UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencia Biológicas e Ingenieria

TÍTULO: Toxicological studies of the fungicides Manzete, Triziman D, and Curamax, using Saccharomyces cerevisiae yeast as a model

Trabajo de integración curricular presentado como requisito para la obtención del título de Biólogo

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Dedicatoria

Esta tesis se la dedico a mis padres, por estar ahí en los buenos y malos momentos, por ser mi motivo y lucha para seguir adelante.

A mis compañeros: Israel, Pablo, Mishell, Andre, Catalina, Eli, Cami, por brindarme todo su amor y apoyo en esta etapa. Gracias por convertirse en mi segunda familia.

Fernando David Vera Erazo

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Fernando David Vera Erazo

Resumen

Los plaguicidas se han convertido un método habitual de lucha contra las plagas que afectan a los cultivos. Por desgracia el uso excesivo y la poca regulación de pesticidas ha traído perjuicios para el medio ambiente y la salud. El objetivo de este estudio es evaluar el riesgo o peligro potencial que pueden causar los fungicidas Manzete (mancozeb), Trizimand D (mancozeb) y Curamax (mancozeb+cymoxanil). El uso de células de levaduras *Saccharomyces cerevisiae* como modelo para medir la toxicidad de los fungicidas puede proporcionar pistas útiles que permitan priorizar análisis más complejos en eucariotas superiores y en un futuro determinar marcadores para biosensores que permitan evaluar la toxicidad de los productos que consumimos y a los que nos exponemos, bien sean alimentarios, cosméticos, o de otro tipo. Los fungicidas usados son de calidad comercial autorizados para su uso en la zona de Imbabura-Ibarra.

Palabras claves: pesticida, eucariotas, levaduras, toxicidad, biosensores, fungicidas, biomarcadores, Mancozeb.

Abstract

Pesticides have become a standard method of fighting pests that affect crops. Unfortunately, the excessive use and poor pesticide regulation have brought environmental and health damage. The objective of this study is to evaluate the potential risk or danger that the fungicides Manzete (mancozeb), Triziman D (mancozeb), and Curamax (macozeb+cymoxanil). The use of Saccharomyces cerevisiae cells as a model for measuring the toxicity of fungicides can provide useful clues to prioritize more complex analyses in higher eukaryotes and the future to determine markers for biosensors to assess the toxicity of the products we consume and to which we are exposed, whether be food, cosmetic or another type. The fungicides used are commercial quality for use in the Imbabura-Ibarra area.

Keywords: pesticide, eukaryotes, yeast, toxicity, biosensors, fungicides, biomarkers, Mancozeb.

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Abbreviations

WHO	The World Health Organization
LD50	Median Lethal Dose
FAO	Food and Agriculture Organization of the United Nations
INEC	National Institute of Statistics and Census of Ecuador
EBDC	Ethylene Bisdithiocarbamates
ETU	Ethylene Thiourea
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
S. Cerevisiae	*Saccharomyces cerevisiae*
ROS	Oxidative Stress Response
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ATP	Adenosine Triphosphate
GPCR	G Protein-Coupled Receptors
ORF	Open Reading Frame
NER	Nucleotide Excision Repair
BER	Base Excision Repair
AP	Abasic Sites
OD	Optical Density

MB	Methylene Blue
Mg/l	Milligrams per Liter
YPD	Yeast Extract Peptone Dextrose
nm	Nanometer

1 INTRODUCTION AND JUSTIFICATION

1.1 General introduction

The world's population continues to grow, and to cope with this demographic growth; agriculture has an indispensable value in supplying food to the population. Pesticides are an important tool in the development of agriculture, and their use has contributed to the production of food and raw materials. Today, high-yield agriculture is not possible without using plant protection measures (Walia et al., 2014). Nowadays, the use of pesticides is conceived within the framework of integrated pest, disease, and weed management, which requires an in-depth knowledge of the properties of these compounds, their residues on crops and in the environment, as well as their toxicological aspects (Bucur et al., 2018; Fatma et al., 2018).

The World Health Organization (WHO) estimates that, without the use of crop protection products, losses due to inefficient production processes in the agricultural sector amount to 53% of the total. With the use of such inputs, losses are reduced to 45%. Furthermore, the use of pesticides is predicted to increase due to changes in climatic conditions, the development of resistance to fungicides, and invasive fungal species (Zubrod et al., 2019).

Pesticides can also be very harmful, can damage the environment, and accumulate in ecosystems (Zubrod et al., 2019). Besides, pesticides have the potential to cause a variety of toxic effects on human health, depending on the dose applied and exposure time; including cancer, reproductive, endocrine, and immune system dysfunction, acute and chronic nervous system damage, and lung damage (Tzanova et al., 2017).The fact is that pesticide molecular targets are frequently shared by pest and non-target species, including humans. Pesticides are the leading method of poisoning in the developing world. It was estimated that annually there were around 1-5 million cases of pesticide poisoning among agricultural workers (Tobar, 2020).

WHO recommends classifying pesticides according to the median lethal dose (LD50). This is the most useful tool when considering the risk posed by their use and the repercussions they may have on people's health (FAO & WHO, 2019). They can also be classified according to their chemical composition: organochlorines, organophosphates, carbamates, pyrethroids, and others. Considering their uses, they are classified into insecticides, fungicides, herbicides, nematicides, acaricides, defoliants, and rodenticides (Lushchak et al., 2018). Of those mentioned above, the most widely used in Latin America are fungicides, insecticides, and herbicides. Among these, we have Mancozeb, a fungicide sold under different trade names and can protect crops from a wide range of phytopathogenic products (INEC, 2013).

The Food and Agriculture Organization of the United Nations (FAO) in collaboration with the WHO in its 2019 report, stated that the maximum amounts of pesticides in foods labeled as extremely hazardous should be equal to or less than 0.01 mg/kg (FAO & WHO, 2019). This amount may vary depending on the toxicity of the pesticide, since for pesticides labeled as highly hazardous, the maximum amount of pesticide ranges from 0.05 to 0.1 mg/kg. To obtain these values, the acute toxicity of the pesticide is taken as a reference and this value is divided into parts per million to ensure food safety (FAO & WHO, 2019).

Yeasts are increasingly used in ecotoxicology as an alternative model because of their low cost and ease of culture (Rica et al., 2010). Different types of response can be detected after exposure of yeasts to contaminants (Dias et al., 2010). For example, the response of yeasts to environmental changes either by changing growth kinetics or adaptation processes can also be related as markers of toxicity, (Dos Santos & Sá-Correia, 2015). The sensitivity of yeasts to a toxicant can be measured by changes in optical density, antimicrobial assays, and different molecular techniques that allow studying the activation or inhibition of different genes. All these parameters are necessary to measure for the development of biosensors (Campuzano, 2011; Skrzypek et al., 2018).

2 PROBLEM STATEMENT

The massive use of synthetic pesticides in modern agriculture has unleashed a series of problems. The intoxications of agricultural workers, the presence of pesticide residues in food, and their persistence in the environment mean that people are increasingly exposed to the action of a large number of substances which, according to their conditions of use, can be harmful. The main problem is that, through our diet, we are exposed not to one but to several agents used to combat weeds, insects, or fungi (Santiago, 2012).

WHO has allowed ranges for pesticide residues in food that ensure, within reasonable limits, their safety for the consumer. Therefore, rapid detection and analysis methods must be sought that allow the monitoring of chemical contaminants found in the environment or food to evaluate their toxic effects (FAO & WHO, 2019). Among the devices capable of meeting this demand is biosensors using microorganisms.

Toxicity studies involve the determination of the lethal dose, which results in the death of several treated animals (Vieira et al., 2020). Animal research also involves major moral and ethical drawbacks and is also affected by economic factors, since the costs of implementing and maintaining animal facilities are high and time-consuming. Therefore, research should be conducted alternative methods to replace or reduce the number of animals used in experiments.

The present research proposes the use of yeast as a toxicity biomarker, measuring the sensitivity response of the yeast to the contaminant. In addition, this work presents a literature review of possible candidates of yeast biomarkers that could be used in biosensors to detect contaminants.

3 OBJECTIVES

3.1 General Objective

- To present an analysis of the use of yeast as a model to study the toxicity of pesticides marketed in Ecuador.

3.2 Specific objectives

- To know the current situation regarding the problem of pesticide poisoning in Ecuador.
- To detail the different mechanisms of toxicity of fungicides with the active center Mancozeb for the environment and people.
- Collect information on the different molecular biomarkers of yeast to detect the toxicity of pollutants.
- Understand the basis of biosensors using yeasts.
- To analyze the yeast sensitivity against main fungicides used by farmers in the area of Ibarra-Imbabura, which are used in their commercial presentation (Manzete, Triziman D, Curamax) with active ingredient Mancozeb.

4 PESTICIDES

Pesticides are composed of one or more active and inert ingredients of natural or synthetic origin, to which other substances (solvents, wetting agents, colorants, repellents, etc.) are added to improve their efficacy and facilitate their use (Lushchak et al., 2018). Binders improve the physical properties of the pesticide (e.g. solubility, spread ability, and stability). Unlike additives and binders are not listed on the pesticide label. The active substance (molecule) constitutes the active ingredient (the one that acts on the pests) of the product (Zubrod et al., 2019).

Pesticides can be classified according to their objective: insecticides (used against insects), herbicides (against weeds), fungicides (against fungi), rodenticides (against rodents), acaricides (against mites), molluscicides (against mollusks), and nematocides (against plant parasites), the most widely used of which are insecticides, fungicides, and herbicides (Walia et al., 2014). Pesticides classified by their chemical structures are organochlorine, organophosphate, carbamate, inorganic pesticides and synthetic pyrethroids (Tobar, 2020).

However, the advance of biotechnology has brought new ways to combat pests with biopesticides (of botanical, microbiological, and pheromone origin) (Kumar et al., 2021). However, biopesticides still cannot replace chemical pesticides because they still face different challenges, such as high production costs, the difficulty of application to large-scale crops, and biopesticides are generally not intended as a "quick fix" and must always be used in conjunction with other strategies. For this reason, chemical pesticides are still the market leader in the pesticide market (Kumar et al., 2021).

Objective	**Molecule**	**Examples**
Insecticides	Dicarboximides	Ronuron
	Dinitrophenol	Cyromaworm
	Pyrimidines	Butaclor
	Halogenated hydrocarbons	Atrazina
		Amunil
		Cyromaworm
Fungicides	Metal dithiocarbamates	Triziman D
	Dinitrophenol	Stratego
	Ureas	Cimazol
	Pyrimidines	Milsana
		Manzete
		Curamax
		Acroplant
Herbicides	Bipyridines	Crisalamina
	Chlorophenols	Actinic
	Glyphosate	Propanil
	Organic nitrogen	Atalar
		Ametrex
		Atraprim

Table 1. The main classification of pesticides with their common molecule uses (Santiago, 2012). Examples are the most commercialized pesticides in Ecuador 2020 (INEC, 2021).

4.1 Pesticide poisoning problem in Ecuador

Agricultural activity plays a crucial role in the country's economy. In Ecuador, agriculture contributes to the generation of capital that allows the mobilization of industries and employment in the rural sector, thus contributing to the reduction of poverty in the countryside. Ecuador's Agricultural Productivity Report indicates that this activity contributes an average of 7% to GDP, making it the sixth-largest contributor to its production (Viteri & Tapia, 2018).

National Institute of Statistics and Censuses of Ecuador (INEC) reported that in 2020 the area devoted to agriculture in Ecuador corresponds to 12 462 614 hectares, this value corresponds to approximately 22% of the national territory (INEC, 2021). Manabí is the province with the largest area devoted to agriculture. In Ecuador, the crops of most excellent production at the national level are sugar cane, bananas, savoy grass, and African palm (INEC, 2021). Ecuador is the world leader in banana production. In 2019, it exported almost 25% of the bananas in the world, just followed by Europe with a 20% (Tobar, 2020). Similarly, it is one of the largest cocoa producers in the world. In 2014, Ecuador registered a total production of 240 thousand tons of cocoa. In addition, Ecuador is the third-largest exporter in cut flower industry in the world (Tobar, 2020).

In Ecuador, most agricultural production is based on monoculture techniques, which causes soil deterioration and makes these crops more susceptible to damage by pests and diseases, forcing the mandatory use of chemical pesticides to avoid economic losses (INEC, 2013). The outbreak of a pest in crops results in lower production or total crop losses. For this reason, farmers are forced to apply pesticides preventively and constantly on their crops to avoid economic losses. As a result, people are in contact with these pesticides due to mishandling and misuse of the pesticide (INEC, 2021). Thus, between 2014-2016 approximately 50% of permanent crops and 75% of transient crops used agricultural products of chemical origin (fertilizers and pesticides), from which at least 25% of the pesticides used are cataloged

in a moderately to a hazardous range (Mollocana-Lara & Gonzales-Zubiate, 2020). Proper pesticide management during storage, application, and disposal might considerably reduce the dangers and health consequences on farmers, consumers, and the ecosystem surrounding the crop, while also improving production costs and output. Despite this, only two out of ten farmer producers are thought to be trained in the use and administration of agrochemicals (Mollocana-Lara & Gonzales-Zubiate, 2020).

