 750 kg. The most common data can be that males range between 800-1200 kg live weight and cows between 500-700 kg live weight. In the case of weight gain, the Indubrasil cattle breed shows excellent values according to management conditions, which can range between 400 grams and 1000 grams per day, with an average of 650 grams. Indubrasil is currently present in several countries in the world such as Venezuela, Colombia, Costa Rica, Panama, Guatemala, Mexico, Thailand, South Africa and Australia (Montes et al., 2009).

Figure 7.7 Indubrasil dark gray

Figure 7.8 Indubrasil red

Source: Chan et al. (2010)

This breed has shown its versatility both as a pure breed and in crossbreeding with other breeds. In the case of Mexico, this breed is used to cross it with European Swiss to increase meat production and adaptability and hardiness, as well as crosses with American Swiss breeds, achieving a greater increase in milk production of Indubrasil cows (Elzo *et al.,* 2003).

Breed Swiss European

The Swiss European breed (also known as Brown Swiss) originates from the middle eastern part of Switzerland in Europe, is famous all over the world and is the second breed for milk yield. In Switzerland it competes with the Simmental for milk and meat supply. Phenotypically it is characterized by its medium size, short, fine and soft hair; pigmented skin; shows a black color on the exposed part as the muzzle, the horns are white with black tips, medium, small or absence of them, directed outward and upward, curving at the tips. The head is broad and moderately long. The back is broad and the dorsal line straight. The chest is deep, ribs arched and the hindquarters are fleshy (Castaño, 2003).

López et al. (2009) reported that the Swiss European breed is recognized for its excellent short, thick, short legs and black hooves, traits necessary for the evolution of the breed in the Swiss Alps, which gives it advantages in grazing. The udder is well developed, attached and has excellent teats. Its outer coat is of a single light to dark brown color (Fig. 7.9 and 7.10).

Figure 7.9 Swiss European light brown

Figure 7.10 Swiss European light Brown

Source: Castaño et al. (2003)

Adult animals are strong and of good weight, cows can weigh from 600 kg to 700 kg and bulls from 950 kg to 1000 kg, but there are specimens of both sexes with more weight. Regarding their milk yield, the current average is 7,200 kg adjusted at adult age with 4.0% fat, these averages are those of the United States of America, which is the highest in the world for this breed. The Swiss-Austrian average is 5,103 kg and Germany 6,030 kg (López *et al.,* 2009). The average for Swiss-Mexican cattle is irrelevant, since this breed is not left as dairy cattle in an intensive system, as in the case of cattle from the United States of America, but is produced as dual purpose cattle (1,500 to 2,000 kg per lactation) although in tropical regions averages of 3,200 to 4,000 kg are reported for this breed, which cannot be doubted given the good adaptation shown in warm climates by Swiss European cattle (Chan *et al*., 2010).

Brahman x Swiss European cross (Suiz - Boo)

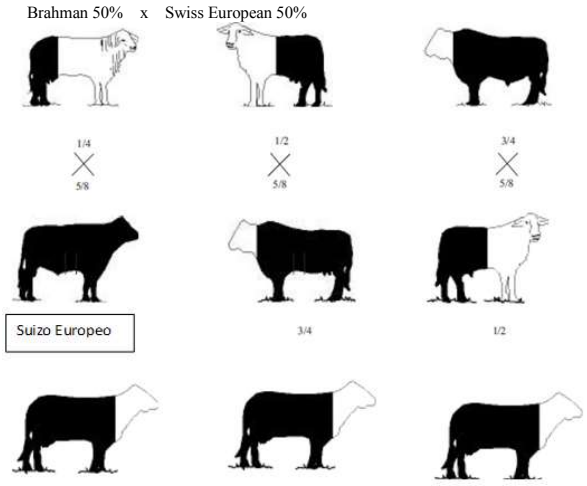
The recognition of the Suiz-Bú breed in Mexico dates back to the 80's. This breed was generated with the purpose of obtaining an animal suitable for tropical conditions and efficient production, contemplating characteristics such as docility, longevity, hardiness, precocity, conversion (fast development), fertility, calving ease, maternal ability, marked pigmentation, as well as adequate milk (rich in fat) and meat production (AMCSB, 2016). Dual-purpose cattle systems are considered a traditional livestock production system in the tropical region, where cows are milked once or twice a day with the support of the calf and their main source of food is green forage. Animal production in the tropics represents one of the most important alternatives that Mexico has to reduce the milk deficit, due to the number of cows available and the amount of basic usable natural resources, such as soil, water and pasture. However, the improvement of milk production in tropical conditions depends on direct factors related to the animal and genotype, as well as environmental factors such as feeding, management, days in lactation and number of calving (Chan *et al*., 2010).

In many countries with tropical climates, crossbreeding of Indian breeds with European breeds (Swiss European, Swiss American, Jersey, Holstein, among others) has been a common practice in dual-purpose herds to improve traits of composition and production of milk, meat, adaptability to the environment, survival and fertility. Among these traits, it has been observed that fertility has the greatest impact on cattle efficiency (López *et al.,* 2009).

The percentages of blood obtained are confractions of 1/2, 3/4, 5/8, with cases up to 15/16. The F1, which provide 50% of the sire and 50% of the dam (Fig. 7.11), are better for tropical conditions. Crossbreeding allows the introduction of favorable genes and takes advantage of racial complementarity and heterosis. Breed complementarity allows breeders to capitalize on the strengths of the different breeds, since no single breed is superior in all traits that affect profitability. In Mexico, the dual-purpose production system is mainly constituted by crosses between Brahman and European Swiss (Cabrera *et al.*, 2013).

A crossbred animal can become purebred over time according to the crossbreeding system used. It is called an absolving system, in which a pure A breed is crossed with a pure B breed and the offspring that is born absorbs the pure genes. The use and implementation of these breeds in the tropics brings with it a series of advantages to be taken into account, more resistant to sanitary conditions, younger age and higher weight at puberty, better birth weights and calving facilities, higher milk and meat production with higher weaning weights (AMCSB, 2016).

Figure 7.11 Type of crossbreeding to obtain the Suiz-Bú breed



Source: López et al. (2009)

In this example we are looking at the crosses necessary to reach a ratio of 5/8 Swiss European and 3/8 Brahman, starting from a pre-existing genetic base. With F1 animals crossed with a 3/4 Swiss European and 1/4 Brahman, they must be crossed with an F1. The dominant proportion of one or the other breed is determined by the breeder based on what he wishes to have for his cattle. This process of crossbreeding must be accompanied by evaluations and selections of the females to be used and a serious choice of bulls to be used in each crossbreeding. To achieve excellent results we must choose excellent specimens, it is a costly procedure, but it will be worth it (López et al., 2009).

Phenotypically it is characterized by its tall stature, short, fine and soft hair; pigmented skin, medium sized black horns, directed outwards and upwards, curving at the tips. The head is broad, moderately straight, with broad back and straight dorsal line, deep chest, arched ribs and the hindquarters are fleshy. Excellent straight and thickly set, black hooves, well-developed udder, well attached and excellent teats. The external coat (Fig. 7.12 and 7.13) is characterized by being is a single light to dark brown color (AMCSB, 2016).

Figure 7. 12 Suiz-Bú light brown

Figura 7. 13 Suiz-Bú dark Brown

Source: AMCSB (2013)

Conclusions

Practice has shown the difficulties of raising European cattle in the tropical region, facing adverse conditions, where cattle decline rapidly and after a few generations no longer have the size of those that preceded them. Meat and milk production is reduced, birth rate decreases and mortality increases, the impossibility of European cattle to eliminate excess body heat makes it difficult to condition them in tropical climates. Therefore, it is necessary to cross zebu breeds with European breeds and simultaneously obtain milk and meat, in addition to increasing the potential for adaptation to tropical conditions.

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Chapter 8 Preparation and use of intravaginal sponges for induction of estrus in hair sheep

Capítulo 8 Elaboración y uso de esponjas intravaginales para la inducción del estro en ovejas de pelo

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Abstract

The aim of the study was to assess the effectiveness of handmade sponges for the induction of estrus in hair sheep. Sixty multiparous females were used, which were divided into three groups: 1) ChronogestCR commercial sponge, 2) FGA commercial sponge and 3) handmade sponge. A sponge was inserted intravaginally to each sheep according to the assigned treatment and remained *in situ* for 12 days, two days before the sponge was removed 300 IU of eCG was administered intramuscularly, and 24 hours after removal of the sponge, estrus was detected using tester ram, and were controlled breeding (morning and afternoon). At 17±1 day, the estrous detection was performed again to obtain the percentage of repetition of estrus. All the sheep in the three groups were in estrus. The percentage of non-return to estrus was 73% in ewes with Chronogest sponge, 65% in ewes with handmade sponge and 63% in ewes with FGA sponge, without statistically differentiating ($p>0.05$). In conclusion, the use of a handmade sponge for the induction of estrus in sheep was as effective as the two commercial sponges in the presentation of estrus.

Sheep, Estrus induction, Intravaginal sponge, FGA

Resumen

El objetivo fue valorar la efectividad de las esponjas elaboradas artesanalmente para la inducción del estro en ovejas de pelo. Se utilizaron 60 hembras multíparas, las cuales fueron divididas en 3 grupos: 1) esponja comercial ChronogestCR, 2) esponja comercial FGA y 3) esponja artesanal. A cada oveja se le insertó intravaginalmente una esponja de acuerdo al tratamiento asignado y permaneció *in situ* durante 12 días, dos días antes del retiro del dispositivo se aplicaron 300 UI de eCG vía intramuscular, y 24 horas después del retiro del dispositivo se detectó el estro utilizando un macho cubierto con mandil, y se realizó la monta dirigida (mañana y tarde). A los 17±1 día se realizó nuevamente la detección del estro para obtener el porcentaje de repetición de celos. Todas las ovejas de los tres grupos presentaron celo. Respecto al porcentaje de no retorno a celo, las ovejas con esponja Chronogest obtuvieron el 73%, las ovejas con esponja casera el 65% y las ovejas con esponja FGA el 63%, sin diferenciarse estadísticamente ($p>0.05$). En conclusión, el uso de una esponja de elaboración artesanal para la inducción del celo en ovejas fue tan efectivo como las dos esponjas comerciales en la presentación del celo.

