Universidade do Minho
Escola de Ciências

Eduarda Maria Oliveira Miranda

Novel Yeast-Based Biosensor for Environmental Monitoring

Dezembro 2021

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Novel Yeast-Based Biosensor for Environmental Monitoring

Dissertação de Mestrado
Genética Molecular

Trabalho efetuado sob a orientação da
Doutora Susana Chaves

Dezembro 2021

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A todos vocês, o meu obrigada!

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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ABSTRACT

The human population has been increasing worldwide, and, with it, food demand and consequently the need for higher agriculture yield. As approximately 35 % of the worldwide crops are lost to pests, pesticides have been used as a solution to this problem, increasing agricultural productivity from 42 to 70 %, improving food quality and quantity, at a reasonable price. However, there are human and environmental safety issues, since most of these chemical compounds affect both target and non-target organisms. With the interest to control and monitor pesticide usage, conventional assays have been used and can achieve low limits of detection, but are expensive, require trained technicians, and are time-consuming. As an alternative to conventional methods, analytical devices that use biological molecules to measure reactions, either biological or chemical, have emerged, named biosensors. The yeast *S. cerevisiae* has been used as a model to develop biosensors, often relying on the activation of an inducible promoter that controls the transcription of a reporter gene, using fluorescence, bioluminescence, and colorimetry as measurable signals.

Bearing this in mind, this project aimed to develop a yeast-based biosensor to detect the presence of a pesticide in aqueous samples, tebuconazole (TEB). TEB is a fungicide, i.e, it inhibits or eliminates fungal pests. TEB is a broad-spectrum, low-cost triazole fungicide that has been detected in vegetables, fruits, as well as wheat products. It is a 14α-demethylase inhibitor, blocking lanosterol conversion into ergosterol, thus causing a depletion of ergosterol, an essential component of the lipid membrane in fungi. The risk of this chemical in humans and ecosystems is well documented since the ergosterol pathway is conserved among several species. During the course of this work, four yeast strains were constructed, using specific promoters induced by TEB to drive the downstream expression of luciferase from a plasmid (expressed in *S. cerevisiae* BY4741), detected by bioluminescence assays. The system was further optimized and characterized, and the final chosen biosensor was able to detect 5 ng/ml of tebuconazole in a 6-hour treatment assay.

Keywords: ergosterol; tebuconazole; biosensor; fungicide; *S. cerevisiae*

RESUMO

A população humana tem vindo a aumentar e, com isso, aumenta a procura por alimento e, consequentemente, a necessidade de aumentar o rendimento agrícola. Como aproximadamente 35 % dos cultivos no mundo são perdidos devido a pestes, os pesticidas são usados como solução para este problema, aumentando a produtividade agrícola de 42 para 70 %, melhorando a qualidade do alimento e aumentando a sua quantidade, a um preço razoável. No entanto, problemas associados à saúde humana e ambiental começaram a surgir, já que estes compostos químicos afetam organismos alvo e não-alvo. Com o interesse em controlar e monitorizar o uso de pesticidas, ensaios convencionais de deteção têm vindo a ser usados, conseguindo atingir baixos limites de deteção, sendo, contudo, técnicas dispendiosas, morosas e que requerem técnicos especializados. Como alternativa aos métodos convencionais, surgiram os biossensores, dispositivos analíticos que usam moléculas biológicas para medir reações químicas ou biológicas. A levedura *S. cerevisiae* tem vindo a ser usada como modelo genético para o desenvolvimento de biossensores, muitas vezes baseando-se na ativação/repressão de um promotor indutível que controla a transcrição de um gene repórter, usando fluorescência, bioluminescência e colorimetria para medição de sinal.

Posto isto, este projeto teve como objetivo desenvolver um biossensor de levedura, para detetar a presença de um pesticida em amostras aquáticas, o tebuconazol (TEB). TEB é um fungicida azólico, de amplo espetro e de baixo custo, que inibe ou elimina pestes fúngicas, sendo regularmente detetado em amostras de vegetais, frutas e trigo. Este composto químico é um inibidor da desmetilase 14α, bloqueando a conversão de lanosterol em ergosterol, causando assim uma depleção de ergosterol, um componente essencial da membrana lipídica nos fungos. O risco deste químico para os humanos e para os ecossistemas está bem documentado, uma vez que a via de síntese do ergosterol é conservada entre espécies. Durante este projeto, quatro estirpes de levedura foram construídas, usando promotores específicos, induzidos pelo TEB, para ativar a expressão da luciferase num plasmídeo (expresso em *S. cerevisiae* BY4741), detetado através de ensaios de bioluminescência. O sistema construído foi posteriormente otimizado e caracterizado, e o biossensor final selecionado foi capaz de detetar 5 ng/ml de TEB num ensaio de 6 horas de tratamento.

Palavras-chave: ergosterol; tebuconazol; biossensor; fungicida; *S. cerevisiae*

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INTRODUCTION

AGRICULTURE AND THE CROP LOSS PROBLEM

For the past decades, the human population has been increasing worldwide, leading to a rapid increase in food demand, and consequently the need to increase the agriculture yield (Malik & Grohmann, 2012). In addition to this problem, approximately 35 % of the worldwide crops are lost to pests, and thus protecting crops from these losses is key to improve yield. There are several types of pests. For example, weeds compete with crops for nutrients. In cereals, the main loss-causing agents are fungal and animal pests, each responsible for a 10 % loss in the total crop. In potatoes, fungal pathogens and animal pests result in a 25 % and 5 % reduction in productivity, respectively. In sugar beet, fungal pathogens and animal pests cause a crop reduction of 5 % and 10 %, respectively (Jozsef Popp, 2011; József Popp et al., 2013).

Due to large losses in production and the high demand to increase agricultural productivity, there has been an increase in the use of a wide variety of agrochemicals. Indeed, the most effective method to enhance agriculture productivity is pest control, which can increase agricultural productivity from 42 to 70 % (Fig. 1). This results in 2 million tons of pesticides used per year worldwide, of which Europe alone is responsible for 45 %, for agricultural purposes (Abhilash & Singh, 2009; Keulemans et al., 2019; Malik & Grohmann, 2012; József Popp et al., 2013; Sassolas et al., 2012; Verma & Bhardwaj, 2015). Among many other countries, India has been reported as a famine country in the past but has been able to

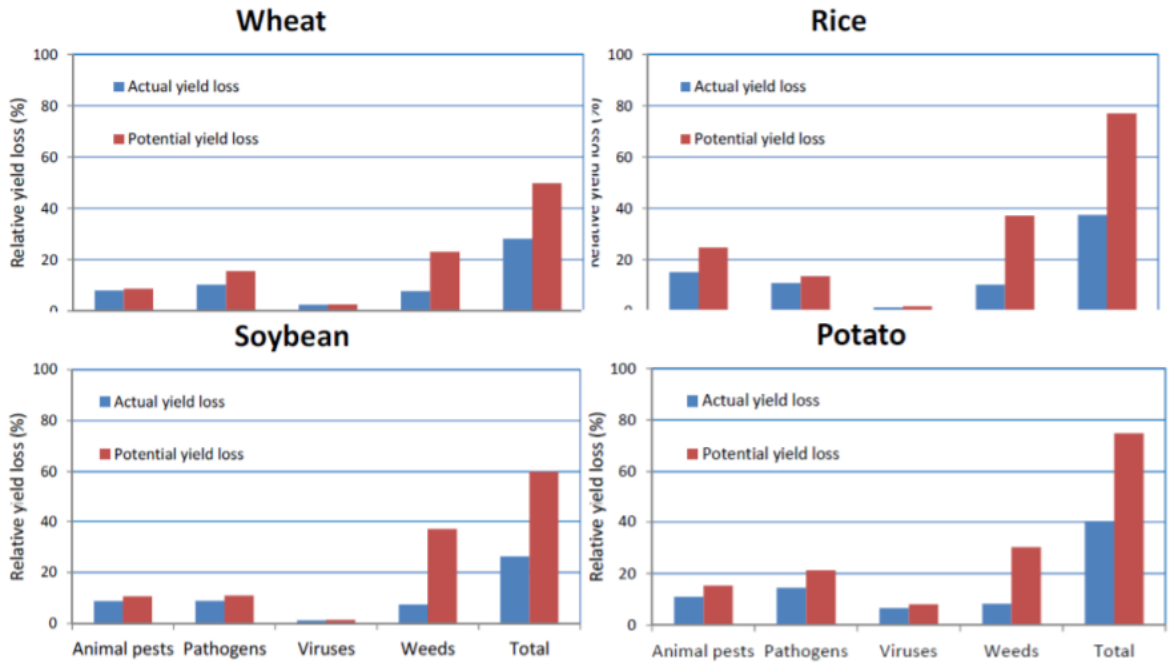

Figure 1. Effects of pests in actual vs. potential crop losses in different crops. Representation of the actual yield losses (blue) comparing with the potential yield losses (red), if no pesticide or other protection products were applied in agriculture. In the total loss, pesticides have saved approximately 20 % of yield losses in wheat, 50 % in rice, 30 % in soybean and 35 % in potato. In general, potential crop losses in Southern Europe (where Portugal is localized), would be 63 %, while the actual crop losses are 25 % (Keulemans et al., 2019).

increase agriculture productivity and even export food, as a result of pesticide usage (Austin, 1999; Malik & Grohmann, 2012). Other than agriculture, many fields can benefit from chemical usage, like aquaculture, the food or wood industries, in their processing, transportation, and storage. Pesticides can also be found in our homes, schools, and gardens (Akashe et al., 2018; Weiss et al., 2004). Since the Green Revolution in 1950, the use of fertilizers has greatly increased, and it is expected that its application will continue to increase in the next years. In summary, food quality and quantity improved as a result of pesticide use (Beketov et al., 2013; Yongbo Liu et al., 2014). Nevertheless, pesticides are also associated with negative effects on human and non-human health (József Popp et al., 2013).

PESTICIDES

According to the Environmental Protection Agency (EPA), pesticides are a combination of chemical compounds that are commonly used to prevent or eliminate any pest, including bacteria, nematodes, insects, among others, at a reasonable price (Malik & Grohmann, 2012; Sassolas et al., 2012). Although the benefits of pesticides in agriculture yield are clear, reports evidencing potential problems started to arise and, concomitantly, the scientific interest and publications addressing this subject. In 2010, around 7,000 papers were published regarding pesticides, while in 2020 approximately 10,000 papers were published (Pubmed data).

The transfer and breakdown processes that a pesticide may be subjected to are crucial to decide its final fate. Most chemicals present in pesticides are degraded, but some are commonly released in the air, water, or soil, polluting and jeopardizing the environment. In addition, they can be absorbed by plants or in the soil, reaching underground waters, and even entering food chains. Some pesticides are mutagenic, tumorigenic, carcinogenic, and genotoxic, and exposure to these chemicals has been associated with health problems in humans, animals, plants, and microorganisms (Kumar et al., 1996; Kuroda et al., 1992; Malik & Grohmann, 2012; Rehana et al., 1995; Verma & Bhardwaj, 2015).

Impact of pesticides

As the pesticides move through air, soil, and water, they enter the food chain and can be consumed by humans, often with carcinogenic and cytotoxic effects. When these chemicals are consumed, they can cause diseases involving the respiratory, nervous, immune, or reproductive systems. Moreover, exposure of children and pregnant women to these chemicals has been associated with childhood cancer. On average, about 1 million events of pesticide poisoning are reported per year (Cohen,

2007; Malik & Grohmann, 2012; Verma & Bhardwaj, 2015). In developing countries, these poisoning events are related to the lack of awareness and training in its use, as well as the lack of control regulation (Akashe et al., 2018). The degree of exposure to pesticides is not the same for all individuals, thus the impact on humans is not the same, and nor are the consequences. Farmworkers, sprayers, and pesticide manufacturers, for example, have direct and chronic exposure and a higher risk of pesticide inhalation. Other people are still indirectly exposed to pesticides, through their diet. Even if food is produced on a farm where no pesticides or other chemicals are used, food is not always pesticide-free, since they are present in the soil and the rain (Cohen, 2007; Malik & Grohmann, 2012). Some studies have connected exposure to toxic chemicals, such as pesticides, with the development of Autism Spectrum Disorder (ASD), consisting in a deficit or lack of communication and social skills (Ongono et al., 2020). A case study carried out in the United States of America (USA), involving approximately 7000 children, showed that the risk of ASD development increased with the decreasing distance of the mother's residence from agricultural fields during pregnancy, and decreased the farther the mother lived (Roberts et al., 2007).

Like human beings, other animals suffer from exposure to pesticides. After the pesticide molecules reach the soil, they move through aquatic ecosystems where they can either sediment and accumulate, or dissolve and associate with organic matter (Fig. 2) (Malik & Grohmann, 2012; Pirsaheb & Moradi, 2020). Once pesticides reach aquatic fields, they are highly toxic to numerous species, from algae to fish, decreasing their levels. Exposure of aquatic species can either be dermal, oral, or inhaled; with the decrease of aquatic species, a consequent decrease in the oxygen content occurs, leading to a further decrease in fish levels (Rani et al., 2020). As the pesticides applied in agriculture can accumulate in the soil, they can interfere with its normal microbiota, important for fertilization and the growth of plants. These important microorganisms are eliminated with the applied agrochemicals, threatening the sustainability of crops, with the effect depending on the concentration used (Malik & Grohmann, 2012; Rajagopal et al., 1984). Only around 0.1 % of the applied pesticides affect the target organism, with the rest affecting and decreasing the normal microbial biodiversity. Furthermore, these chemicals interfere with enzymes present in the soil involved in cycles such as the carbon, nitrogen, sulfur, and phosphorus cycles. When in contact with pesticides, the activity of such enzymes is blocked, interrupting those cycles that are important for soil fertilization and, consequently, its quality, essential features for the normal growth and development of crops (Rani et al., 2020). Since most pesticides are broad-spectrum, they have a large effect on non-target terrestrial and aquatic microorganisms, animals, and plants (Rani et al., 2020).

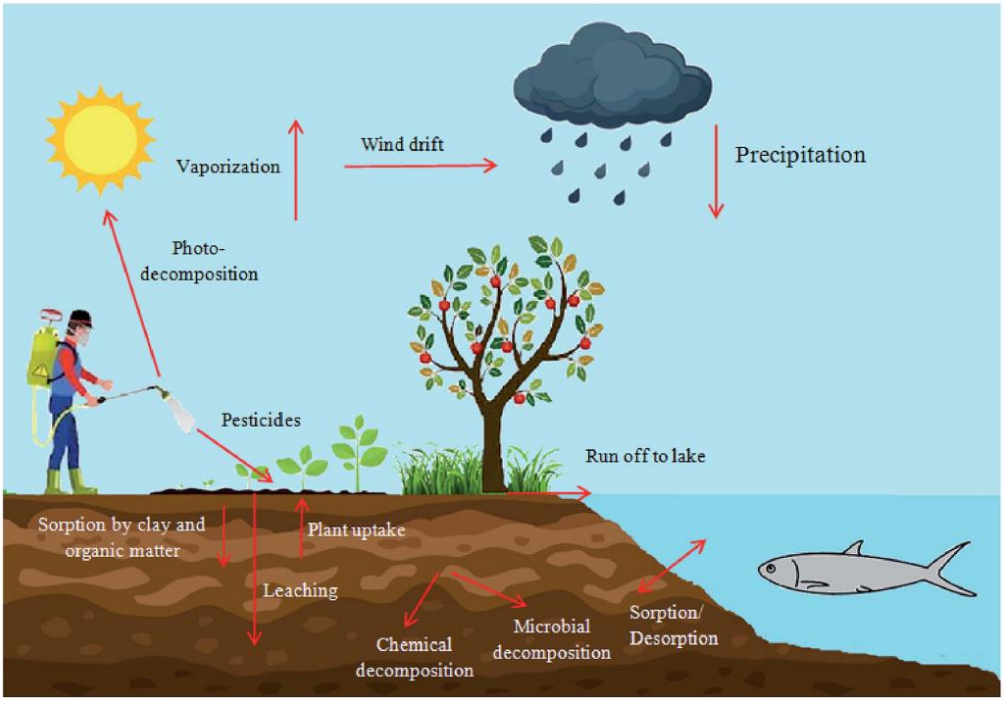

Figure 2. Pesticide cycle in the ecosystems. Depending on the breakdown process of the pesticide, its fate can be hazardous to the environment. If the pesticide is sprayed into the soil or surface water, it can be absorbed or it can evaporate, being then further released in the form of precipitation, directly exposing humans, animals, and microorganisms through food or water (Pirsaheb & Moradi, 2020).

Despite its negative impact, the use of these chemicals cannot be fully eliminated from agriculture (Amaral, 2014; Odukkathil & Vasudevan, 2013). Indeed, a drastic decrease in pesticide usage could result in a serious productivity decline, to about one to two-thirds of current levels (Malik & Grohmann, 2012). On the other hand, the use of pesticides needs to be increased in some countries of Africa and Asia, to fulfill the needs of the population, while a decrease is needed in most European and American countries, to preserve and protect the environment and public health. For this reason, the use of pesticides needs to be optimized according to the requirements of each region, combined with the detection and quantification of pesticides to control their use (Yongbo Liu et al., 2014; Verma & Bhardwaj, 2015).

CLASSIFICATION OF PESTICIDES

All the used pesticides have the same final goal and consequence, to kill or inhibit the growth of its target, though their mechanism of action differs, as does the chemical composition of each pesticide (Weiss et al., 2004). Pesticides can be differentially classified according to several parameters, such as toxicity, origin, chemical composition, target organism, and mode of action (Akashe et al., 2018).

Exposure to pesticides can be either acute or chronic, taking into consideration the dosage and time of exposure to the chemical. Acute toxicity refers to an event with a high pesticide concentration, commonly affecting those who work directly with these chemicals, and can even lead to the death of the organism. A chronic effect involves a delayed, long-term exposure to low concentrations that can be either oral, dermal, or inhaled. These effects include decreased reproductivity and behavior changes. Only acute toxicity is considered by the World Health Organization (WHO) when classifying pesticides, which are divided into extremely, highly, moderately or slightly hazardous, or unlikely to present acute hazard (Akashe et al., 2018; Weiss et al., 2004; Wendt-Rasch et al., 2003).