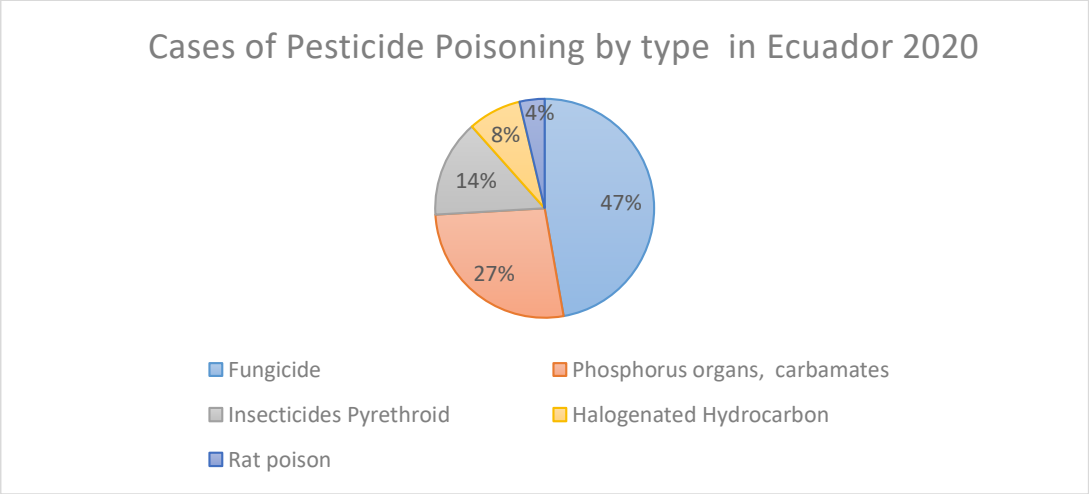

Figure 1. In Ecuador in 2020 most cases of pesticide poisoning have been caused by fungicides. Data adapted from (MSP, 2020).

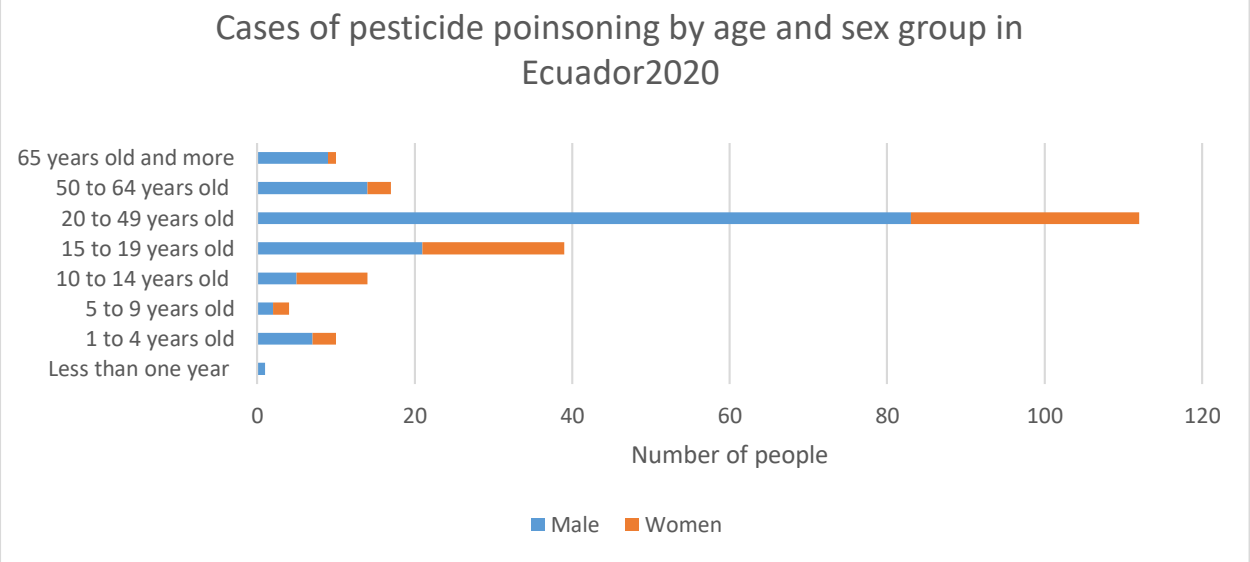

Figure 2. Most of the people poisoned by pesticides in Ecuador were men between 20 and 49 years old. A total of 216 people were poisoned in 2020. Data adapted from (MSP, 2020).

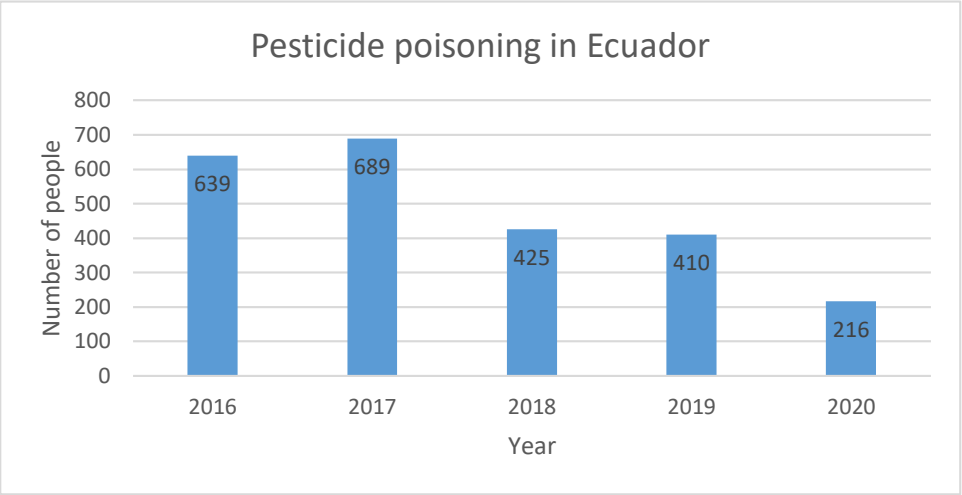

Figure 3. Represents the cases of poisoned people per year in Ecuador. Although in 2020 the number of cases of people poisoned by pesticides decreased considerably, this may be due to the pandemic caused by the Covid-19 virus, since many people lost their jobs. Data adapted from (MSP, 2020).

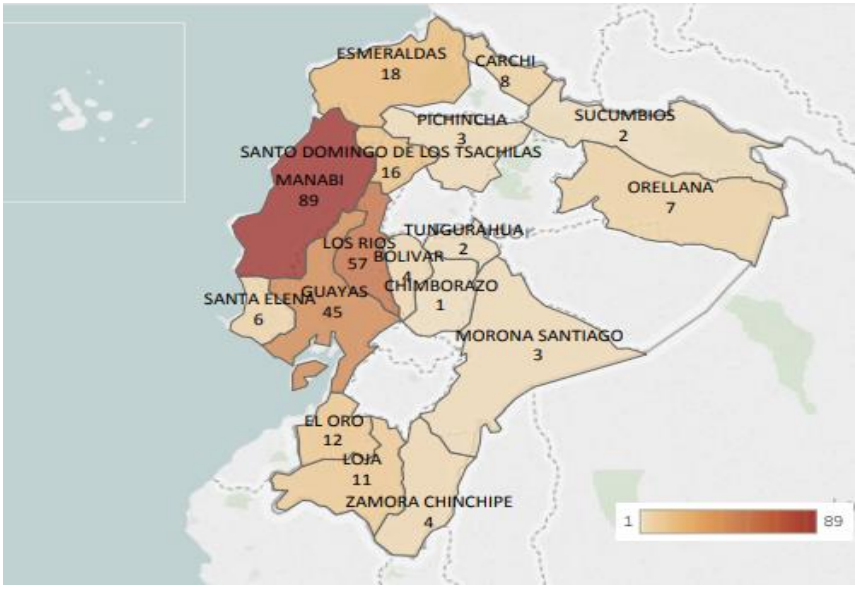

Figure 4. Distribution of pesticide poisoning cases in Ecuador. The province of Manabí is where most cases of poisoning occur (MSP, 2019).

In Ecuador, pesticides are generally classified by risk of hazard based on the classification criteria of the World Health Organization (WHO). This categorization includes four different classes I, II, III, and U. Each class represents a hazard level: extremely hazardous (Ia), highly hazardous (Ib), moderately hazardous (II), slightly hazardous (III), unlikely to present an acute hazard (U, sometimes referred to as to

IV) more than 50% of pesticides used in Ecuador belong to classes III and IV (Mollocana Lara & Gonzales-Zubiate, 2020). In 2008, one of the largest recorded cases of poisoning by fungicides with the active ingredient Mancozeb occurred in Ecuador. The evidence gathered by the Ombudsman's Office served as the basis for the Latin American Human Rights Association to file a lawsuit against ten Ecuadorian companies related to the fungicide Mancozeb in banana plantations in Ecuador (Santiago, 2012). The re-evaluation process had several intermediate resolutions that slowed down the process until August 2012, when 7 products containing Mancozeb, which paradoxically were listed in category III (slightly toxic), were removed from the market (Santiago, 2012). According to several studies, the perception of risks to pesticides in the banana sectors of Machala city suggests that various health concerns in banana workers and their families may be related with prolonged and uncontrolled irrigation of pesticides in light aircraft carried out by large banana corporations (Mollacana-Lara, 2020). Another investigation was carried out in the Pichincha province, in floricultural community, and studied neurobehavioral disorders in children who did not work in floriculture but live near flower farms or with at least one worker (Mollacana-Lara, 2020).

The use of pesticides in Ecuador has increased due to the need to satisfy local food needs, as well as to improve export goods to foreign markets. In Ecuador lacks relevant regulations that allow all the actors involved to take adequate management measures (Mollocana Lara & Gonzales-Zubiate, 2020). Although there are programs for the implementation of Good Agricultural Practices, they neglect the reality that many small farmers lack the expertise and experience to handle them properly and safely. This problem is exacerbated by the country's fragmented pesticide registry, the availability of outmoded pesticides on the market, and the agroindustry's lack of control over its environmental duty and pesticide management in vulnerable communities (Mollocana Lara & Gonzales-Zubiate, 2020). State agencies such as AGROCALIDAD take charge of the regulation of pesticides considering that the crops that require a

higher amount of agrochemicals are those included in the Ecuadorian basic food basket and the ones produced for export (Mollacana-Lara, 2020). On the other hand, the lack of current information and studies about the adverse impacts on the health of living beings and ecosystems associated with pesticide mishandling and accumulation in Ecuador prohibits acquiring a complete picture of the problem, reducing its importance. Furthermore, economic interests sometimes take precedence over health hazards, probably because agriculture is many small farmers' sole source of income, and it is not acceptable to risk losing harvests (Mollacana-Lara, 2020).

4.2 Mancozeb active ingredient

The scientific name of Mancozeb is Ethylene bisdithiocarbamates (EBDC), and its molecular formula is (C4H6MnN2S4)x(Zn)y. They are a group of contact fungicides that have been used since the 1940s (Vieira et al., 2020). EBDCs are most widely used in agriculture as contact fungicides that provide a broad spectrum of protection and with preventive activity against endoparasitic fungal diseases by inhibiting spore germination (Fatma et al., 2018). These compounds are characterized by metal or metalloid-carbon bonds forming an organometallic polymeric complex with zinc and salt (Vieira et al., 2020). Due to the presence of two sulfur atoms in the molecule, dithiocarbamate ligands show a robust binding capacity forming covalent bonds and occurrring between soft acidic metals. These compounds can also exhibit catalytic (strong Lewis acid) and redox characteristics when bound with anions (Fatma et al., 2018).

EBDC has lipophilic properties which allow it access through the molecular membrane. In addition, due to its ability to form complexes with metals, it can inhibit cofactors causing the inactivation of enzymes, resulting in the interruption of several biochemical processes at the cellular level involved in the production of adenosine triphosphate (ATP), causing the inhibition of mitochondrial respiration. For this reason, EBDCs are known as Group M fungicides (multi-site chemical inhibitors) (Fatma et al., 2018).

Ethylene thiourea (ETU), ethylene urea (EU), and ethylenebis sulphide (EBIS) are metabolites produced by the hydrolysis of EBDC. Mancozeb is an unstable molecule in water and can be decomposed by the presence of heat, humidity, and UV light (Mutic et al., 2017). Likewise, all these metabolites are responsible for creating an unfavorable microenvironment for fungal growth and increasing the toxicity of the fungicide (Fatma et al., 2018).