Inducción de estro, Esponja intravaginal, Ovejas, FGA

1 Introduction

Lamb meat in Mexico is mainly consumed in the form of barbacoa and mixiote, typical dishes from the central region of the country (Martínez *et al*., 2011). Among the main sheep meat producers, the State of Mexico stands out nationally. Per capita consumption of sheep meat is estimated between 0.800 and 1.0 kg and imports approximately 10,379 tons (SAGARPA, 2017; Valadez et al., 2020) from New Zealand and Australia, where they have production subsidies and large forage extensions superior in quantity and quality with respect to the Mexican ones (Bobadilla-Soto *et al*., 2017; Valadez *et al*., 2020).

The need to supply the demand for sheep meat makes the search for production alternatives, one of these alternatives is reproductive biotechnologies.

Sheep are seasonal polyestrous, which means that their reproductive behavior is linked to the time of year, with photoperiod being the main environmental factor that influences the onset or cessation of reproductive activity; therefore, there is a reproductive period during short days and an anestrus period when the length of the day is longer, which affects production (Porras *et al*., 2003). The induction of estrus and ovulation in ewes consists of the use of effective and easily applicable pharmacological methods (Lozano *et al.,* 2012), which allow manipulating the reproductive physiology of ewes, allowing the implementation of reproductive programs and optimizing production and reproduction (Córdova-Izquierdo *et al*., 2008).

There are two situations in which exogenous hormone treatments can be applied: the first is when ewes are in anestrus and their ovaries are not active, generally this occurs in the months of March, April and May in all breeds, therefore, estrus or oestrus is induced.

The usefulness of inducing estrus is to have lambs at times when there are generally no births, reducing the interval between lambing ewes and increasing the number of offspring born. The second situation is when the ewes are in the reproductive season, therefore, their ovaries are active and the ewes present estrus every 17 days, in this case the hormonal treatment only groups the presentation of estrus, that is, synchronizes estrus, which allows having groups of ewes of similar reproductive status and the use of technologies such as artificial insemination or embryo transfer (Trejo, 2016). The most commonly used methods for induction and/or synchronization of estrus and stimulation of follicular growth in ewes include prostaglandins, progesterone, progestogens and intramuscular administration of equine chorionic gonadotropin (eCG). The use of progestogens is the simplest artificial method to induce estrous behavior and ovulation in ewes, since it mimics the presence of a corpus luteum of a natural estrous cycle (Mejia, 2019). The synchronization of the estrous cycle associated with artificial insemination schemes, constitute a useful tool to improve reproductive efficiency, flock productivity, concentrate lambing at pre-established times, favor the spread of specific genotypes and improve flock genetics (González-Stagnaro, 1993). In rural production systems with low technology, synchronization is not applied due to the low availability of the products, mainly because of the cost, since these are imported products and have high costs, which makes their acquisition difficult for producers.

2 Material and Methods

The research was conducted in a Livestock Production Unit located in the municipality of Tihuatlán, Veracruz, which is located in the northern part of the state at coordinates 18° 27' north latitude and 96° 21' west longitude at an altitude of 60 meters above sea level. Its climate is warm-regular, with an average annual temperature of 22°C; its average annual rainfall is 1,076.2 mm (INEGI, 2015).

Sixty hybrid ewes (*Katahdin x Pelibuey),* multiparous, with body condition of 3 on a scale of 0-5 (Russel et al., 1969) were used. All were fed wet orange silage (2.7 kg daily), 2 kg of hayed insurgent grass (*Brachiaria brizanta*) and 0.5 kg of balanced feed at 18% crude protein and water at free access. The ewes were divided proportionally into three groups: the first group (n=20) was given a commercial sponge, Chronogest CR®, a controlled-release sponge containing 20 mg of cronolone. The second group (n=20) was inserted with a commercial sponge, FGA-30, an intravaginal sponge containing 40 mg of fluorogestone acetate (FGA). The third group (n=20) was inserted with a handmade sponge, made of polyurethane, measuring 4 cm wide by 3 cm high, sterilized and impregnated with 20 mg of progesterone (Progesterona®).

The induction protocol for the three groups lasted 12 days, considering the day of application as day 0. On day 10, 300 IU of equine chorionic gonadotropin (eCG; Novormon 5000®) were applied to each ewe and on day 12 the sponge was removed. Oestrus detection was performed 24 hours after sponge removal, in the morning and afternoon, using a male covered with an apron. Once estrus was detected, directed mating was performed with previously evaluated males.

To determine the percentage of females that did not repeat estrus, at 17±1 days after directed mating, estrus detection was performed again with a male covered with an apron. Statistical analysis was performed using the SPSS 24 for MAC statistical package (IBM SPSS, 2016). The variable hours of estrus presentation was analyzed with the univariate general linear model and differences between means were analyzed using Tukey's test. The variables percentage of estrus presentation and percentage of non-return to estrus were analyzed using the Chi-squared test. The significance level considered was $p<0.05$.

3 Results

According to the results obtained, it was demonstrated that the use of handmade intravaginal sponges for the induction of estrus in hair ewes was as effective as the use of commercial sponges, since 100% of the ewes of the 3 treatments presented estrus, however, in the time of estrus presentation statistically significant differences were observed ($P<0.05$) among the treatments, the ewes with Chronogest CR commercial sponge presented estrus in less time (29.62 ± 1.45 hours after the removal of the intravaginal sponge) with respect to the ewes of the group with handmade sponge that presented estrus at 30.94 ± 1.94 hours, while the ewes with FGA commercial sponge did not present differences with the two previous protocols by presenting estrus at 30.21 ± 1.11 hours (Table 8.1).

Table 8.1 Hours of estrus presentation (mean ± standard deviation) after intravaginal sponge removal in ewes with estrus induction. Different literals in the column indicate statistical difference (P<0.05)

Group	Oestrus presentation times
Chronogest CR	29.62 ± 1.45[a]
FGA	30.21 ± 1.11[ab]
Handmade	30.94 ±1.94[b]

In the variable rate of non-return to estrus, the Chronogest group had the highest percentage of ewes that did not return to estrus (73%), and the FGA commercial sponge group had the lowest percentage (65%), while the handmade sponge group had 68% of ewes that did not return to estrus, with no statistically significant differences between the groups.

4 Discussion

In this study, 100% of the ewes presented estrus, both the ewes in the groups with commercial sponges and the group with handmade sponges with 20 mg of progesterone, which means that the homemade sponges are as effective for estrus induction in hair sheep as the commercial sponges. The results obtained in the groups with commercial sponges coincide with Martinez et al. (Similarly, Córdova-Izquierdo *et al*. 2007) who performed an estrus synchronization protocol with intravaginal sponges impregnated with 65 mg of medroxyprogesterone acetate (MPA) for 12 days and application of 200 U. I of eCG 48 h before removal of the sponges during the low fertility period (March-April) and under tropical climate conditions in Mexico, also reported 100% estrus presentation during the first 72 hours after removal of the sponges. (1999) obtained a 100% response when synchronizing Creole ewes in the months of May-June, i.e., during the seasonal anestrus period, by using sponges impregnated with 30 mg of FGA and application of 460 IU of pregnant mare serum gonadotropin. Likewise, Cruz (2018) when comparing a long protocol of 12 days and a short protocol of 6 days in the months of March-April with polyurethane sponge with 20 mg of chronolone (Chronogest CR®), 100% of the ewes in both groups presented estrus.

Regarding the time of estrus presentation after the removal of the intravaginal sponge, statistically significant differences were found between the commercial Chronogest sponge and the handmade sponge; ewes with commercial sponge presented estrus at 29.62±1. 45 h and ewes with handmade sponge came into estrus at 30.94±1.94 h, being normal in both cases, since the manifestations of estrus can start from 20 hours after removal of the device and take place, on average, between 30 to 36 hours (González et al., 2010). Cruz, (2018) obtained similar results when using the polyurethane sponge with 20 mg of cronolone (Chronogest CR®), recording estrus behavior 30.31±0.5 after device removal.

The percentage of ewes that did not return to estrus was higher in the protocol with the Chronogest sponge 73%, followed by the handmade sponge with 65% and FGA with 63%, without statistical differences. Martinez et al. (2007) obtained a gestation percentage of 66.6% when using a 12-day protocol with 65 mg of medroxyprogesterone acetate and the application of 200 I.U. of eCG. In contrast to what was obtained by Perez, (2015) who obtained a gestation percentage of 78.95% when using a 12-day long protocol with FGA and 500 IU of eCG considering that this study was carried out during the reproductive season. In relation to the results obtained in the percentage of non-return Ishida et al. (1999) mentioned that generally the gestation percentages of estrus induced during the low fertility period are lower (40-60%) than those induced during the reproductive period. In the same way Ungerfeld and Rubianes, (1999) and Viñoles (2011) agree that fertility results improve when short treatments of 6 days duration are applied with respect to long treatments, both in seasonal anestrus and in the reproductive season.