Based on their origin, pesticides can be chemical – toxic and rarely biodegradable –, or biopesticides – when they are naturally produced, less toxic, and easily degradable. Chemical pesticides are the most common and toxic class, being further divided into their chemical composition. According to chemical constituents, pesticides can be divided into four main groups: carbamates and organophosphates (inhibit acetylcholinesterase, affecting the nervous system), organochlorines (depolarize the nerve membrane), and pyrethroids (disrupt the nerve membrane permeability to sodium ions) (Akashe et al., 2018).

Based on the target, among others, pesticides can be insecticides, herbicides, rodenticides, or fungicides. Herbicides are used mainly in agriculture to control and prevent the growth of weeds and potentially dangerous plants. Insecticides are used to destroy insects, spiders, or mite pests. Rodenticides are used to kill rats and related animals (Koh & Jeyaratnam, 1996; Odukkathil & Vasudevan, 2013; Verma & Bhardwaj, 2015; Weiss et al., 2004). Fungicides, one of the most used pesticides in Europe, are a type of pesticide used to inhibit or eliminate any fungal pest, including molds, rots, and plant diseases, and are mainly used for agricultural purposes. These chemicals can be classified according to their mode of action (MOA), and are divided into 13 different categories by the Fungicide Resistance Action Committee (FRAC), 6 of which are represented in Fig. 3 (Petit et al., 2012). One of these categories includes sterol biosynthesis inhibitors. These inhibitors affect the ergosterol biosynthetic pathway, leading to the accumulation of sterol precursors, and a depletion of the cellular content of ergosterol, an essential fungal component (Makvandi et al., 2021).

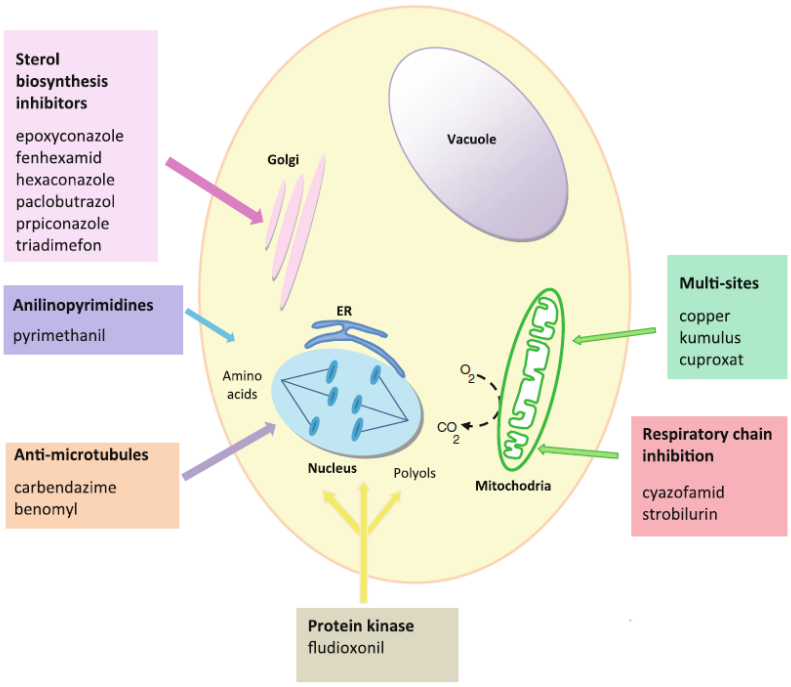

Figure 3. Fungicide classification categories according to their mode of action (MOA). Fungicides can act in many different ways in the fungal cell, including the inhibition of sterol biosynthesis and respiratory chain, can have multi-sited activity (affecting many biochemical processes), can inhibit amino acid and protein synthesis (anilinopyrimidines) or the cytoskeleton and motor proteins (anti-microtubules). There are other categories of fungicides (13 in total) classified by FRAC, interfering with nucleic acid metabolism, signal transduction, melanin synthesis in the cell wall, etc. These 13 categories can be further subdivided in several groups according to their composition (Petit et al., 2012).

ERGOSTEROL PRODUCTION AS A TARGET OF FUNGICIDES

Ergosterol is a component of the cellular membrane of fungi and is involved in the regulation, permeability, fluidity, and binding of enzymes to the membrane. This sterol is stored in lipid droplets in the cytoplasm, in order to regulate the intracellular balance of sterols. A perturbation in the biosynthetic pathway of ergosterol, such as the decrease of its levels, can have lethal consequences, by increasing permeability of the fungal membrane and leading to endocytosis, cell wall assembly, and cell fusion (Chen et al., 2019; Hu et al., 2017). Due to its importance in the development and proper growth of fungi, the ergosterol pathway is the main target of antifungal chemicals, both in the agriculture and medicine fields (Jordá & Puig, 2020; Pierson et al., 2004).

The biosynthetic pathway of ergosterol is commonly studied in *S. cerevisiae*, due to its conservation among eukaryotic species (Jordá & Puig, 2020). This pathway is complex and subdivided into three energy-consuming modules (Fig. 4). It starts in the vacuole with two acetyl coenzymes A (acetyl-CoA) molecules, further reduced to mevalonate in the mitochondria, being this first module (green) highly conserved among eukaryotes. In the second module (blue), mevalonate is transformed into farnesyl

pyrophosphate (farnesyl-PP) throughout six reactions in the vacuole. Farnesyl-PP is an intermediate that is present in the biosynthesis of several molecules, including sterols, hemoglobin, and benzoquinone, important metabolites. The third and last module (orange) corresponds to consecutive reactions from farnesyl-PP to the synthesis of ergosterol in the endoplasmic reticulum (ER), involving many essential enzymes for yeast growth, development and function, and even survival.

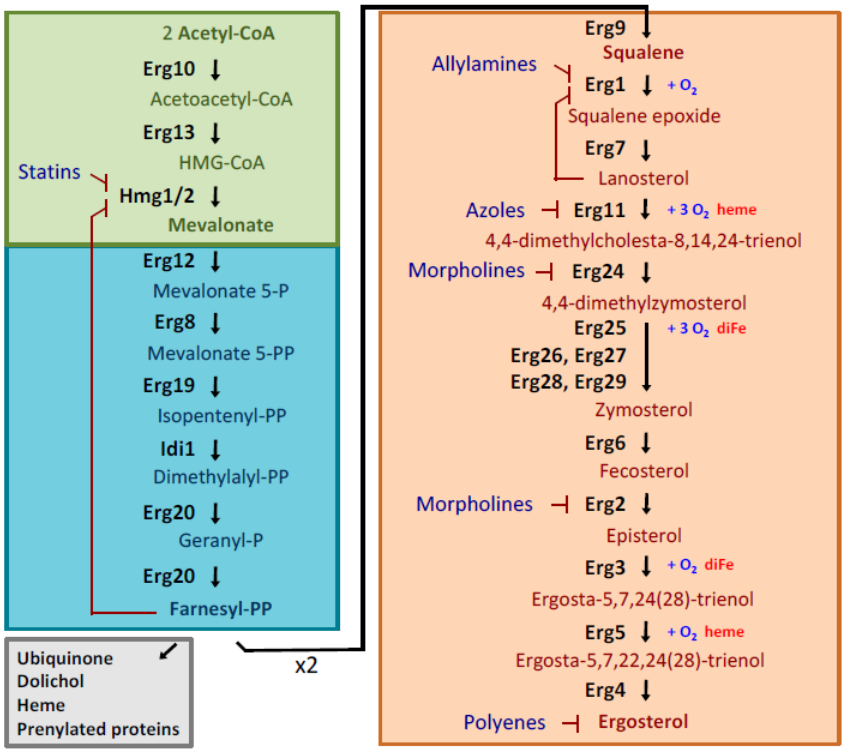

Figure 4. Ergosterol biosynthetic pathway in *S. cerevisiae*. The biosynthesis of ergosterol is divided in three different sections: mevalonate synthesis (green) in the mitochondria, where 2 molecules of acetyl-CoA are reduced into mevalonate; farnesyl-PP biosynthesis (blue) in the vacuole; and the final ergosterol biosynthetic pathway (orange), that converts farnesyl-PP into ergosterol through consecutive intermediate reactions in the ER (Jordá & Puig, 2020).

AZOLE COMPOUNDS

Azole compounds are synthetic antifungal agents of the sterol biosynthesis inhibitor class. According to their chemical structure, they can be classified into imidazoles or triazoles, with two or three nitrogen atoms in the azole ring, respectively, both with the same molecular mechanism of antifungal activity (Georgopapadakou, 1998; Zarn et al., 2003). These chemicals were first used in agriculture in the 70s and, due to low cost and high effectiveness (wide broad-spectrum), are of preferential use (Price et al., 2015). Despite the great efficacy of these agrochemicals, several molecular mechanisms of resistance to azoles have been reported in yeast and fungi, as a result of continuous exposure. This resistance to pesticides leads to uncontrolled diseases in crops, significantly decreasing target fungi

susceptibility and sensitivity to triazoles, and consequently to larger amounts being applied in crops (Fraaije et al., 2007; Joseph-Horne & Hollomon, 1997; Ma et al., 2006; Verweij et al., 2009).

Mode of action

Azole compounds are antifungal agents that act through the blockage of fungal ergosterol biosynthesis, an essential component of the membrane, disturbing the assembly of the cellular membrane in yeast and fungi (Fig. 5). Sterol 14α-demethylase (*ERG11*), part of the cytochrome (CYP) P450 superfamily of enzymes, is the most common target enzyme inhibited. Azole compounds, or demethylase inhibitors, therefore block lanosterol conversion into ergosterol, causing a depletion of ergosterol and the accumulation of lanosterol. This depletion promotes the disruption of the plasma membrane, rendering fungi more susceptible to damage (Espinel-Ingroff, 1997; Georgopapadakou, 1998; Makvandi et al., 2021; Verweij et al., 2009; Zarn et al., 2003).

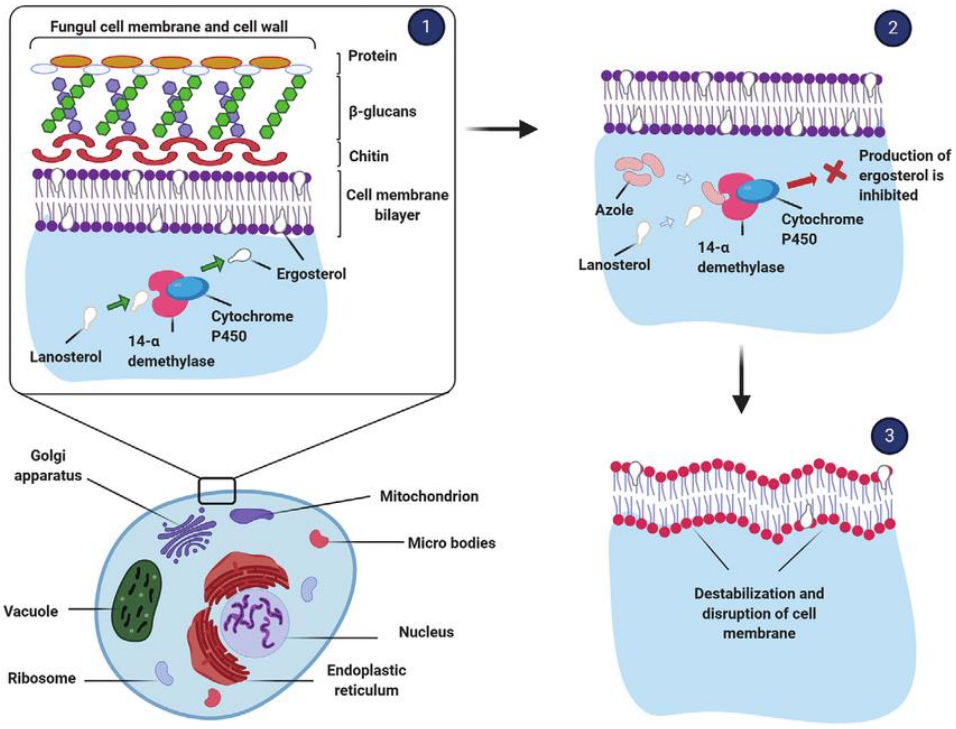

Figure 5. Mechanism of action of azole compounds in fungal cells. (1) Normal biosynthetic pathway of ergosterol biosynthesis in fungal cells. (2) 14α-demethylase, an intermediate of the biosynthesis of the ergosterol pathway, is the main target of azole compounds; when it is blocked, there is inhibition of ergosterol production, resulting in the accumulation of lanosterol in the cell and (3) promoting cellular destabilization and inhibition of growth (Makvandi et al., 2021).

PESTICIDE DETECTION

The continuous exposure of plants to pesticides, their toxicity to humans and soil microorganisms promoted the determination of a limit in the use of these agrochemicals. In the European Union (EU), the EPA established a maximum residue limit (MRL) of 0.1 μg/l for each pesticide and a total of 0.5 μg/l of pesticides (Pérez-Fernández et al., 2020; Rodriguez-Mozaz et al., 2006). Pesticides are usually detected through conventional assays such as gas and liquid chromatography (GC and HPLC, respectively), mass spectrometry (MS), immunoassays, and capillary electrophoresis (CE). Although these methods can achieve low limits of detection, they are expensive, require trained technicians, and are time-consuming. Besides, they often require the treatment of samples and are not adequate for on-site detection (Rani et al., 2020; Uniyal & Sharma, 2018). With the interest to more easily control and monitor pesticide usage, the development of biosensors emerged as an alternative to conventional methods (Rodriguez-Mozaz et al., 2006; Sassolas et al., 2012).

Biosensors as an alternative to conventional detection methods

Biosensors are defined as analytical devices that use biological molecules to measure reactions, either chemical or biological. They combine a biological receptor used as a recognition element that is in direct contact with a transduction element, in order to measure an analyte concentration in a reaction, through a signal proportional to its concentration. These systems are widely used in drug discovery, monitoring of diseases in health care, pollutant detection in environmental and agricultural applications and the food industry, and to detect disease-causing microorganisms in body fluids such as urine, blood, sweat, or saliva (Bhalla et al., 2016). Biosensors are low-cost techniques for simple and rapid analysis, and thus the interest in the field has been increasing (Verma & Bhardwaj, 2015).

Biosensors can be differentially classified according to their biological receptor used as a recognition element, and to the transduction method used for detection (Fig. 6) (Ying Liu et al., 2012; Makvandi et al., 2021; Singh et al., 2008). Enzymes, antibodies, whole cells, or nucleic acids can be used as recognition elements, and so the biosensor can be classified as enzymatic, immunochemical, whole-cell, or DNA biosensor. Enzymatic biosensors usually rely on the inhibition of enzymes when exposed to certain compounds, leading to a decrease in their activity. Immunochemical biosensors play on the high affinity of antibodies binding to their respective antigens, being a sensitive and selective approach. Regarding whole-cell biosensors, whole cells or tissues of organisms are used, like bacteria, fungi, animals, or plants. These are used as recognition elements and are useful to measure the toxicity

of compounds in a certain cell. DNA biosensors rely on hybridization of sequences to detect, for example, infections by microorganisms (Rodriguez-Mozaz et al., 2006; J. Wang et al., 1997)

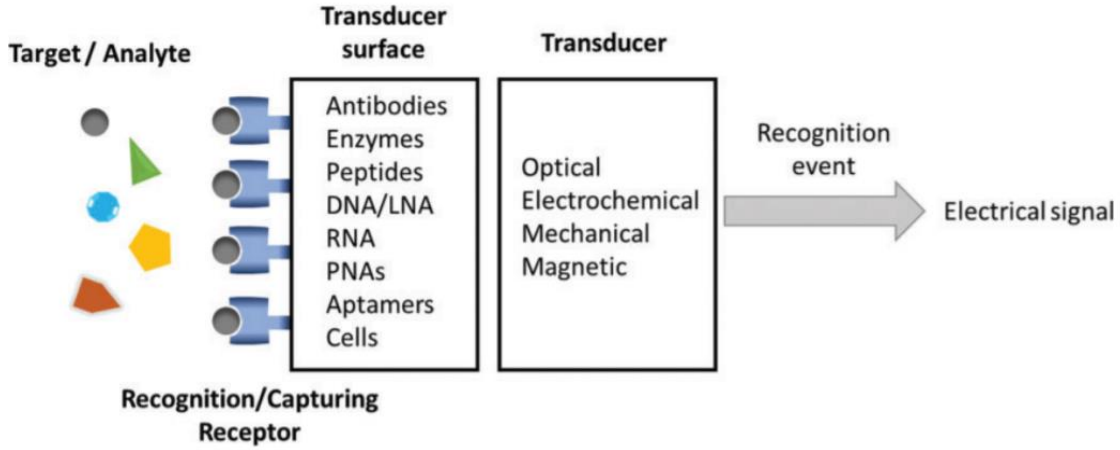

Figure 6. Principal components of a biosensor. Biological molecules (target/analyte) are recognized by a biological receptor, interacting with it, which leads to an alteration that is detected and converted into a measurable electrical signal, by the transducer. Biosensors rely on the affinity of the target molecule to the respective receptor (Makvandi et al., 2021).

Regarding the transduction method, biosensors can be optical, electrochemical, piezoelectric, or thermal. Optical sensors are based on light absorption, fluorescence, and luminescence. An electrochemical sensor measures a biological reaction through an electronic signal. Piezoelectric methods rely on changes of frequency, which can be associated with mass changes. Thermal biosensors measure the heat in biological reactions (Hassani et al., 2017; Rodriguez-Mozaz et al., 2006; Velasco-Garcia & Mottram, 2003)

There are several reported traits associated with a biosensor that are usually optimized in order to enhance the performance of the biosensor. Characteristics such as selectivity, reproducibility, stability, sensitivity, and linearity are the main focus to develop a good biosensor. Selectivity is related to the specific detection of a certain analyte in a mixture, being the most important trait. Reproducibility is the feature responsible for generating a similar output in duplicate experiments. Stability shows whether the biosensor is independent of surrounding disturbances or external factors such as pH or temperature. Sensitivity, or limit of detection (LOD), is the minimum concentration of the analyte that can be detected, usually measured in ng/ml or even fg/ml. Linearity is the accuracy of the response (Bhalla et al., 2016; Mehrotra, 2016).