4.2.1 Toxicity of Mancozeb in the environment

The fate of a contaminant in the environment can be influenced by its physical, chemical, biological properties and its interaction with the environment (Zubrod et al., 2019). Likewise, the amount, mode of application, and geographical area where the pesticide was applied may influence the fate of the contaminant in the environment (Walia et al., 2014).

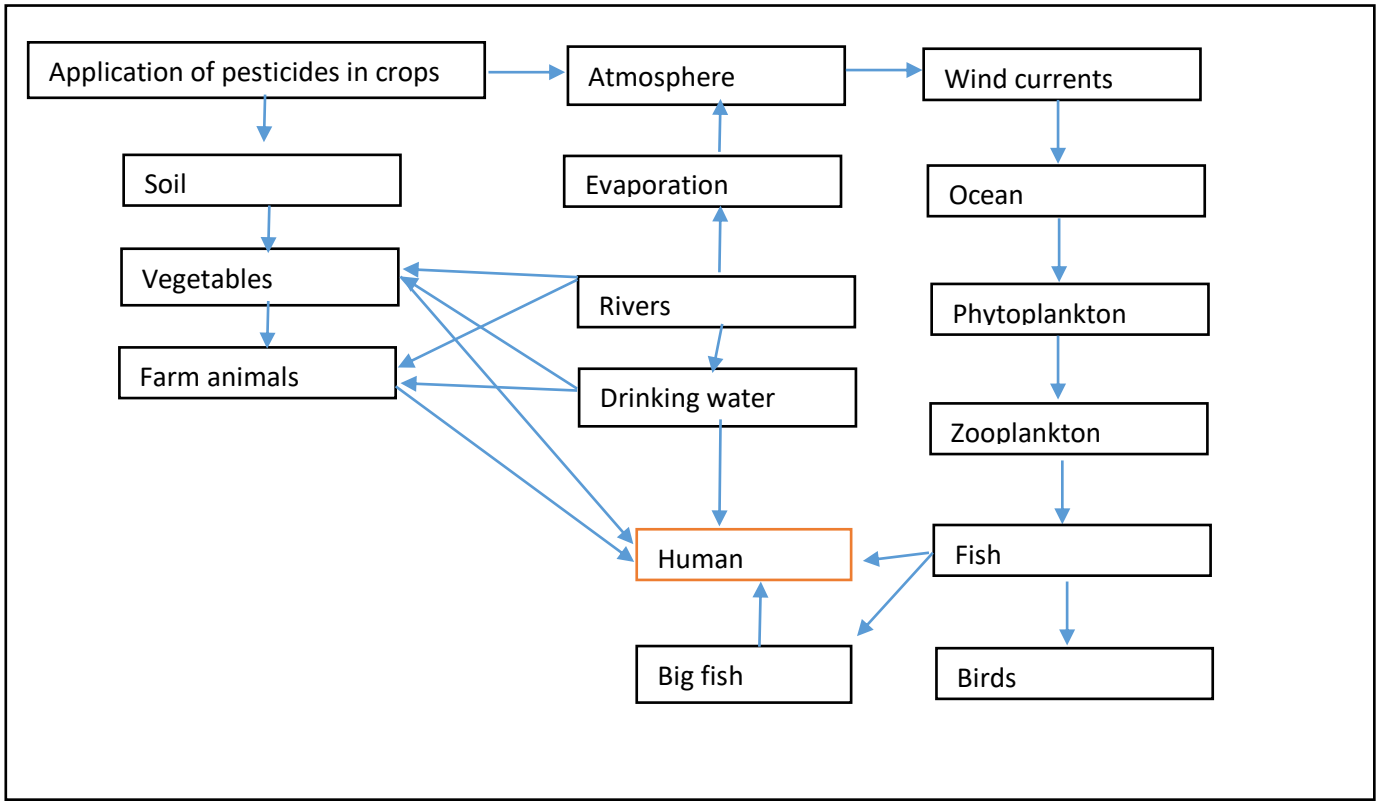

Figure 5. Fates of pesticides, adapted from: (Lushchak et al., 2018).

The soil plays an essential role in the distribution and fate of organometallic compounds, as it often serves as an important reservoir and sinks for these pollutants due to its high absorption capacity, damaging the soil microbiota and thus causing an imbalance in the ecosystem (Vieira et al., 2020). Pesticides can be transported through the air, water, and soil, endangering water quality in areas near crop fields and even affecting aquatic ecosystems (Walia et al., 2014).

4.2.1.1 Soil toxicity

Low concentrations of Mancozeb in the soil are detrimental to beneficial populations of fungi and actinomycetes, while higher concentrations (1000 and 2000 ppm) are detrimental to soil bacteria. Mancozeb raises the nitrogen-nitrate concentration by lowering the pH of the substrate (Walia et al., 2014). The concentration of enzymes synthesized by animal and plant microorganisms such as amylase and invertase phosphatase decreases in the presence of Mancozeb in the soil (Fatma et al., 2018). Likewise, the amount of CO2 present in the soil decreases, making it a non-viable soil for agriculture in the future (Elizabeth Ruiz Suárez et al., 2013). The constant adsorption values (Koc = 283 to 2279 ml/g) for Mancozeb indicate that it is moderately to slightly mobile in soils. However, ETU is mobile in soils (Koc = 35 to 855 ml/g). It can contaminate surface water through runoff and groundwater through leaching (Elizabeth Ruiz Suárez et al., 2013).

4.2.1.2 Water toxicity

Water contamination by pesticides occurs when they are carried by water from agricultural fields to rivers and seas where they enter food chains, causing the death of various forms of life necessary in the balance of some ecosystems (Zubrod et al., 2019). Fungicides have the potential to adsorb organic carbon. Consequently, fungicides can adsorb on sediments and organic surfaces in aquatic systems (Zubrod et al., 2019). Fungicides in rivers and lakes can cause eutrophication, increasing the number of algae covering the surface of the water and decreasing the oxygen present in the water (Elizabeth Ruiz

Suárez et al., 2013). In laboratory studies, it has been observed that crustaceans also reduce their survival at low concentrations (35 µg/L) of fungicides in the medium (Zubrod et al., 2019).

After being exposed to the environment, Mancozeb can decompose into one of its secondary metabolites, ETU, in approximately seven days (Elizabeth Ruiz Suárez et al., 2013). ETU is mobile in soils and waters and can be absorbed into the tissues of crustaceans. For example, ETU accumulation has been detected in rainbow trout, even affecting the fertilization rate of trout eggs (Tzanova et al., 2017). The amount of ETU present in rainbow trout was obtained by liquid chromatography in the range of 0.045 - 10 mg/l, making it dangerous for human consumption (Tzanova et al., 2017). The LD50 dose for trout was 1.1 mg/l given per ETU metabolite. Similarly, studies were conducted in zebrafish, but in this case, Mancozeb commercial formulations were used so that the amount of adjuvant increased the toxicity. The studies showed that from a concentration of 50 µg/l fish showed deformities such as pericardium edema, yolk sac edema, tail deviations (Vieira et al., 2020).

4.2.2 Toxicity of Mancozeb in humans

The reactions produced by pesticides according to the time of exposure can vary between acute (short-term) and chronic (long-term). Chronic exposure's effects may not usually leave visible signs; they can appear months to years after exposure and are linked to the development of several types of cancer, reproductive issues, developmental abnormalities, and nervous system ailments (Mollocana Lara & Gonzales-Zubiate, 2020). Mancozeb is considered a slightly toxic pesticide with a lethal oral dose of 6,250 mg/kg and a lethal dermal dose of 12,500 mg/kg. However, Mancozeb has been associated with several negative effects on various organs upon long-term contact with the fungicide. Upon skin contact, the fungicide causes dermatitis and dermal sensitization (Lushchak et al., 2018). Mice (*mus musculus*) orally exposed to Mancozeb showed thyroid hyperplasia, probably through its ability to inhibit thyroxine synthesis, toxicity manifested as altered thyroid hormones, increased thyroid weight, and presence of

microscopic lesions in this gland. In addition, prolonged exposure to Mancozeb may produce neurotoxicity through an as yet unknown mechanism and Mancozeb has a strong link to neurodegenerative disorders. Mancozeb has been reported to inhibit complex III of the mitochondrial electron transport chain to disrupt the glutathione antioxidant system with is often associated with the generation of reactive oxygen species (ROS) (Lushchak et al., 2018).

Mancozeb has also been linked with abnormal fetal development and significant physiological, biochemical, and pathological alterations may occur, leading in infertility. Exposure to Mancozeb also alters reproductive and endocrine structures, leading to decreased fertility. Furthermore, Mancozeb possesses chelating effects as well, allowing it to potentially disrupt multiple metal-containing enzyme systems, such as zinc, copper, and iron (Mutic et al., 2017).

ETU is a water-soluble heterocyclic compound that would facilitate its rapid absorption in the gastrointestinal tract. EBDCs are generally considered a probable human carcinogenic due to their metabolite ETU. The carcinogenicity classification of Mancozeb is based in part on that of its major metabolite, ETU (Mutic et al., 2017). According to published studies, Mancozeb is reported to be a neurotoxic pro-oxidant that promotes increased intracellular concentrations of reactive oxygen forms. In vitro studies in human lymphocytes and CHO cells showed induction of DNA strand breaks, suggesting its carcinogenic potential in the case of surviving and propagating cells, and single-stranded DNA breaks were demonstrated in rat fibroblasts exposed to Mancozeb thus demonstrating the risk it may be if people come into prolonged contact with fungicides having Mancozeb as their active center (Lushchak et al., 2018).

5 *SACCHAROMYCES CEREVISIAE (*S. CEREVISIAE) AS A MODEL FOR TOXICITY STUDIES.

In 1996, the S. cerevisiae genome became the first eukaryotic genome to be completely sequenced. The density of protein-coding genes in the yeast genome is higher than the density of genes

in the human genome (S. van Leeuwen et al., 2012). The high gene density is partly explained by the relatively low number of intron-containing genes in *S. cerevisiae* (Rica et al., 2010). Nevertheless, yeast remains a good study model for assessing stress mechanisms and gene function in response to environmental toxins as it has a high level of functional conservation within the human genome and other more complex eukaryotes (Dias et al., 2010). Yeast and humans are separated by a billion years of evolution, yet thousands of recognizable orthologous genes exist between the two species (Dos Santos & Sá-Correia, 2015). In addition, there are hundreds of genes from one species that can functionally replace (complement) their orthologues in the other; such functional complementation between genes from evolutionarily distant species indicates significant conservation of function (Skrzypek et al., 2018).

There are several pieces of evidence in which yeast has played an essential role in research. For example, the Nobel Prize in Medicine and Physiology in 2001 was awarded jointly to researchers Paul Nurse and R. Timothy Hunt, and Leland Hartwell, for their discoveries on cell cycle regulators, using yeast as a model, allowing a better understanding of normal cell growth and division, and also of possible alterations that lead cells to become cancerous (Hohmann, 2016). The Nobel Prize in Chemistry in 2004 was awarded to Aaron Ciechanover, Avram Hershko, and Irwin Rose, in which yeast played an important role in discovering how ubiquitin-mediated proteolysis occurs. Recently, Randy Schekman and Yoshinoro Ohsumi, both Nobel Prize in physiology and medicine, in 2013 and 2016 respectively, for their fundamental work, on vesicular trafficking and the mechanisms of autophagy (Hohmann, 2016).

Likewise, Yeast was one of the first organisms in which Green fluorescent protein (GFP) was shown to be a suitable marker for protein localization and helped in the discovery that chromosomes are protected by telomeres (Hohmann, 2016). To this day, the yeast continues to be studied to understand the molecular basis of eukaryotic transcription and to improve biochemical molecular processes, as is the

case with RNA polymerase II, which allows the purification and characterization of components of the gene expression machinery (Hohmann, 2016).

These are some of the characteristics that make yeast such a competent model system (Rica et al., 2010; S. van Leeuwen et al., 2012; Skrzypek et al., 2018):

- High level of functional conservation within the human genome and other more complex eukaryotes
- The Saccharomyces genome database (http://www.yeastgenome.org/), provides detailed and up-to-date information on every yeast gene.
- It is a non-pathogenic single-celled microorganism with rapid and economic growth.
- In comparison to higher eukaryotes, yeasts are easier to handle, and yeasts have a quick generation time of about 1.5 hours in rich medium that contains a carbon source, a nitrogen source, salts, vitamins and essential minerals.
- These cells can be easily stored short- or long-term in plates at 4 °C or in glycerol at -80 °C, respectively.
- Well-established protocols for yeast gene modification.
- Survival and growth of yeast cells under stress conditions.
- They are also helpful for determining the outcome of protein mutations.

