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Mestrado em Bioquímica Aplicada

Trabalho efetuado sob a orientação da
Doutora Ana Preto e da Doutora Susana Chaves

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Declaração

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Abstract

The improvement of pesticides and its increased use was highly influenced by the growth of the world population in the last centuries. The use of pesticides improved agri-food production by decreasing the loss of crops caused by many pests and diseases. The production of wine is important for the Portuguese culture and economy and has increased in the last decades. Since many grapevine diseases are caused by fungi, various fungicides like tebuconazole and cymoxanil are used to control such diseases. Fungicides often represent a higher risk for mammals, namely humans. Despite their broad use, the possible impact of both fungicides on human health and in the ecosystem is poorly understood. Since ingestion represents one of the most important ways of human exposure to surrounding chemicals, the digestive tract is one of the most affected. The incidence of different pathologies in the colon has increased in the last years namely colorectal cancer. In this work, we aimed to explore the effect that two of the most commonly used fungicides (tebuconazole and cymoxanil) may have in the human colon, investigating the possible effects related to acute and chronic exposure. For this purpose, we used normal human colon-derived cells to assess cytotoxic concentrations of the fungicides in the human colon, and to uncover their effects and mode of action. We also used yeast *S. cerevisiae* reporter strains to assess the genotoxic potential of the two fungicides as a way to evaluate possible long-term effects resulting from chronic exposure.

Our results indicate that cytotoxic concentrations of both fungicides induce colon cell death, which is associated with increased production of ROS. Concerning the genotoxic potential, we found that tebuconazole increased mutation rates in yeast. Our data indicate that there is no risk in terms of acute toxicity of fungicides for food consumers when concentrations are below legal limits. Our results suggest that tebuconazole might have chronic effects even at concentrations within the legal limits for food, however more studies are required to confirm this.

Resumo- alterar o português de acordo

O desenvolvimento de pesticidas e o seu crescente uso foi altamente influenciado pelo crescimento da população mundial nos últimos séculos. O uso de pesticidas melhorou a produção de produtos agroalimentares diminuindo a perda de colheitas causadas por pestes e doenças. A produção de vinho é importante para a cultura e economia Portuguesa, e tem vindo a crescer nas últimas décadas. Uma vez que muitas doenças das vinhas são causadas por fungos, vários fungicidas, tais como o tebuconazol e o cymoxanil, são usados para controlar essas doenças. Os fungicidas representam um risco maior para mamíferos, e particularmente para a saúde humana. Apesar do seu uso extensivo, o possível impacto de ambos os fungicidas para a saúde humana e para o ecossistema não está bem estudado. Sendo a ingestão uma das principais formas de exposição dos humanos às substâncias químicas que o rodeiam, o trato digestivo é um dos mais afetados. A incidência de diferentes patologias no cólon tem vindo a aumentar nos últimos anos, nomeadamente o cancro colorretal. Neste trabalho, pretendemos explorar os efeitos que dois dos pesticidas mais usados (tebuconazol e cimoxanil) possam causar no cólon humano, investigando os possíveis efeitos relacionados com exposição aguda e crónica. Para este fim, usamos células normais do cólon para avaliar as concentrações citotóxicas dos fungicidas no cólon e explorar os seus modos de ação. Também usamos uma estirpe repórter de levedura *S. cerevisiae* para avaliar o potencial genotóxico dos dois fungicidas como forma de avaliar possíveis efeitos a longo prazo como resultado de uma exposição crónica.

Os nossos resultados indicam que em concentrações citotóxicas, ambos os fungicidas induzem morte celular nas células do cólon, e que está associado a um aumento da produção de ROS. Acerca do potencial genotóxico, descobrimos que o tebuconazole potencialmente aumenta a taxa de mutações em leveduras. Os nossos dados sugerem que não há risco em termos de toxicidade aguda para consumidores quando os fungicidas estão presentes na comida a concentrações abaixo dos limites legais. Os nossos resultados sugerem que o tebuconazole poderá ter efeitos crónicos mesmo dentro do limite de concentrações legais para os alimentos, no entanto são necessários mais estudos para o confirmar.

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List of abbreviations and acronyms

Apaf-1	Apoptotic protease activating factor 1
APO1	Apoptosis antigen 1
APP	Acute pesticide poisoning
ATP	Adenosine triphosphate
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous killer
Bax	Bcl-2-associated X protein
BCFA	Branched chain fatty acid
Bcl-2	B-cell lymphoma 2
Bid	BH3 interacting-domain death agonist
CAT	Catalase
CD95	Cluster of differentiation 95
CFSE	Carboxyfluorescein succinimidyl ester
CRC	Colorectal cancer
CSM	Complete supplement mixture
CYP	Citocromo P450
CYP17	Steroid 17α-monooxygenase
CYP19	Aromatase
CYP1A1	Cytochrome P450 1A1
CYP3A4	Cytochrome P450 3A4

Cyp-51	Lanosterol 14α-demethylase
DAPI	4',6-diamidino-2-phenylindole
DCF	2', 7'-dichlorofluorescein
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
dUTP	Deoxyuridine triphosphate
EDTA	Ethylendiamine tetraacetic acid
EFSA	European Food Safety Authority
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GPX	Glutathione peroxidase
GSH	Glutathione
H_2DCFDA	2', 7'-dichlorodihydrofluorescein diacetate
IC_{50}	Half-maximal inhibitory concentration
LC_{50}	Median lethal dose
LCB1	Long chain base biosynthesis protein 1
LC-MS	Liquid chromatography–mass spectrometry
MIC	Minimum inhibitory concentration
MLH1	MutL homolog 1

mRNA	Messenger RNA
NADH	Reduced form of nicotinamide adenine dinucleotide
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodine
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SCFA	Short chain fatty acid
SD	Standard deviation
SOD	Superoxide dismutase
SRB	Sulphorhodamine B
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal transferase dUTP nick end labeling
URA1	Orotidine-5'-monophosphate decarboxylase
WHO	World Health Oganization
YPD	Yeast extract peptone dextrose
YPDA	Yeast extract peptone dextrose agarose

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1. Introduction

This project is under the scope of EcoAgriFood, a project that aims to develop and apply innovative "eco-friendly" products and processes in the northern region of Portugal, with the final goal of developing the agriculture and food bio-economy in that region. Understanding the behavior and interactions of living systems and their responses to global change is one of the priorities of the project. EcoAgriFood promotes research in the biological sciences, seeking to understand the impact of agriculture and the food industry on biodiversity and human health.

This thesis focused on the study of two fungicides currently on the market and frequently used in some crops in Portugal, namely vineyards, and their impact on human health mainly through exposure by ingestion. The main objective of this work was to evaluate the potential impacts of pesticides for the human colon, in terms of acute or chronic exposure.

1.1. Pesticides: risks and benefits on the agricultural production

The world population is increasing every year. From the 1 billion people registered in 1804, the world population surpassed the 7 billion mark in this century and the 9 billion mark is expected to be surpassed by 2050 (**Figure 1**). The populational increase resulted in an increase in food production by around 70% (Popp, Pet and Nagy, 2013). This was only possible due to an increase in the use of fertilizers, pesticides, new crop strains and the development of new technologies. However, humanity needs to keep improving in the future, because about half of the usable land of the planet is already being used for pastoral or intensive agricultural proposes. Furthermore, it is a challenge to keep improving food production with minimal environmental impact, not only avoiding the use of more land for food production, but also minimizing the contamination of soils and fresh water, using less water and restricting the use of fertilizers and pesticides (Tilman *et al.*, 2002; Zimmer and Renault, 2003).

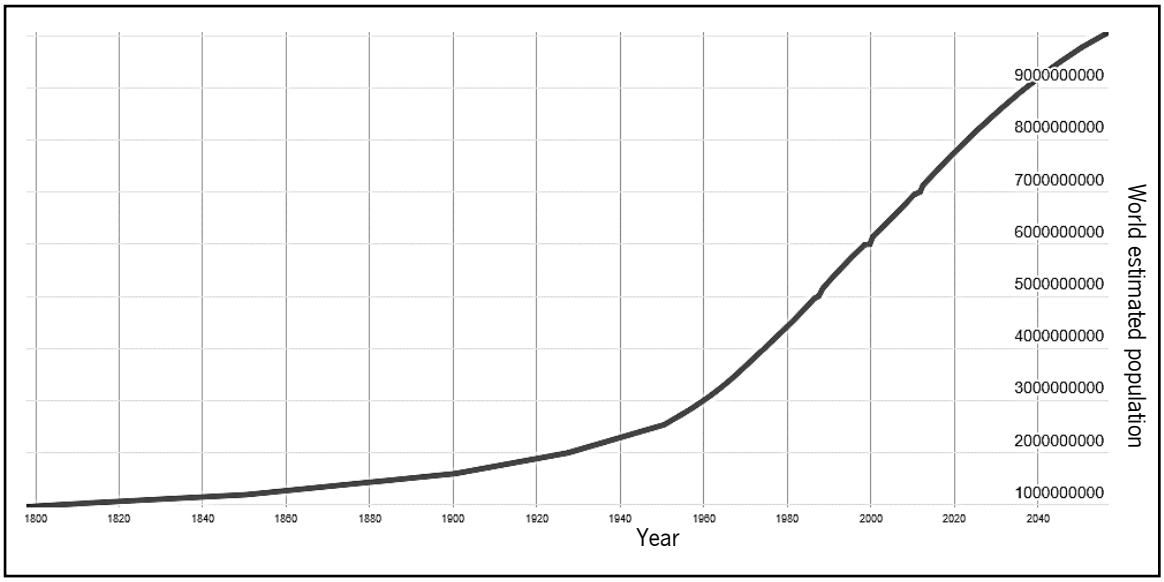


Figure 1. Estimated world population over the years, from 1800 to 2050. Graph adapted from http://www.worldometers.info/world-population, accessed the last time on 1/10/2018.

In agriculture, the use of pesticides aims to control plagues, diseases and undesired characteristics of harvested or processed food. Some diseases affect the healthy development and/or growing of pants, while others may be as an issue for human health. This results in a need to control such diseases and, except for diseases that might already be controlled through other means, like genetically engineered plants resistant to the most common diseases, the use of pesticides was the main tool in the last decades (Forget, 1994; Wolfenbarger *et al.*, 2000; Abrunhosa, Calado and Venâncio, 2011; Popp, Pet and Nagy, 2013).

The use of pesticides, however, must be restricted and well regulated. Organochlorides, one of the first groups of pesticides used on agriculture, were introduced in 1940 but no significant effects were seen up to 1974 when the use of pesticides was already a common practice. Although beneficial effects were not observed in every crop, in some cases, namely rice and tomatoes, its use resulted in a substantial increase in production (Forget, 1994). Nonetheless, pesticides are toxic chemicals, and even those are designed to affect other targets, they might represent a risk to users and all surrounding living beings. In 1972, the World Health Organization (WHO) estimated that 500 000 cases of poisoning were occurring every year worldwide, and this number increased to 1 million annually by 1986, accompanying the popularization of its use, with 20 000 resulting in death (Forget, 1994). Nowadays, WHO estimates that 18,2 per 100 000 full-time workers may suffer from acute pesticide poisoning (APP) in developed countries, where the use of pesticides is regulated and most of the harmful pesticides were already banned. In developing countries, the lack of regulation and restrictions on the use of pesticides is reflected in a much higher rate of APP, while countries like Sri Lanka and

countries from Central America reach values as high as 180 and 35 per 100 000 for APP of the general population, respectively (Thundiyil, Stober and Pronczuk, 2008). It is important to mention that full-time workers are exposed to much higher doses and concentrations of pesticides in comparison with the rest of the population, namely consumers, and the frequency at which they are exposed to pesticides is also greater, which makes them highly susceptible to APP.

Neurodegenerative diseases, such as Alzheimer and other kinds of dementia, many kinds of cancer, hormonal changes resulting from endocrine disruption, loss of fertility and development problems are some examples of risks that dietary intake or occupational exposure to pesticides represent for human health (Gauthier *et al.*, 2001; Cocco, 2002; Alavanja, Hoppin and Kamel, 2004; Remor *et al.*, 2009; Zaganas *et al.*, 2013; Zhang *et al.*, 2013). This strongly increases the importance of investigating and uncovering potential secondary effects of the use of each individual pesticide on human health and on the environment to prevent such occurrences.