Yeast as a biosensor

Despite the main focus of prokaryotic hosts in most reported biosensors, the interest in yeast as a biosensor has been rising (Qiu et al., 2019). There are many advantages of using yeast as a biosensor. For example, yeast is inexpensive to culture and storable for long periods. Much is known about yeasts as *S. cerevisiae*, allowing for easy genetic manipulations, it is easy to grow and was considered safe by the Food and Drug Administration (FDA). Compared to bacteria, yeast is more resistant to environmental changes, allowing for a wider range of experiments, and has a higher degree of conservation, so experiments involving yeasts can be more easily transposed into higher-eucaryotic organisms, as opposed to prokaryotic bacteria.

S. cerevisiae has been used as a genetic model to develop biosensors, with a special focus on the activation/repression of a promoter gene. These biosensors are entitled transcription factor-based biosensors (TF-based biosensors) and they rely on the activation of an inducible promoter that controls the transcription of a reporter gene. The induction of a promoter is usually associated with the presence of a ligand that activates a subsequent cascade, making the biosensor sensitive to the intra- or extracellular presence of that metabolite or ligand. For instance, the presence, and hence the binding of a ligand to a transcription factor can change the DNA binding domain, promoting/inhibiting downstream transcription (Adeniran et al., 2015; N. A. Da Silva & Srikrishnan, 2012; Qiu et al., 2019). These promoters are usually fused with reporter genes that generate a detectable output such as fluorescence, bioluminescence, and colorimetry (Adeniran et al., 2015). The use of GFP, a well-known fluorescent protein, is a rapid procedure (4 to 6 hours) and procedure steps and substrate addition are not required. Fig. 7a represents a whole-cell biosensor using GFP to detect the uptake of exogenous fatty acids by the yeast cell (Baumann et al., 2018; Beck et al., 2005; Bovee et al., 2004). Colorimetric systems use β-galactosidase and less expensive equipment and substrates. Fig. 7b represents a colorimetric system based on yeast two-hybrid to detect perturbations in the interaction between c-Myc (a transcription factor involved in some types of cancer) and its regulator Max (Fox et al., 2008; Heller et al., 2017).

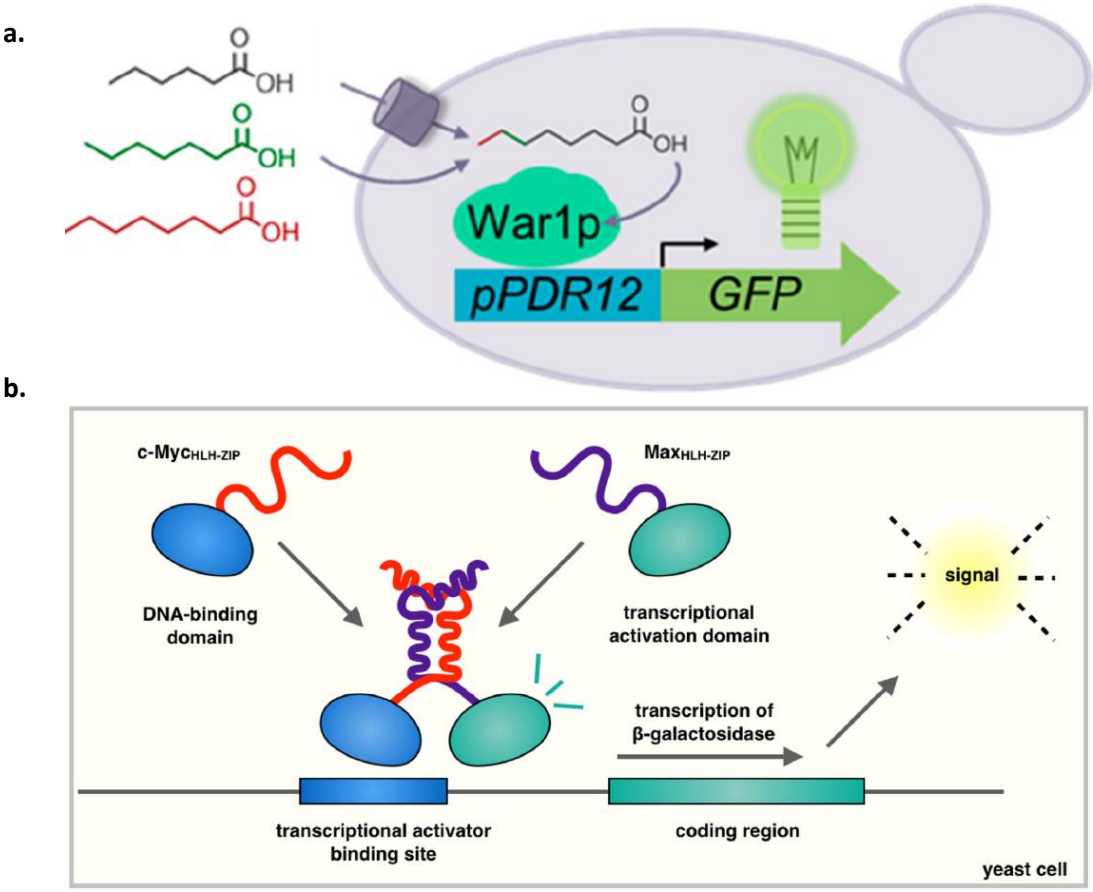

Figure 7. Yeast-based biosensors. (a) Whole-cell biosensor using the reporter gene system: the promoter of the gene *PDR12* was cloned upstream of the gene encoding GFP, in a multicopy vector transformed in *S. cerevisiae*. This sensor is based on the fatty acid uptake of yeast cells, that activates War1p, promoting the transcription of *PDR12* and consequently leading to GFP production. It detects the uptake of exogenous fatty acids, through the presence or absence of fluorescence (Baumann et al., 2018). **(b) Colorimetric screening technique using the two-hybrid system:** detects protein-protein interactions of the transcription factor c-Myc, with its regulator Max. c-Myc is fused with the DNA-binding domain (BD) of the yeast transcription factor Gal4, and Max is fused with its activation domain (AD). Only upon the interaction of these two proteins, and their dimerization, there's an activation of the transcription activator binding site, promoting the activation of *β*-galactosidase transcription. It detects small molecules, that interact with each one of these proteins, hence blocking their dimerization and consequently, inhibiting *β*-galactosidase expression (adapted from Heller et al., 2017).

Bioluminescence assays use luciferase as substrate, being a rapid, simple, and sensitive monitorization system. The production of light by bioluminescence occurs naturally in many living organisms. This phenomenon has been observed in nature, mostly in the ocean, for thousands of years, but it was only in the past hundred years that its mechanism was clearly understood. Bioluminescence is a chemical process (chemiluminescence) involving an enzyme (luciferase) and a substrate (that differs depending on the luciferase) that interact with each other, leading to the production of light (Fig. 8). NanoLuc (NLuc) luciferase, originally found in *Oplophorus gracilirostris* (deep sea shrimp), is a recently commercialized luciferase with a high-intensity luminescence, using furimazine as substrate (England et al., 2016; Widder & Falls, 2014).

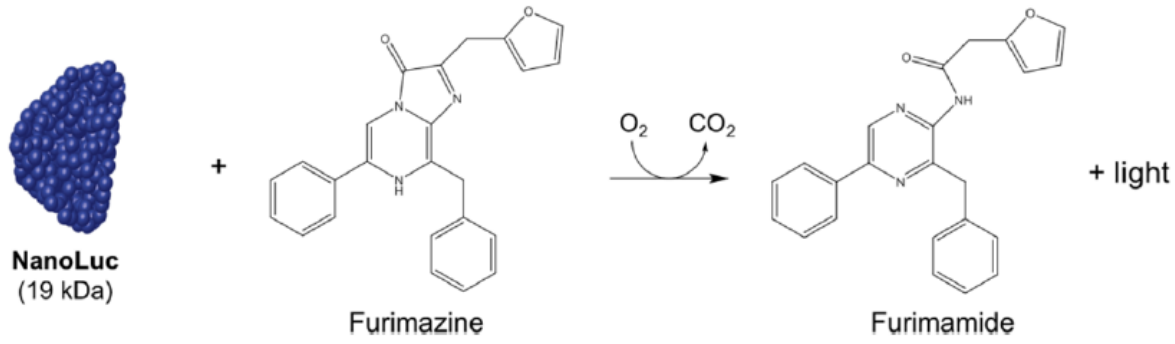

Figure 8. Catalytic mechanism underlying bioluminescence production by NanoLuc, an enzyme that catalyzes the conversion of furimazine to furimamide, in the presence of oxygen. Upon the interaction with furimazine, NanoLuc catalyzes its transformation in furimamide, releasing CO_2 and producing light, which can be further detected. Some applications using the NLuc technology involve the monitorization of protein stability, bioluminescence imaging, protein-protein and protein-ligand interactions, gene regulation and cell signaling (adapted from England et al., 2016).

Biosensors to detect the presence of pesticides

The interest in biosensors has increased over the past decades, being now worldwide used in many different fields, mainly in medicine (in drug discovery or disease detection and monitoring), in the food industry (in food quality monitoring), and in the environment (in the detection of toxins and water quality management) (Bhalla et al., 2016). As an example of the latter, Fig. 9 is a schematic representation of an enzymatic biosensor to detect the presence of pesticides in water samples, using an optical transducer method. Acetylcholinesterase (AChE) is one of the most used enzymes in enzymatic biosensors for the detection of organophosphorus (OP). AChE hydrolyses acetylcholine (ACh), leading to the production of choline and acetic acid, decreasing the pH due to the presence of an acid. In the presence of OP or carbamate pesticides, the serine residue in the active center of AChE is phosphorylated, inhibiting this hydrolysis, thus the amount of the acid produced is lower. The presented sensor (Fig. 9) consists of a filter paper with a pH indicator, dyed with bromocresol purple. The enzyme (AChE) and the substrate (ACh) are separated with a gap to prevent the reaction from starting before the sample is added. After the sample is added, the paper is folded and the reaction starts. This biosensor is a simple and rapid single-use colorimetric system to determine the presence of pesticides, and the only requirement is the addition of the sample since all the reagents needed are immobilized in the device. The limit of detection achieved was 0.24 and 2 ng/ml of carbaryl (a carbamate pesticide) and chlorpyrifos (an OP pesticide), respectively (Fernández-Ramos et al., 2020; Pohanka et al., 2010). Many different biosensors were constructed, relying on this enzyme-inhibition system, with different LODs, due to different affinities of the selected enzyme to the correspondent pesticide compound (Pérez-Fernández et al., 2020).

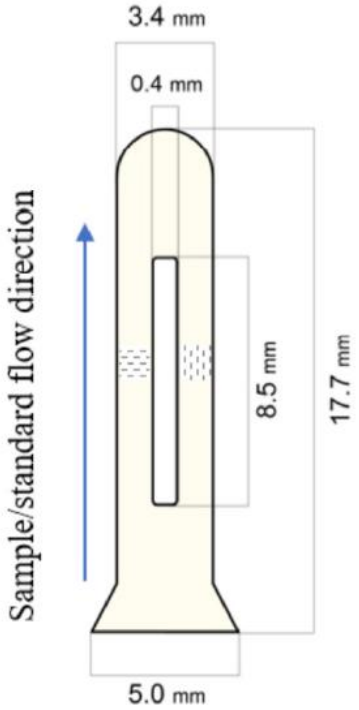

Figure 9. Schematic representation of a colorimetric biosensor to detect pesticides in water samples. This device is composed by three sections: one for the addition of the sample, a section with two transporter channels with a gap in between (one with AChE and another with ACh, immobilized), and the top section, for the detection, with a pH indicator. The system detects pH variations, related to the presence or absence of pesticides containing chlorpyrifos or carbaryl. In the presence of such pesticides, the detection zone of the paper turns yellow (indicating an increase in the pH), while in the absence of a pesticide it turns purple (indicating a decrease in the pH) (adapted from Fernández-Ramos et al., 2020).

In previous work in the group, a biosensor to detect the presence of tebuconazole (TEB) was constructed. As mentioned above, TEB is a broad-spectrum, low-cost triazole fungicide, commonly used since 1992 in agriculture and in medicine to treat and prevent fungal infections. Among other countries, TEB is one of the most used azoles in Portugal and its danger for humans and ecosystems has been well documented (S. Li et al., 2019). Its presence has been detected in vegetables, fruits, as well as wheat products, constituting a possible human health hazard. Additionally, it has been detected in high concentrations in aquatic samples and it can be detrimental for aquatic ecosystems (Cuco et al., 2017; S. Li et al., 2019; Price et al., 2015; Q. Zhang et al., 2015). As an azole compound, it suppresses ergosterol biosynthesis by inhibiting the activity of 14α-demethylase, a CYP P450 (Ghannoum & Rice, 1999). CYPs enzymes are also expressed in mammals, and thus these compounds can affect the activity of mammalian enzymes. As they are broad-spectrum, adverse effects in non-target organisms are relatively common (Rendic & Guengerich, 2018; Taxvig et al., 2007). TEB is classified by the EPA as a possible human carcinogen (type C) due to results in mice studies, suggesting a reprotoxic effect (US EPA, 1996; Yang et al., 2018). Tebuconazole was first classified by WHO in 1994 in the Joint of Food

and Agriculture Organization (FAO)/WHO Meeting on Pesticide Residues (JMPR) as being unlikely to present acute hazard (low acute toxicity), but in their last meeting, in 2010, it was re-evaluated as having a low to moderate acute toxicity (FAO & WHO, 1994, 2010). The constructed biosensor was based on a whole-cell system, using a reporter gene under the control of an inducible promoter. The reporter gene was the nanoluciferase encoding gene and the selected promoter was *ERG11*, which encodes for the lanosterol 14α-demethylase, the main target of azole compounds such as tebuconazole. The constructed biosensor was responsive to the presence of tebuconazole but required optimization and improvement for a yeast-based biosensor to monitor aqueous samples (Carvalho, 2018).

OBJECTIVE

Despite the well-known advantages of agrochemicals in society, consequent negative impacts arose due to their uncontrolled use. Since many pesticides are broad-spectrum, they can have many off-target effects. One example is azole fungicides, which inhibit the ergosterol biosynthetic pathway, a conserved pathway in many other organisms. As such, target and non-target fungi are affected by azoles in general and tebuconazole in particular. Taking into consideration the need to improve monitoring systems, this project aimed to:

A – Construct yeast reporter strains to function as biosensors to detect the presence of tebuconazole in environmental samples. For that purpose, promoters of *ERG* genes that are induced in the presence of azole compounds were cloned in a plasmid upstream of the reporter gene Nluc, resulting in luciferase protein production upon the activation of the *ERG* promoters.

B – Test the reporter strain and optimize the biosensor, so that it would detect low azole concentrations. For that purpose, the constructed strains were tested in the presence of increasing concentrations of tebuconazole, at different time points. Furthermore, several strategies were pursued to improve the biosensor, such as the use of mutants, strains harboring two vectors simultaneously, and integration of the system in the genome.

Part of the results were obtained in collaboration with F. Mendes and were submitted for publication:

Filipa Mendes, Eduarda Miranda, Leslie Amaral, Carla Carvalho, Bruno B. Castro, Maria João Sousa, Susana R. Chaves. Novel yeast-based biosensor for environmental monitoring of tebuconazole.

MATERIALS & METHODS

1. STRAINS AND PLASMIDS

The *S. cerevisiae* and *E. coli* strains and the plasmids that were used in this project are listed in Tables 1 and 2, respectively. *S. cerevisiae* diploid BY4741xBY4742 (BY(2N)) was obtained by mating haploid BY4741 and haploid BY4742.

Table 1. List of strains used in the present study.

Strain	Genotype	Reference/Source
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB + lac LqZ* ΔM15 Tn 10 (TetR)]	Lab stock
S. cerevisiae BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	EUROSCARF
S. cerevisiae BY4742	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*	EUROSCARF
S. cerevisiae BY(2N)	*MATa/α*, *his3Δ1/his3Δ1*, *leu2Δ0/leu2Δ0*, LYS2/*lys2Δ0*, *met15Δ0/MET15*, *ura3Δ0/ ura3Δ0*	Present study

Table 2. List of plasmids used in the present study.

Plasmid	Features	Source
pRS426yNluc	*URA3*, *ampR*	Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P.
pRS426*ERG3*pryNluc	*ERG3* promoter, *URA3*, *ampR*	Present study
pRS426*ERG6*pryNluc	*ERG6* promoter, *URA3*, *ampR*	Present study
pRS426*ERG10*pryNluc	*ERG10* promoter, *URA3*, *ampR*	Present study
pRS426*ERG11*pryNluc	*ERG11* promoter, *URA3*, *ampR*	Present study
pRS426*ERG25*pryNluc	*ERG25* promoter, *URA3*, *ampR*	F.Mendes
pRS425*ERG25*pryNluc	*ERG25* promoter, *LEU2*, *ampR*	Present study

1.1. Plasmid construction

1.1.1. Genomic DNA purification

S. cerevisiae BY4741 cells were grown overnight in 5 ml of liquid YPD [YPD; 1 % (w/v) yeast extract, 2 % (w/v) bactopeptone, 2 % (w/v) glucose and 2 % (w/v) agar] at 30 °C with orbital shaking of 200 rpm. Cells were collected, washed in sterile water, and resuspended in 100 µl of 1 M Sorbitol containing 100 mM EDTA (pH=7.5). 2 µl of 20 mg/ml zymoliase were added and the cells were incubated

at 37 °C for 20 minutes. 100 µl of 50 mM Tris-HCl containing 20 mM EDTA (pH=7.4) and 5 µl of 10 % SDS were added. The cells were incubated at 65 °C for 5 minutes and transferred to ice. 80 µl of 5 M KAc were added and the tubes were incubated on ice for 5 minutes. The cells were collected at maximum speed for 30 minutes at 4 °C. The supernatant was transferred into new eppendorfs, and 1 volume of isopropanol was added. Cells were incubated for 5 minutes at room temperature and collected for 15 minutes at maximum speed (4 °C). The pellets were then washed twice with 70 % ethanol and once with 100 % ethanol. The pellets were dried at room temperature and resuspended in 20 µl of TE (pH=8.0). The DNA was quantified and stored at -20 °C.