5 Conclusion

Handmade sponges impregnated with 20 mg of progesterone are a viable alternative for the induction of estrus in hair sheep by obtaining a level of response comparable to commercial sponges in estrus presentation and fertility.

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Chapter 9 Evaluation of an alternative nixtamalization method in maize landraces from Chiapas

Capítulo 9 Evaluación de un método alternativo de nixtamalización en maices criollos de Chiapas

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Abstract

In the State of Chiapas, México there is a great diversity of maize landraces (*Zea mays L.*) among which there are pigmented grain variants that have been little used in nixtamalization. The objective of this research was to evaluate an alternative cold method using four different creole genotypes corn (white, yellow, red and purple) in order to propose a process alternative focused on reducing the process time and having similar product characteristics to traditional nixtamalization. A pre-treatment was used in the corn with an excess of water with calcium hydroxide (2%) at room temperature called "Cold Nixtamalization". Different treatments were evaluated with soaking times before cooking (8, 10 and 12 h) and repose time in the nejayote water (6, 8 and 12 h). The variables studied were dry grain moisture, wet grain (nixtamal) and dough, coccion time, pH of the nejayote and dough yield. The best treatment to reduce the coccion time was 12 h of soaking up at room temperature before coccion and 8 h of repose, which caused a decrease in the consumption of gas or firewood, the physicochemical characteristics of dough and tortillas were obtained similar to that of traditional nixtmalization, as well as in pH conditions, process performance.

Pigmented corn, Physicochemical characteristics, Time and process performance

Resumen

En el Estado de Chiapas, México existe una gran diversidad de variedades criollas de maíz (*Zea mays L.*) entre las que se encuentran variantes de grano pigmentado que han sido poco utilizadas en la nixtamalización. El objetivo de esta investigación fue evaluar un método alternativo en frío utilizando cuatro diferentes genotipos de maíz criollo (blanco, amarillo, rojo y morado) para proponer una alternativa de proceso enfocada a reducir el tiempo del mismo y que tenga características de producto similares a la nixtamalización tradicional. Se utilizó un pretratamiento en el maíz con un exceso de agua con hidróxido de calcio (2%) a temperatura ambiente denominado "Nixtamalización en frío". Se evaluaron diferentes tratamientos con tiempos de remojo antes de la cocción (8, 10 y 12 h) y tiempo de reposo en el agua de nejayote (6, 8 y 12 h). Las variables estudiadas fueron humedad del grano seco, grano húmedo (nixtamal) y masa, tiempo de cocción, pH del nejayote y rendimiento de la masa. El mejor tratamiento para reducir el tiempo de cocción fue 12 h de remojo a temperatura ambiente antes de la cocción y 8 h de reposo, lo que provocó una disminución en el consumo de gas o leña, se obtuvieron características fisicoquímicas de la masa y de las tortillas similares a las de la nixtmalización tradicional, así como en las condiciones de pH, rendimiento del proceso.

Maíz pigmentado, Características fisicoquímicas, Tiempo y rendimiento del proceso

1 Introduction

From a food point of view, corn is one of the most important crops in Mexico because the national consumption of corn has registered a sustained growth since 2012. For the years 2019/2020, a record domestic consumption of 44.5 mdt was projected, almost 400 thousand tons above the consumption of the immediately previous cycle. (FIRA, 2020). In Mexico and some Central American countries, corn is consumed mainly in the form of a tortilla, a food that is obtained through out a very old process called “Nixtamalization” (FAO, 1993). However, it is well known that the process has several variants, among which the excessive use of water, energy inefficiency, and long process times, among others.

Currently, it has not been possible to develop a technology that completely replaces the traditional nixtamalization process, since the results obtained in the dough and tortillas do not meet the physicochemical and rheological characteristics required by consumers. Therefore, a new alternative process must be generated that allows the improvement and optimization of the traditional process without affecting the final quality characteristics of the processed products. It is also important that these process alternatives are used in natives corns to give added value to what the region produces.

A pre-treatment of corn genotypes from the State of Chiapas, Mexico (white, yellow, red and purple) was evaluated before starting the traditional nixtamalization process, it consisted of exposing the corn to an excess of water with lime at room called "Cold Nixtamalization", in order to increase cohesion, improve water absorption and therefore reduce coccion times and process costs.

Tests were carried out on the final product (dough and tortillas), process performance, and pH evaluation of the nejayote under the norms that regulate the consumption of nixtamalized corn foods, in addition to its comparison with the traditional nixtamalization process.

2 Theoretical framework

Corn is the most important crop in Mexico and is essential in the diet of Mexicans, it is present in the production of more than 4 thousand products (starch, fructose, oils, cardboard, chocolates, biofuels, animal feed); It occupies little more than half of the sown area of the country and unlike other cereals, it can be grown in almost all climates, almost all altitudes and all soils. It grows early, it is easy to store and to keep for a long time; It is prepared with simplicity and does not require complex equipment to consume it. (Fuentes, 2012).

Mexico is considered as the center of origin and diversity of corn, which has led farmers to apply many of the practices taught by their ancestors, in addition to conserving native materials, the knowledge and practices that reflect a great evolution among crops and human populations.

During 2009 and 2010, maize collections of landraces werw collected in Chiapas, Mexico with subsistence farmers and collected 700 varieties finding a wide diversity of races (18) and grain color (8), most of them are used in the Nixtamalization process. (Coutiño *et al*., 2015). There are currently 59 unique Mexican landraces varieties registered.

Sierra-Macías *et al*., (2014) classified the varieties in the following groups:

Conical Group or races from the highlands of central Mexico: those whose outstanding characteristic is the pyramidal shape of their ears; they are predominantly distributed in regions with elevations of more than 2,000 m mainly and the races of this group are: Arrocillo, Cacahuacintle, Conic, Conical corn, Chalqueño and Mushito.

Group of eight-row corn or races of western Mexico: includes those that are cultivated at low and intermediate elevations, from the central valleys of Oaxaca to the glens of northwest Mexico (CONABIO, 2011; Sánchez *et al*., 2000), It is grown especially for consumption as corn and for various special uses (cookies, pozole, huachales, tejuino, etc.), the main races are Onaveño and Bolita.

Precocious or early maturing tropical maize races: they are grown mainly in the dry tropics and semi-arid regions of the country (100-1300 m), adapted to limited humidity regimes that have given them a short or early maturation cycle. The Raton race is the main one of this group and has been widely used as a material in the development of improved materials. (Sierra - Macías *et al*., 2018).

Group of tropical toothed maize: they are agronomically very important races of southern Mexico, distributed mainly in intermediate and low-altitude regions. These and their hybrids are probably the most used in genetic improvement programs worldwide, the races considered in this group are: Tuxpeño, Tepecintle, Vandeño, Celaña, Pepitilla and Nal-Tel de Altura (Sánchez *et al*., 2000).

Group of late maturing maize: includes races that are grown in wide areas at different altitudes (Aragón *et al*., 2006). Their range of adaptation has facilitated the cultivation of some of them from sea level to high hillsides, a humidity and cloudy condition in the southeastern mountains and center-east of the country (Ortega, 2003; CONABIO, 2011). The races mainly considered in this group are Olotillo and Coscomatepec.

The Olotón race dominates in the upper parts of the southeast of the country, generally above 1,900 m, in the state of Chiapas, it is typical of the Altos, Selva and Soconusco regions, it has also been collected in Oaxaca (Aragón *et al*., 2006, CONABIO 2011); and it extends to Guatemala, where it presents a great variation from which several races have been differentiated (Wellhausen et al. 1957). It also constitutes the food base of the indigenous and mestizo communities of the State of Chiapas, as well as of the North and South Sierras of Oaxaca (Aragón *et al*., 2006, CONABIO 2011, Wellhausen *et al*., 1951).

Figure 9.1 Maize landraces from Mexico

Source of reference: Courtesy CIMMYT Maize Germplasm Bank, 2019

Normally, the nixtmalization is carried out with white and yellow corn, either by culture or custom or consumer demand, however in some states such as Chiapas, due to having a diversity of pigmented corn maizes, they tend to use this technology to obtain corn products nixtamalized, in addition to being foods with great potential for the supply of colorants and beneficial antioxidants for health, such as anthocyanins (Cadena-Iñiguez, 2018).

Nixtamalization is the process by which the corn is cooked with water and calcium hydroxide, to obtain the nixtamal that, after grinding, gives rise to the nixtamalized dough used to make tortillas, tamales, etc. There are some documents that indicate that nixtamalization was originated in Mesoamerica (specifically in the Mexican highlands).

Alternative processes in Nixtamalization

Bressani *et al*., (1962) evaluated a procedure based in the coccion corn under pressure of 0.35 and 1.05 kg/cm^2 in dry and humidity conditions, for 15, 30 and 60 minutes, without using lime. This method reduced the crude fiber content, which is one of the specific effects of lime, and the calcium content was significantly lower than that of the dry dough produced in the traditional method.

Molina *et al*., (1977), reported the preparation of instant flours by coccing and drying a mixture of corn with water (ratio 1:3) and lime (0.3% w/w) in a double rotating drum. The process conditions were: pressure of 15, 20 and 25 psi, thereby reaching temperatures of 93, 99 and 104 ° C, respectively at 2, 3 and 4 rpm. In this study, the flour was hydrated and tortillas were made, obtaining physicochemical and sensory characteristics similar to the tortillas obtained by the traditional method.

Johnson *et al*., (1980), proposed an instant flour process by micronization, which is a dry processing method, using infrared gas burners. This procedure was to mix the broken corn kernels in a dilute calcium solution and subsequently the mixture was subjected to infrared cooking. The tests obtained for texture and rollability in tortillas were similar to tortillas made from commercial instant flour.