1.1.1. Grapevine, viticulture and the Portuguese wine industry: the needs and hazards of using fungicides

Grapevine (*Vitis vinifera L.*) is one of the oldest crops in the world. It was introduced in Portugal about 5000 years ago. Some cultivars were latter introduced by Greeks and latter explored by Romans, who were also responsible for the introduction of more cultivars (Anderson, 2004; Veloso *et al.*, 2010). Grapevines are the raw material in wine production. The production of wine was developed long ago by Western civilizations and has been accompanying and benefiting from technological advances since the ancient Egyptian times, passing by Greeks and Romans (Kennedy, Saucier and Glories, 2006). Nowadays, the production of wine is important for the Portuguese economy. In 2001, Portugal was the ninth most wine-exporting country in the world (Anderson, 2004) and in 2017 occupied the eleventh place, exporting more than 6.6 million hl of wine. To achieve this, 194 kha of Portuguese land was used towards wine production (OIV, 2018). In order to improve the total amount of wine production without occupying more land, it is necessary to keep some pests and diseases under control and despite the fact that the European Commission (EC) has promoted decreasing use of pesticides in member states, this practice is still the most used way to control agricultural pests and diseases (Hillocks, 2012). Fungi and fungus-like organisms are the cause of many vine diseases and grape

trunk diseases among European wine producing countries, and therefore they are considered as a real problem for the grape crops and wine industry (Giménez-Jaime *et al.*, 2006).

Pesticides are classified according to the pest they aim to control, so in order to control fungal-related diseases, the most common practice is the use of fungicides. It is expected that each pesticide is designed to attack a specific target and has a well-established mode of action in order to avoid unwanted effects on crops and non-specific targets in general. Unfortunately, this is not the reality. Since fungi are eukaryotes, many genes and proteins have homologs in higher organisms, including humans, and many proteins or enzymes whose functions may have changed in the course of evolution still share important structural parts (Burnie *et al.*, 2006; Lehle, Strahl and Tanner, 2006). Therefore, some drugs designed to have inhibitory action against an enzyme or protein of a fungus can have unpredictable effects in an enzyme of higher organisms (Lamb, Kelly and Kelly, 1998; Taxvig *et al.*, 2007; Zhou *et al.*, 2016). Furthermore, fungicides whose target and mode of action is unknown are currently available on the market, and therefore it is highly relevant to study them (Estève *et al.*, 2009; Toffolatti *et al.*, 2014).

Based on its chemical properties and the lack of studies regarding the effects of various fungicides in the colon, or in mammalian cells in general, tebuconazole and cymoxanil were chosen from a list of pesticides commonly used in Portugal. In the following subtopics, some data about what is already known and what is not known about those two compounds will be presented.

1.1.1.1. Tebuconazole

Tebuconazole (**Figure 2**) is an organochloride, a class of frequent pesticides known to cause poisoning of waters due to its persistence (Iwata *et al.*, 2000). It belongs to the triazole antifungal drug family and is used against foliar diseases. This class of fungicides is used on fruit, vegetables, cereals, and seeds to control rust and mildew infections and has pharmaceutical applications since drugs from this group are used in the treatment of local and systemic

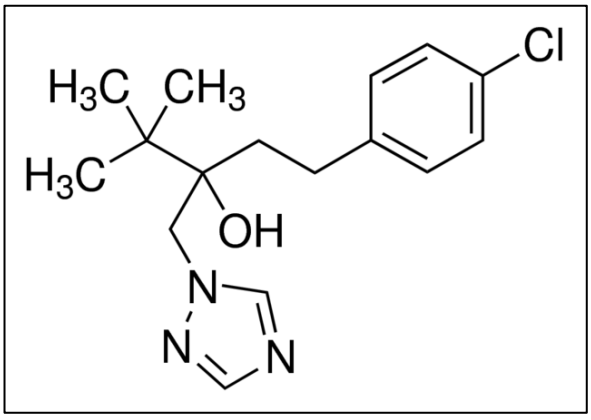


Figure 2. Tebuconazole structure. Source: Sigma-Aldrich®. Available from https://www.sigmaaldrich.com/catalog/product/sial/32013, accessed on 28/09/2018.

infections. This group of drugs acts by inhibiting the fungal enzyme lanosterol-14a-demethylase, also known as cyp-51 (**Figure 3**), which converts lanosterol into 4,4-dimethylcholesta-8(9),14,24-trien-3β-ol, which is a precursor of ergosterol synthesis in fungi. Ergosterol is important for fungi cell membrane stability and permeability, and its inhibition leads to disruption of the fungi cell membrane (Goetz *et al.*, 2006).

Cyp-51 is evolutionarily conserved not only between fungi but also between animals and plants. The same way 4,4-dimethylcholesta-8(9),14,24-trien-3β-ol is required for ergosterol synthesis in fungi, it is also required for cholesterol synthesis in animals (**Figure 3**). Furthermore, cholesterol is a precursor on the *de novo* steroid biosynthesis pathway and this affects the synthesis of androgens, estrogens, progestins, glucocorticoids, and mineralocorticoids (Lamb, Kelly and Kelly, 1998). In animals, by directly or indirectly inhibiting the synthesis of these steroids, a process that is also called endocrine disruption, it potentially leads to problems related to reproduction, growth and development, alterations on sexual differentiation, and the development of certain kinds of cancer (Goetz *et al.*, 2006; Sanderson, 2006).

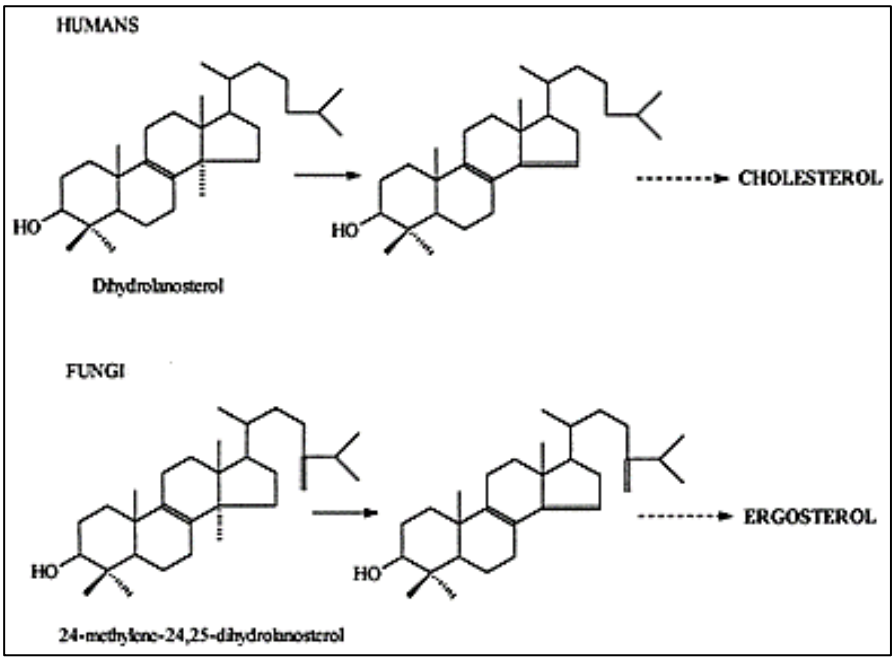


Figure 3. Eukaryotic CYP51-mediated sterol synthesis in humans and fungi. Dihydrolanosterol and lanosterol or 24-methylenne-24,25-dihydrolanosterol are substrates of the animal cholesterol and fungi ergosterol. Cyp-51 is also important on plants, participating on the phytosterol production through the obtusifoliol 14α-demethylation, the same type of reaction in which cyp-51 participates in homologous animals and fungi. Adapted from: Lamb, Kelly and Kelly, 1998.

There is a possibility that tebuconazole may indeed be an endocrine disruptor, like other fungicides from the triazole family. A study with rats focused on the effects that the exposure to some pesticides has since pregnancy until 16 postnatal days during lactation. In that study, it

was observed that tebuconazole caused a feminization in males, with a decrease of testosterone in fetal testis associated with an impact of the CYP17 activity in Leydig cells, and an increased number of nipples. In females, there was an increase of anogenital distance, which was interpreted as a mark of virilization of females (Taxvig *et al.*, 2007).

Cyp-51 and CYP17 belong to a big family of enzymes, called cytochromes P450 (CYPs), which are heme-containing enzymes present in several tissues of our organisms. The family is responsible for the metabolism of drugs and toxins to make them more hydrophilic, so they can be excreted easily, but is also responsible for the oxidation of fatty acids, activation of drugs, or even the metabolism of vitamin D, besides the steroid synthesis as previously described. Triazoles can modulate gene expression and enzyme activity of several P450 enzymes. This increases the need to determine the range of safe concentrations of triazole fungicides that can be used, in order to prevent secondary effects (Sanderson, 2006; Taxvig *et al.*, 2007; Jones, Prosser and Kaufmann, 2014; Omari and Murry, 2015).

It is known that tebuconazole does not affect the activity of aromatase (CYP19 – enzyme involved in the steroidogenesis) in human adrenocortical carcinoma cells (H295R) at concentrations near cytotoxic values (50 μM ≈ 15.39 μg/ml), assessed by different assays, namely Neutral red uptake assay and MTT assay (Sanderson *et al.*, 2002). It also does not affect the activity of CYP3A4 – the most abundant in the gastrointestinal tract, responsible for the oxidation of xenobiotics and either activating or deactivating drugs – at a concentration of 2.5 μg/ml in human epithelial colorectal cancer cells (Caco-2) after a 3 hour incubation, however it does affect the activity of CYP1A1 – enzyme involved in the activation of the major classes of tobacco procarcinogens – with the same previous conditions. This study warns us for the possibility of Tebuconazole being able to have a procarcinogenic effect by indirect route. By inhibiting the activity of an enzyme responsible for combating carcinogenic substances, the organism is more susceptible to the effects of these same substances (Sergent *et al.*, 2009; Omari and Murry, 2015).

As mentioned before, triazole antifungal drugs may affect growth and development; Jinghua Zhou and co-workers found that tebuconazole inhibits proliferation, changes the cell cycle distribution of G1 and G2 phases, induces apoptosis, decreases the invasive and migratory capacities and alters the mRNA levels of key regulatory genes in human placental trophoblasts (HTR-8) (Zhou *et al.*, 2016). These effects were observed after 24h to 72h exposure to a concentration ranging from 20 μM to 80 μM (≈ 6.16 μg/ml to 24.63 μg/ml). They also found a

significant down-regulation of the antiapoptotic Bcl-2 protein and an increase in the levels of the proapoptotic Bax protein expression in the same conditions. These alterations suggest that the observed apoptosis induction was caused by mitochondrial dysfunction.

Robin Mesnage and colleagues studied the effects of several pesticides and compared the results with the effects of some of their commercial formulations in three different cell lines – human hepatocyte carcinoma (Hep G2), human embryonic kidney (HEK293) and human placental choriocarcinoma (JEG3). For most pesticides, the formulation was more toxic than the active molecule. For tebuconazole, particularly, they concluded that the formulation (Maronee) is 1056 times more toxic than the active molecule alone. They also verified that the loss of cell viability was mostly due to necrosis and apoptosis markers (caspase 3 and caspase 7 activation) were decreased in the majority of the cases (Mesnage *et al.*, 2014).

The amounts of tebuconazole absorbed by the skin of winegrowers were estimated through liquid chromatography-mass spectrometry (LC-MS) by monitorization the presence and concentrations of tebuconazole metabolites on the workers' urine, skin and clothes (Fustinoni *et al.*, 2014). It was estimated that the maximum body exposure to this fungicide can potentially be as high as 21500 µg, with a median potential total exposure of 14200 µg all over the body at some point during a working day. In this study, samples were collected before, during, and after each work-shift to quantify the maximum total exposure to this fungicide per work-shift and this led the team to estimate a maximum total exposure of 3680 µg, with a 1410 µg median for each work-shift. Also, the authors concluded that the most abundant tebuconazole metabolite found in urine was TEB-OH, followed by TEB-COOH. They suggest that most tebuconazole was absorbed by the skin, however, to the best of our knowledge, no work has been published concerning the potential intake of that fungicide by humans in any scenario, either by accidental ingestion or inhalation by workers, or even by eating highly contaminated food (Fustinoni *et al.*, 2014). European Food Safety Authority (EFSA) has considered tebuconazole to be "Harmful if swallowed" and of low toxicity by the dermal route and by inhalation. The estimated LC_{50} after oral administration was 1700 mg/kg bw (body weight) in rats, 2000 mg/kg bw for dermal exposure and 5.093 mg/l for inhalation. Also, they estimated that more than 98% of orally administered tebuconazole can be absorbed within 48h. For skin exposure, the absorption is much lower, representing only 13% of the tebuconazole administrated, tested *in vivo* on monkeys (EFSA, 2008a).