1.1.2. Insert preparation

From the extracted gDNA, *ERG3*, *ERG6*, *ERG10,* and *ERG11* promoter sequences were amplified by conventional Polymerase Chain Reaction (PCR), using the primers 1 – 8 listed in Table 3, respectively. For the PCR, 10 µl of 5X Phusion™ HF Buffer, 0.5 µl of Phusion High-Fidelity DNA Polymerase, 1 µl of 10 mM dNTPs mix, 2.5 µl of each 10 µM primer (forward and reverse), 2 µl of DNA template and ultrapure water to the final volume of 50 µl were added to PCR tubes. The PCR consisted of an initial denaturation at 98 °C for 30 seconds, followed by 35 cycles of denaturation (98 °C, 10 seconds), annealing (69 °C for *ERG6* and *ERG10*, and 66.9 °C for *ERG3* and *ERG11* for 10 seconds), and extension (72 °C for 1 minute – 15-30 s/kb), and one cycle of a final extension at 72 °C for 10 minutes. PCR products were purified using the protocol for "PCR product clean up" from Grisp Kit (Appendix A) and subjected to an amplification PCR using primers 8 to 16 listed in Table 3. A negative control was performed for each gene amplification, without a DNA template.

1.1.3. Vector preparation

The plasmid pRS426yNluc was linearized using Hind III restriction enzyme: to an eppendorf, 1 µl of 10x Buffer R, 1 µl of Hind III, 0.5 µg/µl of DNA and ultrapure water to the final volume of 10 µl were added. The digestion was performed overnight at 37 °C, and a negative control was performed, without the Hind III restriction enzyme.

1.1.4. Yeast transformation

BY4741 wild-type cells were transformed with pRS426yNluc linear plasmid and each one of the amplified *ERG* promoters individually, using a high-efficiency yeast transformation protocol. Briefly, a single colony from *S. cerevisiae* BY4741 was grown overnight in liquid YPD medium at 30 °C with orbital shaking of 200 rpm. The culture was diluted to fresh medium of YPD 2x glucose to an OD_{640nm} = 0.2 and grown for 3 additional hours in the same conditions. The culture was collected and then washed in sterile water and resuspended in 100 µl of sterile water, transferred into new sterile eppendorfs, and collected. Cells were resuspended in a mix containing 250 µl of 50 % (w/v) PEG3350, 36 µl of 1.0 M LiAc, 50 µl of 2.0 mg/ml boiled ssDNA and 32 µl of sterile water. 0.1 – 1 µg of DNA (2 µl of the linear vector and 14 µl of each *ERG* promoter) was added and the cells were incubated in a water bath at 42 °C for 40 minutes. In the end, cells were collected at maximum speed for 1 minute and the pellets were resuspended in 100 µl of sterile water, then plated on SC-glucose agar media without uracil [SC-glucose: 0.5 % (w/v) ammonium sulphate, 0.17 % (w/v) yeast nitrogen base without amino acids, 0.14 % (w/v) dropout, 2 % (w/v) glucose, 0.04 % (w/v) leucine, 0.008 % (w/v) histidine, 0.008 % (w/v) tryptophan, 0.008 % (w/v) uracil, 2 % (w/v) agar]. The plates were incubated at 30 °C for two days. The controls performed in the transformation were: a negative control without the DNA template, a control with the empty vector and without the DNA template, the linear vector without the DNA template and the circular vector (non-digested) without the DNA template.

1.1.5. Confirmation PCR

From each transformation plate, a few colonies were selected, cultured and their gDNA was extracted, to be used as template DNA for the confirmation PCR. 12,5 µl of the NZYTaq II 2x Green Master Mix, 0.25 µM of each primer (forward and reverse), 5 pg – 0.5 µg of DNA template (0.25 µl) and ultra-pure water to the final volume of 25 µl were added to PCR tubes. The primers used to confirm the positive clones were the primer forward 25 and the primer reverse of each gene (2, 4, 6 and 8) listed in Table 3. The PCR consisted of one initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds and extension at 72 °C for 2 minutes (15-30 sec/kb), and one cycle of a final extension at 72 °C for 5 minutes. A negative control was performed without the DNA template.

1.1.6. Clone selection

From the selected colonies their plasmids were extracted following the miniprep protocol from the Sigma-Aldrich kit (Appendix B). The purified plasmids were transformed in *E. coli* XL1-Blue, following a high-efficiency transformation protocol. The DNA was added to 100 µl of competent cells (a protocol to prepare competent cells is described in Appendix C). The cells were incubated on ice for 30 minutes, in a water bath at 42 °C for 45 seconds, and then on ice for an additional 10 minutes. The cells were added to 800 µl SOC [2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 20 mM glucose] and incubated for 1 hour at 37 °C with orbital shaking of 200 rpm. The cells were then collected at 3000 rpm for 3 minutes and the supernatant was discarded, leaving just enough to resuspend the cells and plate them on LB-agar plates [0.5 % (w/v) yeast extract, 1 % (w/v) tryptone, 1 % (w/v) NaCl, 1 % (w/v) agar] containing 0.1 % ampicillin. The plates were then incubated at 37 °C overnight. A negative control was performed without the DNA template.

Table 3. Primer sequences – forward (Fw) and reverse (Rv) – used in the construction of the vectors.

	Promoter	Primer sequences (5'→3')
1	*ERG3*_Fw	attgggtacccaccatcgtcgtcctcctgttc
2	*ERG3*_Rv	ccataagcttatctcaaatctagacgaatatttttcttattatc
3	*ERG6*_Fw	ccccccttcgagtgctcgctatcctcgccatc
4	*ERG6*_Rv	ccataagcttcttatgctgcctactatattattattttattcttgtt
5	*ERG10*_Fw	ccccctcgagattgaagcacctgtggagtatttaaaaac
6	*ERG10*_Rv	ccataagctttttgagtacgtctaatctgtataaatatatatgatatgg
7	*ERG11*_Fw	ccccctcgagcttgttctctctcgcttcctacgtt
8	*ERG11*_Rv	ccataagcttccttgtattactcgtttgttctgtttctattc
9	*ERG3*_gap_repair_Fw	tacgactcactatagggcgaattgggtacccaccatcg
10	*ERG3*_gap_repair_Rv	aaaatcttctaaagtaaaaaccataagcttatctcaaatctagacgaa
11	*ERG6*_gap_repair_Fw	ggcgaattgggtaccgggccccccctcgagtgctcg
12	*ERG6*_gap_repair_Rv	aaaatcttctaaagtaaaaaccataagcttcttatgctgcc
13	*ERG10*_gap_repair_Fw	ctcactatagggcgaattgggtaccgggccccccctcgagattgaagc
14	*ERG10*_gap_repair_Rv	aaaatcttctaaagtaaaaaccataagctttttgagtacgtctaat
15	*ERG11*_gap_repair_Fw	ggcgaattgggtaccgggccccccctcgagcttgttctc
16	*ERG11*_gap_repair_Rv	aaaatcttctaaagtaaaaaccataagcttccttgtattactcgttt

17	20_upstream	atccgataagcgcaggcgcctttatatcattcgcgcgtttcggtgatgac
18	20_downstream	Tcttcttagtgcttgtatatgctcatcccgaccttccattcatgattacgccaagcgcgc
19	pRS_*ERG25*_Fw	ggtcgacggtatcgataagcttcctttcatccgtctcgtttatcataa
20	*ERG25*yNluc_RV	ccaacaaaatcttctaaagtaaaaaccataagcttttcctctttttttttatggctgtac
21	20_up_check	ggatgcctatgttcccccc
22	20_down_check	cgttagtatcgtcaaaacactcgg
23	pRS_*CYC1*_Fw	ggatccactagttctagatcatgtaattagttatgtcacgcttac
24	*URA*_5'	tagcagcacgttccttatatgtag
25	T7	taatacgactcactataggg

1.1.7. Sanger sequencing

From all the selected strains, the plasmids were extracted, following the miniprep protocol by Sigma-Aldrich kit (Appendix B), and the DNA was sequenced (Eurofins Genomics).

1.1.8. Storage

The strains *S. cerevisiae* BY4741 and *E. coli* XL1-Blue, each harboring pRS426*ERG3*pryNluc, pRS426*ERG6*pryNluc, pRS426*ERG10*pryNluc, or pRS426*ERG11*pryNluc, were stored in 1 ml of 30 % glycerol at -80 °C.

1.2. Strain construction using genome integration

1.2.1. Construction of the strain

The vector pRS426*ERG25*pryNluc was digested twice, separately, to linearize the plasmid to be used as a template for PCR: the first digestion was performed with the restriction enzyme PvuII (2 µl 10 X Buffer G, 1 µl PvuII, 0.5 µg/µl DNA, and ultrapure water to the final volume of 20 µl) at 37 °C for 1 hour and the second digestion was performed overnight with the restriction enzyme XbaI (2 µl 10 X Buffer Tango, 1 µl XbaI, 0.5 µg/µl DNA, and ultrapure water to the final volume of 20 µl), at 37 °C. In both digestions, a negative control without each restriction enzyme was performed. From the resultant fragments of both digestions, an amplification PCR was performed, using primers 18 and 19 (digestion with PvuII), and primers 17 and 20 (digestion with XbaI), listed in Table 3. For the PCR, 10 µl of 5 X

Phusion™ HF Buffer, 0.5 µl of Phusion High-Fidelity DNA Polymerase, 1 µl of 10 mM dNTPs mix, 2.5 µl of each 10 µM primer (forward and reverse), 1 µl of DNA template, and ultrapure water to the final volume of 50 µl were added to PCR tubes. The PCR consisted of an initial denaturation at 98 °C for 30 seconds, 35 cycles of denaturation (98 °C, 10 seconds), annealing (72 °C for 10 seconds) and extension (72 °C for 2 minutes – 15-30 s/kb), and one cycle of a final extension at 72 °C for 10 minutes. A negative control without DNA template and another of the PCR amplification mixture without Phusion polymerase were performed. After each fragment was amplified, 10 µl of each was transformed in *S. cerevisiae* BY4741 x BY4742 using a high-efficiency transformation protocol. as described above, in "1.1.4. Yeast Transformation". A control without DNA and another of the PCR amplification mixture without Phusion polymerase were used. The SC-glucose without uracil agar plates were incubated at 30 °C for 2 days.

All the obtained colonies were streaked onto both 5-FOA and SC-glucose without uracil agar plates and grown at 30 °C for 2 days. The positive colonies (those which grew on SC-URA but not on 5-FOA) were selected for confirmation.

1.2.2. Confirmation PCR

The potential positive colonies were incubated overnight in SC-glucose without uracil liquid medium at 30 °C with orbital shaking of 200 rpm and their gDNA was extracted for confirmation PCR. 12,5 µl of the NZYTaq II 2x Green Master Mix, 0.25 µM of each primer (forward and reverse), 5 pg – 0.5 µg of DNA template, and ultrapure water to the final volume of 25 µl were added to PCR tubes. Two confirmation PCRs were performed, using two different sets of primers. The first PCR used primers 22 and 23, listed in Table 3, and consisted of one initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 1 minute and 30 seconds (15-30 sec/kb), and one cycle of a final extension at 72 °C for 5 minutes. The second PCR used primers 21 and 24, listed in Table 3, and consisted of one initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute and 30 seconds (15-30 sec/kb), and one cycle of a final extension at 72 °C for 5 minutes. A negative control without the DNA template was performed.

2. BIOLUMINESCENCE ASSAY

S. cerevisiae BY4741 wild-type cells harboring pRS426*ERG3*pryNluc, pRS426*ERG6*pryNluc, pRS426*ERG10*pryNluc, pRS426*ERG11*pryNluc, or pRS426*ERG25*pryNluc, were grown overnight in liquid

SC-glucose with proline and without uracil [SC-glucose with proline: 0.5 % (w/v) proline, 0.17 % (w/v) yeast nitrogen base without amino acids, 0.14 % (w/v) dropout, 2 % (w/v) glucose, 0.04 % (w/v) leucine, 0.008 % (w/v) histidine, 0.008 % (w/v) tryptophan, 0.008 % (w/v) uracil, 2 % (w/v) agar], at 30 °C with orbital shaking of 200 rpm and diluted to an OD_{640nm} = 0.1, transferred to 5 ml of fresh medium and grown for an additional 3 hours. The cultures were then subjected to a 2, 4, 6, 8, and/or 24-hour treatment with 0.001, 0.005, 0.01, 0.05, and/or 0.5 µg/ml tebuconazole. The negative control was assessed with DMSO, the solvent for tebuconazole. After the treatment, the cultures were collected and resuspended in 1 ml of SC-glucose (pH=8.0) with 6.25 mM PEG 3350 to an OD_{640nm} = 0.5, 2.5 or 5.0. A blank with 100 µl of SC-glucose (pH=8.0) containing 6.25 mM PEG 3350 and a negative control for each sample with 100 µl cells of each were the controls performed in this assay. The test samples contained 97.5 µl cells with 2.5 µl of luciferase substrate. The bioluminescence was recorded for 15 minutes, and the results represent the bioluminescence at time 0 of each time point.

3. STATISTICAL ANALYSIS

The results presented in this study were calculated by the mean and standard deviation (SD) values of independent experiments. The statistical analysis was performed using GraphPad Prism Software.

RESULTS & DISCUSSION

1. YEAST BIOSENSOR STRAIN CONSTRUCTION

Azole compounds are widely and successfully used in the agricultural field as antifungal agents. The major target of these agents is cytochrome P450-Erg11p, involved in ergosterol biosynthesis. The exposure of fungi to these compounds causes the inhibition of this target which, in turn, blocks the oxidative removal of the 14α-methyl group of lanosterol, promoting the accumulation of 14α-methylated sterols and diminishing the ergosterol content in the cell. As ergosterol is an essential component of the yeast cell membrane, being required for its survival, exposure of fungi to these compounds leads to growth inhibition (Espinel-Ingroff, 1997; Verweij et al., 2009). Several studies have shown that *ERG* genes can be up-regulated as a response to the ergosterol depletion caused by the presence of azole compounds. Some of these genes include *ERG2*, *ERG3, ERG5, ERG6, ERG7, ERG10, ERG11, ERG25*, and *ERG29* (Q. Q. Li et al., 2018; Rodrigues et al., 2017; Rybak et al., 2017; M. C. Silva et al., 2020). *ERG3, ERG6, ERG11*, and *ERG25* are involved in the last module of the ergosterol biosynthesis, while *ERG10* is involved in the first module, the mevalonate synthesis (Fig. 4) (Hu et al., 2017).

As the aim of the project was to construct a yeast-based biosensor to detect the presence of azole compounds, the first task was to construct the yeast reporter strains. A yeast reporter strain harboring pRS426*ERG25*pryNluc (a vector containing *ERG25* as a promoter, fused with yNluc), had already been constructed and was used as a positive control. Four different *ERG* promoters (*ERG3, ERG6, ERG10*, and *ERG11*) were separately cloned into the pRS426yNluc vector and transformed in yeast. They were cloned upstream of the coding sequence for the yNluc reporter so that it would only be expressed, and further detected, upon the activation of the promoter. In the first attempt, conventional cloning was tried (not shown), but only the gap-repair cloning (GRC) approach was successful (Fig. 10). GRC takes advantage of a DNA repair system that occurs upon a double-stranded DNA break. It relies on the transformation of a gapped vector (linearized) and a linear DNA insert in *S. cerevisiae*, with homologous sequences between them. After transformation, homologous recombination occurs, and a circular vector is obtained, in which the "gap" has been repaired with the insert. This system is highly efficient, because of the high frequency with which homologous recombination occurs in the budding yeast *S. cerevisiae.* For the transformation process, it is important to add a control without the DNA insert, only with the linear vector, in order to control the vector recircularization (Bessa et al., 2012; Chino et al., 2010; Oldenburg et al., 1997).

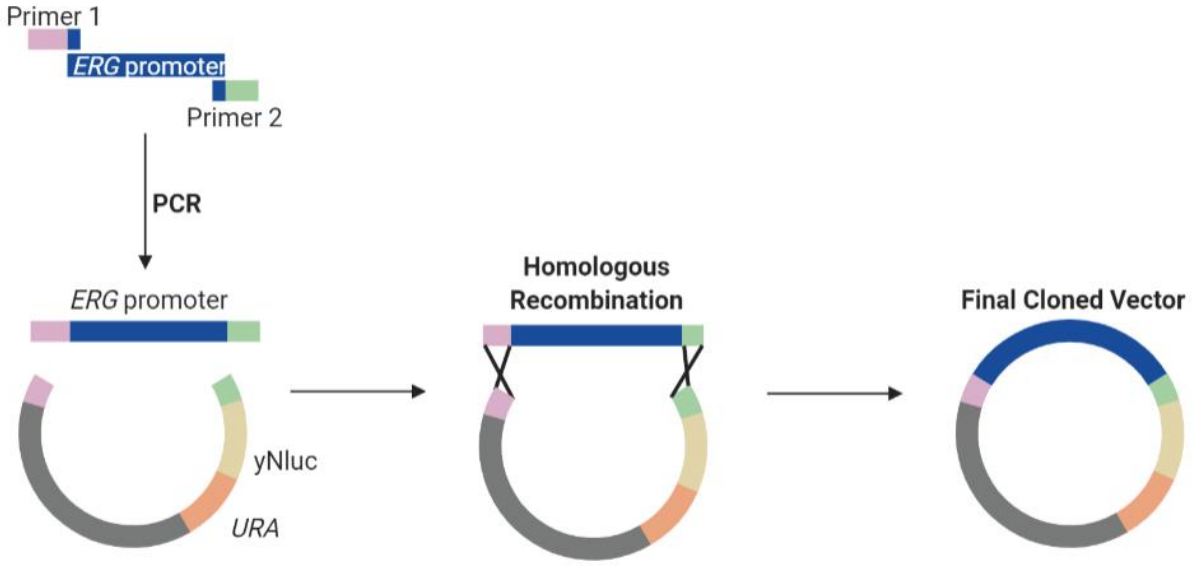

Figure 10. Gap Repair Cloning approach. The *ERG* promoter, that is going to be cloned in the vector, is amplified using hybrid primers (Primer 1 and Primer 2), that will add a region (pink and green) in the end of each *ERG* promoter, homologous to the linearized pRS426yNluc vector on its cloning site. These homology regions will allow for homologous recombination to occur between the linear vector and the amplified sequence. Once homologous recombination occurs, the final vector contains the cloned sequence (blue), in this case, upstream of the yNluc reporter (yellow) (created with BioRender).