5.1 Biomarkers of *Saccharomyces cerevisiae* for the evaluation of toxic agents.

Biomarkers are observable and/or measurable changes at the molecular, biochemical, cellular, physiological, or behavioral level that reveal present or past exposure to a chemical of a contaminant nature (Campuzano, 2011). When large numbers of cells are affected by the effects of toxic substances, toxic effects may be detectable by measuring changes in growth rate, expansion, resource efficiency, and

adaptability. Biomarkers fall into different categories: biomarkers of exposure to xenobiotics, biomarkers of the effects of exposure (biochemical, physiological, or behavioral alteration), and biomarkers of susceptibility. Several parameters can be used as biomarkers; even the absence of the contaminant plays an important role in measuring toxicity (Nguyen et al., 2017).

In the case of yeast, the response to environmental stress is translated into different mechanisms at the molecular and cellular levels so that the yeast can adapt to the environment. To cope with these unfavorable situations, the yeast responds rapidly by synthesizing molecules that allow it to attenuate or repair the damage caused by the stress. In *S. cerevisiae* yeast, different general environmental stress response pathways have been identified, such as protein folding and turnover, response to oxidative stress (ROS), generation of multidrug resistance, generation of transporters in the membrane to expel the contaminant, DNA repair mechanisms, changes in carbon metabolism and regulation of gene expression reflecting the toxic action of the pollutant (Campuzano, 2011; S. van Leeuwen et al., 2012). These yeast stress responses can be used as biomarkers to detect toxicants and help delineate regulatory risk assessment to gather essential information. The use of a simple single-cell experimental model such as *S. cerevisiae* is very useful as a first screening tool, limiting the use of animal models (Campuzano, 2011). However, animal toxicity tests are a great tool to measure toxicity at the organ level, but the use of yeast helps us to see the toxicity that may be present in the environment in small amounts (S. van Leeuwen et al., 2012).

5.1.1 Oxidative stress response (ROS)

ROS occurs when there is an imbalance in our cells due to increased free radicals and/or a decrease in antioxidants. Over time, this imbalance between free radicals and antioxidants can damage tissues (Dias et al., 2010). The electron transport chain of mitochondria, peroxisomes, NADPH oxidase, uncoupled nitric oxide synthase and the cytochrome P450 system are the most important sources of ROS

production (Carvajal Carvajal, 2019). ROS are also formed under the influence of ultraviolet light, ionizing radiation, and xenobiotics. In addition, mitochondrial disease genes are highly conserved among eukaryotes, and yeast genetics has been used to study the mechanisms of mitochondrial toxicity (S. van Leeuwen et al., 2012).

5.1.2 Vacuoles

Yeast vacuoles are acidic compartments with hydrolytic and proteolytic enzymes for the degradation of different types of molecules. They are also responsible for protein turnover, nutrient recycling, and are involved in osmoregulation and ionic homeostasis (Conibear & Stevens, 2002). The vacuole undergoes detectable morphological changes in response to stress, which can be observed by vital dye staining, using green fluorescent protein (GFP) markers, and immunofluorescence microscopy of formaldehyde-fixed cells (Conibear & Stevens, 2002).

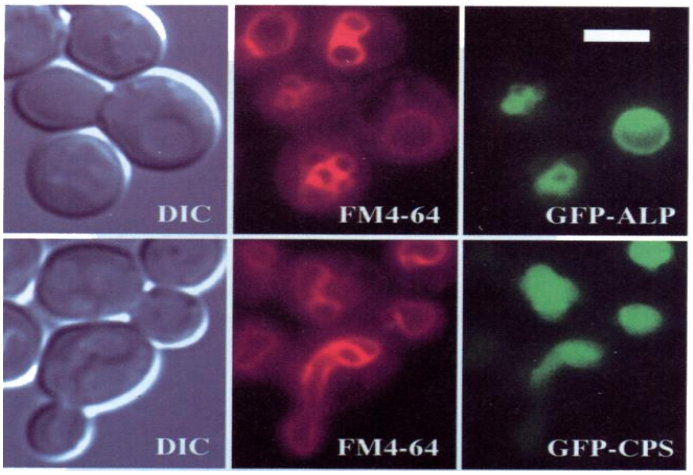

Figure 6. Environmental stresses cause rapid fusion of vacuoles into a single vacuole large and round structure, whereas if the xenobiotic comes in contact during mitosis it makes the vacuole appear elongated and fragmented (Conibear & Stevens, 2002). Dic is control Wild type that contain one to five vacuoles that can be observed in the light microscope. FM4-64 is vital stain to follow bulk membrane-internalization and transport to the vacuole in yeast. GFP-tagged proteins help to follow the endocytic pathway to reach the vacuole (Conibear & Stevens, 2002).

Oxidative stress alters vacuolar enzymes in yeast, so it is assumed that intracellular vacuoles are used as an indicator to detect oxidative stress in yeast caused by contaminants (Dias et al., 2010). Toxicity can be measured as lethal concentration when it interferes with the proper function and growth of vacuoles in yeasts. Detection of toxic chemicals can be employed by using a lysosome-like vacuole in yeast. Vacuoles are susceptible organelles in which lysosomal enzymes are readily activated and released when exposed to stressful conditions (Nguyen et al., 2017).

5.1.3 Membrane transporters

Eukaryotic cells can adapt and survive exposure to a wide variety of exogenous compounds, such as toxins or drugs. Several proteins are involved in the resistance process, mainly multidrug membrane transporters and transcription factors that confer resistance to xenobiotics (Dias et al., 2010). Overexpression of proteins and transcription factors can be used as biomarkers of the presence of toxicity. The pleiotropic drug resistance (PDR) system belongs to the subfamily of ATP-binding ABCG transporters to hydrolyze ATP, which share conserved proteins with higher eukaryotes with similar structures and domains (Karamanou & Aliferis, 2020).

Similarly, the YCF1 transporter found in the yeast vacuole membrane; is a morphological and functional analog of human MRP1 that mediates metal sequestration. The YCF1 transporter in the yeast vacuole exerts the function of fungicide translocation across the membrane for the development of fungicide resistance (Karamanou & Aliferis, 2020).

5.1.4 Intracellular sequestration

In case the contaminant contains metals, the increase of metallothionein proteins can be used as biomarkers to detect these contaminants at the intracellular level. Intracellular accumulation of metals takes place in particular in the cytoplasm by metallothioneins. These are small cysteine-rich proteins that

bind heavy metals at the level of sulfhydryl groups; protect the cell against copper toxicity by strongly chelating copper ions (Ruta et al., 2017).

Thus, they trap metals within the cell to render them harmless. Metallothioneins are present in most eukaryotes (animals, plants, fungi, yeasts). In *S. cerevisiae*, the excretion of glutathione fixes metals by forming metal precipitates in the medium (Ruta et al., 2017).

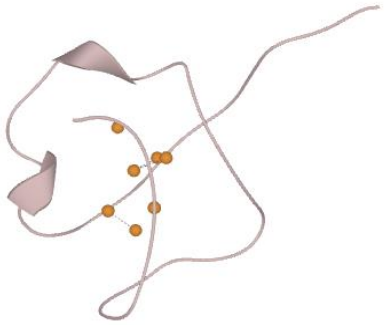

Figure 7. Yeast metallothioneins. Contains 1 metal-binding domain: 6 to 8 copper ions are chelated within a single copper thiolate group and coordinated via cysteinyl thiolate bridges to 10 cysteine ligands. 6 copper ions are trigonally coordinated, while the other 2 are only diagonally coordinated. Retrieved from https://www.uniprot.org/ database

5.1.5 G protein-coupled receptors (GPCRs)

They constitute a large superfamily of eukaryotic membrane proteins that act as signal transducers across the cell membrane: on the outside, they receive a ligand and on the inside of the cell they activate G proteins. Binding of the agonist ligand to a GPCR causes ligand-specific active conformational changes and allows the receptor to couple to G proteins (Nakamura et al., 2018).

GPCRs can selectively detect an incredibly diverse range of molecules including photons, ions, small molecules, and proteins. The different conformational changes of the associated proteins and signal transduction that interact with the *S. cerevisiae* pheromone mating pathway can be measured and used as biomarkers of toxicity (Nakamura et al., 2018).

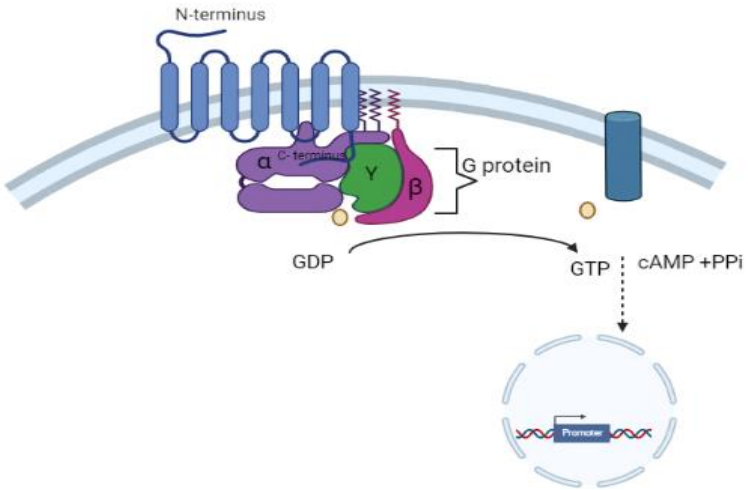

Figure 8. The ability of GPCRs to mediate intracellular changes in response to extracellular signals, Increased cAMP levels in cells results in the upregulation of genes. Adapted from:(Lengger & Jensen, 2020) and create with https://biorender.com/

Similarities between the yeast acylation pathway and signaling mechanisms with higher organisms, including structural and functional similarities between Gpa1, the alpha subunit of the yeast G protein, and the mammalian Gα subunit, are other reasons that make yeast a good model for toxicity (Lengger & Jensen, 2020).

5.1.6 Gene expression variations

Yeast toxic genomics aims to study the cellular response to a given toxicant at the genome, transcriptome, proteome, and metabolome level. Mutagenicity testing in single-cell organisms is generally a preliminary screen before animal testing to assess the safety of a chemical (Dias et al., 2010).

Open Reading Frame (ORF) is an open reading frame of a DNA molecule that is translated into amino acids and contains no termination codons. In the presence of xenobiotics, the reading frame can

undergo mutations (Dias et al., 2010). By having a complete library of yeast genes, ORF changes and mutations can be measured to gauge the toxicity of a compound. The mutations that are tracked are those that confer resistance to the drug thus providing the modes of action of the compound (Dias et al., 2010).

ORF	ID gene	Descripción	Human homolog
EFT2	851993	Catalyzes ribosomal translocation during protein synthesis; contains diphthamide, the unique post translationally modified histidine residue specifically ADP-ribosylated by diphtheria toxin	*EEF2*
YTM1	854446	Constituent of 66S pre-ribosomal particles, required for maturation of the large ribosomal subunit.	*WDR12*
MVD1	855779	Mevalonate pyrophosphate decarboxylase, an enzyme that has a function in the biosynthesis of isoprenoids and sterols, including ergosterol; acts as a homodimer.	*FP17780*
FPR1	855587	Peptidyl-prolyl cis-trans isomerase (PPIase), binds to the drugs FK506 and rapamycin; also binds to the nonhistone chromatin-binding protein Hmo1p and may regulate its assembly or function.	*FKBP1A*
SGN1	854817	mRNA-binding protein that may play a role in modulating the expression of cytoplasmic mRNA	*PABPN1*
NGR1	852513	RNA binding protein that negatively regulates growth rate; interacts with the 3' UTR of the mitochondrial porin (POR1) mRNA and enhances its degradation.	*TRNAU1AP*
PGK1	850370	3-phosphoglycerate kinase catalyzes the transfer of high-energy phosphoryl groups from the acyl phosphate of 1,3-bisphosphoglycerate to ADP to produce ATP; a key enzyme in glycolysis and gluconeogenesis.	*PGK1*
PDR5	854324	ABC transporters, actively regulated by Pdr1p; also involved in steroid transport, cation resistance, and cellular detoxification during exponential growth.	*ABCA9*
URA3	856692	Orotidine-5'-phosphate (OMP) decarboxylase, catalyzes the sixth enzymatic step in the de novo biosynthesis of pyrimidines, converting OMP into uridine monophosphate	*UMPS*

		(UMP); converts 5-FOA into 5-fluorouracil, a toxic compound.	
PDR15	852015	Plasma membrane ATP binding cassette (ABC) transporter, multidrug transporter, and general stress response factor implicated in cellular detoxification.	*ABCA9*
SNQ2	851574	ABC multidrug transporter involved in resistance to singlet oxygen species and Confers also resistance to 4-nitroquinoline-N-oxide	*ABCA9*

Table 2. ORF description is adapted from SGD (www.yeastgenome.org) and human homology was obtained by performing a BLAST at NCBI (www.ncbi.nlm.nih.gov)

This similarity and presence in different species help to find and assign a particular function to a given gene. Paralogous genes are usually not entirely identical because there are duplicated mutations that accumulate along the evolutionary line, but the degree of similarity between them provides a useful measure for calculating evolutionary time and constructing the phylogenetic tree. Comparative genomics uses these similarities and differences between genomes to deduce structural and functional information and serve as biomarkers to study the toxicity of compounds and thus predict the damage they might cause in higher eukaryotes (Dias et al., 2010).