Khan *et al*., (1982) compared three methods: the traditional, a commercial one and pressure coccion in the laboratory. The corn was subjected to sub coccion, optimal coccion and over coccion in order to measure some physical and chemical changes that could occur. Although the traditional method caused the greatest loss of dry matter from the maize, it produced the best tortillas in terms of texture, color, and acceptability. The pressure coccion method in the laboratory gave sticky batter and unsightly looking tortillas and the commercial gave less appetizing looking tortillas.

Contreras (2009) proposed using ohmic heating for the production of instant corn flour. Treatments were evaluated using conditions of humidity of 45%, 53% and 60%, temperatures of 70 °C, 80 °C and 90 °C, and particle sizes of maize 0.5, 0.8 and 1.3 mm.

The best treatment was the one that had similarities in the retrogradation and luminosity of the flour; and the adhesion, cohesion, moisture and yield of the dough. In relation to the tortillas, similarities were found in color and rollability, in addition to the fact that the ohmic process efficiently preserves the protein content present in the original corn. Therefore this method can be a production alternative for instant corn flours.

Contreras (2015), mentions that the cold nixtamalization of corn obtained by an extruction method produces interaction of corn with excess water and calcium hydroxide, which irreversibly modifies its viscosity and structure properties. In addition to the fact that the standing time without heating causes swelling and morphological changes in the corn starch, all these factors can guide a process that improves the quality of processed corn products.

Currently, research has been dedicated to not replacing the traditional nixtamalization process, but on the contrary, seeking improvements either in some modification of the process, use of equipment or emerging technologies in order to optimize the process and obtain quality characteristics. similar to those of a traditional Nixtamalization and acceptable to the consumer.

3 Methodology

The development of the project was carried out in the laboratories of the Technological University of La Selva. The vegetative material was collected in 2019 in different localities of the municipality of Ocosingo, Chiapas. The races landraces evaluated were Olotón of white, yellow, purple and red color.

Description of traditional Nixtamalization

The first step of traditional nixtamalization consisted in coccing the corn kernels in an alkaline solution of calcium hydroxide (1-2%) at a temperature close to the boiling point. After coccion, the corn remained in the broth (nejayote) for 8 h. Coccion and steeping times for corn varied depending on the type of corn, hardness of the grain, and local traditions. In the region of Ocosingo, Chiapas., farmers can be perform the coccion from a few minutes to an hour, and leave it soaking from a few minutes to about a day, it is not yet a standardized process.

To know if the process was successful, it was verified that the corn grain could be easily peeled between the fingers when rubbing it. Subsequently, the grains were completely washed to clean them of the remains of the nejayote, the pericarp was discarded and only the germ of the grain was preserved. Afterwards, the grain was tritured with a manual disk mill to obtain corn dough or flour.

Description of cold Nixtamalization

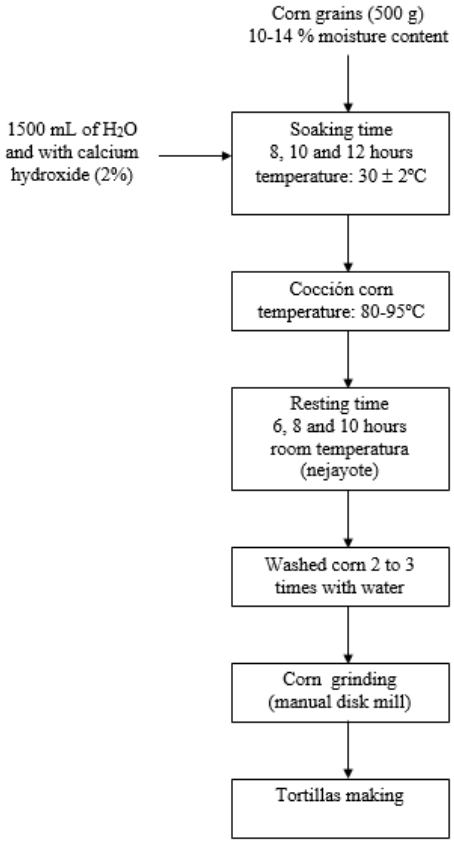
Corn grains (500 g) were placed in plastic containers for a soaking stage with 1500 mL of H_2O and with calcium hydroxide concentrations (food grade) of 2%. The containers were kept closed at room temperature (30 ± 2ºC) for 8, 10 and 12 h.

Afterwards, the cocción of corn grains were made at a temperature of 80-95 °C, the coccion time was verified by cutting a kernel and visually inspecting that it had partially gelatinized on the periphery and softened the grain. Then a resting time of 6, 8 and 10 h of the corn grains was carried out in the nejayote at room temperature. Subsequently, it was washed 2 to 3 times with water until the excess of lime was eliminated.

To obtain the dough, a manual disk mill and worm screw feeder were used, adding an average of 100 mL of water, in each grinding, to obtain the pasty texture and soft, characteristic of the dough produced by the traditional nixtamalization method.

The variables evaluated in the process were: grain moisture, coccion time, yield (dough/corn), pH of the nejayote, dough moisture and number of tortillas obtained.

Figure 9.2 Diagram of cold nixtamalization process



Moisture Analysis (dry grain, nixtamal and dough)

Samples of 0.5-1.0 g, randomly selected from each treatment, were analyzed and placed in an oven at a temperature of 130 ± 3 °C for four hours, then left to cool for 45 minutes and the weight was recorded to calculate the content of moisture (AACC, 2000).

pH determination (nejayote)

It was based on the electrometric measurement of the activity of the hydrogen ions present in a 5-10 ml sample, it was gently stirred for 30 seconds and the pH was measured with a potentiometer.

Experimental design

A completely randomized design with factorial arrangement A x B x C with three replications per treatment was used for the study. The factors evaluated were soaking times with three levels (8, 10 and 12 h), resting times (6, 8 and 10 h) and the maize landraces with 4 levels (factor C). The analysis of the results was processed through an ANOVA and a comparison of means, using the Tukey test ($p \leq 0.05$) using the statistical software R-studio version 3.1.1.

Preparation of tortillas

About 25 g of dough was taken and 11.5-12 cm diameter discs were formed using a metal hand press. The discs were placed on a stainless steel griddle conditioned with a lime solution, heated to a temperature of 200 °C ± 20 °C, the tortillas were cooked alternately on both sides in times no longer than 30 seconds, and later they were deposited in a container covered by a cloth blanket for later analysis. The variables evaluated in the tortillas were: rollability and degree of inflation through hedonic scales.

Tortilla properties analysis

Degree of inflation of the tortilla

The tortillas were evaluated during cooking, through the following scale: 1) tortilla with full inflation, 2) intermediate inflation and 3) without inflation (Jiménez – Juárez *et al*., 2012).

Tortilla rollability

It was determined by rolling the tortilla, through the following scale: 1) tortilla without rupture, 2) with an approximate break of 25%, 3) with an approximate break of 50%, 4) with an approximate break of 75% and 5) with complete rupture (Bedolla, 1984).

4 Results

Before starting the nixtamalization process, the corn grains were characterized, observing that the four genotypes had a standard moisture condition between 10-14%. Moisture is an parameter that indicates that the corn is ready to be stored or used in nixtamalization process. (NMX- FF-034/1-SCFI-2002). The moisture differences between the studied maize landraces were not significant, indicating that the corn grains used were homogeneous at the beginning of the study (Table 9.1).

Table 9.1 Moisture content in maize landraces of Chiapas, Mexico

Maize landraces	Moisture of corn grain (% w.b)
White corn	11.385[a]
Yellow corn	11.498[a]
Red corn	10.195[a]
Purple corn	11.023[a]

Averages with the same letters are not statistically different (Tukey, $p<0.05$)

It is important to mention that in order to evaluate the "Cold Nixtamalization" process, the traditional nixtamalization was first characterized in the four genotypes, since it is a thermal process that requires a combination of temperature and times, in addition, a highly relevant factor is the genotype of corn that is used, since each type has qualities that can give it a different texture and flavor to the final product.

Table 9.2 Traditional Nixtamalization in maize landraces

Genotypes	Moisture content of nixtamal (% w.b)	Coccion time (minutes)	Dough yield (g mass/g dry corn)	Dough moisture (% w.b)	Nejayote pH
White	41.61033[a]	70.06667 [a]	0.9352 [a]	58.4247 [a]	10.65 [a]
Yellow	41.03133 [a]	70.09333 [a]	0.8910 [a]	53.5993 [a]	10.84 [a]
Red	42.50167 [a]	60.3533 [a]	0.9136 [a]	50.8980 [a]	11.80 [a]
Purple	46.18167 [a]	66.8466 [a]	0.9603 [a]	59.6760 [a]	11.53 [a]

Averages with the same letters are not statistically different (Tukey, $p<0.05$)

In Table 9.2 it is observed that there is an increase in the moisture of the corn grain during coccion and rest times of the process, so that the nixtamal reached values around 41- 46% moisture content and is similar to that reported by Serna - Saldivar *et al*., 1990. According to the standard, when a moisture value of no more than 42% is reached, they are very hard maizes, which do not retain much pericarp, since the moisture of the nixtamal is given both by endosperm and by pericarp attached to the grain. The values obtained in the genotypes evaluated indicated that they are very hard maize, where the diffusion of water into the grain is slow due to its structural composition, and to achieve the increase of the moisture in nixtamalization, its necessary to apply prolonged periods of process. It is also observed that in the other variables evaluated, such as coccion time, dough yield, dough moisture and pH of the nejayote there are no significant differences, in addition to being in adequate ranges to obtain tortillas with industrial quality accepted by the consumer. Because when it comes to "quality", it is about tortillas that must meet certain sensory and rheological properties that allow them to have accepted aroma, flavor, flexibility and texture characteristics, as well as that they can be folded and rolled.