S. cerevisiae BY4741 was the selected yeast wild-type strain due to its mutations in the *URA, HIS, LEU*, and *MET* genes. These mutations prevent the yeast strain from growing on a medium lacking uracil, histidine, leucine, or methionine since the genes involved in their production are mutated and non-functional. For that reason, upon the cloning of a vector with the *URA* gene as a selectable marker, for example, the positive transformants would be easily selected since, theoretically, only the strains containing the cloned vector would grow on a medium lacking uracil.

1.1. Vector cloning

Upon gDNA extraction of *S. cerevisiae* BY4741, *ERG3, ERG6, ERG10*, and *ERG11* promoters were amplified using the primers 1-8 listed in Table 3. The results from the amplification are presented in Fig. 11. The negative control was assessed in the same conditions, but without the correspondent template DNA, in order to verify that the used primers were not contaminated with DNA and so they would only amplify upon the presence of the template DNA. The agarose gel electrophoresis showed that the amplification PCR of the *ERG* promoters was successful, since the amplification only occurred in the presence of the template DNA and the amplified fragment had the correct size. These fragments were used for restriction enzyme cloning but this attempt was unsuccessful (not shown).

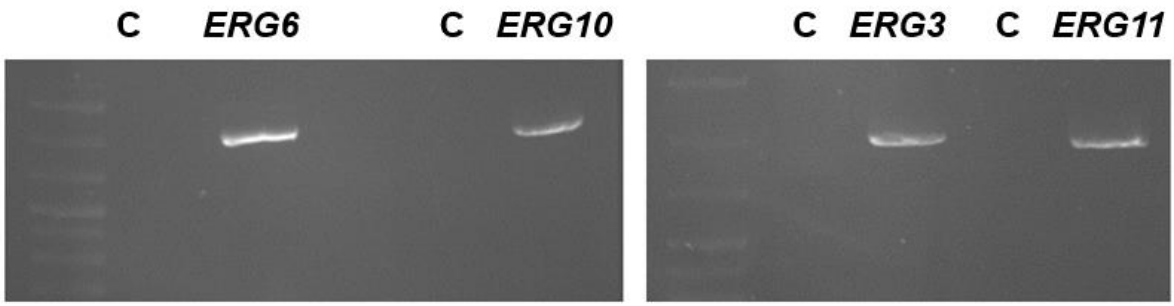

Figure 11. Agarose gel electrophoresis of the amplification PCR of the *ERG* genes. The designed primers amplified fragments of 1020 bp for *ERG3*, *ERG10* and *ERG11* and 1021 bp for *ERG6*. The amplification was successful, as the amplified fragments had approximately 1 KB. The negative control, C, was testing for primer contamination and was performed in the same condition of the *ERG* genes but lacking the correspondent template DNA. As no fragment was amplified in the control, the primers were not contaminated and only amplified in the presence of the correspondent template DNA.

For the GRC method, another PCR was thus performed, in order to add a region homologous with the pRS426yNluc plasmid to the amplified *ERG* sequences. This amplification is represented in Fig. 10, with primers 1 and 2. The primers used in this PCR were the primers 9-16 listed in Table 3 that contained, in the 5' and 3' ends of each primer, a nucleotide sequence homologous to the pRS426yNluc plasmid, allowing for homologous recombination in yeast. After amplification with these primers, the resultant fragments of the *ERG3, ERG6, ERG10*, and *ERG11* promoters were approximately 1 KB, similar to the predicted 1060 bp, 1060 bp, 1070 bp, and 1059 bp of length, respectively.

After amplification of the inserts, the pRS426yNluc plasmid was digested overnight with the HindIII restriction enzyme to create a "gap" in the plasmid sequence. Plasmid digestion was confirmed by agarose gel electrophoresis. Then, the cut plasmid was co-transformed with each of the inserts in *S. cerevisiae* BY4741. The plasmid contains the *URA* selectable marker, which allows the yeast to grow on a selective medium lacking uracil. However, not all the obtained colonies would be positive clones containing the insert. For that reason, several colonies of each transformation were selected for confirmation.

1.2. Confirmation PCR

In order to confirm the success of the cloning strategy, DNA was extracted from selected clones and a confirmation PCR was performed. The primers used in this PCR allowed us to understand if the desired insert was in fact contained in the vector since the forward primer hybridizes with the vector and each reverse primer hybridizes with each of the inserts. Consequently, a fragment would only be amplified in the vector containing each insert. The selected primers amplify fragments of around 1 KB. After the PCR, the samples were subjected to an agarose gel electrophoresis (Fig. 12). As only the samples where

the amplified fragments were around 1 KB would be positive clones, it is possible to conclude that the colonies harboring the circular vector containing each promoter were in *ERG3* 1 and 3, in *ERG6* 1 and 3, in *ERG10* 3 and 5, and *ERG11* 1, 3 and 4. These colonies were used to perform a bioluminescence assay in order to confirm the results.

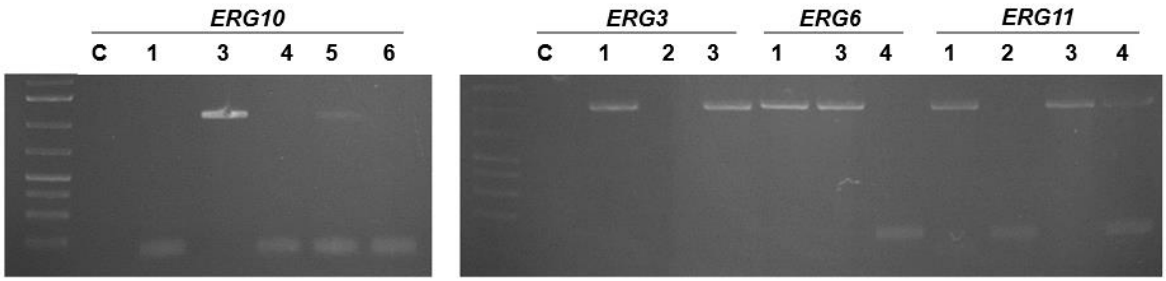

Figure 12. Confirmation PCR agarose gel electrophoresis. From the selected colonies, a confirmation PCR was performed, to select those containing the vector with the desired insert (*ERG* promoter). From *ERG10*, only clones 3 and 5 were positive clones, from *ERG3*, clones 1 and 3, from *ERG6*, clones 1 and 3 and from *ERG11*, clones 1, 3 and 4 were positive clones. The negative control, C, was assessed in the same conditions as the *ERG* constructs, but lacking in the template DNA, as a control for the primers.

1.3. Bioluminescence assay

1.3.1. Starting point

Before the construction of the four vectors, pRS426*ERG25*pryNluc was already available (F. Mendes). The results of the bioluminescence signal emitted by the strain *S. cerevisiae* BY4741 harboring

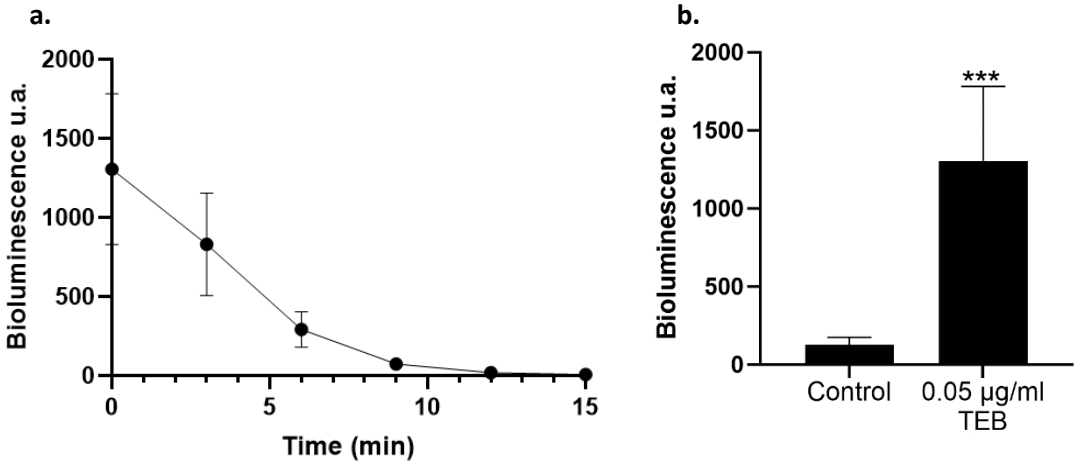

Figure 13. Starting point using a previously constructed vector. *S. cerevisiae* BY4741 wild-type cells harboring pRS426*ERG25*pryNluc were grown overnight and diluted to an OD_{640nm}=0.1, transferred to fresh medium and grown for an additional 3 h. Afterwards, cells were exposed to 0 and 0.05 µg/ml of TEB for 8 hours and 1 ml of culture ($OD_{640\ nm}$=0.5) were collected and resuspended in SC-Glu medium (pH=8) containing 6.25 mM PEG 3350. Bioluminescence was recorded for 15 min (a.) and the results represent bioluminescence at time 0 (b.). The negative control was assessed with DMSO, the solvent for TEB. Values were calculated based on mean ± SD of three independent experiments.

pRS426*ERG25*pryNluc are presented in Fig. 13. After previous optimizations, this biosensor was able to detect 0.05 µg/ml of tebuconazole, but the main goal was to detect 0.001 µg/ml. In order to improve the limit of detection of the biosensor, pRS426*ERG3*pryNluc, pRS426*ERG6*pryNluc, pRS426*ERG10*pryNluc, and pRS426*ERG11*pryNluc vectors were constructed. *ERG3, ERG6, ERG10*, and *ERG11* are four genes that, similar to *ERG25*, may be up-regulated in yeast in the presence of azole compounds. For that reason, these genes were selected as promoters, in order to activate the transcription and further production of NanoLuc luciferase, to which they were fused, so that a bioluminescence signal could be detected.

The fluorometer, a device that detects the emission of bioluminescence, records the bioluminescence signal throughout 15 minutes (at times 0, 3, 6, 9, 12, and 15 minutes). As shown in Fig. 13a, the bioluminescence signal decreases over time, as a consequence of the high concentration of the enzyme in comparison with the substrate, leading to a rapid depletion of the substrate, and consequently a decrease in the emitted bioluminescence. So, for this study, only the signal recorded at 0 minutes immediately after adding the substrate (Fig. 13b) was considered to analyze further results.

1.3.2. Confirmation bioluminescence assay

In the strains harboring the positive constructs, there should be activation of the *ERG* promoters in the presence of tebuconazole, as a response to the decrease in cellular ergosterol content. Since the *ERG* promoters are cloned upstream of the yNluc reporter gene, when the *ERG* promoter is activated,

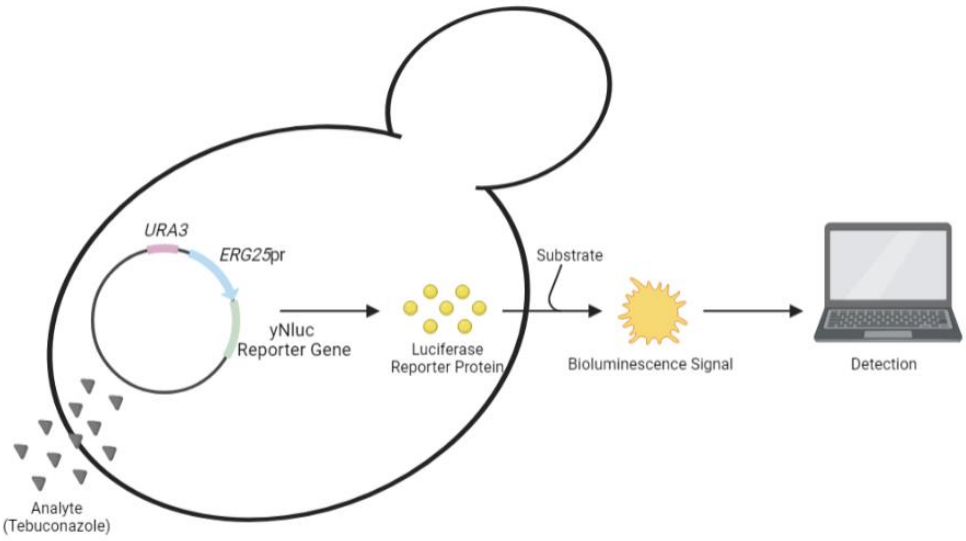

Figure 14. pRS426*ERG25*pryNluc yeast reporter strain. Outline of the developed yeast-based reporter strain biosensor. In the presence of an analyte (tebuconazole), the *ERG25* promoter is activated, promoting the transcription of the luciferase reporter gene, and the consequent production of its protein. Upon addition of a substrate, luciferase protein produces a bioluminescence signal that can be detected by a fluorometer and further analyzed (created with BioRender).

yNluc is expressed, leading to the production of luciferase protein. In the presence of luciferase substrate containing furimazine, luciferase catalyzes the conversion of furimazine to furimamide, leading to the release of CO_2 and the production of light (luminescence) which can be recorded through a fluorometer (Fig. 14). The same colonies that were used to perform the confirmation PCR were used in this assay. The results obtained are presented in Fig. 15. In this case, the positive clones will be those that will have a high bioluminescence signal, when compared with the negative control (assessed with DMSO instead of tebuconazole). The strains that produced a high bioluminescence signal were, 1 and 3 in *ERG3*, 1 and 3 in *ERG6*, 3 in *ERG10*, and 1 in *ERG11*. Combining these results with the results obtained in the agarose gel electrophoresis, the clones *ERG3*.1, *ERG6*.3, *ERG10*.3, and *ERG11*.1 were selected as, in addition to being positive in the confirmation PCR, they had the highest bioluminescence signal (of note, a high concentration of TEB of 4 µg/ml was used to test the colonies). Colonies *ERG11* 3 and 4, used in the PCR confirmation, were not used in this assay, since they did not grow in the overnight culture.

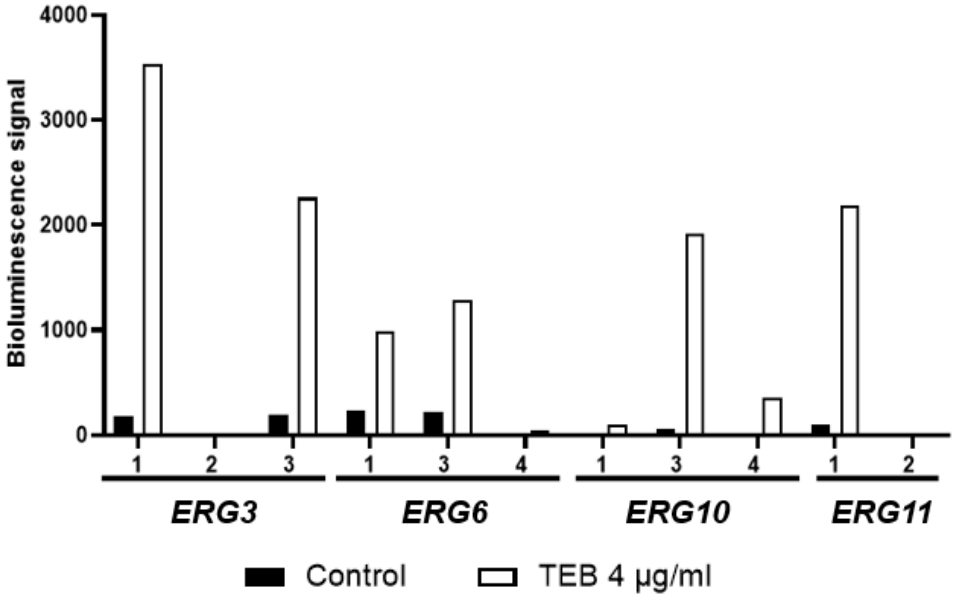

Figure 15. Bioluminescence assay to select one strain from each construct. *S. cerevisiae* BY4741 wild-type cells harboring pRS426*ERG3*pryNluc, pRS426*ERG6*pryNluc, pRS426*ERG10*pryNluc or pRS426*ERG11*pryNluc were grown overnight and diluted to an OD_{640nm}=0.1, transferred to fresh medium and exposed to 0 and 4 µg/ml of TEB for 6 hours. 1 ml of culture (OD_{640nm}=0.5) was collected and resuspended in SC-Glu medium (pH=8) containing 6.25 mM PEG 3350. Bioluminescence was recorded for 15 min and the results represent bioluminescence at time 0. The negative control was assessed with DMSO, the solvent for TEB.

From the selected positive clones, the plasmids were extracted and amplified in *E. coli* XL1-Blue, in order to obtain higher amounts of the plasmid to store and for further analysis (Appendix D).

1.4. Sequencing

For a final confirmation that each *ERG* promoter was indeed inserted in the vector and that no errors were introduced during the cloning strategy, the constructs were sequenced. None of the constructs had mutations, and plasmids were thus transformed in *S. cerevisiae* BY4741, the model organism used for the biosensor. The strains *S. cerevisiae* BY4741 and *E. coli* XL1-Blue, harboring each of the four constructs, were stored at – 80 °C in glycerol stocks.