5.1.7 DNA repair

Yeast exhibits similar DNA repair mechanisms with other eukaryotic organisms, therefore potential genotoxicity can be identified by genetically modified yeast strains responsive to DNA damage (Mollacana-Lara, 2020). When the damage is extensive and severe, human cells undergo apoptosis and are efficiently eliminated from the body so that the cells do not transform into mutant cells. On the other hand, if the damage is mild, the cells undergo a series of DNA repair operations (Tafurt & Morales, 2014). Organisms have many complex strategies that function with the common goal of maintaining the integrity of their genetic material. The cellular repair response to DNA damage by endogenous or exogenous factors is mediated by signaling pathways, requiring multiple sensors, transducers, and effector proteins,

in an interacting network of different repair pathways (Moura & Houten, 2017). Apart from mating error repair (MMR) that corrects errors introduced spontaneously during replication by DNA polymerase, these mechanisms can be biochemically divided into direct and indirect repair (Moura & Houten, 2017).

5.1.7.1 Direct repair

Direct repair is performed by the action of a single enzyme capable of repairing the lesion without replacing the damaged base. For example, the photolyase enzyme reverses the mutagenic effects generated by UV radiation, which captures a photon to reverse the dimer, breaking the covalent bond between the pyrimidines and repairing DNA damage (Tafurt & Marin, 2014). The human genome has two CRY genes (genes coding for cryptochroma protein) homologous to photolyases. In the case of the enzyme guanine methyltransferase, it locates the alteration site and then transfers the methyl group from guanine to a cysteine (Tafurt & Morales, 2014).

5.1.7.2 Nucleotide excision repair (NER)

DNA damage recognition establishes a signaling pathway that optimizes cellular conditions for damage repair. In *S. cerevisiae*, it involves the activation of two central protein kinases, Tel1 (ATM in mammals) and Mec1 (ATR in mammals) (Morita et al., 2010). This process involves enzymes that detect the incorrect DNA sections and perform different functions: they open the double-strand (helicases), cut the damaged fragment (endonucleases), synthesize the correct DNA sequence (polymerases), and bind the new nucleotides (ligases)(Morita et al., 2010). NER operates by two routes that differ in the mechanism of lesion recognition. On the one hand, transcription-coupled specifically recognizes lesions that lead to transcription blockade. On the other hand, the different pathway recognizes these lesions in Introduction any part of the genome globally (GG-NER, Global genomicNER). The lesions are corrected by removing and filling in an oligonucleotide of approximately 20-30 bases containing the damaged bases (Moura & Houten, 2017; Tafurt & Morales, 2014).

5.1.7.3 Base Excision Repair (BER)

Glycosylases, a key enzyme in this mechanism, recognize few lesions and are ATP-independent, giving high specificity and low energy cost. Among the multiple types of lesions that can arise in DNA, one of the most frequent is abasic sites (AP). AP sites can originate from spontaneous nucleotide purification (Morita et al., 2010). The proximity of AP sites on the two strands of DNA can lead to double-strand breaks. BER repair is accomplished by cleavage of the glycosyl linkage by a DNA glycosylase that must recognize the lesion, then incision of the AP sugar (apurinic or apyrimidinic) by an endonuclease and resynthesize of the missing nucleotide by DNA polymerase β, and finally a DNA ligase rejoins the strand. BER involves the change of a single base, caused by oxidative damage (Morita et al., 2010).

6 BIOSENSORS

The standard analytical methods used for the detection of pesticides are chromatographic techniques in conjunction with various detectors (Jiménez & León, 2009). These methods have the advantage of being automated and accurate, with high specificity, and can be used for simultaneous detection (Campuzano, 2011). However, these systems suffer from some drawbacks, such as high costs, expensive equipment for detection, time consumption, the need for sample pretreatment, slow response time, and the need for specialized personnel (Bucur et al., 2018; Jiménez & León, 2009).

A biosensor is an analytical device that combines a biological component with a physicochemical detector and can be used to detect a specific analytic in less time (Rumayor G et al., 2015). Thus allowing small and medium-sized companies with biosensors to carry out their detection analyses since they are portable and perform their tests (Jiménez & León, 2009). This will mean considerable savings in cost and time, and give the company greater peace of mind, autonomy, and operability to follow up and supervise its production. These methods with biosensors must provide data in real-time, allowing control and

traceability of each of the processes involved and ensuring the safety and innocuousness of food products (Campuzano, 2011).

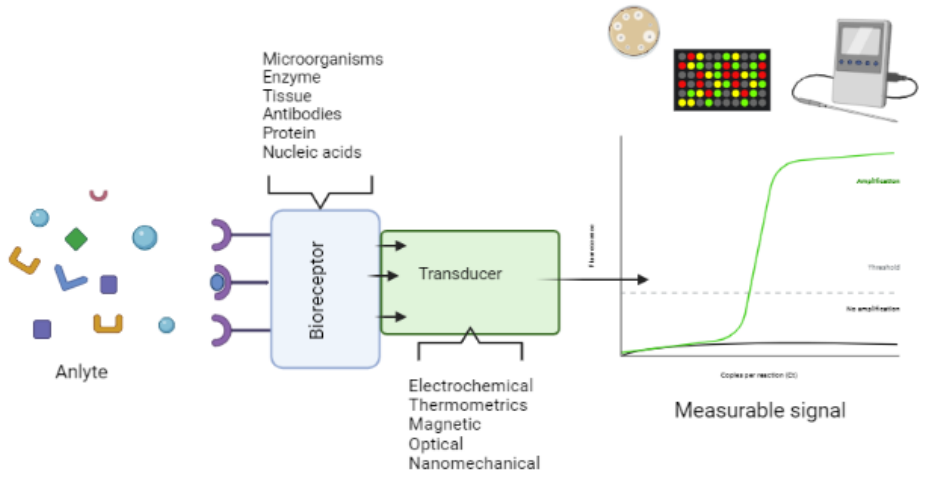

Figure 9. Structural elements of a analyte biosensor adapted from:(Mendoza-Madrigal, A.G. et al., 2013) and created with Biorender.

The bioreceptor specifically detects a substance by taking advantage of the specificity of the biomolecular interactions for the recognition of the analyte resulting in the variation of one or several physicochemical properties (pH, electron transfer, heat transfer, change of potential, mass, variation of optical properties, etc) detected by the transducer (Mendoza-Madrigal, A.G. et al.., 2013).

The transducer is the element that converts the variations of the physical or chemical properties produced by the interaction between the recognition element and the analytic into a signal that can be amplified, stored, and recorded (Campuzano, 2011). There are several types of transducers: electrochemical, optical, piezoelectric (mass, gravimetric, acoustic), thermometric and nanomechanical. Depending on the nature of the interaction between the recognition element and the species of interest, one type of transducer or another may be used (Jiménez & León, 2009). The *Table 3* shows some examples of transducers used in biosensors.

Transducer	Characteristics	Bibliography
Optical	The physical basis of this type of sensor is the changes that occur in absorption, fluorescence, luminescence, scattering, or refractive index when light is reflected from the recognition surfaces.	(Rumayor G et al., 2015)
Thermometric	Measurement in exothermic processes, in which the heat transfer can be related to the concentration of the analyte of interest.	(Jiménez & León, 2009)
Nanomechanics	The determination is made employing the change in the surface tension between the two components	(Jiménez & León, 2009)
Electrochemicals	Determination of electric currents associated with the electrons involved in redox processes. These can be potentiometric, conductimetric, and amperometry	(Rumayor G et al., 2015)
Piezoelectric	They can be mass, gravimetric or acoustic and measure direct mass changes induced by antigen-antibody complex formation.	(Mendoza-Madrigal, A.G. et al., 2013)

Table 3.Transducer alternatives for uptake and quantification in analyte detection

In biosensors technology development, the biosensing element requires to be immobilized with the transducer interface (Tobar, 2020). A proper immobilization will assure the interaction between the biorecognition material and the transducer; hence, the quality of the biosensor. The most used methods for immobilization are physical adsorption at a solid plane, cross-linkage between molecules, covalent attachment to a surface, affinity-based linkage and the entrapment in a membrane system (Tobar, 2020). Furthermore, because a large number of samples must be collected on-field for high-throughput applications, biosensors must have ready-to-use methods and lengthy storage durations without losing their sensing characteristics (Mollacana-Lara, 2020). In this regard, cell encapsulation methods play a critical role in ensuring cell viability and survival. Calcium alginate, polyvinyl alcohol, pectin, gelatin, agar,

and silica gel are examples of cell immobilization polymers that have been shown to keep cells alive for up to 120 days (Mollacana-Lara, 2020).

6.1 Application of biosensors using *S. cerevisiae*

Yeast-Based Biosensors detect a variety of compounds, including odorants, metals, intracellular metabolites, carcinogens, lipids, sugars, alcohols, and other contaminants like pesticides. Furthermore, yeasts can be natively sensitive to analytes or can be made sensitive by introducing a biorecognizing molecule such heterologous proteins (Mollacana-Lara, 2020). Microbial biosensors can interpret or measure the signal by respiration and metabolite changes exerted by xenobiotics. For example, if yeasts can metabolize a substrate, a metabolic reaction occurs with oxygen consumption and the pollutant can be determined by the decrease in gas pressure. If the effluent is toxic, the yeast activity decreases; this signal is very stable since it will react to the slightest disturbance of the natural environment (Campuzano, 2011). On the contrary, some organic compounds increase yeast activity: the signal recorded is then an increase in voltage. This is the case for domestic and agricultural effluents or even hydrocarbons. Most microbial biosensors are electrochemical, detecting the changes generated by the redox reactions caused by pollutants and depending on the oxidation or reduction values can be correlated with the amount of pollutant (Gong et al., 2020; Zhao et al., 2021).

Important issues in the field of creating amperometric (electrochemical) microbial biosensors include increasing the selectivity of analysis; searching for strains that oxidize foreign compounds to create effective environmental monitoring devices (Campuzano, 2011). For this reason, genetic engineering is used to incorporate the genes necessary for contaminant degradation into yeast to increase the analytical potential of microbial sensors (Ostrov et al., 2017). The use of modified microorganisms that incorporate reporter genes containing a contaminant-induced promoter facilitates the quantification of the contaminant (Tian et al., 2017). Examples of commonly used reporter genes encoding the following

proteins: green fluorescent protein (GFP), red fluorescent protein (RFP), luciferase enzyme, β-galactosidase (GAL), β-lactamase, chloramphenicol acetyltransferase (Moscovici et al., 2020; Tian et al.,2017). The promoter in this case plays an important role in regulating the expression of reporter genes as it provides binding sites for RNA polymerase. Functionally, a promoter is a DNA sequence located upstream (towards the 5' end of the coding region of a gene) that includes the binding regions for transcription factors (Tian et al., 2017).

In addition, the native enzymatic responses of yeast are one of the elements used in biosensors to detect the presence of the contaminant by quantifying enzyme activation or inhibition (Bucur et al., 2018; Martin-Yken et al., 2018). Basically, the process to determine the pesticide level is to get a main signal with an enzyme-substrate complex, then measure the reduction of that signal due to the presence of the inhibitor, and match it with the concentration of the pesticide (Tobar, 2020). The main signal is obtained from the enzyme catalyzing the substrate reaction at the most optimal conditions. Tyrosinase-based biosensors are mainly used for rapid screening because the huge number of inhibitors for this enzyme (Tobar, 2020).