There has been an increase in the number of studies on the effects of tebuconazole on humans and other non-target species, and it is now considered a persistent-bioaccumulative-toxic compound, and therefore a Europe Union candidate for substitution after 2019. As far as what was found on the literature, no study about the genotoxic or mutagenic potential of this fungicide has been reported, as well as studies carried out on normal cell lines, which increased our interest in contributing to a full understanding of its mutagenic potential.

1.1.1.2. Cymoxanil

Cymoxanil is a synthetic cyanoacetamide-oxime (**Figure 4**) fungicide usually applied on leaves, but it can act and protect other parts of the plant by permeabilizing leaves and moving through the xylem to untreated parts of the plant. This is only possible because cymoxanil is water soluble – up to 890 µg/ml. The mode of action is still unknown, however, some studies suggest that it is able to block the synthesis of nucleic or amino acids (EFSA, 2008b). It has been reported that it inhibits the oospore germination of some fungi strains such as *P. viticola*, which actually was one of its first uses (Gisi and Sierotzki, 2008).

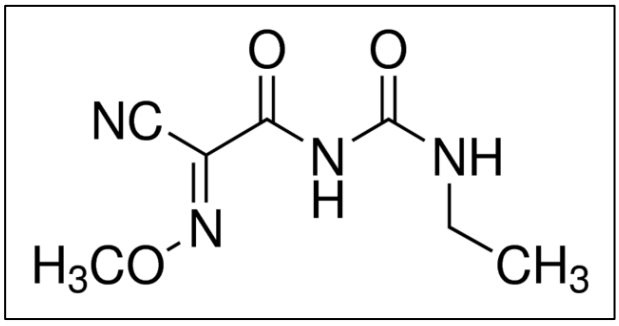


Figure 4. Structure of cymoxanil. Source: Sigma-Aldrich®. Available from https://www.sigmaaldrich.com/catalog/substance/cymoxanil198185796695711, accessed on 28/09/2018

Development of resistance against this pesticide has been reported in vineyards in the north of Italy; the minimum inhibitory concentrations (MIC) were calculated separately for different regions and an increase in resistance to the pesticide observed from 10 to 100 mg/L (10-100 µg/mL) in non-resistance vineyard regions to concentrations over 500 mg/L (500 µg/mL) in some regions where resistance developed. In the same article, it is also referred that the recommended field concentration for cymoxanil is 120 mg/L (120 µg/mL) (Gullino, Mescalchin and Mezzalama, 1997). This means that applying this fungicide at concentrations below the recommended values creates a selective force that promotes the development of fungicide resistance on target fungi.

In a genome-wide screen of yeast heterozygotes, it was reported that 3 mutant strains were significantly affected by a low concentration of cymoxanil (1,25 µg/mL), namely *lcb1*Δ, *ybr226c*Δ, and *ura1*Δ (Lum *et al.*, 2004). Based on available experimental and comparative sequence data, *ybr226c*Δ is not likely to encode a functional protein. *LCB1* is associated with sphingolipid metabolism and Lcb1 protein is a subunit of serine palmitoyltransferase in human cells, which is localized in the endoplasmic reticulum (ER), while *URA1* encodes dihydroorotate dehydrogenase, which carries the fourth step of the *de novo* biosynthesis of pyrimidines in yeast cytosol. The ortholog mammalian protein is present on the mitochondria membrane, where it also participates in the electron transport chain and its activity was already associated with an increase of ROS concentration and induced apoptosis on colon carcinoma cell lines (Nagiec *et al.*, 1994; Vorísek *et al.*, 2002; Yasuda, Nishijima and Hanada, 2003; Hail *et al.*, 2010; North *et al.*, 2012; Schertl and Braun, 2014). In another study, using *S. cerevisiae IGC 3507* and *C. utilis,* it was discovered that cymoxanil lowers the respiration rates at concentrations similar to the LC_{50} published for fish (7,5 µg/mL to 25 µg/mL for yeasts vs 13.5 µg/mL to 18.7 µg/mL for fish) (Ribeiro *et al.*, 2000). These studies suggest that cymoxanil may affect ER or mitochondria in human cells, which could be latter explored.

EFSA considered cymoxanil rapidly but incompletely absorbed by oral administration (75%), leading the agency to classify it as "Harmful if Swallowed". It was considered widely distributed throughout the body after ingestion, based on studies performed in rats, with highest residues on liver and kidney. By dermal exposure or inhalation, it has low toxicity, however it was positive for the Magnusson Kligmann test, through which it was considered that it "may cause sensitization by skin contact". In the same report, cymoxanil was not considered to have potential for bioaccumulation. Based on studies with rats, mice, and dogs, cymoxanil was classified as "Danger of serious damage to health by prolonged exposure if swallowed". These studies found an increase in rat liver weight with associated histological changes. Vacuolar changes of liver cells were also referred, even without affecting liver function. Increased kidney weight and changes in clinical chemistry and hematological parameters were found on rats, in addition to histological alterations on epididymis and spermatid, together with spermatid degeneration. Finally, within these studies for short-term exposure, it seems that dogs were the most affected organisms. Atrophy of testis in the 1-year study was one of the observed effects, in addition to alteration in body weight. On females, a 90-day exposure led to a decrease of erythrocytes at all the tested doses, with no related histopathological changes. Furthermore, anemia was also found

on rats at doses below 50 mg/kg bw/day. Overall, cymoxanil was not considered to present oncogenic or genotoxic potential, however, one *in vitro* study found chromosomal aberrations in lymphocytes (EFSA, 2008b).

Besides regulatory agencies, no particular *in vivo* or *in vitro* published works associating the intake of cymoxanil through ingestion with the wellbeing of human, or even other mammals, were found, either by ingestion of contaminated food or direct exposure by workers. This increased the interest in studying possible side effects of cymoxanil.

1.2. Colon and its place in acute and chronic exposure

Colorectal cancer is the third most diagnosed cancer worldwide and the second leading to death among all the types of cancer (Steliarova-Foucher *et al.*, 2014; GLOBOCAN, 2018). Lifestyle and hereditary predisposition appear to be the two most impacting factors for the development of this disease. (Mathiak *et al.*, 2002; Lee *et al.*, 2007; Peltomaki, 2016).

With a length varying between 90 and 150 cm, the colon is the organ that plays a major role absorbing water and minerals. There resides the largest population of microorganisms within the gastrointestinal tract and where the major production and absorption of short-chain fatty acids (SCFA) occurs, as well as being the site of secretion and storage of residues resulting from digestion (Kararli, 1995; DeSesso and Jacobson, 2001).

The large-scale production of SCFA is the result of the metabolism of the resident

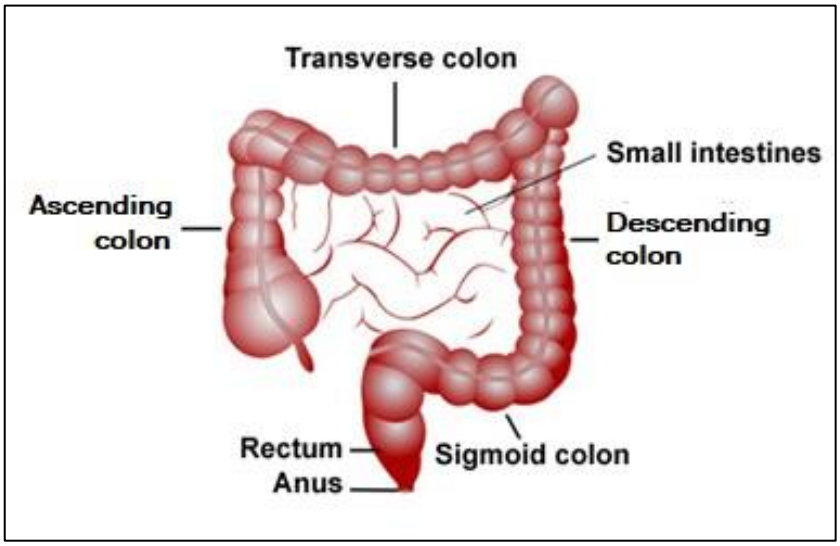


Figure 5. Anatomy of the large intestine. Ascending colon is connected to cecum, where the colon links to the small intestines. This section is followed by transverse colon, descending colon, sigmoid colon and finally, by rectum. Adapted from: Cedars-Sinai®. Available on https://www.cedars-sinai.edu/Patients/Health-Conditions/Sigmoid-Colectomy.aspx, accessed on 25/09/2018

microflora and studies from our group have already shown the relevance of this event to the potential prevention/treatment of colorectal cancer (CRC) (Marques *et al.*, 2013; Oliveira *et al.*, 2015). Besides the SCFA, colonic microorganisms also produce CO_2, H_2, lactic acid, ethanol, branched chain fatty acids (BCFA), ammonia, amines, phenol and indoles. Although some of these metabolism products are potentially toxic, the microorganism metabolism also plays a role in the protection against some toxic chemicals, metabolizing them before their absorption (Roberfroid *et al.*, 1995).The human colon is divided in 4 regions (**Figure 5**) and, like the rest of the gastrointestinal tract, it is histologically constituted by 4 distinct layers from inside to outside, namely mucosa, submucosa, muscular and serosa (DeSesso and Jacobson, 2001).

The mucosa is essentially an epithelial cell monolayer in a crypt shape involving capillary, which increases the area of absorption and facilitates a rapid distribution into the bloodstream (DeSesso and Jacobson, 2001). There is where the microflora is located and where the production and absorption of fatty acids occurs, as well as absorption of some bile acids (Roberfroid *et al.*, 1995; Ajouz, Mukherji and Shamseddine, 2014). The presence of such acids and lipids on the colon, along with the absorption of water, makes this section of the gastrointestinal tract the place where most of lipophilic drugs and toxins are more susceptible to concentrate compared to the remaining regions of the intestinal tract. This feature is currently considered an advantage for drug development, in which the design of some lipophilic liposomes and prodrugs tries to use these local properties (Chourasia and Jain, 2003; Zhang *et al.*, 2012; Zhou *et al.*, 2013).

Regarding toxins, they can be present in this section of the gastrointestinal tract and be harmful to humans due to accidental exposure, occupational exposure faced by farmers or, less frequent in Europe nowadays but still occurring in less developed countries, by dietary intake with bioaccumulation, in which organochloride pesticides, such as tebuconazole, but also heterocyclic aromatic amine pesticides represent an already known risk to the development of cancer, particularly in the colon (Lee *et al.*, 2007; Koutros *et al.*, 2009; Macfarlane, Benke and Sim, 2010).

The levels and the type of exposure, in terms of concentration, time and frequency faced by the epithelial cell from the mucosa, will determine the fate of these cells. That is, they will determine whether these cells may activate different type of processes, such as autophagy, processes of metabolization of toxic agents, or cell death, and in this case by which cell death pathway (Ames and Gold, 1997; Gauthier *et al.*, 2001; Alavanja, Hoppin and Kamel, 2004;

Mehrpour *et al.*, 2014). It is also worth mentioning that some damage caused may be at the genome level, and cells need to have mechanisms to find and recover from that kind of damage. However, some individuals are not able to respond to this type of damage due to mutations in important genes, being more susceptible to maintaining such damage in their genome, with all its consequences. There are studies that show that these mutations are relevant at the colon level since they are part of the hereditary causes that can determine the susceptibility that individuals may have to develop cancer (Mathiak *et al.*, 2002; Deng *et al.*, 2004).

In order to understand the effects of fungicides in human health it is important to understand the effects of the exposure to these compounds in cells. In sections 1.3, 1.4 and 1.5 we will describe the possible cellular and molecular alterations that may be affected in response to external compounds like fungicides exposure. (Fulda and Debatin, 2006; Richardson *et al.*, 2007; Alfadda and Sallam, 2012; Zhou *et al.*, 2016).

1.3. Cell death

1.3.1. Regulated cell death

Regulated cell death is a sequence of biochemical and morphological changes that results in a controlled or regulated cell death (Fulda *et al.*, 2010).This occurs to create and maintain the proper functioning of the organism. This does not only occur when cells get damaged either by an external stimulus or by aging but also occur during the development of different tissues (Danial and Korsmeyer, 2004; W. Ryter *et al.*, 2007). It is not exclusive for multicellular organisms. Regulated cell death was already found in many eukaryotic unicellular organisms. Even in prokaryotes, primitive forms of cell suicide can be found when the genome of bacterial colonies competes against other genomes (viruses, plasmids or other bacterial colonies) or as a final step of bacterial multicellular aggregated bodies formation (Ameisen, 1996, 2002).