2. YEAST BIOSENSOR OPTIMIZATION

2.1. Test yeast reporter strains

2.1.1. Biosensor selection

After all clones were constructed and sequenced, several assays were performed in order to optimize and select the best biosensor. First, *S. cerevisiae* BY4741 cells harboring pRS426*ERG3*pryNluc, pRS426*ERG6*pryNluc, pRS426*ERG10*pryNluc, pRS426*ERG11*pryNluc, or pRS426*ERG25*pryNluc were exposed to 0 and 0.05 µg/ml of tebuconazole (Fig. 16), and bioluminescence was measured after 8 hours. The bioluminescence signal was significantly higher in cells exposed to TEB only in the strains harboring pRS426*ERG3*pryNluc, pRS426*ERG11*pryNluc, and pRS426*ERG25*pryNluc. For that reason,

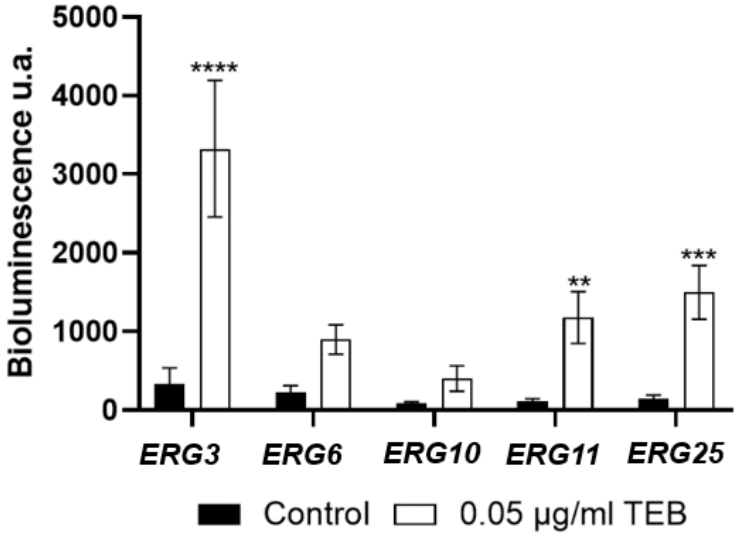

Figure 16. Biosensor selection. *S. cerevisiae* BY4741 wild-type cells harboring pRS426*ERG3*pryNluc, pRS426*ERG6*pryNluc, pRS426*ERG10*pryNluc, pRS426*ERG11*pryNluc or pRS426*ERG25*pryNluc were grown overnight and diluted to an OD_{640nm}=0.1, transferred to fresh medium and grown for an additional 3 h. Afterwards, cells were exposed to 0 and 0.05 µg/ml of TEB for 8 hours. 1 ml of culture ($OD_{640\ nm}$=0.5) were collected and resuspended in SC-Glu medium (pH=8) containing 6.25 mM PEG 3350. Bioluminescence was recorded for 15 min and the results represent bioluminescence at time 0. The negative control was assessed with DMSO, the solvent for TEB. Values were calculated based on mean ± SD of three independent experiments. **, *** and **** represent P ≤ 0.01, 0.001 and 0.0001, respectively.

pRS426*ERG6*pryNluc and pRS426*ERG10*pryNluc were not pursued in the following assays. Although the bioluminescence signal in pRS426*ERG11*pryNluc was significant, the other two strains showed better results. Between pRS426*ERG3*pryNluc and pRS426*ERG25*pryNluc, despite the higher bioluminescence values in pRS426*ERG3*pryNluc, pRS426*ERG25*pryNluc was more stable and with lower error values. Nonetheless, the strains harboring both vectors were used in the next phase.

2.1.2. Sensitivity of the biosensor

After selecting pRS426*ERG3*pryNluc and pRS426*ERG25*pryNluc vectors, a bioluminescent assay was performed to understand if, despite the good bioluminescence signals emitted by the strains harboring both vectors, both were able to detect even lower concentrations. For that purpose, an assay was performed exposing both strains to increasing concentrations of tebuconazole and the results are presented in Fig. 17. The biosensor containing the *ERG3* gene appeared to be the most promising, since it was the one with a higher bioluminescence signal emitted, but the same was not observed with lower concentrations. In fact, when treated with lower concentrations of TEB, the pRS426*ERG25*pryNluc biosensor could detect until 0.005 µg/ml of TEB, while pRS426*ERG3*pryNluc could only detect 0.05 µg/ml. In addition to a lower detection limit, the results obtained with pRS426*ERG25*pryNluc were more

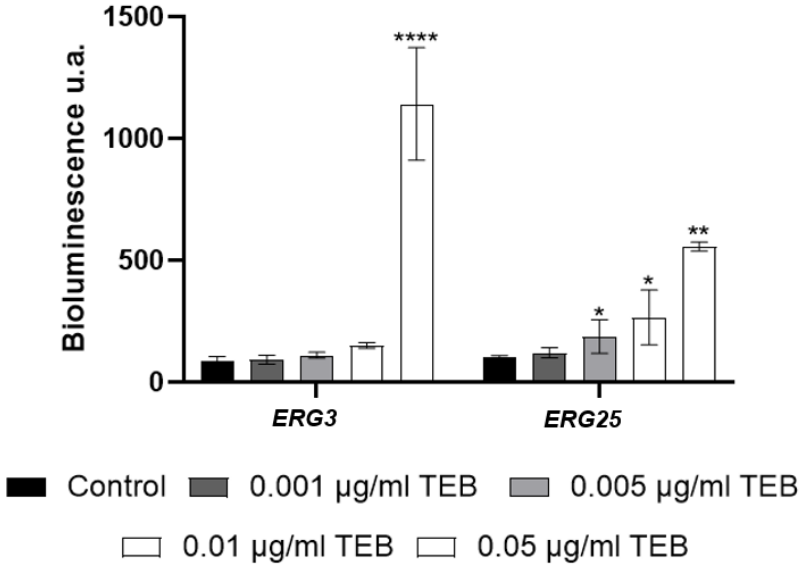

Figure 17. Sensitivity of the biosensor. *S. cerevisiae* BY4741 wild-type cells harboring pRS426*ERG3*pryNluc or pRS426*ERG25*pryNluc were grown overnight and diluted to an OD_{640nm}=0.1, transferred to fresh medium and grown for an additional 3 h. Afterwards, cells were exposed to 0, 0.001, 0.005, 0.01 and 0.05 µg/ml of TEB for 6 hours and 1 ml of culture ($OD_{640\ nm}$=0.5) were collected and resuspended in SC-Glu medium (pH=8) containing 6.25 mM PEG 3350. Bioluminescence was recorded for 15 min and the results represent bioluminescence at time 0. The negative control was assessed with DMSO, the solvent for TEB. Values were calculated based on mean ± SD of three independent experiments. *, ** and **** represent P ≤ 0.05, 0.01 and 0.0001, respectively.

stable and consistent. Since reproducibility is an important trait when developing a biosensor, only pRS426*ERG25*pryNluc was selected to perform the next assays.

2.1.3. Treatment time point selection

After one final biosensor was selected, pRS426*ERG25*pryNluc, a bioluminescent assay was performed after 2, 4, 6, 8, and 24 hours of treatment with 0.05 and 0.1 µg/ml tebuconazole, in order to select the time point at which the biosensor emitted a bioluminescence signal significantly different from control. The results are presented in Fig. 18. From the obtained results, at 6 hours of treatment, the biosensor was able to detect both 0.05 and 0.1 µg/ml of TEB. At 8 hours of treatment, although the bioluminescence was higher than at 6 hours of treatment, the errors were also higher. For a more stable and accurate biosensor, as well as a shorter assay procedure, the selected time point was 6 hours of treatment.

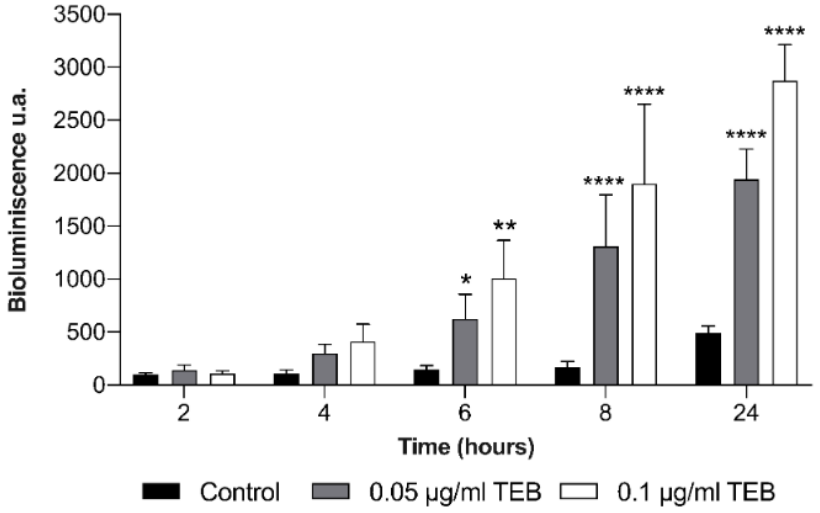

Figure 18. Time point treatment selection. *S. cerevisiae* BY4741 wild-type cells harboring pRS426*ERG25*pryNluc were grown overnight and diluted to an OD_{640nm}=0.1, transferred to fresh medium and grown for an additional 3 h. Afterwards, cells were exposed to 0, 0.05 and 0.1 µg/ml of TEB for 2, 4, 6, 8 and 24 hours and 1 ml of culture ($OD_{640\,nm}$=0.5) was collected and resuspended in SC-Glu medium (pH=8) containing 6.25 mM PEG 3350. Bioluminescence was recorded for 15 min and the results represent bioluminescence at time 0. The negative control was assessed with DMSO, the solvent for TEB. Values were calculated based on mean ± SD of three independent experiments. *, ** and **** represent P ≤ 0.05, 0.01 and 0.0001, respectively.

2.1.4. Specificity of the biosensor

Finally, a bioluminescent assay was performed to understand if the constructed biosensor was specific to detect tebuconazole, or if it could detect other azole compounds as well. 0.162 and 1.62 µM

correspond to 0.05 and 0.5 µg/ml of TEB, two concentrations that the biosensor could easily detect. The results are presented in Fig. 19, and it is possible to conclude that the biosensor can also detect higher concentrations of clotrimazole and myclobutanil (1.62 µM). However, when the biosensor is exposed to low concentrations of those compounds (0.162 µM), still higher than concentrations normally found in the environment, the biosensor is specific for tebuconazole. Therefore, when testing biological samples, it is expected that only tebuconazole will be detected, although other azoles may contribute to the signal.

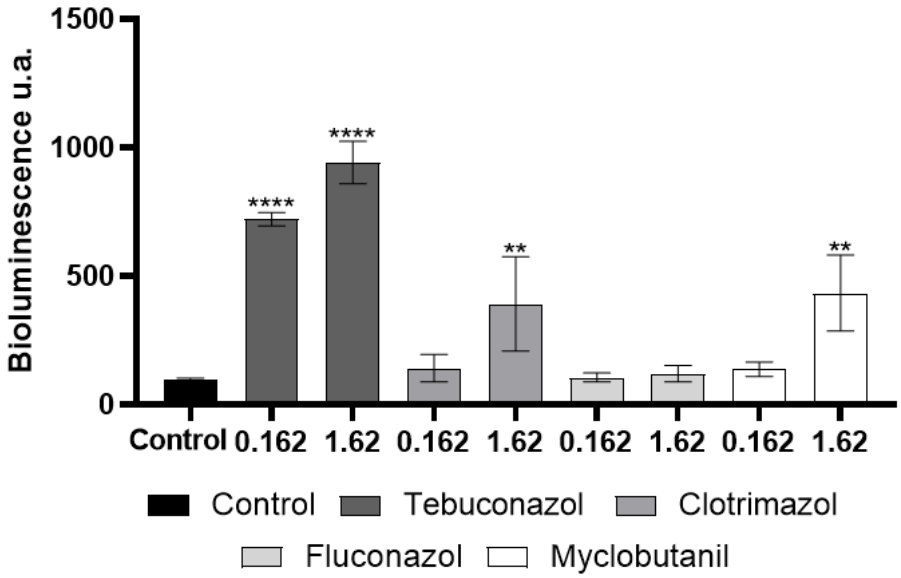

Figure 19. Specificity of the biosensor. *S. cerevisiae* BY4741 wild-type cells harboring pRS426*ERG25*pryNluc were grown overnight and diluted to an OD_{640nm}=0.1, transferred to fresh medium and grown for an additional 3 h. Afterwards, cells were exposed to 0, 0.162 and 1.62 µM of tebuconazole, clotrimazole, fluconazole and myclobutanil for 6 hours and 1 ml of culture ($OD_{640\ nm}$=0.5) were collected and resuspended in SC-Glu medium (pH=8) containing 6.25 mM PEG 3350. Bioluminescence was recorded for 15 min and the results represent bioluminescence at time 0. The negative control was assessed with DMSO, the solvent for TEB. Values were calculated based on mean ± SD of three independent experiments. ** and **** represent P ≤ 0.01 and 0.0001, respectively.

2.2. Sensitivity improvement

2.2.1. Mutant strains

After optimizations in the previous sections, regarding the promoter and assay conditions, several strategies were attempted to increase sensitivity. For that purpose, strains with mutations in lipidic droplets were tested. In order to maintain the balance of ergosterol levels within the yeast cell, ergosterol can either be stored in lipid droplets as a steryl ester (SE) or be secreted into the extracellular matrix as a sterol acetate (Hu et al., 2017). In the presence of an azole compound, ergosterol production is inhibited, but the cell still has basal ergosterol storage in the lipid droplets before the need to activate the *ERG* promoters to start the biosynthesis of ergosterol. Genes involved in the regulation of SE storage

include *TGL3* and *TGL4*, *ARE1*, and *ARE2*. *TGL3* and *TGL4* genes encode lipase proteins that have been found to have a subcellular localization in the lipid droplets (Kurat et al., 2006). *ARE1* and *ARE2* are the enzymes that catalyze the conversion of ergosterol to SE. A double mutant with *ARE1* and *ARE2* deleted has the biosynthesis of SE inhibited, with no effect on its growth (Hu et al., 2017). A Δ*are1*Δ*are2* double mutant was tested in preliminary assays, but the results were similar to wild-type (data not shown).

Δ*tgl3tgl4* and Δ*plg1* mutant strains harboring pRS426*ERG25*pryNluc were then tested. These mutant strains are unable to store ergosterol and require the production of ergosterol after exposure to azole compounds. For that reason, it was hypothesized that using these mutants would improve the sensitivity of the assay. Since the mutant strains were in a *S. cerevisiae* BY4742 strain background, controls included the wild-type strains BY4742 and BY4741, both harboring pRS426*ERG25*pryNluc. The results are presented in Fig. 20.

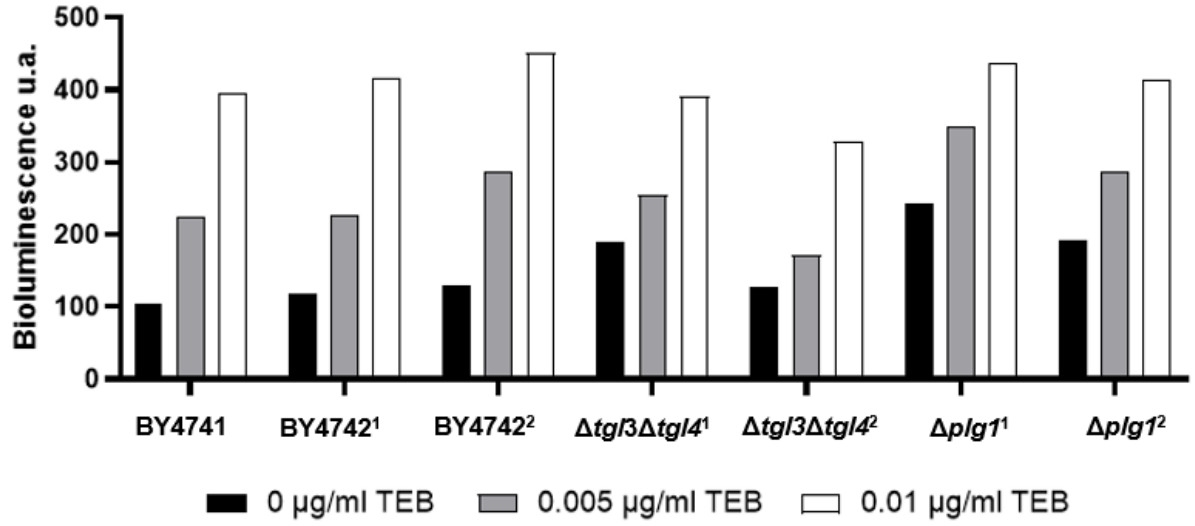

Figure 20. Sensitivity improvement using mutant strains. *S. cerevisiae* BY4742 Δ*tgl3*Δ*tgl4* and Δ*plg1* cells harboring pRS426*ERG25*pryNluc were grown overnight and diluted to an OD_{640nm}=0.1, transferred to fresh medium and grown for an additional 3h. Afterwards, cells were exposed to 0 and 0.005 and 0.01 µg/ml of TEB for 6 hours and 1 ml of culture (OD_{640nm}=0.5) were collected and resuspended in SC-Glu medium (pH=8) containing 6.25 mM PEG 3350. Bioluminescence was recorded for 15 min and the results represent bioluminescence at time 0. The negative control was assessed with DMSO, the solvent for TEB. (¹ and ² represent two different colonies from the same transformation).

Almost no difference was observed in the emitted bioluminescence signal between wild-type cells and the mutants. Furthermore, the negative control was often even higher, when compared with the previous wild-type BY4741 strain cells harboring pRS426*ERG25*pryNluc. For that reason, these mutants were not pursued.

2.2.2. Double-transformants

One other attempt was performed to improve the sensitivity of the biosensor, testing BY4741 cells harboring two vectors simultaneously. Since the biosensors with better outputs were pRS426*ERG25*pryNluc and pRS426*ERG3*pryNluc, they were each transformed with pRS425*ERG25*pryNluc, which has a different selection marker. As a control, pRS425*ERG25*pryNluc was simultaneously transformed with the empty vector (*ERG25*+Ø). The results are presented in Fig. 21, and it is possible to observe that the results of the strain harboring the *ERG25* cassette expressed from two vectors were very similar to the strain harboring only one. When the *ERG25* plasmid was combined with the *ERG3* plasmid, not only was the emitted bioluminescence lower at higher concentrations of TEB, but the negative control was significantly higher. For that reason, this strategy was not pursued further.