Target Molecule	**Mechanism**	**Transducer**	**Sensitivity**	**Bibliography**
Cu^{2+}	ADE2 gene deleted from the genome by the insertion of the kanMX cassette. The AMP pathway is interrupted, leading to the accumulation of the Ade2p substrate which is oxidized in the presence of the Cu^{2+}	Red coloring strain in the presence of Cu^{2+}	1–100 µM	(Nakamura et al., 2018)
Cadmium (cd)	Incorporation of the producer Phytochelatin synthase gene from Arabidopsis. This enzyme requires metals to be activated	Fluorescence derived from pyrene-labeled as the product Phytochelatin synthase	0.2-1.0 µM	(Matsuura et al., 2013)

Genotoxic compounds	RAD54 promoter that regulate yEGFP reporter vectors	Fluorescence signal	0.016-0.5 µg/ml	(Tian et al., 2017)
Endocrine disrupting	Strains are incorporated plasmids (pUTK407 and pUTK420) that containing human estrogen nuclear receptor (hER) or the human androgen receptor (hAR) and GPD and ADH1 yeast promoters.	Fluorescence signal	0.012–200 µg/l	(Moscovici et al., 2020)
Ciguatoxins	Measuring the enzymatic activity of β-galactosidase with the incorporation of the PFKS2 promotor	β-galactosidase is determined by a colorimetric assay	0.1-125 µg/mL	(Martin-Yken et al., 2018)
Glucose	Incorporation of synthetic glucose dehydrogenase genes derived from Aspergillus oryzae TI (denoted GDH1)	Cells are immobilized on gold electrodes to build an electrochemical biosensor	1.4-33.3 mmol/l	(Zhao et al., 2021)
Candida albicans	GPCR´s yeast transmembrane receptors can recognize fungal pheromones that induce the transcriptional activation of biosynthetic genes for the production of red lycopene pigment visible to the naked eye.	Colorimetric detection	5-40 µM	(Ostrov et al., 2017)
Chlorothalonil	Measuring caspase 3 Activity	Colorimetric assay	0.004-2.5 µg/ml	(Gong et al., 2020)

Table 4. Examples of biosensors using yeast for the detection of different analytes

7 METHODOLOGY

7.1 Materials

Yachay Tech University provided yeast strains, reagents, and laboratory equipment. The yeast strain used throughout this study was *S. cerevisiae* type W303. In the case of fungicides, interviews were conducted in the commercial areas of agricultural products to obtain the most used fungicides in the area of Imbabura Ibarra. The fungicides most recommended by the sellers and those most commercialized in the area were: Manzete and Triziman D and Curamax. The fungicide Curamax is characterized by having

mancozeb and cymoxanil in its formula. Cymoxanil is a sealant that helps the fungicide to remain longer on the plants and thus avoid fungicide losses due to rain.

Raw material	*S. cerevisiae* Type W303
Material	Petri dishes, micropipette, gloves, cuvette, distilled water, Erlenmeyer, sterile test tubes, alcohol burner, glass slide, glass spreader, inoculation loop, Neubauer Counting Chamber, Manzete, TrizimanD, Curamax, Bacto-yeast extract, Bacto-peptone, Glucose, Bacto-agar.
Equipment	Balance, microscopy, laminar airflow, autoclave, incubator, centrifuge, shaker, spectroscopy.

Table 5. Materials and equipment necessary to perform toxicity study of fungicides with yeast

7.2 Preparation of culture medium

Yeast cell propagation at laboratory level was performed in a sterile broth consisting of: yeast extract, glucose, and peptone (YPD) under aerobic conditions using sterile air, strict temperature control at 32°c and 150 rpm. For the preparation of solid medium, Agar was added to the broth to solidify the medium.

Components	**Composition**
1% Bacto-yeast extract	10 g
2% Bacto-peptone	20 g
2% Glucose	20 g
2% Bacto-agar	20 g
Distilled water	1000 ml

Table 6. YPD medium for routine growth of Yeast (Sherman, 2002)

7.3 Spectrophotometry analysis

The spectrophotometer wavelength was set at 600 nm (OD600) and blanked in a cuvette containing 1 ml of sterile YPD medium. The 4 samples: control (yeast only), yeast with Manzete, yeast with Triziman D, and yeast with of Curamax started the growth curve with an OD of approximately 0.2. The OD was recorded for each 4 samples at an interval of 2 hours until it reached 6 hours. The procedure

was repeated, but with different concentrations of fungicides (5, 10, 20 mg/l) in the medium. To calculate the fungicide concentration in the medium and the initial OD, the following equation was used:

$$C_1V_1 = C_2V_2$$

7.4 Optical microscope cell counting

Cell counting was performed in the Neubauer chamber in which grids are engraved. The cell was counted under an optical microscope with a 40x magnification objective. The entire chamber can be placed on the microscope stage. Dye was prepared by diluting in 9ml in distilled water with 1 ml of methylene blue. 1000ul of diluted dye was mixed with 1000ul of culture in which the yeasts reside (1:1 mixture) and allowed to stand for 10 minutes. After the 10 minutes had passed, the culture was homogenized by stirring well and 10 ul of the sample was taken with a pipette and the Neubauer chamber was mounted. The tip of the pipette was placed in one of the two slots of the chamber and, by capillary action, the yeasts were distributed in the chamber; covered with coverslips, and mounted on the light microscope stage for observation. It was first focused on the 10X objective and then switched to 40X to observe the yeast cells in the grids for counting. This procedure was repeated for the control and the cells incubated with the different fungicides; starting with an OD of 0.4 and waited two hours to mount in the Neubauer chamber. Finally, the cells stained with methylene blue are dead and the unstained cells are alive. Counting was performed by averaging the yeast contained in the 5 quadrants of the diagonal of the Neubauer chamber as shown in *Figure 10.*

A) B)

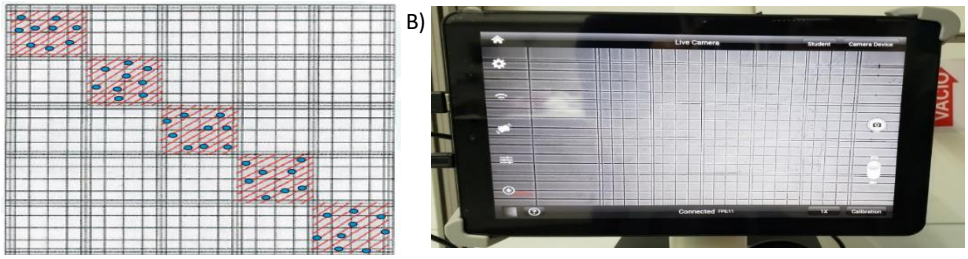

Figure 10. A) The marked areas correspond to the cell counting area at 40x magnification in *Neubauer chamber. B) View of the Neubauer chamber under the microscope*

The cell count was repeated after 24 hours of incubation in a shaker, but in this case, the culture samples were diluted with 900 ul of distilled water and 100 ul of culture. The diluted culture sample was mixed with methylene blue (9 ml distilled water and 1 ml methylene blue) in a 1:1 ratio. To see the viability of the cells, the equation was used. The calculation of the cell concentration was performed according to the $Viability$ equation:

$$Viability\ (\%) = \frac{live\ yeast\ count}{live + dead\ yeast\ count} x100$$

7.5 Plate extension for CFU counting

It was inoculated 300ul of the cell suspension onto the surface of the plate, the sample is dispersed over the agar surface using a glass spreader. The plate is incubated at a temperature of 32°c for 24 hours. The procedure was repeated for the control and yeast inoculated with the different study fungicides (*Figure 11.*). A concentration of 5mg/l of fungicide was added to the yeast culture.

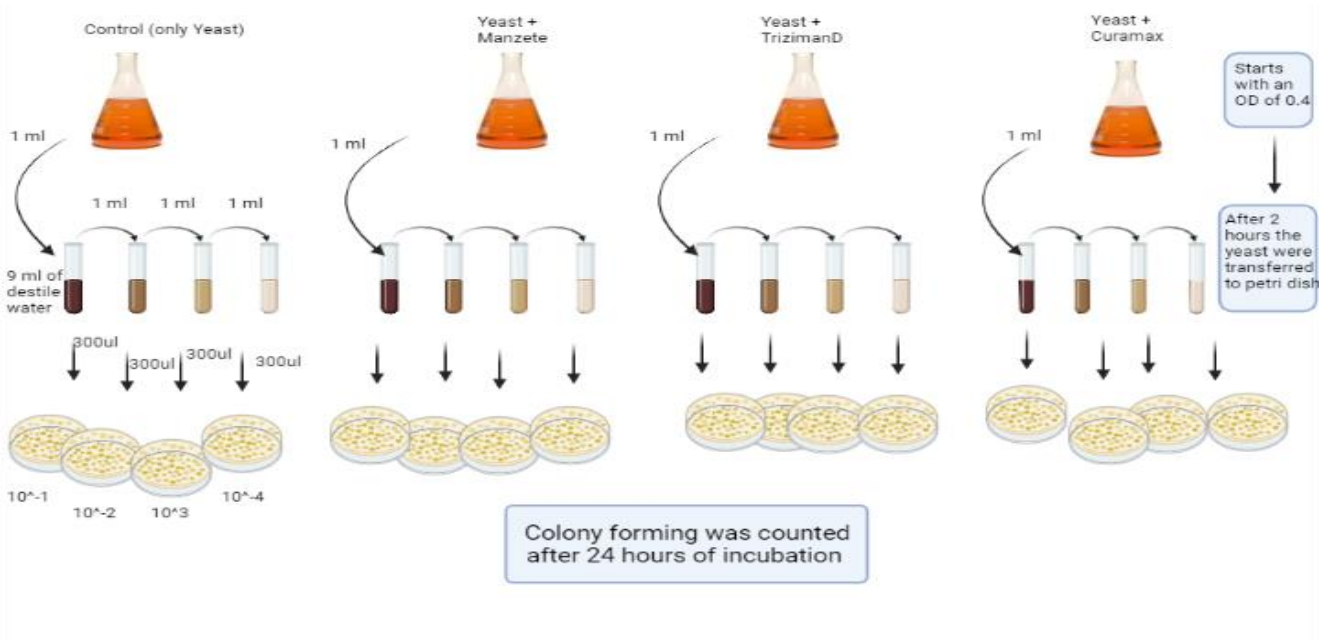

Figure 11. Procedure for CFU determination. Created with Biorender.

7.6 Halo of inhibition

300 ul of yeast was seeded in a Petri dish and spread equally over the entire plate; 45 minutes had waited for the yeast to dry in the solid medium. Then, 6 absorbent paper discs were embedded in each plate. To evaluate toxicity at different concentrations, each disk contained 20, 15, 10, 5, 2.5, 1.25 mg/l of fungicide. The same procedure was repeated for the remaining two fungicides. Finally, after 24 hours of incubation, the inhibition halo was measured by passing through half of the discs as shown in the *Figure 12*.

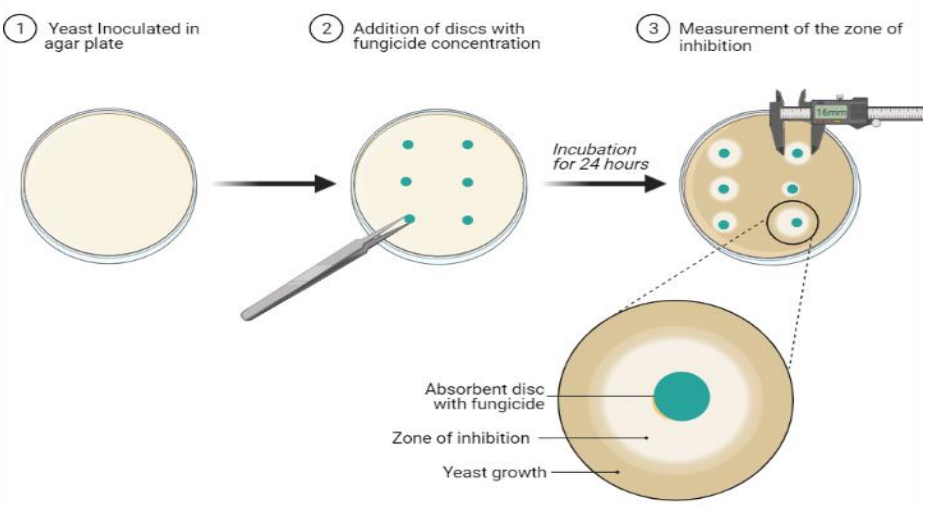

Figure 12. Procedure for measuring the inhibition zone caused by different fungicide concentrations.

7.7 Dry weight

To determine of dry weight, 4 samples of liquid medium were taken and incubated for 24 hours in a shaker at 150 RPM at 32°C. The samples contained each one separately: yeast (control), yeast with 5 mg/l of Manzate, yeast with 5 mg/l of Trizimand D, and yeast with 5 mg/l of Curamax. The cells were suspended with pipettes and 10 ml of each sample was taken and transferred to Falcon tubes. The Falcon tubes were placed in a centrifuge where they were spun at 4000 RPM for 10 minutes (*Figure 13*), the supernatant was discarded and the samples were washed with sterile water and centrifuged again, the

washing process was repeated once more, centrifuged and the supernatant discarded. The pellet formed by centrifugation was transferred with a micropipette to a previously weighed Erlenmeyer flask (figure 13). Subsequently, the Erlenmeyer with the cells was placed in a drying oven at a temperature of 50°c for 24 hours. After this time, the weight of the Erlenmeyer flasks with the dried cells was recorded.

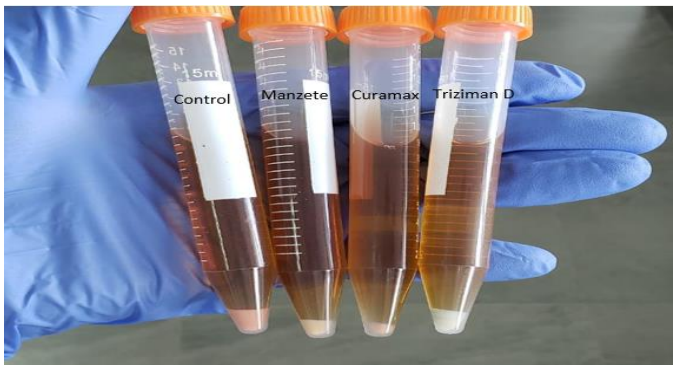

Figure 13. Pellet formed after centrifugation of the 24-hour incubation culture of yeasts (control) and yeasts with fungicide.