Generally, this kind of cell death prevents the surrounded cells of the same tissue, or other cells from near tissues, from being damaged by the unregulated release of cell content by dying cells, keeping the homeostasis and the integrity of the tissue (Ameisen, 2002; Danial and Korsmeyer, 2004; W. Ryter *et al.*, 2007). Depending on the nature, amount and duration of the aggression, different kinds of regulated cell death can be triggered by the same type of cell. Also, different types of cells can react differently to the same stressor, according to the capacity of the cells to handle the induced stress and, in more complex organisms, the possible interplay between different cells may influence the fate of affected cells (W. Ryter *et al.*, 2007; Fulda *et al.*, 2010).

1.3.1.1. Apoptosis

Apoptosis is an energy-dependent cell death process coordinated by cysteine aspartyl proteases, called caspases, through which the cell "self-destructs" under certain conditions. This process is required not only to respond to several external insults or prevent the development and spread of some diseases but also during development, and as a homeostasis mechanism to control cell population in tissues (Elmore, 2007).

During apoptosis, organelles, proteins, and even DNA are degraded by a sequence of events that releases the product of this degradation in vesicles, so-called apoptotic bodies, through exocytosis. This process prevents the cell from exploding or releasing all its content in a non-regulated manner, preventing it from affecting the surrounding cells. This outtake of

apoptotic bodies as well as the condensation of chromatin and alterations in the cell cytoskeleton result in a decrease in cell size, named pyknosis, and is the most characteristic feature of apoptosis (Ameisen, 2002; Elmore, 2007). Other characteristics of apoptosis are plasma membrane blebbing without complete loss of cell membrane integrity, mitochondria outer membrane permeabilization, nuclear chromatin condensation and fragmentation (Ameisen, 2002). Furthermore, there is a flip-flop at the plasma membrane. This process exposes phosphatidylserine, which usually is located on the inner leaflet of the plasma membrane due to the action of aminophospholipid transferase, to the cell surface (Bratton *et al.*, 1997). Surrounding cells are capable of recognizing and rapidly ingesting the dying cell remains (Ameisen, 2002).

Apoptosis can be triggered either by internal or external stimuli through which the cell will activate the intrinsic or mitochondrial pathway, or the extrinsic or death receptor pathway. However, these two main pathways are linked and activation of the extrinsic pathway can lead to the activation of the intrinsic pathway (Elmore, 2007).

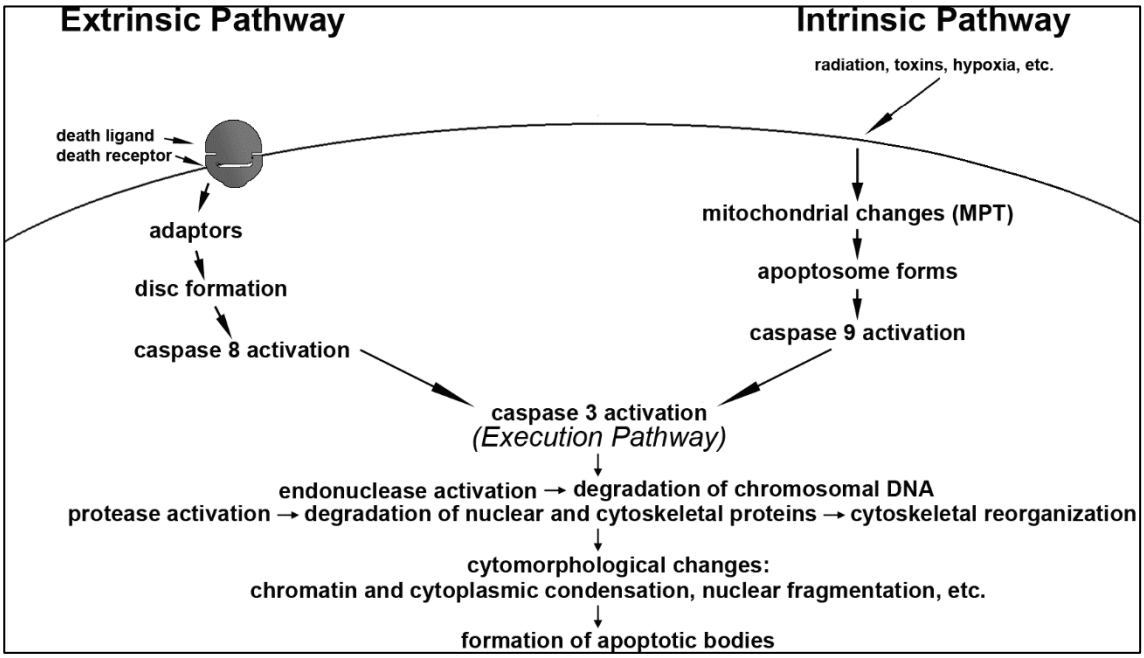


Figure 6. Schematic representation of the two main pathways of apoptosis and its main morphological and metabolic events. The extrinsic pathway starts with an activation of a death receptors, such as tumor necrosis factor-α (TNFα), Fas receptor (CD95 or APO1) and TNF related apoptosis inducing ligand receptors (TRAIL R1/2), proteins from the TNF receptor superfamily whose receptor is localized on the cell membrane. This activation occurs after the death receptor links with a death ligand (Ameisen, 2002; Lemke et al., 2014). When these receptors interact with its corresponding ligand recruit the adapter Fas-associated death domain and form a death-inducing signalling complex (DISC) in addiction with the inactive forms of caspase 8 and caspase 10. The proximity of those caspases to this complex result on its activation and release into the cytoplasm, where they will find and activate caspase 3, 6 and 7 that will conclude the apoptosis (Fulda and Debatin, 2006; Elmore, 2007; Ashkenazi, 2008). Adapted from: Elmore, 2007

In the intrinsic pathway, one of the main affected structures is the mitochondria, with permeabilization of the outer mitochondria membrane by pro-apoptotic members of the Bcl-2 protein family (Fulda and Debatin, 2006). Multiple factors can trigger this pathway such as a lack of growth factors, hormones or cytokines, exposure to radiation, hypoxia, hyperthermia, viral infections and, more importantly for this work, toxins, free radicals or even the detection of damaged DNA. By increasing the permeabilization of the mitochondria outer membrane, several proteins, including cytochrome *c*, which are usually found between the inner and the outer membrane, are released into the cytosol, continuing the cell death process, usually by activating the caspase cascade (Flores, Tsai and Crowley, 2002; Fulda and Debatin, 2006; Elmore, 2007). For instance, the release of cytochrome *c* into the cytosol will allow the formation of a pro-apoptotic complex (apoptosome) by binding with Apaf-1. The resulting complex is able to activate caspase 9, which subsequently activates the caspase 3 (Kim *et al.*, 2005). The activation of Bax/Bak-dependent apoptosis, either by Bad or Bid, proteins from the Bcl-2 family, can also occur. In this case, mitochondria permeabilization is caused by the formation of channels on the outer mitochondrial membrane (Martinou and Green, 2001).

1.3.1.2 Necrosis

Necrosis is the process through which the cell dies by lysis, generally associated with an excessive uptake of water and loss of control of ionic balance. It has been considered an unregulated kind of cell death for many years (Fulda *et al.*, 2010) and generally the result of an acute, accidental or non-physiological insult, however it is now known that necrosis can also be regulated (W. Ryter *et al.*, 2007; Nikoletopoulou *et al.*, 2013; Feoktistova and Leverkus, 2015).

Morphologically, a cell undergoing necrosis has mitochondria swelling and its membranes disturbed, and a gain in cell volume. This results in rupture of the plasma membrane and consequently the release of cellular content into the extracellular space in an unregulated manner. The distension of the endoplasmic reticulum, the formation of cytoplasmic blebs and vacuoles, and the disaggregation and detachment of ribosomes are also characteristics of necrotic cell death (Elmore, 2007; Fulda *et al.*, 2010).

Regarding what triggers this kind of cell death, some authors defend that this is a result of similar insults that trigger apoptosis, but when the cell is low on ATP. Apoptosis is an energy-dependent process of cell death, mainly because caspases use ATP to perform the cleavage of the target proteins, and thus without sufficient ATP cells die by necrosis. It is already accepted

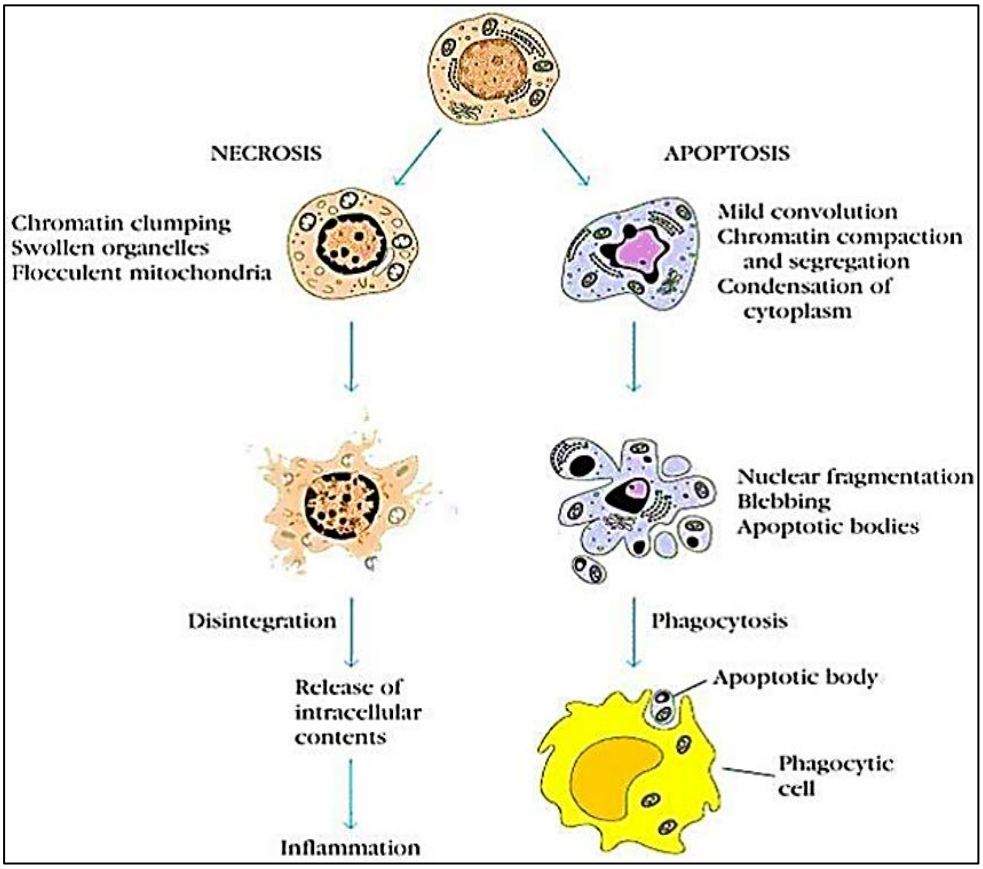


Figure 7. Main morphological features and differences between necrosis and apoptosis. Source: Choudhury et al., 2012

that, for instance, when the intrinsic pathway of apoptosis is activated, cytochrome *c* binds Apaf-1 and tries to recruit and activate caspase 9 to continue the process. In the absence of ATP, the apoptosome cannot be formed, apoptosis cannot proceed, and the cell changes the mechanism of death towards necrosis. Furthermore, extensive DNA damage, oxidative stress and loss of calcium homeostasis have a direct impact on mitochondria function. Since mitochondria are responsible for ATP production, the mitochondria function may determine whether the cell dies from apoptosis or necrosis, and also that there is an interplay between apoptosis and necrosis in, at least, some situations (Nicotera and Leist, 1997; Martinou and Green, 2001; Danial and Korsmeyer, 2004; Nikoletopoulou *et al.*, 2013). Necrosis, however, should not be considered exclusively as an alternative for apoptosis when energy is lacking. This type of cell death can be regulated and is required to initiate inflammatory responses in the human organism. Furthermore, necrosis has its own activation mechanisms and, similarly to apoptosis, necrosis can be executed in a regulated manner, also called necroptosis. This kind of cell death is

activated to responses against virus infections, avoiding the spread of the virus and promoting an inflammatory response by releasing its endogenous content, which will promote the production of cytokines, which will consequently initiate the inflammatory response (Cho *et al.*, 2009; Choudhury *et al.*, 2012; Lemke *et al.*, 2014).