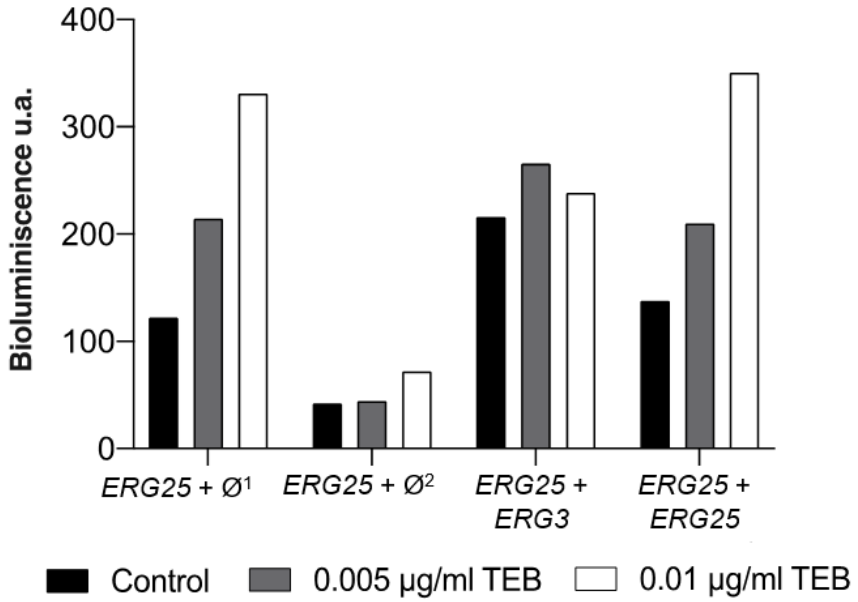

Figure 21. Sensitivity improvement using strains harboring two vectors simultaneously. *S. cerevisiae* BY4741 wild-type cells harboring pRS425*ERG25*pryNluc and pRS426pryNluc (*ERG25*+Ø), pRS425*ERG25*pryNluc and pRS426*ERG3*pryNluc (*ERG25*+*ERG3*), pRS425*ERG25*pryNluc and pRS426*ERG25*pryNluc (*ERG25*+*ERG25*) were grown overnight and diluted to an OD_{640nm}=0.1, transferred to fresh medium and grown for an additional 3h. Afterwards, cells were exposed to 0, 0.005 and 0.01 µg/ml of TEB for 6 hours and 1 ml of culture ($OD_{640\ nm}$=0.5) were collected and resuspended in SC-Glu medium (pH=8) containing 6.25 mM PEG 3350. Bioluminescence was recorded for 15 min and the results represent bioluminescence at time 0. The negative control was assessed with DMSO, the solvent for TEB. (^{1} and ^{2} represent two different colonies resultant from the same transformation).

3. YEAST STRAIN CONSTRUCTION USING GENOME INTEGRATION

Multi-copy expression vectors can promote unstable results since they rely on the number of copies present in cells (Baumann et al., 2018). Therefore, a final approach aimed to insert the

*ERG25*pryNluc cassete in the diploid *S. cerevisiae* BY4741 x BY4742 genome so that, in all assays, only one copy of the gene was being expressed, to promote higher stability and consistency of the biosensor.

3.1. Construction of the strain

The fragment that was inserted into the genome of *S. cerevisiae* BY47441 x BY4742 contained the *URA* gene as a selectable marker, the *ERG25* promoter (to activate the transcription in the presence of tebuconazole), the yNluc reporter gene (Fig. 22), and the *CYC1* terminator. This entire fragment was 3929 bp long and was too long for a single amplification using Phusion™ High-Fidelity DNA Polymerase. For that reason, two smaller fragments (1940 and 3077 bp) were amplified so that the fragments – containing a 1054 bp overlapping region – would overlap and the entire fragment would be inserted into the yeast genome. pRS426*ERG25*pryNluc digested with XbaI or with PvuII was used as a template, to reduce false positives stemming from uncut vector in the PCR reaction transformed in yeast. The primers used to amplify both fragments (18 and 19 for PvuII digested fragment and 17 and 20 for XbaI digested fragment, listed in Table 3), contained, in the 5'-end, a homologous region of 34 and 30 bp, respectively, with the *S. cerevisiae* BY4741 X BY4742 genome, in order to recombine in a specific locus.

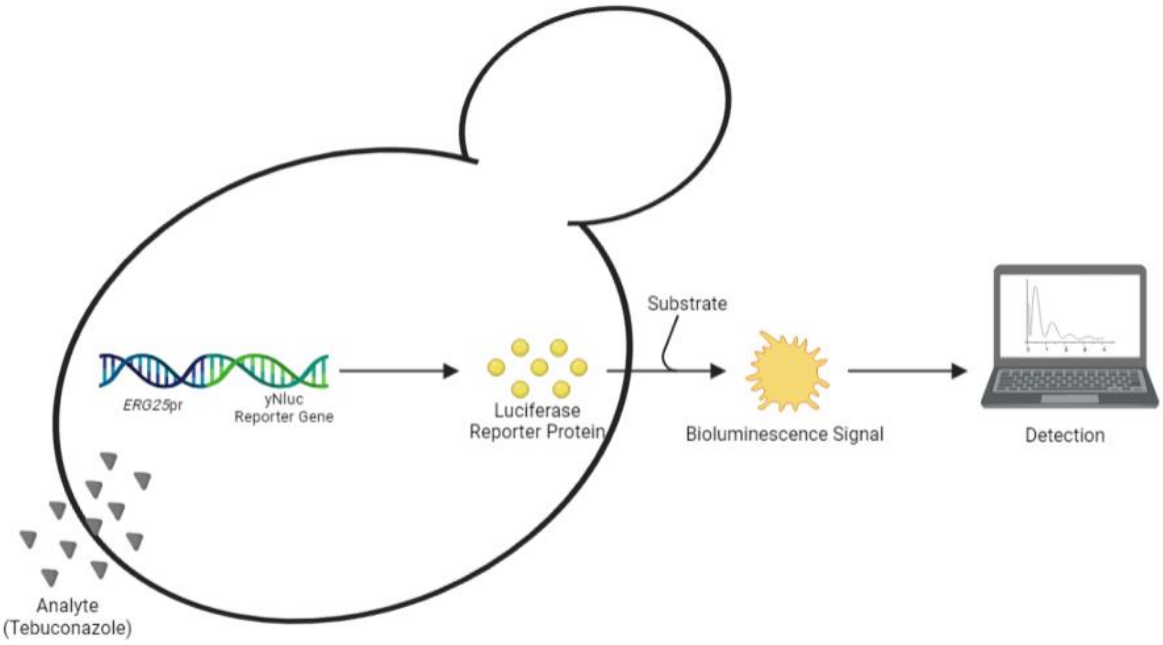

Figure 22. Yeast-based biosensor system integrated in the genome. Function of the developed yeast-based reporter strain biosensor. In the presence of an analyte (tebuconazole), *ERG25* promoter is activated, promoting the transcription of the luciferase reporter gene, and the consequent production of its protein. Luciferase protein, upon the addition of a substrate, produces a bioluminescence signal that can be detected by a fluorometer and further analyzed (created with BioRender).

3.2. Confirmation PCR

Two controls were performed in the transformation, a negative control without DNA and a control with the PCR mix but without Phusion polymerase (with the cut pRS426*ERG25*pryNluc template). The ratio of colonies obtained in the test transformation compared with the transformation without polymerase was 149:119, being too difficult to select positive clones. Since the BY4741 x BY4742 strain has the *URA* gene mutated, it is unable to produce uracil and grow in a medium lacking uracil. However, some false positives were observed since, in the PCR mix transformation control, some colonies were able to grow. In the presence of uracil, 5-FOA becomes toxic and loose fragments or vectors containing the *URA* gene are discarded from the strain so that it can grow. Therefore, only the true positive clones, with *URA3* inserted in the genome, would fail to grow in 5-FOA plates, since they cannot discard their own genome. From the 149 colonies obtained, 131 colonies were streaked on 5-FOA agar medium and on SC-glucose agar medium lacking uracil, in parallel. Eight colonies (numbers 19, 28, 40, 71, 73, 113, 115, and 120) did not grow on the 5-FOA plates, suggesting that they could be true positives. Those strains were then streaked on SC-glucose plates without uracil for 2 days at 30 °C. After two days, one single colony of each was incubated overnight in SC-glucose liquid medium without uracil, diluted to fresh medium and their gDNA was extracted in order to perform a confirmation PCR. Two sets of primers (22/23 and 21/24, listed in Table 3) were used for confirmation, to test whether homologous recombination occurred in the genome and if the overlap was successful. In the first set of primers, primer 22 hybridizes with the yeast genome, while primer 23 hybridizes with the cloned fragment in the *CYC1* terminator sequence. In the second set of primers, primer 24 hybridizes with the yeast genome while primer 21 hybridizes with the cloned fragment in the *URA* gene sequence.

The results are presented in Fig. 23. From the agarose gel electrophoresis results, it is possible to infer that only clone 19 was a positive clone since it was the only one in which the fragment was amplified. Clone 28 could be a possible positive clone since the target fragment was amplified in one of the confirmation PCRs. However, in clone 28, homologous recombination could have occurred only with one of the fragments, without the overlapping and homologous recombination with the other fragment. Nevertheless, both clones were used to perform the following assays.

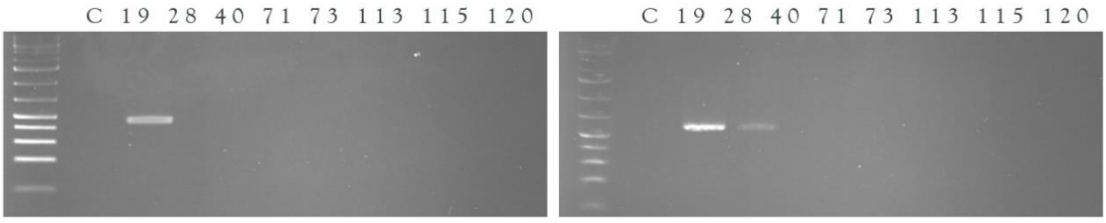

Figure 23. Confirmation PCR agarose gel electrophoresis. From the selected colonies, a confirmation PCR was performed, to check for colonies that would contain the desired fragment (*URA* selectable marker, *ERG* promoter gene, yNluc reporter gene and *CYC1* terminator) in their genome. From all the eight possible positive clones that did not grow on 5-FOA agar plates, only clone 19 was positive using the two pairs of confirmation primers. Clone 28 was only positive using one of the two pairs of confirmation primers, suggesting that the entire cassette may not have been correctly inserted.

3.3. Bioluminescence measurements

To confirm if the clones were indeed positive, a bioluminescence assay was performed in order to determine if the system was working properly, and the constructed strains could detect tebuconazole. The assays were also performed using *S. cerevisiae* BY4741 harboring pRS426*ERG25*pryNluc as a positive control. A preliminary assay was performed where both clones 19 and 28 were tested, however, the bioluminescence detected with clone 28 was very low when compared with the negative control (data

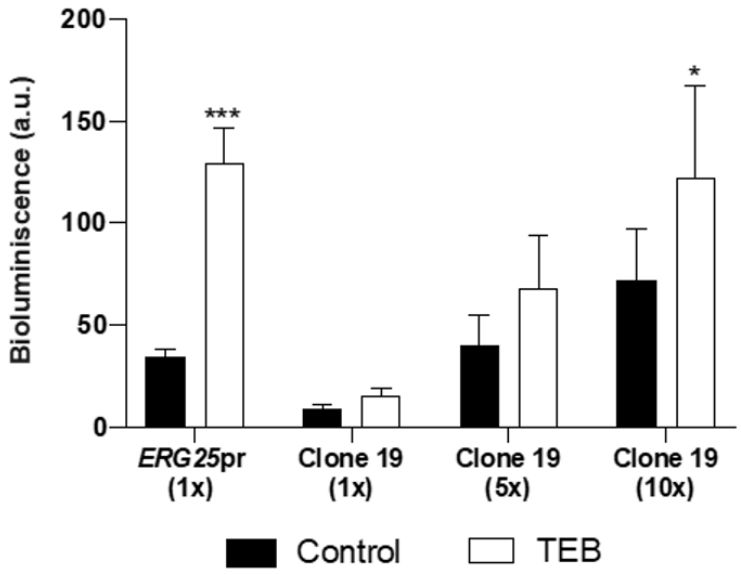

Figure 24. Bioluminescence assay performed with the strain constructed using genome integration. *S. cerevisiae* BY4741 cells harboring pRS426*ERG25*pryNluc and clone 19 cells were grown overnight and diluted to an OD_{640nm}=0.1, transferred to fresh medium and grown for an additional 3h. Afterwards, cells were exposed to 0 and 0.05 µg/ml of TEB for 6 hours and 1 ml of culture (OD_{640nm}=0.5, 2.5 and 5.0) were collected and resuspended in SC-Glu medium (pH=8) containing 6.25 mM PEG 3350. Bioluminescence was recorded for 15 min and the results represent bioluminescence at time 0. The negative control was assessed with DMSO in which TEB was diluted. Values were calculated based on mean ± SD of three independent experiments. * and *** represent P ≤ 0.05 and 0.001, respectively.

not shown). So, three additional independent experiments were performed, where only clone 19 was considered, and the results are presented in Fig. 24.

From the obtained results, it is possible to observe that in the same conditions as the performed assays previously (1x), that is, with the same concentration of cells used, clone 19 could not detect 0.05 µg /ml of tebuconazole. The signal was only significant when 10 times more cells ($OD_{640\,nm}$=5.0) was used, i.e, 10 times higher than the strain harboring the vector pRS426*ERG25*pryNluc. This was not unexpected, considering that, in a vector, multiple copies are being activated and promoting a higher production of luciferase, while in the genome only one copy is activated and so the luciferase protein concentration is significantly lower. It was for that reason that cell concentration was increased, but along with the increased bioluminescence signal, the negative control (assessed with DMSO instead of TEB) also increased. Thus, and going against results obtained in other studies (Baumann et al., 2018), a multi-copy system showed to be necessary in order to obtain a higher sensitivity of the biosensor.

In conclusion, the final selected biosensor was the *S. cerevisiae* BY4741 wild-type strain harboring pRS426*ERG25*pryNluc, with 6 h of treatment, that could detect 0.005 µg/ml of TEB.

CONCLUSION & FUTURE PERSPECTIVES

Fungicides (and pesticides in general) have been associated with serious problems, such as human health issues, as well as interference with soil natural fertilization and with a number of aquatic species. Despite the proven benefits of fungicides for society, it is necessary to control the amount that is used in agriculture. Tebuconazole, an azole fungicide commonly used in agriculture, inhibits the production of ergosterol, and thus decreases ergosterol content (Amaral, 2014; S. Li et al., 2019; Makvandi et al., 2021; József Popp et al., 2013). This results in *ERG* promoter genes being activated and thus this study aimed to construct a biosensor based on that premise. Five different biosensors were constructed, differing in the promoter inserted. *ERG3, ERG6, ERG10,* or *ERG11* promoters were cloned upstream of the yNluc reporter gene, so that, along with the activation of the *ERG* promoter, yNluc would be transcribed and translated, being further detected using a fluorometer (a plasmid with the *ERG25* promoter was available). From all the biosensors, pRS426*ERG25*pryNluc proved to be the best, with high and stable signals, capable of detecting low concentrations of tebuconazole, from 5 ng/ml. Several attempts were made to try to improve the biosensor, including using lipidic droplet mutants and double transformations. Neither of these promoted a higher bioluminescence signal, and thus no further assays were performed using those strategies. One last attempt was made, by inserting the reporter cassette in the *S. cerevisiae* BY4741 x BY4742 genome. Having only one gene copy, the results should be more stable and consistent, but the signal was significantly lower, and the negative control was higher, so no further assays were performed. pRS426*ERG25*pryNluc was therefore the final selected yeast-based biosensor.

The procedure optimized in this study was sufficient for the biosensor to detect tebuconazole in concentrations of 5 ng/ml or higher, with an exposure of 6 hours. TEB is frequently detected in water samples from 0.001 to 0.03 ng/ml, being sometimes found in 0.175 to 0.2 ng/ml (Kahle et al., 2008). A set of biosensor mechanisms to detect the presence of pesticides are described in the paragraph below, and listed in Table 4, for comparison.

Due to poor practices by beekeepers, if flowers contain pesticide residues, these residues can be transposed to the hives, thus contaminating the honey. A study has shown that 75 % of collected honey samples were contaminated with at least one pesticide. A rapid MS technique was used to detect atrazine, benalaxyl, and pirimicarb (three of the reported pesticides in honey) and the LODs were, respectively, 0.6, 1, and 0.5 ng/ml (Choi et al., 2020). Carbofuran is a pesticide of broad-spectrum and high solubility in water, contaminating water, thus potentially causing an environmental hazard. The most common techniques for its detection are the conventional ones, which as previously referred are complex and expensive. Since carbofuran can inhibit esterase activity, an enzymatic biosensor was developed to detect

its presence, with immobilized esterase, by measuring its activity (an increase in the pesticide concentration is proportional to a decrease in the enzyme activity). This biosensor could detect concentrations above 1.69 ng/ml, being the maximum residue limits (MRL) of carbofuran concentration allowed in water samples, 7.0 ng/ml (Grawe et al., 2015). Another biosensor showed a LOD of 0.02 nM to 20000 nM (0.007 to 7012 ng/ml), using chitosan and AChE, in a low-cost assay, with a 10-minute incubation period. In this biosensor, similar outputs were observed when detecting pesticides in a controlled laboratory experiment and environmental samples (X. Liu et al., 2020). A biosensor used to detect captan, a fungicide agent, in fruit samples, was developed relying on the inhibition of AchE activity by captan, showing a decrease of the enzyme activity upon an increase of the pesticide concentration. It showed a LOD of 107 nM (32.16 ng/ml) after 15 minutes of incubation (Nesakumar et al., 2015). Carbendazim (CDM) is a fungicide applied in agriculture, being the MRL in citrus fruits in EU of 100 – 700 ppb (100 – 700 ng/ml), while it was completely banned in Australia and the USA. An electrochemical biosensor detected carbendazim fungicide in concentrations above 3.0 nM (0.57 ng/ml) (Kokulnathan & Chen, 2020). A paper published in 2021 reviewed the current detection methods of pesticide detection, namely dithiocarbamate fungicides (DTFs), their advantages and limitations. They report that although chromatographic-based methods are highly sensitive, having very low detection limits, even below the MRL (10 – 25000 ng/ml, although higher levels have been found in some fruits), the equipment costs and complexity of the processes are a huge limitation. Biosensors seem to be a good alternative, with low incubation times (examples of 2.5 – 20 min of total incubation) (Fanjul-Bolado et al., 2021). Each detection technique must adapt its LOD to the MRL of the specific pesticide that it proposes to detect. To increase the LOD of the biosensor to detect TEB, several approaches could be pursued.