Figure 14. Weight of the Erlenmeyer flasks before introducing the pellet formed from the samples.

Figure 15. Samples after having been dried 24 hours in drying oven.

The following equation was used to determine the dry weight:

$$Dry\ cell\ weight\ (\frac{g}{ml}) = \frac{(dry\ cell\ weight + Erlenmayer\ weight) - (Erlenmayer\ weight)}{volume\ of\ culture\ sample}$$

7.8 pH measurement of the medium

The samples were cultured in a shaker with the same parameters previously mentioned. The pH was measured at the beginning of the incubation with the different concentrations of fungicides and the pH of the medium was measured after 24 hours.

8 RESULTS AND DISCUSSION

8.1 Growth curves

The specific growth rate is characteristic for each type of microorganism and culture medium (substrate). Yeast cells isolated and cultured in a finite volume of culture medium use the nutrients available to them as efficiently and rapidly as possible. Each time an interval of time elapses, the number of cells doubles, thus following an exponential growth until the nutrients in the medium are exhausted. The increase in absorbance (OD) correlates with the increase in the microbial population. When the nutrients in the medium are depleted, the microorganisms enter a stationary phase followed by a cell death phase.

The *Figure 16* shows that the dormancy phase or adaptation period ends one hour after the yeasts are placed in the culture medium. After one and a half hours, the exponential growth phase begins. The application of a concentration of 5 mg/l of fungicide already affects the growth velocity of the yeasts. However, from a concentration of 10 mg/l of fungicide, it can be observed that yeast growth decreases by half compared to the controls. In addition, it can be observed that the samples treated with Manzate and Triziman D at low concentrations of these fungicides (5 mg/l), the yeasts adapt and create resistance to the fungicide after 4 hours of cultivation. With Curamax, yeasts are more sensitive to this fungicide from low concentrations.

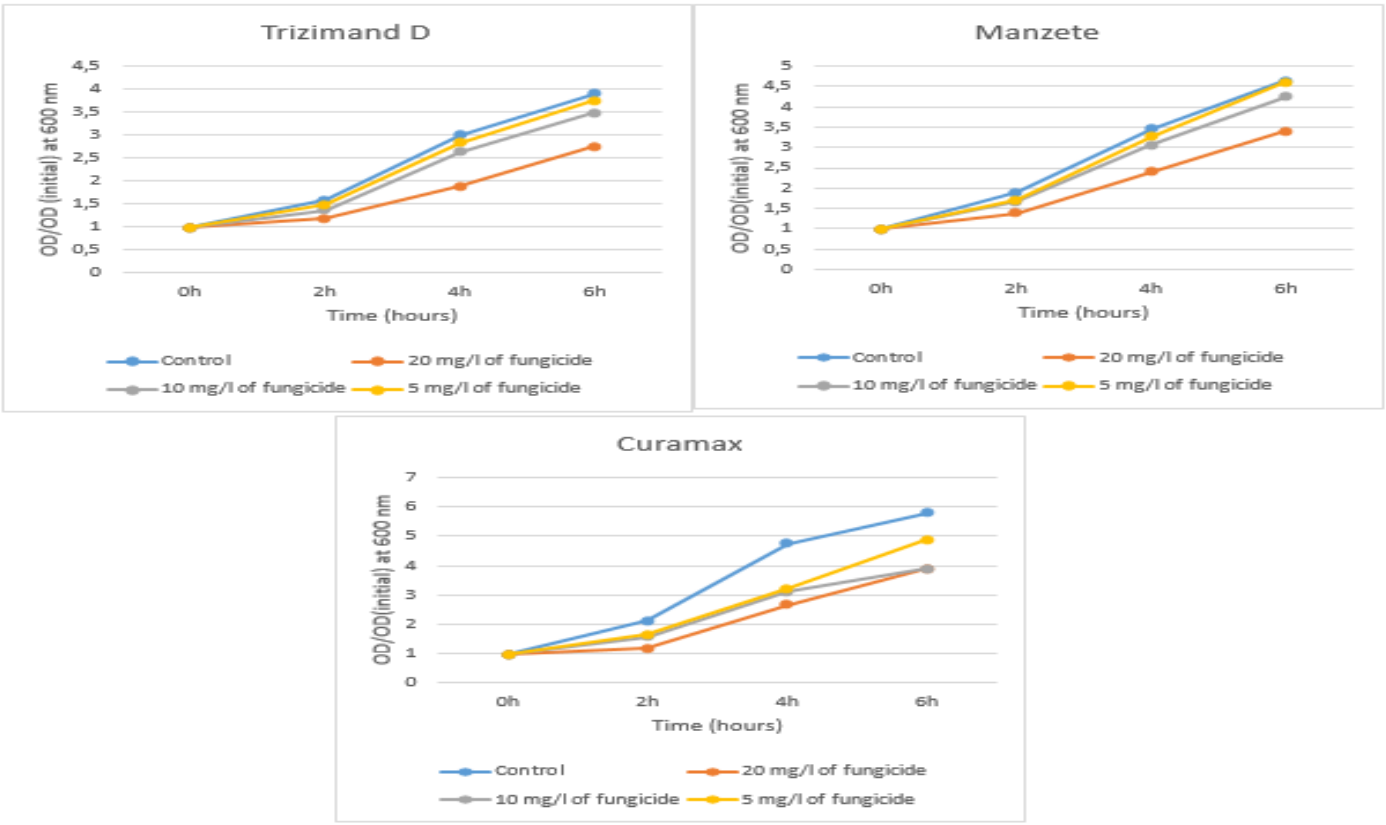

Figure 16. Growth dynamics and behavior of yeasts against different fungicides at different concentrations.

A)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Fungi	2	26692	13346	0.12	0.887
Horas	1	17206600	17206600	154.34	<2e-16 ***
Residuals	56	6243100	111484		

B)

-	diff	lwr	upr	p adj
Manzate-Curamax	13.74285	-240.4620	267.9477	0.9907053
Trizimand D-Curamax	50.00230	-204.2026	304.2072	0.8839866
Trizimand D-Manzate	36.25945	-217.9454	290.4643	0.9371358

Figure 17. Statistical tests performed in RStudio with a significance level of 0.05. A) Anova test, B) Tukey test.

In Figure 17. A) in the indicated red box, since the P value is greater than the significance level of 0.05; the null hypothesis is accepted and the mean of the variables studied is the same in the different groups. In Figure 17. B) compares in pairs the treatments and affirms the similarity between the different groups. Although from the studies shown below, it is determined that Curamax is a little more toxic to yeasts.

8.2 Staining of cells with methylene blue

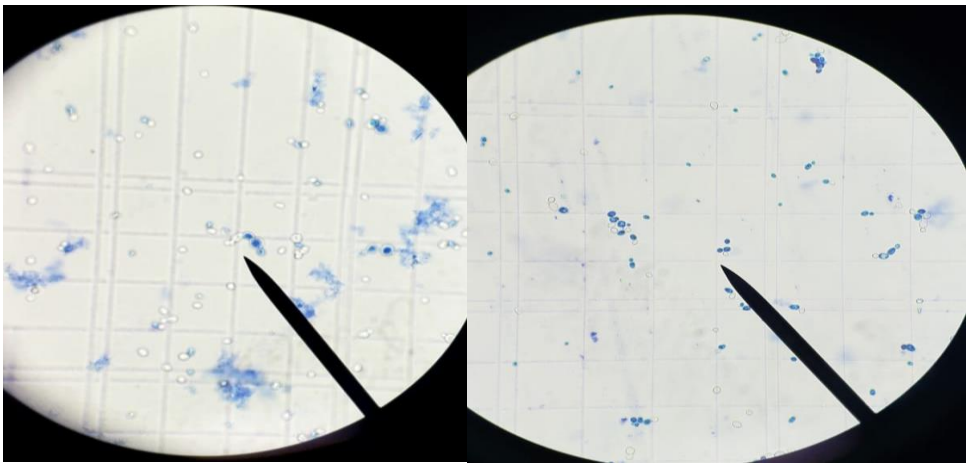

Figure 18. Yeast saw under a microscope at 40x magnification. Transparent and light blue cells are counted as alive since they can metabolize methylene blue dye. Dark blue cells are counted as dead.

2 Hours	
Tests substance	Viability (%)
Control	98.71
Trizimand D	93.33
Curamax	88.70
Manzete	89.07

24 Hours	
Tests substance	Viability(%)
Control	75.22
Trizimand D	62.06
Curamax	55.77
Manzete	61.29

Table 7. It shows the influence on the viability of yeast cells when 5 mg/l concentration of fungicide is introduced to the culture medium. The control refers to no fungicide exposure.

Living yeasts contain enzymes capable of reducing methylene blue (MB) dye. Staining of dead yeasts occurs as the MB dye penetrates through the cell walls and stains negatively charged molecules in the cell, including DNA and RNA. Viability describes how many live cells there are in a cell population.

High viability tells us the general state of the yeast in the medium and if these cells have good vitality, the yeast will increase its population, as it will tend to reproduce from the dormant phase (metabolic inactivity) to the log phase and thus increase the number of living cells (biomass). In the table, it can be seen that concentrations of 5 mg/l of fungicides already affect the viability of the cells and this effect is prolonged since the next day it is seen that the cell cultures with fungicides continue to be

affected by fungicides. These results may show that yeasts at low fungicide concentrations may already be subjected to stress conditions that cause a drop in viability up to 24 hours after fungicide application.

8.3 Zone of Inhibition

The table: shows the susceptibility of yeasts to different fungicide concentrations. In addition, yeasts tend to form strains resistant to the fungicide, since at concentrations lower than 2.5 mg/l of fungicide the inhibition halo begins to appear semi-diffuse. The zone of inhibition with larger diameters can be observed above a concentration of 10 mg/l of fungicide, which indicates that between 20 ≥ 10 mg/l the IC50 of these fungicides is found.

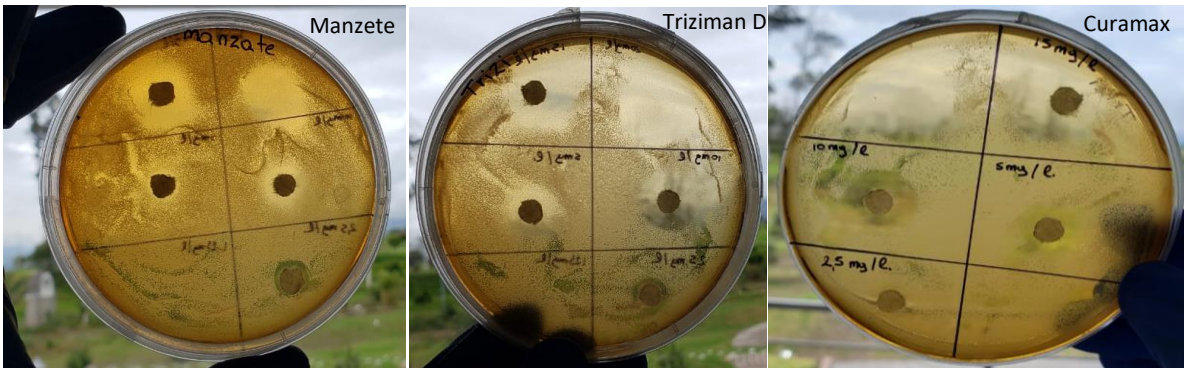

Figure 19. Inhibition of yeast growth in solid medium caused by the presence of fungicides at different concentrations

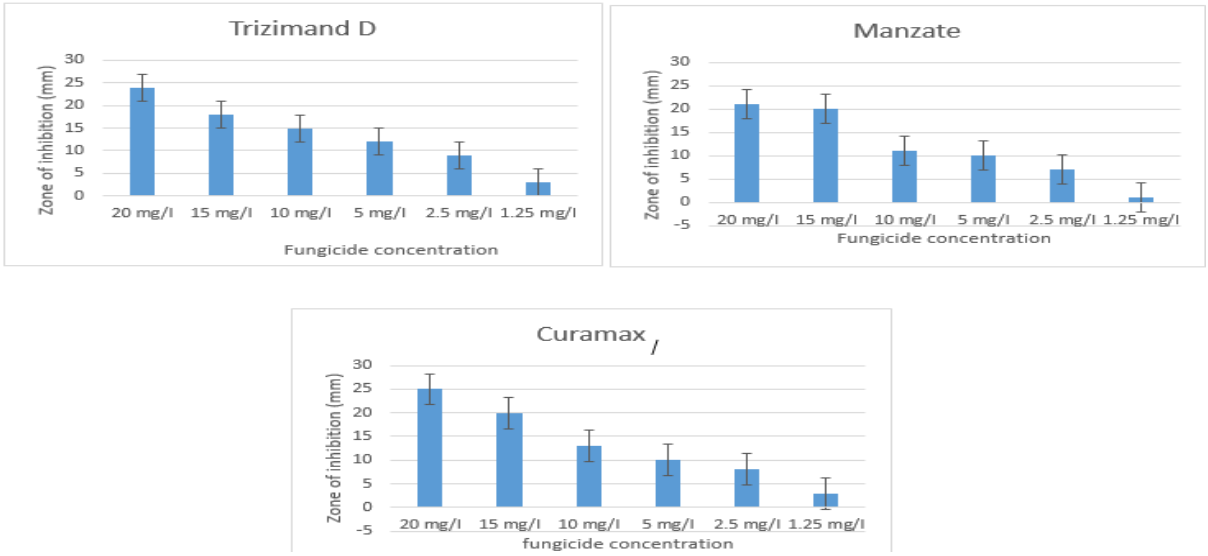

Figure 20. Diameter of zone of inhibition growth caused by different fungicide concentrations in yeasts.