1.4. The role of reactive oxygen species production

Normal cell metabolism generates reactive oxygen species (ROS). Most of this production occurs within the mitochondria aerobic metabolism. *In vitro*, under normal conditions, 1-2% of the total oxygen consumption within the cell is converted into superoxide radicals, particularly in complex I and complex III of the electron transport chain, through NADH dehydrogenase and ubiquitinone-cytochrome *c* reductase, respectively. Excess of ROS is dangerous to cells and, therefore, to maintain the levels under control, cells possess an antioxidant defense system that includes enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH) and catalase (CAT) (**Figure 8**). ROS accumulation arises when the capacity of those enzymes reaches the limit, which results in a loss of cell redox homeostasis, ending up damaging several parts of the cell. This can be a consequence of the activity of enzymes responsible for the metabolization of external compounds. In this case, the activity of enzymes from the Cytochrome P-450 family, xanthine oxidase (XO) and cyclooxygenase is generally associated with an increase of intercellular concentration of ROS (Dostalek *et al.*, 2008; Gisi and Sierotzki, 2008; Fulda *et al.*, 2010; Vergeade *et al.*, 2012; Martínez.Revelles *et al.*, 2013).

Mitochondria is very sensitive to ROS, excess of ROS results in cardiolipin oxidation, which consequently decreases the binding of cytochrome *c*, leading to an increase of free cytochrome c within the cytosol (Ott *et al.*, 2007). Under normal conditions, this results in apoptotic cell death (Kim *et al.*, 2005). However, as previously discussed, in some situations this energy-dependent cell death process cannot occur, either because of the presence of apoptotic inhibitors or by low ATP levels. This may result in a shift to necrosis (Li *et al.*, 1999; Nikoletopoulou *et al.*, 2013).

Excess of cellular ROS levels may affect proteins, lipids and DNA, compromising the cell viability and potentially causing mutations when DNA repair mechanisms are not efficient. Excess of ROS is associated with carcinogenesis, type II diabetes, chronic inflammatory diseases, and neurodegenerative diseases (Apel and Hirt, 2004; Ott *et al.*, 2007).

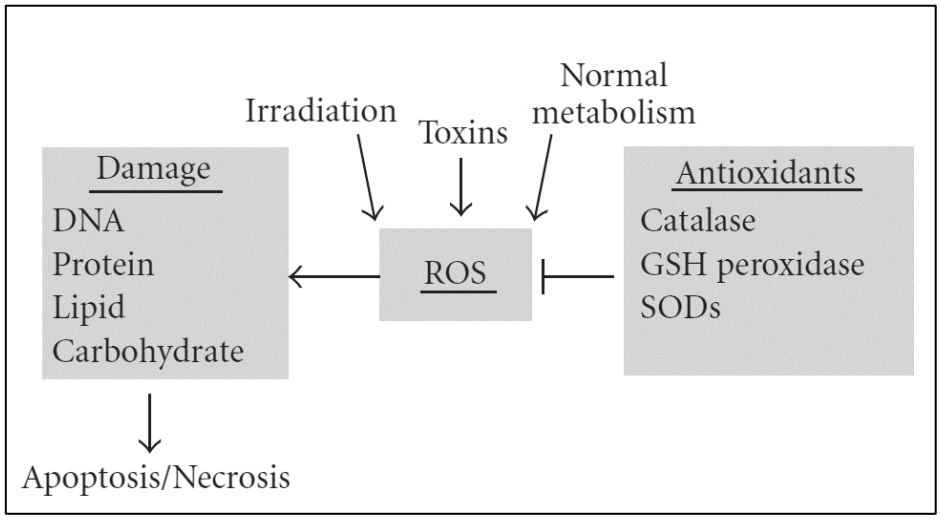


Figure 8. Reactive oxygen species (ROS) origins and its cellular targets. SOD converts superoxide into hydrogen peroxide which is further converted into water by GPX or other enzymes that can be either on mitochondria or in the cytosol (Finkel and Holbrook, 2000; Apel and Hirt, 2004; Ott et al., 2007). Adapted from: Fulda et al., 2010

1.5. Genotoxicity and mutagenicity

The term genotoxicity and mutagenicity both refers to the capacity that some chemical agents, like drugs or toxins, possess to damage the genome of a cell, potentially resulting in mutations when the cell cannot revert the damage. Although both of terms are similar, mutagenicity is widely used to describe the occurrence of actual mutations, either genomic or chromosomal, while the term genotoxicity also refers to other kinds of damage, such as DNA adducts or DNA strand breaks; however, it is not considered that cells suffering from genotoxicity are able to pass damage to further generations (Richardson *et al.*, 2007).

Many types of DNA damage may occur as a result of exposure to drugs, either by direct interaction of the drug with DNA, or a result of alterations induced by the drug within the cell, such accumulation of ROS, whose effects were previously discussed. Covalent changes, noncovalent anomalous structures, loops or bubbles resulting from a series of base pair mismatches are some examples of the changes that genotoxic compounds potentially induce in the DNA (Sancar *et al.*, 2004). It is also possible that some of those chemicals increase the

chance of mutations without directly or indirectly interact with the DNA, for instance, by inhibiting proapoptotic proteins or enzymes involved in the DNA-repairing machinery, once naturally some errors occur in the duplication of the DNA. If those errors cannot be repaired or the cell is unable to undergo apoptosis this results in an increase of chance to develop genomic anomalies. Although this type of damage is associated with carcinogenesis, many cancer drugs, such as cisplatin and etoposide, are based on genotoxic effects as a tool, once cancer cells proliferate faster and thus this increases the chances of having a greater effect on target cells through checkpoint signaling, which ultimately activates apoptosis with the accumulation of genome damage (Sancar *et al.*, 2004; Helleday *et al.*, 2008).

The ability of the cell to recognize and repair the damage induced by a genotoxic or a mutagenic compound decides whether the cell continues proliferating or dies, activating regulated cell death - generally apoptosis. Failure of the repair mechanisms and cell death may result in disease since these genome alterations will remain for the subsequent generations of cells (Demir, Creus and Marcos, 2014). From cancer to neurodegenerative and chronic inflammatory diseases, there is a wide range of possible pathological implications of this kind of damage, which makes it very important to study the impact that every drug or toxin for which there is possible occasional or chronic human exposure may have in the genesis of such diseases (Remor *et al.*, 2009).

Mammalian cells have many response reactions against DNA damage (**Figure 9**). Defects in proteins responsible for those processes are not always the cause of genomic diseases but they are considered a shield of the cell against them. Since mutations may occur naturally during

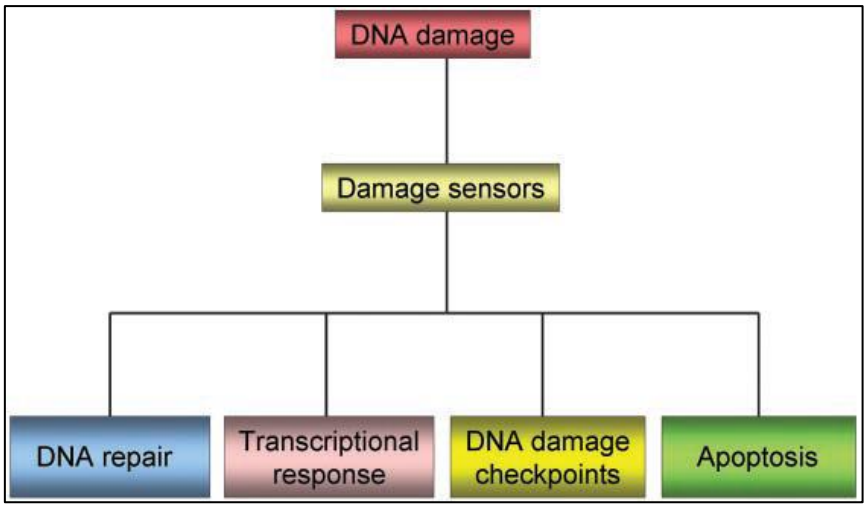


Figure 9. Overview of the four known responses on mammalian cells against DNA damage. DNA repair, transcriptional responses, DNA damage checkpoints and apoptosis. Many proteins involved in one of those reactions can interact with the others, but all those reactions generally start in one step; the recognition of the damage through damage sensors. Source: Sancar et al., 2004.

DNA replication any time a cell divides, inhibition (partial or complete) of such proteins indirectly contributes to an increased susceptibility to mutations and, consequently, related diseases. For instance, the *MLH1* gene is present in many organisms, such as yeasts and mammals, and it is responsible for producing an important protein that helps correct errors in the genome. Mutations in this gene are associated with an increased development of specific types of cancer, namely some forms of colorectal cancer like Lynch syndrome or Muir-Torre syndrome, which are associated with individuals who carry one or two dysfunctional genes involved in the repair of DNA while it is duplicating, consequently leading to microsatellite instability (Mathiak *et al.*, 2002; Martinez and Kolodner, 2010; Peltomaki, 2016).

2. *Rationale* and aims

Over the two last centuries, the growth of the world population led to an increased need to produce more food. Food production has suffered several alterations, such as the introduction of pesticides to control plagues and plant diseases and therefore avoid the loss of crops and improve the overall quality of food (Forget, 1994; Popp, Pet and Nagy, 2013; Blair, Wesseling and Freeman, 2015).

Fungicides are produced to attack fungi (eukaryotic organisms), and therefore prevent their spread or even kill them. Fungicides may interfere with other organisms, namely higher eukaryotes, such as humans, which have an increased chance to suffer secondary effects as a result of their misuse (Sergent *et al.*, 2009; Mattiazzi, Petrovič and Križaj, 2012; Zhou *et al.*, 2016). In fact, even though some agencies restrict and regulate the use of pesticides in general in developed countries, the occurrence of acute pesticide poisoning still occurs among full-time workers (Thundiyil, Stober and Pronczuk, 2008), generally as a result of bad practices on managing and applying pesticides. The north of Portugal is rich in vineyards, and since many of the diseases in the vineyards are caused by fungi, the use of fungicides constitutes a common practice.

Based on the chemical characteristics of various fungicides and the scarce reports and knowledge regarding their potential effects on human health, we chose two of the most commonly used fungicides (tebuconazole and cymoxanil) in order to study their impact on human health. Since ingestion represents one of the main human exposure pathways to fungicides, the digestive tract is one of the most affected sites, namely the colon. The incidence of colon-related diseases, such as cancer, has been increasing over the last decades, especially in developed countries (Horton, Corl and Fishman, 2000; Alavanja, Hoppin and Kamel, 2004; Bailey *et al.*, 2015; Peltomaki, 2016). Particularly in Portugal, colorectal cancer is one of the most frequently diagnosed and the second leading to death among all the types of cancer (Steliarova-Foucher *et al.*, 2014; GLOBOCAN, 2018). These diseases seem to be caused by lifestyle and hereditary genetic predisposition (Mathiak *et al.*, 2002; Lee *et al.*, 2007; Peltomaki, 2016).

Our general aim was to uncover the effect, mechanism of action and genotoxic potential of the fungicides tebuconazole and cymoxanil in order to explore the consequences related to acute and chronic exposure.

Specifically, we aimed to answer the following questions:

- What is the cytotoxic concentration of these fungicides in human colon cells?
- Which are the effects of these fungicides on the human colon?
- Which are the possible mechanisms of action of these fungicides?
- What is the genotoxic potential of these fungicides?

To achieve these aims, we used "normal" colon-derived cells NCM460 to evaluate acute consequences and the yeast model as a tool to assess genotoxic potential, in order to evaluate a possible long-term effect as a consequence of chronic exposure.

3. Materials and methods

3.1. Cell lines and cell culture conditions

The cell line used in this work was NCM460 which is a normal colon-derived cell line obtained from INCELL, San Antonio, TX, USA (Moyer M.P, Manzano L.A, Merriman R.L, Stauffer J.S, Tanzer L.R. NCM460), a normal human colon mucosal epithelial cell line (Moyer *et al.*, 2014).

One vial of frozen cells from NCM460 cell line, stored either in liquid nitrogen or at - 80 °C, was thawed and the content, cells blended with freezing mixture (DMSO and fetal bovine serum (FBS), 1:4 (v/v), respectively), was transferred to a falcon tube where it was, carefully, resuspended in complete medium. The cell suspension was then centrifuged at 1200 rpm for 5 minutes and the pellet resuspended in fresh complete medium, which was transferred into a sterile culture flask.