Table 4. Limits of detection (LOD) of reporter biosensors for pesticide detection

Pesticide	Technique LOD	Source
Tebuconazole	5 ng/ml	Present study
Atrazine, benalaxyl, and pirimicarb	0.6, 1, and 0.5 ng/ml	Choi et al., 2020
Carbofuran	1.69 ng/ml	Grawe et al., 2015
	0.007 to 7012 ng/ml	X. Liu et al., 2020
Captan	32.16 ng/ml	Nesakumar et al., 2015
Carbendazim	0.57 ng/ml	Kokulnathan & Chen, 2020

One alternative could be to insert a multi-copy system in the genome of *S. cerevisiae*. For this purpose, the ribosomal DNA (rDNA) cluster could be selected as an integration site. rDNA is a naturally repetitive sequence present in the genome of *S. cerevisiae* and, due to its high number of repeats, has been targeted for multi-copy editing. The rDNA repeated sequence is represented in Fig. 25a. As NTS1 and NTS2 are non-transcribed sequences, they can be used as insertion sites. The proposed system uses the CRISPR/Cas9 machinery to precisely edit the genome. The guide RNA (gRNA) guides the Cas9 endonuclease to the target site, where it recognizes the PAM sequence. Upon matching with the complementary region, Cas9 unwinds the target DNA and cleaves on both strands, creating a double-stranded DNA break (DSB). This break can be repaired with non-homologous end joining (NHEJ) or homologous recombination, the latter promoting precise editing (L. Wang et al., 2018). Cas9 can be cloned in an inducible vector and transformed with the vector harboring the gRNA cassette targeting the rDNA repeats and the DNA editing template with 1 KB up- and downstream homologous arms (UHA and DHA) to the target site, NTS1. Upon induction of Cas9 expression, the gRNA guides the endonuclease to NTS1, containing the PAM sequence followed by 20 nucleotides (Fig. 25b), where it produces a DSB. This break can be repaired by homologous recombination, with the donor DNA editing cassette (in this case it would be the *ERG25* promoter, the yNluc reporter gene, and the *CYC1* terminator, in between the UHA and DHA). After the integration is complete, the expression of Cas9 would be suppressed. Hopefully,

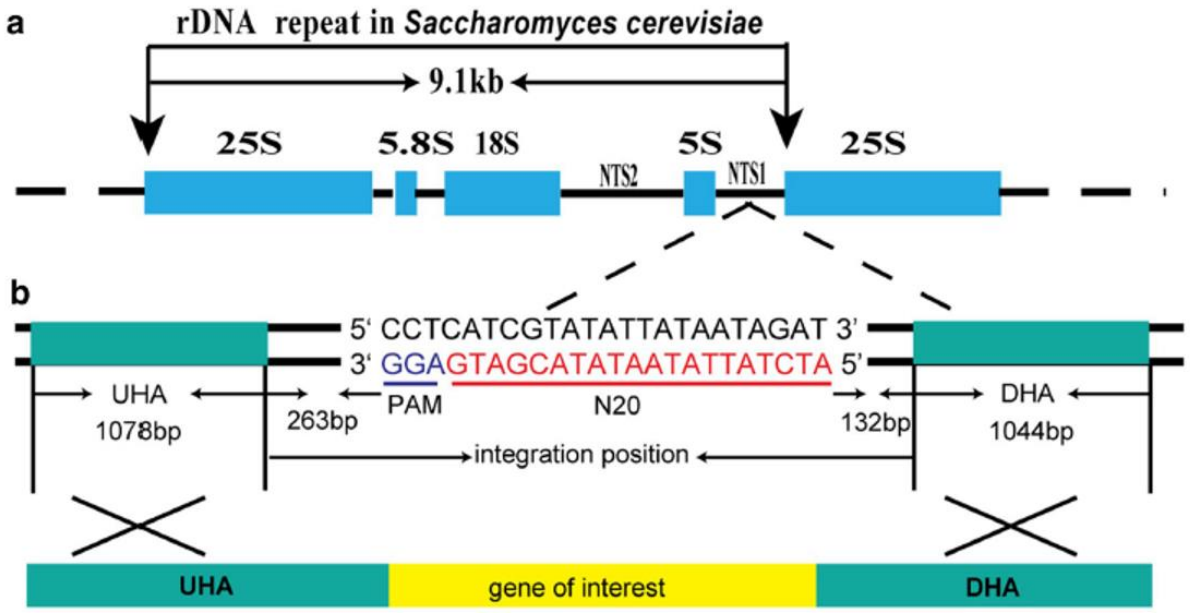

Figure 25. (a) rDNA repeats present in *S. cerevisiae*: 25S, 5,8S, 18S, 5S and 25S rDNA sequences, and two non-transcribed sequences, NTS1 and NTS2; **(b) NTS1 sequence as insertion site:** NTS1 sequence, containing approximately 1 KB of an overlapping region before and after the integration position (UHA and DHA), with the PAM sequence followed by 20 nucleotides, to be further replaced by the gene/sequence of interest (L. Wang et al., 2018).

this approach would promote a high increase in the bioluminescence signal, improving the sensitivity of the biosensor, while maintaining its stability.

A study in 2017 in *Candida parapsilosis* showed that it was not only *ERG* genes being up-regulated in the presence of azole compounds, but the gene encoding the transcription factor Upc2 was also up-regulated. When there is a loss of the sterol desaturase activity, there is a depletion of the ergosterol in the membrane, which leads to activation of Upc2. This transcription factor is not directly involved in the biosynthetic pathway of ergosterol, but it is activated upon a decrease in its content in the membrane (Rybak et al., 2017). Another study conducted in 2018 in *Candida glabrata* showed that, in the presence of an azole, again not only *ERG* genes were overexpressed, but also two transporter genes, *AUS1* and *TIR3,* and sterol metabolism regulators *SUT1* and *UPC2* in fungal cells. Aus1 is responsible for the external import of ergosterol and cholesterol into the cell (Q. Q. Li et al., 2018). It would be interesting to test these genes as an alternative to the *ERG* promoters used in this study, to see if they are more induced in the presence of TEB than *ERG25*, and to try to improve the sensitivity of the biosensor.

In addition to these known genes, microarrays could detect other genes induced in the presence of tebuconazole. Microarrays are commonly used to analyze hundreds of genes at the same time, in a single experiment, possibly analyzing the whole transcripts of an organism. It consists of a two-dimensional DNA chip (microscope slide) with thousands of spots, each one with a control DNA labeled sequence or gene (probe) immobilized on a solid surface, with a certain known pattern. After the addition of the sample DNA, it hybridizes with the complementary probe. This hybridization can be detected by fluorescence since the sample and the control DNAs are labeled with different colors. If the sample DNA is labeled with a red dye and the control DNA is labeled with a green dye, only if the genes are expressed in the sample DNA, the sample and the control DNAs will hybridize, and the spot will appear yellow, a mixture of the two colors. In this case, each spot would contain one of the approximately 6000 *S. cerevisiae* genes, labeled in green. After treating *S. cerevisiae* with different concentrations of tebuconazole, RNA would be extracted and used as a template for cDNA synthesis. The cDNA would be labeled in red and added to the DNA chips. Only upon gene expression, hybridization with the control will occur, the DNA will remain attached in the spot and the spot will have a yellow fluorescence light (Bilitewski, 2009). The gene with the higher overexpression level in the presence of tebuconazole would be selected to clone in the presented system. This microarray system has already been performed, with the simultaneous analysis of the expression of 5935 genes in *S. cerevisiae*, after treatment with amphotericin B and nystatin (L. Zhang et al., 2002).

Recently, aptamers have garnered interest as recognition elements in biosensors (aptasensors), to detect small molecules. Aptamers are single-stranded oligonucleotides (DNA or RNA) or peptide molecules, with 15 to 100 nucleotides, able to recognize and bind, with high affinity and specificity, to several ligands/targets, due to their three-dimensional structure. These ligands include proteins, viruses, bacteria, and amino acids. Aptamers are known as the "antibody substitute" since antibodies have been used as recognition molecules. In comparison, many are the advantages of aptamers. For instance, they are highly stable: antibodies, as proteins, are denatured at higher temperatures and can lose the tertiary structure, while aptamers, as oligonucleotides, are thermally stable and can maintain the structures at denaturation and renaturation cycles. Also, the process of aptamer production is more cost-effective, since the processes to identify and produce antibodies are laborious and expensive, and an activity confirmation assay is needed for each batch, while aptamers are synthesized in high quantities through chemical reactions, with long-term storage. Finally, they are more versatile, since there is a wider spectrum of targets to which they can bind, showing affinity and specificity to targets, that antibodies cannot recognize. Aptamers have already been used in many applications, including diagnostic and drug discovery and delivery (Hassani et al., 2017; Pérez-Fernández et al., 2020; Phopin & Tantimongcolwat, 2020; Song et al., 2012; Uniyal & Sharma, 2018). Aptamers can be isolated through the systematic evolution of ligands by exponential enrichment (SELEX), with a high affinity for a certain ligand. It consists of 10 to 15 rounds of three steps: library generation, binding and separation, and amplification. First, an initial library is broken down into random single-stranded nucleotides that will or not bind to a given target. The fragments that do not bind to the target are washed and those that do are eluted to be further amplified by PCR, originating a new library that will be used in the following round. Aptasensors can be used to detect several molecules through several mechanisms. For instance, for a fluorescence aptasensor, the aptamers are labeled with a fluorophore and a quencher that will change conformation only upon the presence and binding of the target, joining the fluorophore to the quencher, leading to a decrease in the signal (Song et al., 2012). An alternative to this study would be to test the efficacy of an aptasensor with a high affinity for tebuconazole and compare it with the biosensor developed in the present study.

Colorimetric biosensors are designed to detect the presence of an analyte through a color change, in a more economical manner, since there is no specialized equipment required. The system constructed in this work could be transposed into a colorimetric biosensor. For example, the *ERG25* promoter could be cloned upstream the β-galactosidase encoding gene, controlling its expression. Upon the presence of tebuconazole, just like in the present study, the promoter would be activated, and the transcription of the

reporter gene would consequently be activated, promoting the expression of β-galactosidase. Ortho-nitrophenyl-β-D-galactopyranoside, or ONPG, is a colorless substrate that, in the presence of β-galactosidase, is hydrolyzed into galactose and ortho-nitrophenol, the latter having a yellow color, as represented in Fig. 26 (Labus, 2018)., β-galactosidase would be expressed only in the presence of TEB, promoting a color change on the medium upon the addition of the ONPG substrate. However, this system relies on the change of color of the compound, having low sensitivity and being only qualitative.

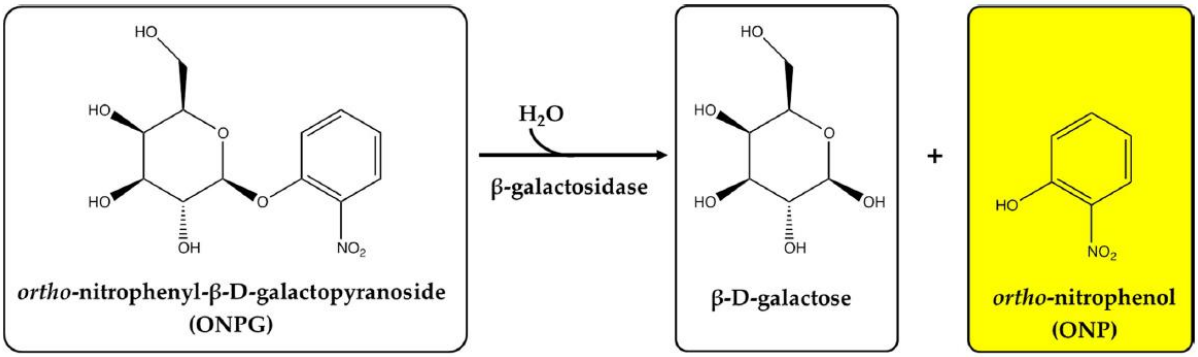

Figure 26. Schematic representation of β-galactosidase reaction using ONPG substrate. In the presence of β-galactosidase, ONPG substrate is hydrolyzed into galactose and ortho-nitrophenol (ONP). ONP has a yellow color, that allows for the monitorization of enzymatic reactions, since the color only changes into yellow in the presence of β-galactosidase (Labus, 2018).

In the future, it is important to test this biosensor in environmental samples, to see if the results obtained in the lab can also be achieved in biological samples. The environmental samples should derive from agriculture fields in which fungicides are used, having as negative control samples from fields that do not use fungicides, and a positive control with non-environmental samples, with the addition of 5 ng/ml of tebuconazole.

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APPENDIXES

APPENDIX A. PCR PRODUCT CLEAN UP FROM GRISP KIT

Transfer up to 100 µl of the PCR reaction solution to a 1.5 ml microcentrifuge tube. Add 5 volumes of Gel Solubilization Solution and mix by vortexing. Place the DNA fragment mini spin column in a 2.0 ml collection tube and transfer the sample mixture to the column. Centrifuge at 14000 to 16000 g for 30 seconds. Discard the collection tube containing the flow-through and place the spin column back in the collection tube. Add 600 µl of Wash Buffer 2 and let stand for 1 minute. Centrifuge at 14000 to 16000 g for 30 seconds and discard the flow-through. Place the column back in the collection tube and centrifuge 14000 to 16000 g for another 3 minutes to dry the matrix of the column. Transfer the spin column to a new 1.5 ml microcentrifuge tube and pipette 20 – 50 µl of Elution Buffer directly to the center of the spin column without touching the membrane. Incubate at room temperature for 2 minutes. Centrifuge for 2 minutes at 14000 to 16000 g to elute purified DNA. Discard the spin column and use DNA immediately or store at -20 °C.

APPENDIX B. MINIPREP PROTOCOL FROM SIGMA-ALDRICH KIT

Harvest & lyse bacteria: pellet cells from 1 to 5 ml overnight culture 1 minute and discard the supernatant; resuspend cells in 200 µl of Resuspension Solution and vortex or pipette up and down; add 200 µl of Lysis Solution, invert gently to mix (do not vortex) and allow to clear for 5 minutes. **Prepare cleared lysate:** add 350 µl of Neutralization Solution and invert 4 to 6 times to mix; pellet debris 10 minutes at maximum speed. **Prepare binding column:** add 500 µl of Column Preparation Solution to the binding column in a collection tube; spin at ≥ 12000 xg for 1 minute and discard the flow-through. **Bind plasmid DNA to column:** transfer cleared lysate into the binding column; spin* for 30 seconds to 1 minute and discard the flow-through. **Wash to remove contaminants:** add 750 µl of Wash Solution to the column; spin* 30 seconds to 1 minute and discard the flow-through; spin* for 1 minute to dry the column. **Elute purified plasmid DNA:** transfer the column to a new collection tube; add 100 µl of Elution Solution and spin* for 1 minute (if a more concentrated plasmid DNA is required, reduce the elution volume to a minimum of 50 µl).

*All spins at 12000 xg, except as noted.

APPENDIX C. COMPETENT CELL PROTOCOL

1 single colony of *E. coli* XL1-Blue was grown overnight in 30 ml of Luria-Bertani broth (LB) medium [0.5 % yeast extract, 1 % tryptone, 1 % NaCl]. The day after, 400 ml of the overnight culture was inoculated in 10 ml of fresh LB medium and incubated at 37 °C at orbital shaking of 180 rpm until OD_{600nm}=0.3. From the previous culture, 4 ml were inoculated in 100 ml of fresh LB medium and incubated at 37 °C at orbital shaking of 180 rpm until OD_{600nm}=0.3. The culture was incubated on ice for 5 minutes and centrifuged at 2500 rpm (4 °C) for 5 minutes. The supernatant was discarded, and the pellet was gently resuspended in 20 ml of cold TFBI [30 mM KOAc, 50 mM MnCl2, 100 mM RbCl, 10 mM CaCl2, 15 % Glycerol] and once again centrifuged. The supernatant was discarded, and the pellet was gently resuspended in 2.5 ml of cold TFBII [10 mM NaMOPS, pH 7.0, 75 mM CaCl2, 10 mM RbC1, 15 % Glycerol] and incubated on ice for 5 minutes. The cells were divided into 200 µl aliquots, frozen in liquid nitrogen, and stored at -80 °C.

APPENDIX D. PLASMID DNA QUANTIFICATON (NANODROP)

Host	Promoter	DNA (ng/µl)	A260/A280	A260/A230
S. cerevisiae[1]	*ERG3*	35.60	1.98	2.40
	ERG6	34.90	1.93	2.66
	ERG10	28.40	1.81	1.40
	ERG11	43.90	2.04	2.74
E. coli	*ERG3*	293.70	1.83	2.46
	ERG6	379.30	1.84	2.60
	ERG10	281.00	1.87	2.55
	ERG11	225.60	1.88	2.79

[1]Plasmids extracted from *S. cerevisiae* BY4741 before transformation in *E. coli* XL1-Blue.