8.4 CFU counting

	10^-1	10^-2	10^-3	10^-4
Control	uncountable	1438	593	134
Yeast+Manzete	uncountable	1153	448	112
Yeast+Triziman D	uncountable	1139	440	109
Yeast+Curamx	uncountable	1013	336	98

Table 8.CFU count of serial dilutions with a concentration of 5 mg/l of fungicide

Viable cells are those that form colonies. The quantification of cultivable yeasts allows us to identify and isolate directly the yeasts that survived or adapted to the fungicides present in the medium. The plating was performed after leaving the fungicide to act for two hours and, as can be seen in the table: after this time, the fungicide already affects the survival of the yeasts. For counting, the 10^-4 dilution is the most recommended because the cells are more dispersed and are not agglomerated.

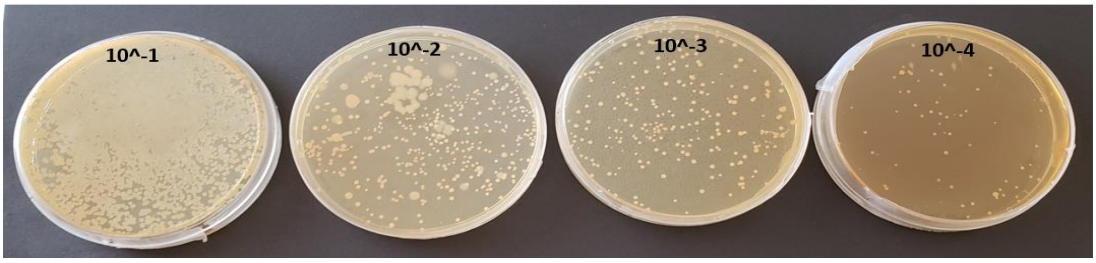

Figure 21. Results were obtained after culturing 300ul of yeast with 5mg/l of Curamax fungicide in Petri dishes with solidified agar medium.

The disadvantage of using this method is that it consumes too much material and several repetitions are needed to validate the method.

8.5 Yeast dry weight

	Weight only Earlenmayer (g)	Weight Earlenmayer + Dry yeast (g)	Dry cell weight (g/ml)
Contol	36.5146 ± 0.0001	36.5910 ±0.0001	0.00764 ±0.0001
Triziman D	41.7816 ±0.0001	41.8500 ±0.0001	0.00684 ±0.0001
Manzete	41.8729 ±0,0001	41.9421 ±0.0001	0.00692 ±0.0001
Curamx	42.0567 ±0,0001	42.0838 ±0.0001	0.00271 ±0.0001

Table 9.Determination of the dry weight of yeasts after 24 hours of exposure to a concentration of 5 mg/l of the different fungicides under study.

Biomass determination can be used as an indication of the yeast growth environment, since microorganisms can also act as a sensitive indicator of contamination, as in this case of fungicides. The determination of yeast dry weight provides information on the yeast growth kinetics. This is a key variable in establishing the production rate. However, the disadvantage of this method is that we are also taking into account the weight of already dead cells and inert material.

The lower biomass production can be attributed to introducing 5 mg/l of Curamax and this may be closely related to the fact that this fungicide affects the metabolic pathways for the uptake of micro and macro nutrients from the medium and therefore does not allow the correct development and reproduction of the yeasts, thus affecting the final biomass production.

8.6 Acidification of the medium due to the presence of the fungicide

Tests substance	pH time 0	pH time 24
Control	6.66	5.96
yeast+Trizimand D (5mg/l)	6.63	5.71
yeast+Curamax (5mg/l)	6.61	5.69
yeast+Manzete (5mg/l)	6.64	5.80

Tests substance	pH time 0	pH in 24 hours
Control	6.60	6.00
yeast+Trizimand D (10 mg/l)	6.13	5.56
yeast+Curamax (10mg/l)	5.98	5.42
yeast+Manzete (10mg/l)	6.15	5.64

Tests substance	pH time 0	pH in 24 hours
Control	6.58	5.93
yeast+Trizimand D (20 mg/l)	5.62	5.38
yeast+Curamax (20mg/l)	4.86	4.13
yeast+Manzate (20mg/l)	5.68	5.33

Table 10. It shows the influence of pH changes in the culture medium due to the presence of fungicides at different concentrations.

In the Tables: it can be observed that the higher the concentration of fungicides, the pH in the medium acidifies. After 24 hours of measuring the pH, it is observed that the influence of the fungicides is still present in the medium, causing more rapid acidification compared to the controls. Consequently,

it causes changes in the medium, resulting in inhibition or failure of yeast growth. The table indicates that low concentrations of 5 mg/l of fungicide, already affect the pH of the medium. In the case of Curamax fungicide, it is the one that tends to acidify the culture medium the most at different concentrations.

The pH and acidity are other of the most important environmental parameters that condition the growth and survival of microorganisms. The various species of bacteria also have a maximum pH, a minimum pH, and an optimum pH. The pH limits at which microorganisms can grow vary greatly depending on the type of microorganism in question: the further the pH of the medium is from the optimum pH of a given microorganism, the slower the growth of that microorganism will be.

The aging and death of yeast cells cause acidification of the medium. Similarly, the acidification of the medium can be caused by the production of organic acids, the fermentation of glucose to ethanol, and the utilization of ethanol as an energy source when glucose is finished. However, the presence of fungicides in the environment can cause the acceleration of replicative aging, decreasing the lifespan of the cells.

Chemical substances with acidic properties can destroy and denature proteins and the superficial layers of the skin, causing an increase in the absorption of the pollutant by this route. External factors, such as high ambient temperatures and humidity, increase the absorption of acidic substances. Therefore, all safety measures should be taken when handling these compounds using gloves, eye protection, respiratory mask, boots, and thick clothing that fully protect arms and feet.

9 CONCLUSIONS AND RECOMMENDATIONS

Agriculture is the basis of the country's economy and food supply. However, the demand for agriculture has driven the increase in the use of pesticides. Most pesticides, being toxic synthetic substances, foreign to the environment, produce diverse negative effects on environmental health and to

people if pesticides are not used and handled in the correct way. In Ecuador, the largest cases of pesticide poisoning recorded in people come from the coastal region, in the province of Manabí. Therefore, the handling of pesticides should be controlled and improved, and pesticides should be handled by trained personnel.

The most commercially available fungicides have the active ingredient mancozeb molecule. These fungicides are broad-spectrum fungicides to treat foliar fungal diseases. Although mancozeb is presented as a low toxicity fungicide; the literature review shows that the secondary metabolite produced by mancozeb, ETU, is more toxic to the environment and has a greater persistence in the ecosystem. Likewise, ETU presents greater toxicity and risk to human health, presenting consistent evidence of neurotoxicity and carcinogenic potential.

Using yeast as a study model, the biomarkers present a first evaluation of the risk that several xenobiotics could have on human health. Different biomarkers have been identified and validated as a tool for their classification, sensitivity detection, determination of sensitivity mechanisms, construction of the dose-response relationship to evaluate the toxicity of pollutants. Biomarkers are becoming increasingly specific as a result of technological advancements. and are fundamental in the development and application of biosensors. Current and future trends are directed towards developing technologies that allow the applications of these biosensors to be expanded in the market. Fortunately, with the development in recent years of biotechnology, micro/nano-technology, microbial biosensors are becoming increasingly powerful in solving practical analytical problems.

When measuring the sensitivity of yeasts to fungicides, it can be concluded that the fungicides have similar toxicity. However, the fungicide Curamax is the one that most affected the growth, adaptation, and development of yeasts in the medium. This may be influenced by the presence of another molecule in its formula (cymoxanil). The toxicity of the fungicides from the most toxic to the least toxic

could be: Curamax > Triziman D ≥ Manzete. The yeasts were able to sense small amounts of fungicides, making them excellent candidates for application in microbial biosensors to detect pesticides. However, after 4 hours, the yeasts could adapt to low concentrations of the fungicide, which opens the possibility of studying more fungicide resistance genes and in the future and using them as biomarkers of toxicity. In addition, the higher the fungicide concentration, the more acidified the medium becomes, making it more toxic at higher concentrations. Despite the acidification of the medium, the yeasts continued to grow between a pH of 4.15 to 6.6; the optimum being between 5.5 to 6.5. Finally, we can conclude from the inhibition halo tests that from a lower concentration of 1.25 mg/l the fungicides do not present a great risk to the yeasts, since the inhibition halos were very small and semi-diffuse, which correlates with the values allowed for pesticides in food by the FAO, which is between 0.05 to 0.1 mg/kg for slightly toxic pesticides (FAO & WHO, 2019).

Recommendations:

- For the staining of yeasts with MB dye, it is recommended to filter the MB dye with filter paper to avoid the appearance of lumps in the microscope and make cell counting difficult.
- In the development of yeast growth curves against a commercial fungicide; it is recommended not to use very high amounts of fungicide since the OD and turbidity of the fungicide interfere in the reading of yeast growth.
- When you are taking the samples to measure the OD in the spectrophotometer, it is recommended to pipette and shake the samples well before placing them in the cuvettes, to move the cells that are at the bottom and thus avoid making errors in the OD reading.
- For a better statistical analysis, it is recommended to do them in triplicates.
- For the year 2021, the European Commission allowed the non-renewal of fungicides with the active center Mancozeb and its derivatives (maneb, metiram, propineb, and zineb); due to their

potential to create ETU (EFSA, 2020). It is therefore recommended to repeat the sensitivity studies with yeasts, but in this case, before applying the fungicides directly to the yeasts, let the fungicides degrade for about two weeks to get ETU and thus see if there is a difference in sensitivity between Mancozeb and ETU in yeast.

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11 ANNEX

Trizimand D		
Time (hours)	Fungicide concentration	OD
0	0mg/l	0,272
	20mg/l	0,287
	10mg/l	0,285
	5mg/l	0,275
2	0mg/l	0,434
	20mg/l	0,340
	10mg/l	0,388
	5mg/l	0,407
4	0mg/l	0,817
	20mg/l	0,541
	10mg/l	0,747
	5mg/l	0,778
6	0mg/l	1,061
	20mg/l	0,786
	10mg/l	0,995
	5mg/l	1,032
24	0mg/l	1,509
	20mg/l	1,391
	10mg/l	1,498
	5mg/l	1,492

Curamax		
Time (hours)	Fungicide concentration	OD
0	0mg/l	0,185
	20mg/l	0,202
	10mg/l	0,230
	5mg/l	0,225
2	0mg/l	0,394
	20mg/l	0,238
	10mg/l	0,362
	5mg/l	0,372
4	0mg/l	0,881
	20mg/l	0,540
	10mg/l	0,715
	5mg/l	0,728
6	0mg/l	1,071
	20mg/l	0,79
	10mg/l	0,894
	5mg/l	1,102
24	0mg/l	1,784
	20mg/l	1,501
	10mg/l	1,524
	5mg/l	1,550

Manzate		
Time (hours)	Fungicide concentration	OD
0	0mg/l	0,228
	20mg/l	0,238
	10mg/l	0,233
	5mg/l	0,230
2	0mg/l	0,432
	20mg/l	0,329
	10mg/l	0,391
	5mg/l	0,397
4	0mg/l	0,788
	20mg/l	0,572
	10mg/l	0,713
	5mg/l	0,752
6	0mg/l	1,060
	20mg/l	0,812
	10mg/l	0,988
	5mg/l	1,058
24	0mg/l	1,612
	20mg/l	1,513
	10mg/l	1,532
	5mg/l	1,542

Values obtained in the spectrophotometer at an OD of 600 nm for the yeast growth curve with fungicides.