The cells were maintained at 37 °C under a humidified atmosphere containing 5% CO_2. Cells grew in Roswell Park Memorial Institute (RPMI) 1640 medium with stable glutamine (Biowest®) supplemented with 10% FBS (v/v) (Biowest®) and 1% penicillin/streptomycin (v/v) (Biowest®). Cells were subcultured once a week at a dilution of 1:5 until 80% of confluence. To maintain the stock of the cell lines used, during the first subcultures some of the unused cell suspension was centrifuged at 2000 rpm for 10 minutes. The pellet was resuspended in 1 ml of the freezing mixture and transferred to a cryotube which was then stored in liquid nitrogen or at -80 °C. Contamination with Mycoplasma is one of the main contaminations found in cell culture, essentially due to improper handling. It is difficult to detect and causes changes in several cellular characteristics that may interfere with the results. In order to test for mycoplasma contamination, 200 µl of cell supernatant was collected from culture plates with almost 100% confluence for a 1.5 ml microtube, which was heated in a dry bath at 95 °C for 10 minutes. After that, the samples were stored at 20 °C. The test was performed by polymerase chain reaction (PCR) using VenorTMGeM Mycoplasma Detection Kit (Sigma-Aldrich®). Cells that showed positive results for Mycoplasma contamination were discarded.

3.2. Determination of IC_{50} through Sulforhodamin B assay

The determination of IC50 of both fungicides was performed through Sulforhodamine B (SRB) assay. Tebuconazole was dissolved in DMSO, while cymoxanil was diluted in water for this assay. Both were stored at 4 °C and protected from light.

NCM460 cells were plated in 24-well plates, three wells per condition, at a concentration of 3×10^5 cells/ml. 24 hours after the seeding cells were treated with different concentrations of individual fungicides. For each experiment, two controls were always used: one containing only cells with growth medium and one with the same volume of the solvent used to dissolve each pesticide. In this case, DMSO was used on the second negative control for tebuconazole experiments and water was used on the second negative control for cymoxanil experiments. This aims to discard any influence of the solvents in the results.

48 hours after the treatment cells were washed PBS 1x twice and then fixed in methanol containing 1% acetic acid (v/v) at -20 °C. After, at least, 90 minutes the fixing solution was carefully removed. The plates were then left drying at room temperature and, when completely dried, incubated with 0.5% (w/v) SRB dissolved in 1% acetic acid (v/v) at 37 °C protected from light. 90 minutes after the incubation with SRB the solution was removed, and plates were washed with 1% acetic acid (v/v) in order to remove the excess of SRB. Plates were then left air-drying at RT. Finally, 1 ml of 10 mM Tris pH 10 was pipetted to solubilize the SRB, plates were agitated carefully and protected from light and afterward, 200 µl of the final solution were placed in a 96-well microplate where the absorbance was read at 540 nm in GENESYS™ 20 Visible Spectrophotometer, Thermo Scientific™, https://www.thermofisher.com.

Statistical analysis was performed using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Each IC_{50} was calculated based on the results obtained from at least three independent experiments and expressed as mean ± SD. The IC_{50} was determined applying a dose vs response (variable slope) nonlinear regression.

3.3. Detection of DNA double-strand breaks by TUNEL assay

Levels of apoptotic cell death were firstly assessed through Terminal transferase dUTP nick end labeling (TUNEL) assay using In Situ Cell Death Detection Kit, Fluorescein (Roche©).

Cells were seeded in 6-well plates at a concentration of 3×10^5 cells/ml. For negative control, only one well was seeded, while for all the other condition two wells were used to ensure enough cells.

24 hours after seeded, the cell medium was changed, and cells were exposed to 250 μM hydrogen peroxide for positive control, and the IC_{50} and the double of the concentration of IC_{50} of both tested pesticides. The negative control was treated with the maximum volume of DMSO used on conditions with pesticides (0,5 % v/v).

48 hours after the exposition cells were collected. Firstly, the medium was collected to a 15 ml tube. Cells were then washed twice with PBS 1x and detached from wells with trypsin-EDTA 0.05% (v/v). After that cells were washed again with PBS 1x and collected to respective falcons. Every solution resulting from washing was also collected to prevent loss of cells, ensuring live and dead cells were collected equally.

Tubes were then centrifuged for 10 minutes at 2000 rpm, the supernatant was discarded and the pellet was washed with PBS 1x before repeating the centrifuge step. After the second centrifugation cells were fixed with paraformaldehyde 4%, for 15 minutes, washed with PBS and centrifuged again with the same previous conditions. Finally, the supernatant was not completely collected, 500 μl was used to resuspend the pallet and the resulting content was collected to 1,5 ml microtubes and stored at 4°C.

250 μl of cells were used to create cytospins. Using Cytospin™ 4 Cytocentrifuge (Thermo Fisher Scientific©), cells were centrifuged for 5 minutes at 500 rpm, the resulting cytospins were detached with hydrophobic pen, washed 3 times with PBS 1x, each one for 5 minutes, and then incubated with permeation solution (0.1% Triton X-100 in 0.1% sodium citrate), for 2 minutes on ice. The washing cycle was repeated, and cells were further incubated with 20 μl of the previously prepared TUNEL reaction mix (dilution buffer, label solution and enzyme solution in a 10:9:1 ratio, respectively) in a dark box, at 37°C for 1 hour, with wet paper under the slides to prevent dehydration.

Once the incubation time was complete the slides were washed 3 times with PBS 1x, 5 minutes each, on an immunohistochemical box protected from light and the coverslip was mounted with 2 μl of VECTASHIELD Antifade Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories®).

Photos were obtained in an Olympus IX71 Inverted Microscope (Olympus©) with 400x of magnification. The results were obtained from at least three independent experiments and

expressed as mean ± SD. For each condition were counted at least 700 cells using ImageJ 1.51j8 software.

Data from more than independent experiments were treated on GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com by one-way ANOVA followed by Dunnett's multiple comparisons test.

3.4. Annexin V/PI assay

To study if pesticides induced cell death by apoptosis or necrosis, we performed the annexin V/PI co-staining by flow cytometry (Figure 10).

Cells were seeded into 6-well plates at the concentration of 3×10^5 cells/ml, 2 wells per condition except for negative control where only one well was used. In the first experiment, instead of 6-well plates, 60 mm Petri dishes were used, one per condition. 24 hours after plating the cells, the medium was changed, and cells were exposed to the conditions under test. A negative control with untreated cells (only medium), positive control with 250 µM H_2O_2, the IC_{50} and the double of the concentration of IC_{50} of both pesticides were the tested conditions in this experiment.

Cells were collected 48 hours after treatment. The medium was collected to previously identified 15 ml tubes, cells were washed twice with PBS 1x and it was collected to the respective tubes. Then, cells were detached with trypsin-EDTA 0.05% (v/v), resuspended with the previously collected medium, washed again with PBS 1x and collected to the respective tube.

Once collected, tubes were centrifuged for 10 minutes at 2500 rpm, the supernatant was discarded, and cells were resuspended with 1 ml PBS 1x. At this point, in the first experiment, cells were equally divided into 4 different tubes per condition: 1 for autofluorescence, 1 for mono-staining with annexin-V, 1 for mono-staining with PI and 1 for the experiment with both annexin and PI staining. After that, cells were counted in the Neubauer's chamber to guaranty more than $2x10^5$ cells/ml. Then, the cell suspension was transferred to 1,5 ml Eppendorf, which were previously identified, Eppendorf were centrifuged at 2500 rpm for 10 minutes, the supernatant was discarded, the pellet was resuspended with 100 µl of "binding buffer" and cells were incubated for 15 minutes with 5 µl of annexin and 5 µl of PI, from Annexin V-FITC Apoptosis Detection Kit (Abcam), protected from light. Eppendorfs for autofluorescence and mono-staining were treated the same way, but annexin and/or PI were not used in those. Finally, after

incubation, 200 µl of "dilution buffer" was added and mixed with the cells and the reading was performed on the flow cytometer.

The flow cytometer used was a CytoFLEX Flow Cytometer, BECKMAN COULTER, Life Sciences, https://www.beckman.com. Annexin was read with the FITC filter and PI was read with the ECD filter. Results were treated with FlowJo version 7.6.5 for Windows, https://www.flowjo.com, and the analysis was performed on GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com, using the results obtained from 3 independent experiments by two way ANOVA, comparing the rows of each column with values obtained from the negative control using the Tukey's multiple comparisons test.

3.5. Reactive Oxygen Species determination by DCFH-DA analysis by flow cytometry

Cell-permeant 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is widely used as a cellular ROS production indicator (Hail *et al.*, 2010; Alfadda and Sallam, 2012). Under the presence of ROS non-fluorescent H_2DCFDA is oxidized into fluorescent 2', 7'-dichlorofluorescein (DCF), which can be detected by flow cytometry.

For this assay, cells were seeded in 6-well plates, 2 wells per for each condition, except for negative control were only 1 well was used, at a concentration of 3×10^5 cells/ml.

24 hours after being plated medium on each well was removed and replaced for new medium with the desired treatments. Negative control had the maximum volume of DMSO used to other conditions (0,5% v/v), positive control had 250 µM H_2O_2 and the conditions tested were the IC_{50} and the double of the concentration of the IC_{50} of tebuconazole and cymoxanil (2xIC_{50}).

After 48 hours of exposure to the conditions, cells were collected. First of all, the medium of each condition was transferred to a 15 ml tube previously identified. After that, cells were washed with PBS 1x twice, detached with trypsin-EDTA 0.05% (v/v), resuspended with the previously collected medium and washed again with PBS 1x. Each time the wells were washed with PBS 1x the content was collected to the respective tube.

The content of each tube was equally resuspended and divided into 2 different tubes. Tubes were then centrifugated at 130 g for 5 minutes, the supernatant was removed, and cells from a tube of each condition were resuspended in 1 ml of a 0.1 µM H_2DCFDA solution diluted in

DPBS 1x. Cells from the other tubes were resuspended on 1 ml of DPBS 1x for latter autofluorescence measurement.

Tubes were then left at 37 °C for 30 minutes protected from light. After that time centrifuged again at 130 g for 5 minutes, resuspended with 500 µl DPBS 1x. The last centrifugation and resuspension were repeated and finally, cells were ready for the flow cytometer acquisition.

The signal was read with the FITC filter using the CytoFLEX Flow Cytometer, BECKMAN COULTER, Life Sciences, https://www.beckman.com. Results were treated using FlowJo version 7.6.5 for Windows, https://www.flowjo.com, in 2 different ways; one for the percentage of DCF positive cells and another for the average DCF intensity per cell. Data from 3 independent tests were further analyzed with GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com by ordinary one-way ANOVA followed by Dunnett's multiple comparisons test, comparing the results of each condition with the values obtained from the negative control.

3.6. Colony formation assay

Colony formation assay was performed to check if the pesticides under study have any kind of influence on cell proliferation.

Only 300 cells/ml were seeded into 6-well plates and left for 24 hours until the medium was removed and changed by the medium with treatments diluted previously diluted into it. Only one well was used for negative control, while the other conditions had two wells each.

After 48 hours of treatment, the medium was removed, and new untreated complete medium was used to fill the wells. The medium was further changed twice a week for the following 14 days, the time required so the colonies could grow until some were visible to the naked eye. To avoid undesired variables, in all the replicates the time left for cells to growth and divide was the same.

When colonies were ready the medium was removed, cells were washed twice with PBS 1x, incubated with a 6% glutaraldehyde and 0.5% crystal violet solution for at least 30 minutes. After that time the solution was removed, the excess of the solution was removed by softly immersing the plates in a bowl full of water. Finally, plates were left to dry at room temperature and photos of individual wells were taken. Photos were further cut, the colors have been changed to grayscale and colonies were counted manually using ImageJ 1.51j8 software. Results were

treated with GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com by ordinary one-way ANOVA followed by Dunnett's multiple comparisons test, comparing the results of each condition with the values obtained from negative control and comparing the different concentrations of each fungicide.

3.7. Carboxyfluorescein diacetate succinimidyl ester (CFSE) analysis to assess cell proliferation using cytometry

To confirm the results obtained from the colony formation assay we used the CFSE assay we started by adapting the protocol provided by the supplier (BD Biosciences, www.bdbiosciences.com/pharmingen/protocols), which is optimized to use with single-cell suspensions of primary lymphoid cells, with some adjustments made previously in a recently published work performed in the Department of Biology of the University of Minho. These changes on the protocol aimed precisely to adjust the technique to monolayer cell cultures (Guedes *et al.*, 2018).

Cells were counted, and the necessary pipette volume was calculated in order to obtain a final concentration of 3×10^5 cells/ml. The same volume was removed and plated for autofluorescence conditions.