Evaluation of Cold Nixtamalization

Based on the results in traditional nixtamalization, a single genotype (white) was selected to standardize the cold nixtamalization process through the study of soaking times before coccion and rest times in the nejayote, parameters considered critical during nixtamalization, and they are variable that are sometimes determined based on the experience and customs of the farmers (Milan-Carrillo *et al*., 2004). In Table 9.3, the best treatments are presented in relation to the parameters evaluated in the standardization of cold nixtmalization process.

Figure 9.3 Genotype of white corn with cold nixtamalization

Table 9.3 Standardization of cold nixtamalization

Soaking and resting time (hours)	Cocción time (minutes)	Corn weight (g)	Dough weight (g)	Tortillas number	Tortillas weight (g)	Tortilla rollability (% break)	Degree of inflation of the tortilla
12-6	43.0 ^{b}	791.50 ^{a}	1023.00 ^{b}	40.0 ^{a}	736.60 ^{b}	75 % ^{b}	Intermediate
12-8	40.0 ^{c}	804.00 ^{b}	1075.25 ^{d}	41.0 ^{b}	738.10 ^{b}	100% ^{a}	Full
12-10	43.0 ^{b}	861.00 ^{d}	1057.00 ^{c}	40.0 ^{a}	785.50 ^{d}	100% ^{a}	Intermediate
10-6	43.5 ^{b}	802.00 ^{b}	1011.00 ^{a}	41.0 ^{b}	715.50 ^{a}	100% ^{a}	Full
8-6	50.5 ^{a}	816.75 ^{c}	1074.00 ^{d}	43.0 ^{c}	765.75 ^{c}	100% ^{a}	Full

Averages with the same letters are not statistically different (Tukey, $p<0.05$)

Table 9.3 shows that the combination consisting of 12 hours of soaking and 8 hours of rest, is a condition that minimizes the coccion time and also presents a statistically similar performance to the other treatments, which makes it the selected scheme. The results show that in the study with very hard maize, the soaking time before cooking contributes to the diffusion of the water towards the grain, softening and detaching the husk from the maize. This pretreatment with the combination of the standing time facilitates the absorption of water in the grain, and this contributes towards a reduction in the coccion time and in turn with the fuel consumption. From the parameters selected in the Cold Nixtamalization, The table 9.4 shows the results of the four maize landraces using both processes, the traditional and the cold one.

Table 9.4 Comparison of cold and traditional nixtamalization of the four maize landraces

Nixtamalization												
	Traditional method						Alternative method (Cold)					
Genotypes	Coccion time (minutes)	Dough yield (g mass/g dry corn)	Tortillas (#)	Rollability (%)	Degree of inflation (%)	Nejayote pH	Coccion time (minutes)	Dough yield (g mass/g dry corn)	Tortillas (#)	Rollability (%)	Degree of inflation (%)	Nejayote pH
G1	70.07 ^{a}	1.04 ^{a}	39 ^{a}	100 ^{a}	94.0 ^{a}	10.65 ^{a}	40.15 ^{a}	1.12 ^{a}	38 ^{a}	100 ^{a}	100 ^{a}	9.86 ^{b}
G2	70.09 ^{a}	1.11 ^{a}	35 ^{a}	100 ^{a}	98.2 ^{a}	10.84 ^{a}	41.80 ^{a}	1.07 ^{a}	37 ^{a}	100 ^{a}	100 ^{a}	10.98 ^{a}
G3	68.43 ^{a}	0.97 ^{a}	37 ^{a}	100 ^{a}	93.0 ^{a}	11.80 ^{a}	41.10 ^{a}	0.99 ^{a}	38 ^{a}	100 ^{a}	100 ^{a}	11.65 ^{a}
G4	66.84 ^{b}	1.11 ^{a}	38 ^{a}	100 ^{a}	100 ^{a}	11.53 ^{a}	39.50 ^{a}	1.12 ^{a}	38 ^{a}	100 ^{a}	100 ^{a}	11.40 ^{a}

Averages with the same letters are not statistically different (Tukey, $p<0.05$)
(Maize landraces: G1= White, G2= Yellow, G3= Red, G4= Purple)

Figure 9.4 Genotype of yellow corn with cold nixtamalization

Figure 9.5 Comparison of red corn tortillas with traditional and cold nixtamalization

Taking the results of Table 9.4 as a reference, it is observed that there is a decrease in the coccion time by the cold nixtamalization method around 40%, this is due to the fact that the resting time (pre-treatment) and soaking contributes to soften the corn grain and allows both water and lime to perform nixtamalization more easily since biochemical reactions, cross-linking and molecular interactions occur in it. These changes modify the physicochemical, structural and rheological characteristics of the dough, as well as the structural and textural properties of the tortilla produced. In addition, this reduction in time contributes to reducing coccion time, fuel costs and therefore process costs.

In relation to the other variables evaluated such as dough yield, number of tortillas, rollability, inflation and pH of the nejayote, in most of them, no significant differences are observed between the four genotypes, and those differences observed as in the case of the nejayote of the white genotype could be attributed to experimental errors. It is also observed that there is no significant difference in traditional and cold nixtamalization in relation to the characteristics of the final product, which allows positive results in the tortilla industry sector.

5 Acknowledgments

The authors thank the Department of Technological Research-Development and the Agro-Food Division of the Technological University of La Selva for the facilities provided for the development of this research.

6 Conclusions

Through the evaluation of the four maize landraces, key processes of traditional nixtamalization were detected and similar results can be obtained if the process is carried out with a control of the process parameters and similarity of the physicochemical characteristics of the corn grain.

An alternative method called "Cold Nixtamalization" was evaluated considering a pretreatment before the process and standardization of the resting time of the corn in the water of the nejayote, and according to the results of physicochemical tests carried out on the mass and tortilla, it was verified that it can be a suitable method to be implemented because it reduces the coccion time, guaranteeing a decrease in fuel consumption in the process.

With the specifications of the alternative method, the evaluation was carried out in the four genotypes (white, yellow, red and purple) and their comparison with the traditional method, obtaining rheological and sensory characteristics of similar tortillas in the two methods.

The implementation of this alternative method favors the integral improvement of the process, reducing energy consumption during coccion time, without the need to use additives or equipment to maintain the quality of the dough and tortillas, similar to that of traditional nixtamalization process.

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Chapter 10 Growth promotion and productivity of tomato using two plant biostimulants: Arbuscular mycorrhizal fungi and seaweed extract

Capítulo 10 Promoción de crecimiento y productividad de tomate utilizando dos bioestimulantes de plantas: Hongo micorrízico arbuscular y extracto de alga marina

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DOI: 10.35429/H.2021.3.158.169

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A. Marroquín, J. Olivares, D. Ventura and L. Cruz (Coord) Agricultural Sciences and Biotechnology. Handbooks-©ECORFAN-México, Querétaro, 2021.

Abstract

Plant biostimulants include different substances, compounds, and formulations of growth-promoting microorganisms, such as those derived from arbuscular mycorrhizal fungi (AMF) or seaweed extracts (SWE), which are used to regulate or enhance physiological and morphological processes in plants. This study analyzed the morphological implications of the addition of two biostimulants, AMF *Rhizophagus intraradices* and a SWE obtained from *Ulva lactuca* (both alone and in combination), in tomato plants (*Solanum lycopersicum*). The responses evaluated were related to plant growth, flowering, and crop productivity. Likewise, the success of AMF colonization in plants was also assessed. The application of AMF increased the length and root area of the plants. The SWE induced an early flowering and thus a greater number of fruits with greater weights. However, the combination of both biostimulants (AMF + SWE) was less beneficial for the plants, which was reflected in a decrease in both foliar and root growth as well as in the number of flowers and fruits. In addition, it was also observed that the SWE showed a positive effect over mycorrhizal establishment, as evidenced by greater root colonization. In the present study, evidence is presented of the benefits of using SWE to improve plant performance, in addition to the positive effects observed in the establishment of mycorrhizal symbiosis between *R. intraradices* and tomato plant roots. These results constitute an important contribution to the research on biostimulants, their development, and functional design, highlighting those complementary effects and the action mechanisms of each biostimulant should be considered.

Marine Algae, Biostimulant, Mycorrhizal symbiosis, Tomato, *Ulva lactuca*

Resumen

Los bioestimulantes de plantas incluyen diferentes sustancias, compuestos y formulaciones de microorganismos promotores del crecimiento, como los derivados de hongos micorrícicos arbusculares (HMA) o extractos de algas marinas (EA), que se utilizan para regular o potenciar los procesos fisiológicos y morfológicos en las plantas. Este estudio analizó las implicaciones morfológicas de la adición de dos bioestimulantes, el HMA *Rhizophagus intraradices* y/o extracto obtenidos a partir del alga *Ulva lactuca* (tanto solos como en combinación), en plantas de tomate (*Solanum lycopersicum*). Las respuestas evaluadas se relacionaron con el crecimiento de las plantas, la floración y productividad del cultivo. Así mismo, se observó el éxito de la colonización de la raíz en la plantas por el HMA. La aplicación del HMA incrementó la longitud y área radicular de las plantas. El EA indujo una floración temprana y por lo tanto mayor número y peso de frutos. Sin embargo, la combinación de ambos bioestimulantes (HMA + EA) resultó menos benéfico para la planta, reflejándose en una disminución tanto en el crecimiento foliar y radicular como en número de flores y frutos. Además, también se observó que el EA mostró un efecto benéfico sobre el establecimiento del HMA, mostrando una mayor colonización de raíces. En el presente trabajo, se presenta evidencia de los beneficios del uso de los EA para mejorar el rendimiento de la planta. Así como el efecto positivo sobre el establecimiento de la simbiosis micorrícia entre *R. intraradices* y las raíces de las plantas de tomate. Los resultados obtenidos son una contribución importante a la investigación de bioestimulantes, al desarrollo y diseño funcional de los mismos, en donde debe considerarse los efectos complementarios y los mecanismos de acción de cada bioestimulante.

Algas marinas, Bioestimulantes, Simbiosis micorrícica, Tomate, *Ulva lactuca*

Introduction

Recent decades have borne witness to substantial increases in the use of biostimulants for agricultural purposes, and biostimulant research has primarily intensified in the search for new compounds that increase crop yield and quality (Calvo *et al*., 2014). A plant biostimulant can be defined as any substance or microorganism that improves nutritional efficiency, tolerance to abiotic stress, or the quality of particular traits, regardless of its nutrient content. Biostimulants may be obtained from a wide variety of natural sources and are mainly categorized as follows: humic and fulvic acids, protein hydrolysates and other N-containing compounds, botanical and seaweed extracts, inorganic compounds, and beneficial fungi and bacteria (du Jardin, 2015).

Even in small amounts, biostimulants improve plant growth and performance by modulating metabolic responses like respiration, photosynthesis, protein synthesis, nutrient absorption, and biotic and abiotic stress responses (Posmyk and Szafrańska, 2016). Biostimulants may also improve crop sustainability by preventing the excessive application of fertilizers and consequently their negative impacts that result in environmental pollution (Halpern *et al*., 2015).

Marine algae have been used for thousands of years to improve soil fertility and crop productivity through either direct application or as soil amendments after composting (Craigie, 2011). After the first process to produce liquid seaweed extracts (SWE) was developed in the 1950s, a variety of commercial SWE products are now available worldwide for agricultural and horticultural purposes (Khan *et al*., 2009). In Mexico, marine macroalgae are abundantly available in temperate and tropical waters and thus constitute a low-cost, local resource for coastal agricultural production with great potential for eventual commercial exploitation (Hernández-Herrera *et al*., 2014 a, b). Algal extracts have been found to act as chelators, improving the structure of the soil and the use of mineral nutrients in plants after composting, which favors root growth. As biostimulants, SWE promote seed establishment and germination and increase growth, yields, flower and fruit production, resistance to biotic and abiotic stress, and postharvest shelf life (Khan *et al*., 2011, Craigie, 2011). SWE are comprised of a complex mixture of components that vary depending on the source seaweed, harvest time, and extraction process (Shekhar *et al*., 2012). Moreover, they contain a wide range of organic and mineral components, including unique and complex polysaccharides that are not present in terrestrial plants like laminarins, fucoidans, and alginates (Khan *et al*., 2009).

The biostimulant effects of SWE are often attributed to plant growth hormones and low molecular weight compounds (Tarakhovskaya *et al*., 2007), although larger molecules like polysaccharides and polyphenols have also been implicated (Zhang *et al*., 2003; Battacharyya *et al*., 2015). Most commercial kelp extracts are made from brown kelp, including *Ascophyllum nodosum*, *Fucus*, *Laminaria*, *Sargassum*, and *Turbinaria* spp. (Hong *et al*., 2007). Although commercial manufacturing processes for extracts are generally patented, they include the use of water, acids, or alkalis as extractants (with or without heating) or the physical manipulation of seaweeds using low-temperature or high-pressure grinding. The final products are prepared in either liquid form or as dry formulations and can be combined with plant fertilizers and micronutrients (Craigie, 2011). In Mexico, six seaweed species are used to produce 14 biostimulant products, which are commercialized as biofertilizers or root promoters (Hernández-Herrera *et al*., 2018). According to our research, the production of these commercial products is based on conventional solvent extraction and hydrolysis under hydrothermal treatment using several methods with acid, neutral, and alkaline conditions. SWE can be applied near the root of the plant by mixing extracts with irrigation water, which can then be applied with drip irrigation systems to crops. They can also be used as foliar sprays with a variety of flowers, vegetable crops, and trees (Battacharyya *et al*., 2015). The algal extracts have biostimulant activity at low concentrations (i.e., diluted to 1:1,000 or more), which suggests that the observed effects are different from those associated with a direct nutritional function.

Arbuscular mycorrhizal fungi (AMF) are one of the most important groups of soil fungi, and they form symbiotic associations with the roots of ~ 80% of all plant species (Aguilera *et al*., 2007; Buendia *et al.,* 2016). AMF positively stimulate plant growth, promote abiotic stress tolerance, and improve resistance to both pests and diseases. Likewise, AMF improve the nutritional status of plants, which is reflected in increased yields and fertilization efficiency (Alfonso and Galán, 2006). Symbiosis between AMF and host plants requires a sequence of molecular recognition events leading to the morphological and physiological integration of the two symbionts (Barea *et al*., 2008). Several factors influence the establishment and functioning of mycorrhizal symbiosis. In particular, edaphological characteristics (e.g., soil texture, pH, organic matter content, humidity levels, nutrient levels, and the organisms present), climatic conditions (e.g., light, temperature, and geographic location), and biotic factors (e.g., interactions with other organisms) affect spore germination, root colonization, and AMF efficiency (Khalil *et al.,* 1992).

The importance of AMF to plant nutrition has provided new insights into the contributions of these symbionts to nutrient assimilation. According to Barea (1991), after AMF establish themselves and develop their hyphae, plants increase their radical exploration area and the absorption of nutrients like N, K, Ca, Mg, B, Fe, and especially phosphate. In tomato plants, various AMF species have been found to positively affect growth (Rodríguez-Yon *et al*., 2004).

Similarly, tomato plants under nutrient- or water-limiting conditions in the presence of mycorrhizal symbiosis can produce the same quality and quantities of fruits as those of plants grown under non stressed conditions. The use of AMF seems a promising option to stabilize tomato production incresing farmers earnings by reducing costs of water irrigation and nutrient supply (Zouari *et al.,* 2014; Fracasso *et al*., 2020).

Nonetheless, few studies have evaluated how plant development is affected by the application of seaweed extracts in conjunction with AMF. Seaweed extracts have been found to positively modulate mycorrhizal interactions by promoting the development of AMF. Mannitol and carrageenan obtained from *Laminaria japonica* were found to induce the growth of AMF *Gigaspora margarita* and *Glomus caledonium* hyphae and root colonization in the trifoliate orange (*Poncirus trifoliata) in vitro* (Kuwada *et al*., 2005 and 2006b). Similarly, favorable results have been observed in the development and growth of papaya, passion fruit, and cucumber when these two biostimulants were applied (Kuwada *et al.,* 2006a; Suhail, 2013a, b). The simultaneous application of two biostimulants can enhance the benefits that are obtained from an individual application, and these may be either additive or synergistic. González-González *et al*. (2020) reported that the joint application of a seaweed extract from *Padina gymnospora* and the AMF *Rhizophagus intraradices* resulted in enhanced root development and increased carbohydrate and protein content in the leaf tissues of tomato plants. Moreover, the individual application of each biostimulant produced an observable synergistic effect on the appearance and number of flowers.

The present study aimed to analyze tomato plant (*Solanum lycopersicum* L. var. Rio Fuego) development under the combined application of extracts from the green algae (*Ulva lactuca* L.) and the AMF *R. intraradices* to identify possible functional links reflected in either positive (i.e., additive or synergistic) or negative effects on plant growth, productivity, and mycorrhizal interactions. The results of this study will be relevant for assessing the importance of the joint use of these two biostimulants for tomato production and the development of environmentally friendly agricultural management strategies with lower costs.

Materials and Methods

Biological material

The experiments were performed with tomato plants (*S. lycopersicum*) using certified seeds (Kristen Seed®, Guadalajara, Mexico). Inoculations of the AMF *R. intraradices* were conducted using the commercial product INIFAP mycorrhizal BIOfertilizer® (Celaya, Guanajuato, Mexico), which consists of spores, hyphae, and root fragments colonized by AMF (64 spores/g of inoculum). The seaweed *U. lactuca* was collected in February 2018 from an intertidal zone of the Port of Topolobampo, Ahome, Sinaloa (25º 36' 00" N, 109º 04' 00" W). The collected algae were washed with tap water, dried in the sun, and pulverized. The extracts were prepared at 0.2% following the methodology of Hernández-Herrera *et al*. (2014a) starting with 2 g of seaweed powder. The SWE were sterilized, filtered using Whatman No. 40 paper, and refrigerated at 4 °C until use.

Greenhouse plant growth conditions and experimental design

Tomato seeds that had been previously germinated in Petri dishes (Copetta *et al*., 2011) were sown in 1-L plastic pots that had been filled with a sterile substrate mixture (sand/vermiculite 1:1 v/v). Two groups of plants were established. The first group consisted of non-inoculated plants, whereas 50 g of the AMF product was incorporated into the individuals of the second group. Inoculation was performed according to the methodology of Menge and Timer (1982), which consists of placing the inoculum in bands measuring 3–5 cm under the surface of the substrate. The plants were kept under greenhouse conditions for 90 d (March–May 2019) at an average temperature of 30 ± 2 °C and an average photosynthetically active radiation (PAR) of 3100 µmol m^{-2} s^{-1}. The pots were watered daily with 100 mL deionized water to maintain humidity, and 100 mL of algae extract at 0.2 % was applied twice a week with or without Rorison nutritive solution: ($MgSO_4$•$7H_2O$, 0.248 g·L^{-1}; $Ca(NO_3)2$•$4H_2O$, 0.476 g·L^{-1}; K_2HPO_4•$3H_2O$, 0.05 g·L^{-1}; FeNaEDTA, 0.025 g·L^{-1}; $MnSO_4$•$4H_2O$, 2.24 mg·L^{-1}; H_3BO_3, 2.88 mg·L^{-1}; $(Na_4)_6Mo_7O_{24}$•$4H_2O$, 0.2 mg·L^{-1}; $ZnSO_4$•$7H_2O$, 0.44 mg·L^{-1}; $CuSO_4$•$5H_2O$, and 0.4 mg·L^{-1}) adjusted with K_2HPO_4 to favor mycorrhizal colonization (Hajiboland *et al*., 2010; Cervantes-Gámez *et al*., 2016). A random block experiment was established with 4 treatments with 14 repetitions each (n = 56 plants).