The volume of cells needed to match all conditions with CFSE was pipette to a 15 ml Falcon and the cells were centrifuged (500 rpm, 10 minutes), the supernatant was removed, and the cells were resuspended in 1 ml of PBS 1x. This process was repeated once before we joined 2 µl of 10 mM CFSE to the cell suspension, obtaining a final concentration of 20 µM CFSE. From this moment on, everything must be done with the smallest possible light to prevent the loss of fluorescence.

The solution was then incubated at 37 °C for 15 minutes. During this incubation, the non-fluorescent dye passively diffuses across cell membranes being subsequently cleaved by intracellular esterases and binding covalently with amine groups of proteins in viable cells

After the 15 minutes of incubation we have, in theory, the point of the highest fluorescence intensity of the signal in each cell and for each division that occurs, the parent cell divides the dye, and therefore the signal strength, to half.

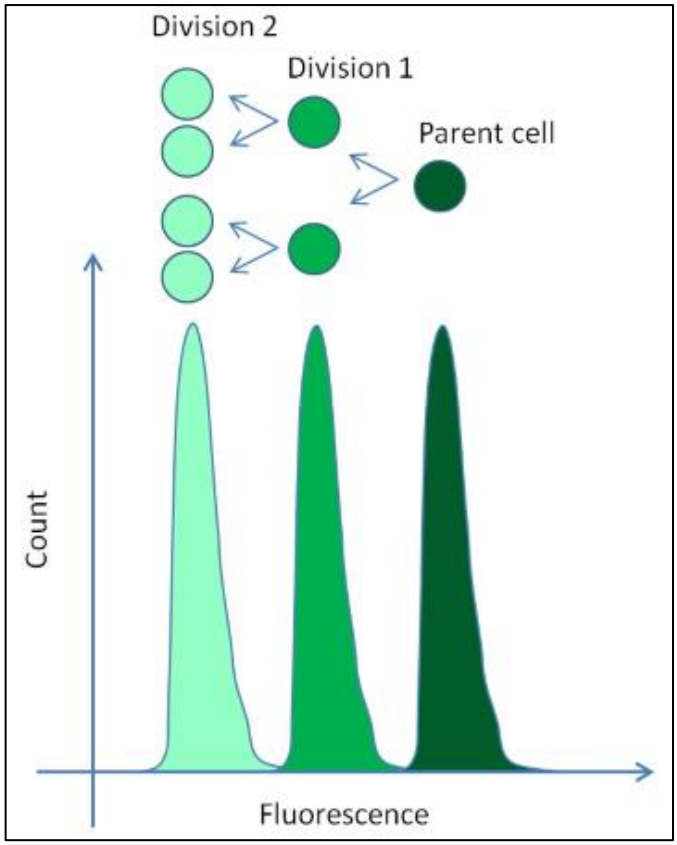


Figure 10. Representation of the loss of signal intensity per cell, as the cells divide during the experiment. Adapted from: https://shop.chemometec.com/product/viafluor-cfse-cell-proliferation-kit-3/, accessed in 23/11/2018.

Two negative controls were incubated for the conditions in which the cells were incubated with CFSE and for the autofluorescence conditions. One was collected and used to read the signal shortly after incubation of the cells with the conditions under test (C- 0 h) and another that was collected along with other conditions (C- 48 h).

After the incubation, we repeated the centrifugations and wash the cells (2 times, 500 rpm, 10 min), removing the supernatant and suspending the cells in 1 ml of PBS 1x between centrifuges. After the second and last centrifugation, the supernatant was removed, and the cells were resuspended in the required volume of cell media for being plated in 6-well plates. The cells used for autofluorescence went through all these processes, including the bath at 37 °C, and the only difference between these cells and cells with CFSE is that these cells do not have CFSE, and therefore do not have fluorescence.

24 hours after plating the cells, the medium of cells was removed and a new medium prepared previously containing the conditions to be tested was added to the respective wells (1 well per condition). As mentioned above, at that point we collected the cells from both negative controls for 0 hours (with and without CFSE). To collect the cell, we discarded the cell medium, cells were detached with trypsin-EDTA 0.05% (v/v) and wells were washed twice with 500 µl PBS

1x, collecting them to previously identified 2 ml Eppendorfs and finally, it was performed the readings of the negative control with the FITC filter using the CytoFLEX Flow Cytometer, BECKMAN COULTER, Life Sciences, https://www.beckman.com.

48 hours after incubation with the conditions under test, the recoil and readings of the cells were performed following the same method used for the negative controls at time zero.

Results were treated using FlowJo version 7.6.5 for Windows, https://www.flowjo.com, through which was taken the mean signal intensity of each condition to evaluate the rate of proliferation held in each condition applying the following formula:

$$\frac{[(C-\ 0\ h)-(Condition)]x100}{[(C-0h)-(C-48h)]}$$

(C- 0 h) refers to the mean signal intensity of negative control at 0 hours, (C- 48 h) refers to the mean signal intensity of the negative control at 48 hours and (Condition) refers to the mean fluorescence of the condition under test. Considering that the difference between these two picks represents the normal loss of signal resulting from successive divisions in a normal rate on the cell line. The remaining conditions were considered to be represented by the difference between the mean fluorescence between the negative control at 0 hours (C- 0h) and the mean fluorescence on cells exposed to each condition for 48 hours (condition). All the resulting values were normalized to negative control to represent an increase or decrease in proliferation in relation to this control, as a percentage.

3.8. Yeast growth and treatment conditions

The *S. cerevisiae* haploid strains used in this study were: RDKY3615 (*MAT* a, *ura3-52*, *his3Δ200*, *leu2Δ1*, *trp1Δ63*, *lys2ΔBgl*, *hom3-10*, *ade2Δ1*, *ade8*, *hxt13*::*URA3*) and RDKY3615$_{\Delta mlh1}$ (RDKY3615 *mlh1::URA3*).

An isolated colony was picked, diluted in yeast extract peptone dextrose (YPD: Table 1) liquid medium and grown overnight at 30 °C in an orbital shaker at 200 rpm. In the next day, cells were diluted to OD_{640nm} 0.1 and divided into 3 tubes. Different concentrations of the fungicide were added to 2 tubes (5 µg/ml and 20 µg/ml), while no fungicide was applied in the third as a control. The concentration of the solvent (DMSO) was the same in all tubes to avoid its influence on the experiment (0.05%). Cultures were then grown at 30°, 200 rpm, and the OD_{640nm} was read every hour for 8 hours, and additionally after 24-hours.

Experiments were repeated at least 3 times. The average OD for each checkpoint was calculated and growth curves were generated in Microsoft Excel 2016, from Microsoft Office 365.

3.9. Yeast viability assessed by spot assay

For each condition described in section 3.8, 5 µl of cell suspension and serial dilutions (10^{-1} to 10^{-4}) were spotted on yeast extract peptone dextrose agarose (YPDA) plates **(Table 1)** at 0 and 24 hours after exposure and left incubating at 30°C for 3 days, after which a picture of each plate was taken.

3.10. Fluctuation Assay

An isolated colony of RDKY3615 and RDKY3615$_{\Delta mlh1}$ from fresh streaks was inoculated into 5 ml YPD and grown overnight. In the following day, the OD was read, and the OD_{640nm} of each strain was adjusted to 0.1 in 15 ml YPD. Both strains were then treated with the same conditions as described in section 3.8. The concentration of the solvent (DMSO) was the same in all tubes to avoid its influence on the experiment (0.05%). Cultures were grown from 10 to 12 hours with these conditions and then centrifuged (3000 rpm, 5 minutes), washed in 1 ml ddH_2O, centrifuged again (14000 rpm, 1 min) and resuspended in 1 ml ddH_2O. From the final cell suspension, serial dilutions in ddH_2O were prepared, up to a factor of 10^{-6}. 100 µl of the 10^{-6} dilution for each condition were plated on CSM **(Table 1)**. 700 µl of the undiluted RDKY3615 samples and 200 µl of the 10^{-2} dilution of Δ*mlh1* samples were plated on CSM^{-thr} **(table 1)**. Cells were spread with the aid of glass beads and incubated for 3 days at 30 °C. After this time, the resulting individual colonies on each plate were manually counted and registered.

The numbers of viable and mutated cells were calculated based on the initial and plated volumes, as well as the number of colonies counted on CSM and CSM^{-thr} plates, respectively. From these numbers, we achieved the frequency of mutations (cell/cell) as described (Shell, Putnam and Kolodner, 2007), which was further used to calculate mutation rates using a macro "calcM" provided by V. Pennaneach. The mean mutation rate was calculated with results from 3 independent experiments.

Table 1. Constitution of the mediums used in the model *S. cerevisiae* (250 ml).

	Medium			
	YPD (g)	**YPDG (g)**	**CSM (g)**	**CSM^{thr} (g)**
Glucose	5	5	5	5
Peptone	2.5	2.5	-	-
Yeast extract	2.5	2.5	-	-
Agar	5	5	5	5
$(NH_3)SO_4$	-	-	1.25	1.25
Yeast nitrogen base	-	-	0.425	0.425
Dropout	-	-	0.179	0.179
Leucine	-	-	0.1	0.1
Histidine	-	-	0.2	0.2
Tryptophan	-	-	0.2	0.2
Uracil	-	-	0.2	0.2
Lysine	-	-	0.2	0.2
Arginine	-	-	0.2	0.2
Threonine	-	-	0.2	-

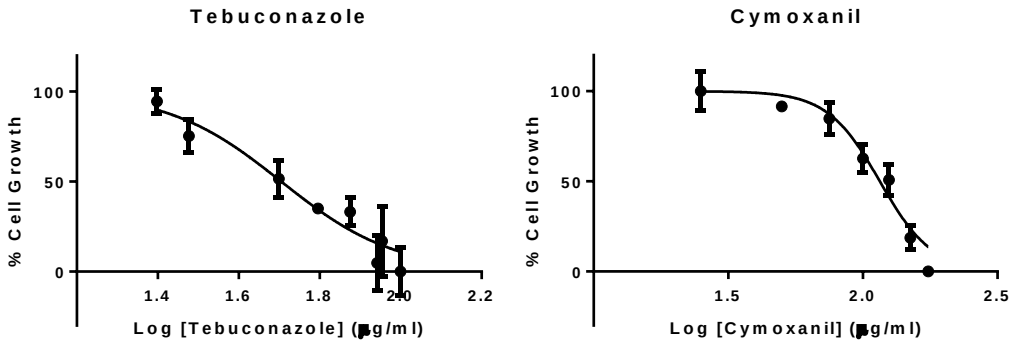
4. Results

4.1. Determination of the IC_{50} concentrations of fungicides in normal colon cells

The determination of the concentrations of fungicides required to decrease the cell growth in 50% (IC_{50}) was assessed by SRB assay in "normal" colon-derived cells NCM460. Different concentrations of tebuconazole, ranging between 1 µg/ml and 10000 µg/ml were tested. We could observe that tebuconazole started affecting cell growth at values between 1 µg/ml and 100 µg/ml. Concentrations in this range were further tested in several experiments until it was possible to calculate the tebuconazole IC_{50} value. Our results showed that the IC_{50} concentration of tebuconazole was 50.7 µg/ml **(Figure 11 A)**.

For cymoxanil, we started by testing concentrations that we previously tested with tebuconazole, but it was necessary to increase the concentrations in order to determine the IC_{50} on NCM460 cells. This effect only started being evident when cells were exposed to 100 µg/ml (69.6% cell growth; *n*=4) and higher concentrations (**Figure 11**). A figura esta desfornmatad

A



B

	IC_{50} (µg/ml)
Tebuconazole	50.7 ± 2.6
Cymoxanil	115.6 ± 2.2

Figure 11. Effect of Tebuconazole and Cymoxanil on NCM460 cell line, determined by SRB assay. NCM460 were incubated for 48 hours, with increasing concentrations of tebuconazole (*n*=5) and cymoxanil (*n*=4) on independent experiments. (A) The percentage of cell growth relative to the negative control was determined after 48h and is expressed as a mean ± SD for each treatment, from at least four independent experiments. (B) IC_{50} values determined using GraphPad Prism 6 software, applying a dose vs response non-linear regression.

4.2. Fungicides tebuconazole and cymoxanil induce cell death

After determination of the IC_{50} of tebuconazole and cymoxanil, we determined their effect on cell death. We performed TUNEL assay by fluorescent microscopy to assess DNA strand breaks, characteristic of cells undergoing apoptosis. We exposed NCM460 cells to the IC_{50} of both pesticides and double the IC_{50} concentration ($2xIC_{50}$): 50.72 µg/ml (IC_{50}) and 101.44 µg/ml ($2xIC_{50}$) of tebuconazole, and 115.60 µg/ml (IC_{50}) and 231.20 µg/ml ($2xIC_{50}$) of cymoxanil. As a positive control, cells were treated with 250 µM of H_2O_2, a condition that was reported to cause apoptosis (Fleury, Mignotte and Vayssière, 2002; Oliveira *et al.*, 2015).