The treatments were: 1) plants watered with nutritive solution (Control), 2) plants inoculated with AMF (RI), 3) plants treated with the seaweed extract (SWE), and 4) plants inoculated with AMF and treated with the algal extract (RI + SWE).

Mycorrhizal colonization

Ninety days after sowing, six plants were randomly chosen from the treatments inoculated with AMF (RI and RI + SWE), and their roots were carefully removed. The root samples were washed with tap water and stored in ethanol (50%) until analysis. Mycorrhizal colonization was evaluated according to the staining technique of Kormanik and McGraw (1982). The roots were cut into ~ 1-cm fragments and clarified for 5 min at 90°C in KOH (10%). Then, the roots were acidified by immersion in HCL (0.1 M) for 24 h and stained with trypan blue (0.05%) at 90 °C for 40 min. After which, the roots were placed in lactoglycerol (lactic acid: glycerin: deionized water, 14:1:1 v/v/v) for 15 d. The magnified intersection method (McGonigle and Miller, 1990) was used to quantify the percentage of AMF colonization in the roots. The roots were horizontally mounted on slides, and each root was methodically scanned using the 40x objective of an Axiostar plus microscope (Carl Zeiss, Jena, Germany) aligned to the axis of a square lattice. The presence or absence of mycorrhizal structures (i.e., arbuscules, vesicles, or hyphae) that touched a grid axis and crossed the root was recorded, and at least 100 fields per sample were counted. The counts are presented as the percentage of the length of the colonized root (% LCR):

$$\% \, LCR = 100 \times \frac{Number \; of \; intersections \; with \; HMA \; structures}{Total \; number \; of \; counted \; intersections} \quad (1)$$

Growth and yield parameters in tomato plants

Multiple morphological parameters of growth and yield were evaluated in the 14 plants of each treatment 90 d after sowing. Whole plants were carefully removed from their pots, and their roots were washed with tap water. Then, the plants were scanned, and the root area (cm^2), leaf area (cm^2), root length, stem length (cm), number of leaves, and fresh weight (g^{-1}) were measured with the help of ImageJ v. 1.52a software (https://imagej.nih.gov/ij/download.html). The following performance parameters were also evaluated: the number of flowers, number of fruits, and fruit weight (g^{-1}) regardless of maturity.

Statistical analysis

Normality and homoscedasticity of the data were evaluated using the Shapiro-Wilk and Bartlett tests, respectively, and the data were analyzed by one-way analysis of variance (ANOVA) and a post-hoc Tukey HSD mean comparison test to compare the means of the different treatments and conditions. The analyses were conducted with R-Commander (R Foundation for Statistical Computing, Version 3.5.1).

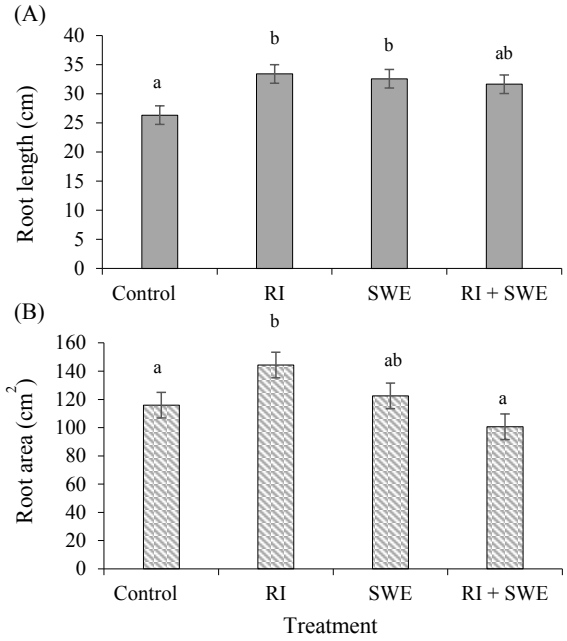
Results and Discussion

Growth promotion in tomato plants

Algal extract application and AMF inoculation significantly increased root length. However, no significant differences were present among plants that were treated with both biostimulants (RI + SWE), the control plants, or plants in either the SWE or RI treatments (Graphic 1A). When analyzing the root area, a positive effect was only observed in the plants in the RI treatment (144.3 cm^2) when compared to those of the control and RI + SWE treatments (Graphic 10.1B). Root growth promotion has been widely documented in response to AMF inoculations. In particular, Copetta *et al*. (2011) found that inoculation with *Glomus mosseae*, *G. caledonium*, *G. viscosum*, *G. intraradices*, and *G. coronatum* promoted root development in tomato plants. In addition to increasing the root area, symbiosis also optimizes nutrient transport, particularly that of phosphate (Gamalero *et al*., 2004). However, AMF can stimulate root formation due to the physiological activity of these endophytes that promotes the synthesis of growth regulators like auxins and cytokinins (Gianinazzi-Pearson *et al*., 1991). Root proliferation in response to algal extract treatment is believed to be related to the presence of bioactive compounds (e.g., thiamine, riboflavin, vitamin K, auxins, cytokinins, gibberellins, polysaccharides, and minerals) in the extracts that play important roles in regulating cell division (Gollan and Wright, 2006; Zodape *et al*., 2008).

The results obtained in this study differ from the additive effects reported from the joint application of AMF *R. intraradices* and a *Padina gymnospora* extract (González-González *et al*., 2020), which may be due to differences in the composition and origin of these extracts.

Graphic 10.1 Root growth in tomato plants inoculated with the arbuscular mycorrhizal fungi (AMF) *Rhizophagus intraradices* (RI), a seaweed extract (SWE) from *Ulva lactuca*, or treated with both the AMF and extract (RI + SWE). The values correspond to the mean ± standard deviation (n = 14). Different letters denote significant differences (P ≤ 0.05) based on Tukey tests



None of the treatments resulted in positive growth promotion in aerial tissues. The foliar area, leaf number, stem length, and shoot fresh weight were lower in the treated plants when compared to those of the control plants (Table 10.1). These results do not correspond to what was reported for both biostimulants when applied individually. It is possible that the growing conditions (particularly the substrate) utilized to evaluate mycorrhization limited plant development.

Table 10.1 Effect of the arbuscular mycorrhizal fungi (AMF) *Rhizophagus intraradices* (RI), seaweed extract (SWE) from *Ulva lactuca*, and both the AMF and algal extract (RI + SWE) on the growth of aerial tissue in tomato plants. The values correspond to the mean ± standard deviation (n = 14). The different letters within columns denote significant differences (P ≤ 0.05) between treatments using a Tukey test.

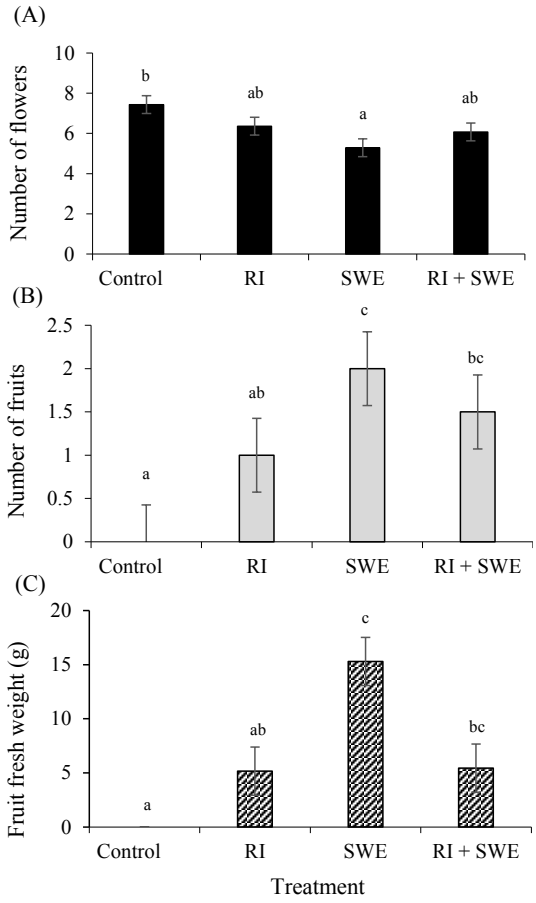
	Foliar area (cm^2)	Number of leaves	Stem lenght (cm)	Sprout fresh weight (g)
Control	185.3 ± 11.6[a]	70.9±8.7[a]	54.6±7.9[a]	16.2±1.6[a]
RI	179.8 ± 10.5[a]	73.3±5.4[a]	56.7±7.7[a]	15.5±1.6[a]
SWE	170.5 ± 12.3[a]	62.3±10.3[a]	47.2±5.0[a]	15.4±1.5[a]
RI + SWE	161±15.6[a]	63.7±13.8[a]	48.3±5.4[a]	14.4±2.1[a]

Tomato plant productivity

Parameters related to productivity showed significant differences between treatments. The number of flowers per plant was significantly lower in the SWE treatment compared to that of the control but similar to the number of flowers registered in the RI and RI + SWE treatments (Graphic 10.2A).The number of fruits per plant was significantly higher in all biostimulant treatments, with the highest number of fruits being found in the SWE treatment (2), followed by the RI + SWE (1.5) and RI (1) treatments. The control plants did not show fruit development on the day of the evaluation (Graphic 10.2B). The analysis of fruit fresh weight indicated a similar trend to that of the number of fruits per plant.

The fruits of the SWE treatment were significantly heavier compared to those of the plants of the RI or RI + SWE treatments (Graphic 10.2C). It is important to highlight that all productivity parameters were evaluated 90 days after sowing, and thus the lowest number of flowers observed in the SWE treatment corresponds to the highest number of fruits in the same treatment.

Graphic 10.2 Productivity of tomato plants inoculated with the arbuscular mycorrhizal fungi (AMF) *Rhizophagus intraradices* (RI), an algal extract from *Ulva lactuca* (SWE), or treated with both the AMF and the extract (RI + SWE). The values correspond to the mean ± standard deviation (n = 14). The different letters denote significant differences ($P \leq 0.05$) between treatments using a Tukey test.



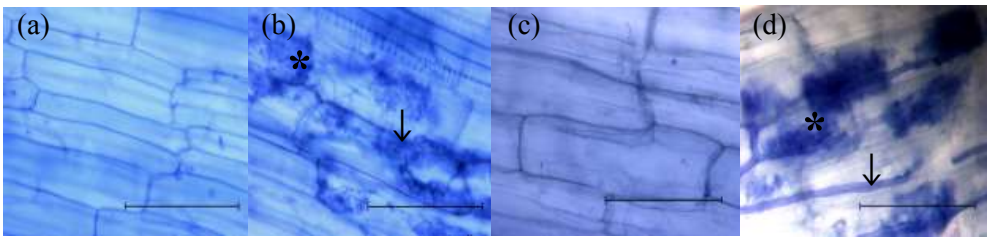
Our results agree with those previously reported for *Ecklonia maxima* seaweed extracts in which accelerated flowering and/or fruiting in tomato plants was observed due to the presence of macro- and micronutrients and growth regulators (Hamed *et al.,* 2018). Furthermore, AMF inoculation has been found to increase tomato yields. For example, William (2007) reported a 40% increase in tomato yield in the nursery after inoculation with *Glomus mosseae* and *Scutellospora calospora*. In other studies, inoculation with autochthonous AMF was found to increase yield by 26% (Regvar *et al*., 2003) while inoculation with *G. fasiculatum* resulted in an increase of 13% (Mohandas, 1987). Similar to what was observed with regard to the promotion of root growth, no synergistic or additive effects were found in the AMF + SWE treatment. This contrasts with previous studies that have found positive effects on crop productivity when two biostimulants were applied. For example, cucumbers inoculated with the AMF *G. intraradices* and *Acoulospora laevis* in combination with the foliar application of a commercial seaweed-based product were found to significantly increase the number of fruits per plant (52.28%), yield per plant (82.42%), and total yield (82.46%) with respect to those of the control plants (Suhail, 2013b). In tomato plants, the joint application of *R. intraradices* and a *P. gymnospora* seaweed extract resulted in a greater number of flowers when compared to those of plants treated with only one of the two biostimulants (González-González *et al*., 2020).

The results of this study differ from those reported for cucumber and tomato plants due to the differences in experimental conditions, particularly those related to the algal species employed.

Colonization of AMF Rhizophagus intraradices

In the roots, where the RI and RI + SWE treatments were applied, the presence of fungal structures characteristic of symbiotic arbuscular-type associations were observed, highlighting intra-radical and arbuscular hyphae (Figure 10.1b and 10.1d). The presence of vesicles was not observed in any field analyzed. As expected, the roots of the non-inoculated plants (control and SWE treatment) did not show the presence of AMF. The results indicated that no significant differences were present in the number of intra-radical hyphae or arbuscules between the plants of the RI and RI + SWE treatments. However, when analyzing the number of arbuscles, significant changes were found between the treatments, with the RI + SWE treatment showing 8-fold the number of arbuscles than those of the RI treatment. Similar results were obtained when analyzing the colonized root length percentage (CRL%) with significantly higher values observed in the (RI + SWE) treatment (Table 2). These results indicate that the *U. lactuca* extract had a positive effect on the establishment of mycorrhizal symbiosis between *R. intraradices* and the plant roots.

Figure 10.1 *Rhizophagus intraradices* fungal structures in tomato plant roots: (a) control, (b) inoculated with the arbuscular mycorrhizal fungi (AMF) *Rhizophagus intraradices* (RI), (c) treated with a *Ulva lactuca* algal extract (SWE), and (d) inoculated with the AMF and treated with the extract (RI + SWE).



Symbols:*, arbuscles; ↓, intraradical hypha; scale bars represent 100mm 40X

Table 10.2 Mycorrhizal colonization of tomato roots. Registration of arbuscles, hyphae, and the colonized root length percentage (CRL%). The values correspond to the mean (n = 6). The different letters denote significant differences ($P \leq 0.05$) using a student t-test.

	Hypha	Arbuscles	%CRL
RI	49.5[a]	8.8[a]	49.2[a]
RI + SWE	52.3[a]	65.5[b]	82.6[b]

The information available regarding the influence of algal extracts on the growth, colonization, and establishment of AMF includes the involvement of low molecular weight compounds like 5-deoxy-5-methylamino-adenosine, alginic acid, mannitol, and a variety of polysaccharides present in marine algae that facilitate phosphorus acquisition (Kuwada *et al.,* 2005; Kuwada *et al*., 2006b; Khan *et al*., 2009; Paszt *et al*., 2015). *Ulva lactuca* extracts contain amino acids, fatty acids, vitamins (Ortiz *et al*., 2006; Frikha *et al*., 2011), polysaccharides, polyphenols, organic acids (Violle *et al*., 2018; Dominguez and Loret, 2019), mucilage (Abirami and Kowsalya, 2011), flavonoids, and terpenoids (Sava and Sîrbu, 2010; Elmegeed *et al*., 2014; Alagan *et al*., 2017). It is probable that some of these compounds directly intervene in the establishment of mycorrhizal symbiosis, favoring spore germination and the growth and branching of germinative hyphae. Furthermore, vitamin E, which is present in *U. lactuca* (Khan *et al*., 2009; Ortiz *et al*. 2006) and *Chlorella pyrenoidosa* extracts, has been found to stimulate the growth of *G. margarita* and *G. caledonium* (Kuwada *et al*., 2006a). In addition, the enhanced fungal growth and root colonization may have been due to the polysaccharides or oligosaccharides contained in the SWE. Another possible explanation for the improvement in lateral root growth by the SWE may have been a change in the hormonal balance as a consequence of the auxins contained in the SWE, which are root hair growth regulators that promote elongation through the up-regulation of associated root epidermis genes, and thus mycorrhization may have consequently been improved as a result of greater root development (Gonzalez-Gonzalez *et al*., 2020).

Conclusions

When AMF and SWE were used individually, each was found to positively stimulate plant growth and performance in different but complementary ways. AMF promoted growth and root development, whereas SWE promoted flowering and tomato fruit formation. In addition, the *U. lactuca* extract stimulated the development of fungal structures and *R. intraradices* colonization (%) in tomato plant roots. However, no advantageous effects were observed from the joint application of the two biostimulants. In agriculture, the application of biostimulants like AMF and SWEs could substantially improve sustainability efforts. However, in Mexico, the production and use of plant biostimulants is still limited, and they have rarely been incorporated into established cultivation practices, partly due to a lack of understanding regarding their usefulness and application. Therefore, accurate information is needed for biostimulants to replace organic and agrochemical fertilizers. Consequently, greater collaboration between farmers, the industrial sectors, researchers, and government entities is required to improve production systems and the quality of biostimulants to develop and implement improved, environmentally friendly agricultural practices.

Acknowledgements

This study was supported by the University of Guadalajara [grant number 238634 UDG-PTC-1329] and the National Council of Science and Technology (CONACyT) through a Master's scholarship awarded to IGBS.

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Variable	Descripción	Valor
V_V	Volumen de Venta	20000
P_V	Postura de venta	490.61
V_C	Volumen de Compra	20000
P_c	Postura de Compra	485.39
P^{Uh}	Precio último Hecho	491.61
V_o	Volumen Operado	1241979
P_u	Precio/Utilidad	0
P^{VL}	Precio/Valor Libro	0
U_a	Utilidad p/Acción	0
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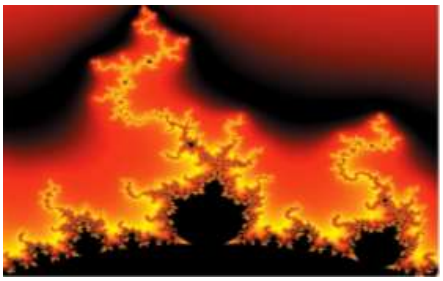

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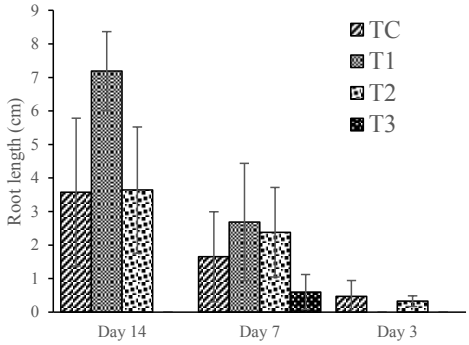

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ISBN 978-607-8695-55-3