The highest tested concentrations of both pesticides led to an increase in TUNEL positive cells (60.66 % and 46,29 % for tebuconazole and cymoxanil, respectively, n=4), which suggests that cells could be dying through apoptosis in those conditions. However, for lower concentrations, although we could observe an increase in the TUNEL positive cells relatively to the negative control, namely in cells exposed to cymoxanil, a statistically significant increase of positive cells was not observed (3.81 % and 6.64 % for tebuconazole and cymoxanil, respectively, *vs.* 2.02 % for negative control; n=4) (**Figure 12**).

The Annexin V/PI assay was performed by flow cytometry to compare with the results obtained with TUNEL assay, in order to differentiate early apoptosis from late apoptosis and necrosis. In the three experiments, after 48 hours of exposure, the presence of a high percentage of cells positive for both annexin V and propidium iodide (PI) was evident for all the conditions, with exception of IC_{50} of tebuconazole (73.47% of double negative cells) (**Figure 13**). Cells positive for annexin V and propidium iodide (PI) are commonly interpreted as being cells in late apoptosis or necrosis, and therefore our results suggest that cells exposed to both concentrations of cymoxanil or to greater concentrations of tebuconazole, for a period of 48 hours or more are in late apoptosis or necrosis.

These results also suggest that tebuconazole induces less cellular death than cymoxanil comparing the results obtained with the IC_{50} of both fungicides. Additionally, at the highest concentration ($2xIC_{50}$) of tebuconazole, in our results was the only condition in which cells positive for PI and negative for annexin V (generally considered necrotic cells) were more representative than cells positive for annexin V and negative for PI (generally considered early apoptotic cells), what similarly occurred on cells treated with the positive control.

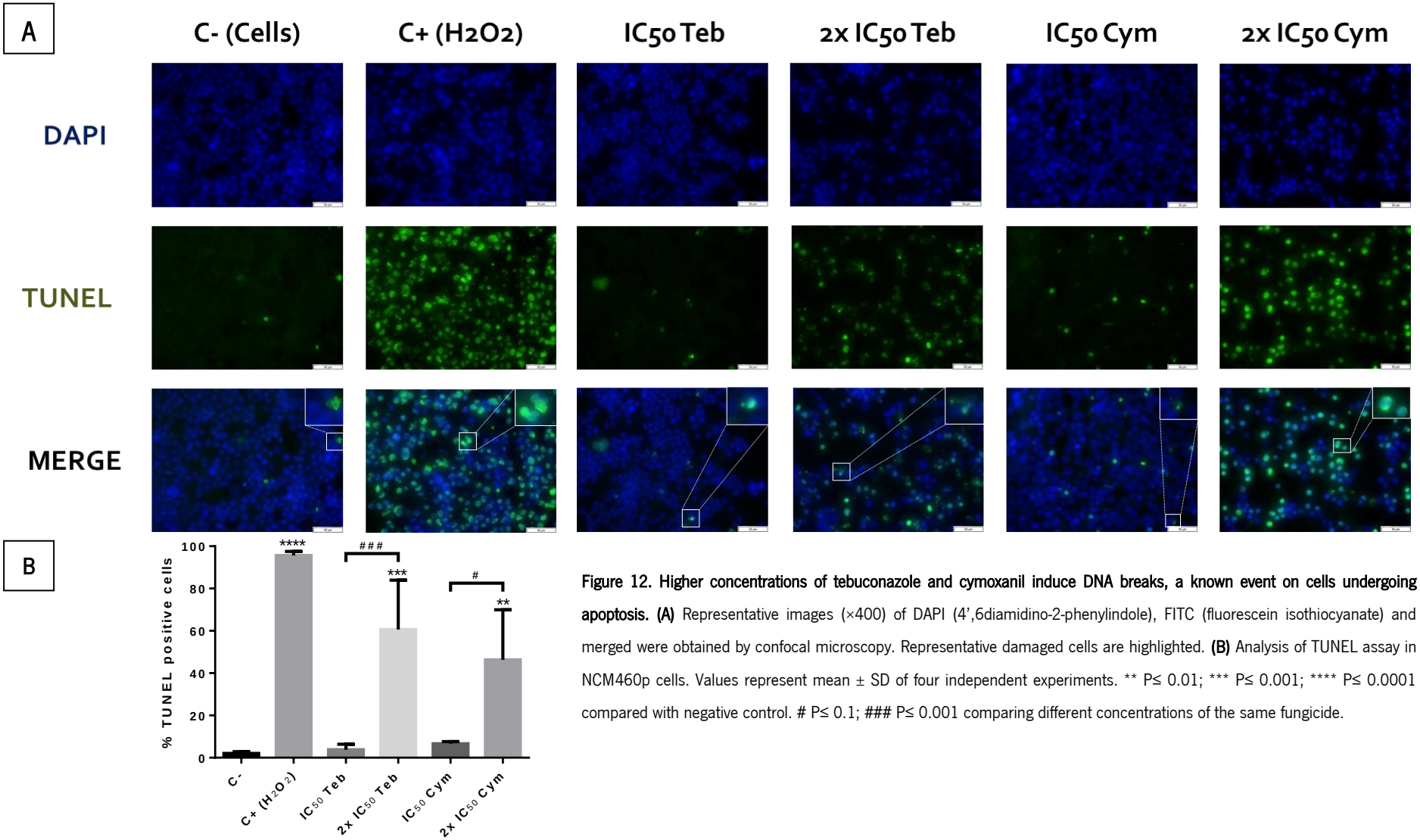


Figure 12. Higher concentrations of tebuconazole and cymoxanil induce DNA breaks, a known event on cells undergoing apoptosis. (A) Representative images (×400) of DAPI (4',6diamidino-2-phenylindole), FITC (fluorescein isothiocyanate) and merged were obtained by confocal microscopy. Representative damaged cells are highlighted. **(B)** Analysis of TUNEL assay in NCM460p cells. Values represent mean ± SD of four independent experiments. ** P≤ 0.01; *** P≤ 0.001; **** P≤ 0.0001 compared with negative control. # P≤ 0.1; ### P≤ 0.001 comparing different concentrations of the same fungicide.

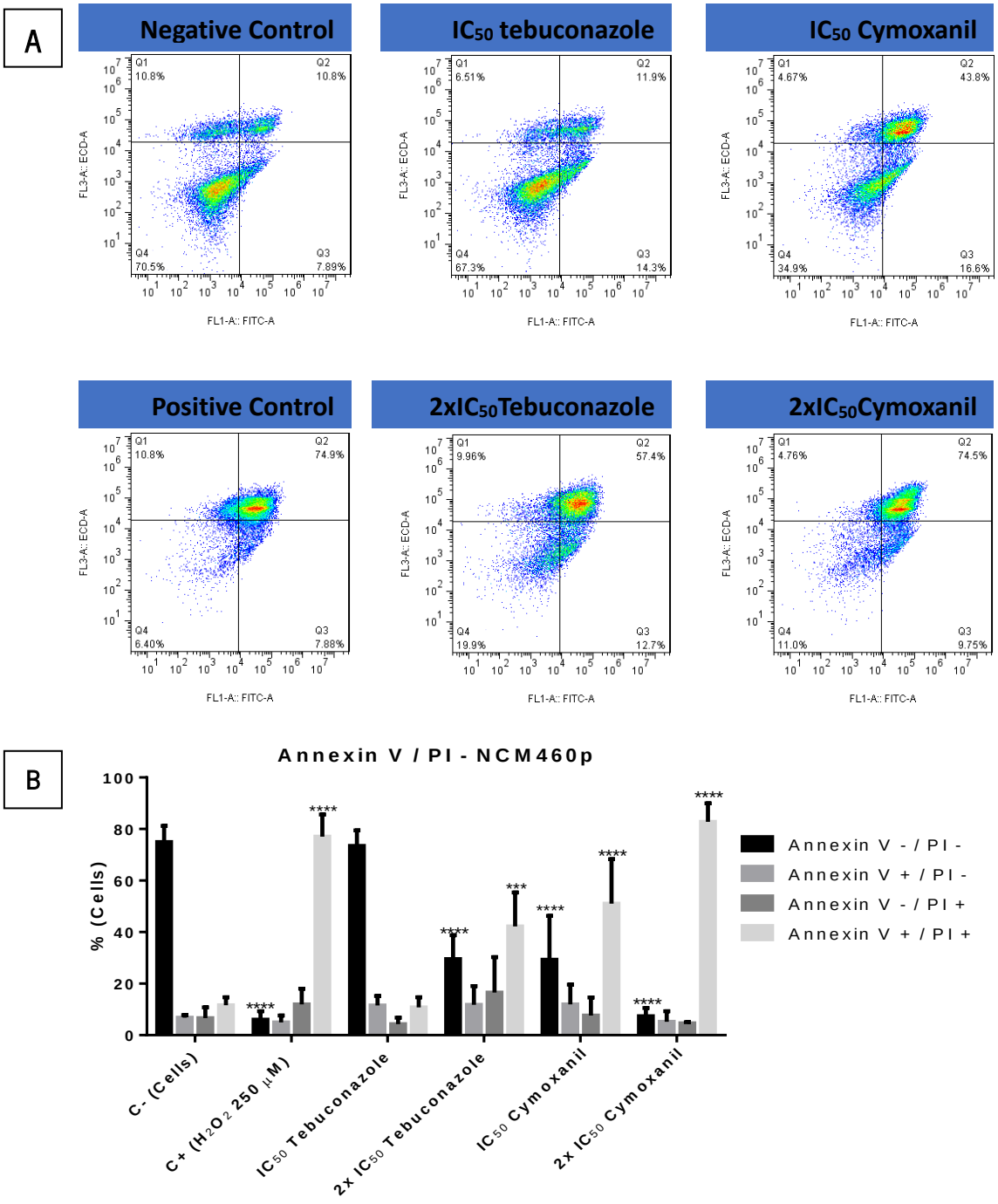


Figure 13. NCM460p cells tend to be in late apoptosis or necrosis when exposed to the tebuconazole or cymoxanil for 48 hours. Annexin V/PI assay was performed by flow cytometry, after cells being exposed to two different concentrations of tebuconazole and cymoxanil, separately. **(A)** Representative dot plots obtained using FlowJo 7.6.1 version software. **(B)** Analysis of Annexin V/PI assay in NCM460p cell line. Values represent mean ± SD of four independent experiments. ** P≤ 0.01; *** P≤ 0.001; **** P≤ 0.0001 compared with negative control. # P≤ 0.1; ### P≤ 0.001 comparing different concentrations of the same fungicide.

4.3. Fungicides increase ROS production

To evaluate if tebuconazole and cymoxanil may interfere with excessive ROS formation, the previously tested conditions were used and after 48 hours were stained for 30 minutes with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), a reduced form of fluorescein used as an indicator of oxidative stress. This assay was performed with flow cytometry analysis.

Our results for the IC_{50} of both compounds show that both pesticides induce the formation of ROS in NCM460 cells. This suggests that these conditions have a correlation with the action of both fungicides. A percentage of positive cells for DCF signal reached values near those obtained with cells exposed to 250 μM of H_2O_2 (positive control). These results were significantly different when compared with the negative control ($p<0.0001$ for tebuconazole and $p<0,001$ for cymoxanil).

Concerning the $2xIC_{50}$ concentrations of each fungicide, the results were different, while cells exposed to $2xIC_{50}$ concentration of tebuconazole still had a significant increase in ROS formation compared to the negative control ($p<0.001$), the $2xIC_{50}$ concentration of cymoxanil showed results similar to the negative control (**Figure 14, 15 (A)**).

In terms of fluorescence intensity, statistically significant values were not obtained. Despite the fact that this analysis shows a similar tendency as the analysis of the percentage of positive cells, the irregularity of the intensity among the 3 replicas resulted in high standard deviations (**Figure 15 (B)**). Significant differences ($P\leq 0.01$) were only obtained for cells treated with $2xIC_{50}$ of tebuconazole. With the IC_{50} we could observe a slight increase in the fluorescence, but due to the small number of replicates of this experience, it was not possible to determine significant differences.

For the $2xIC_{50}$ concentration of cymoxanil, the signal decreased in both analyses (for the percentage of DCF positive cells and for the DCF fluorescence).

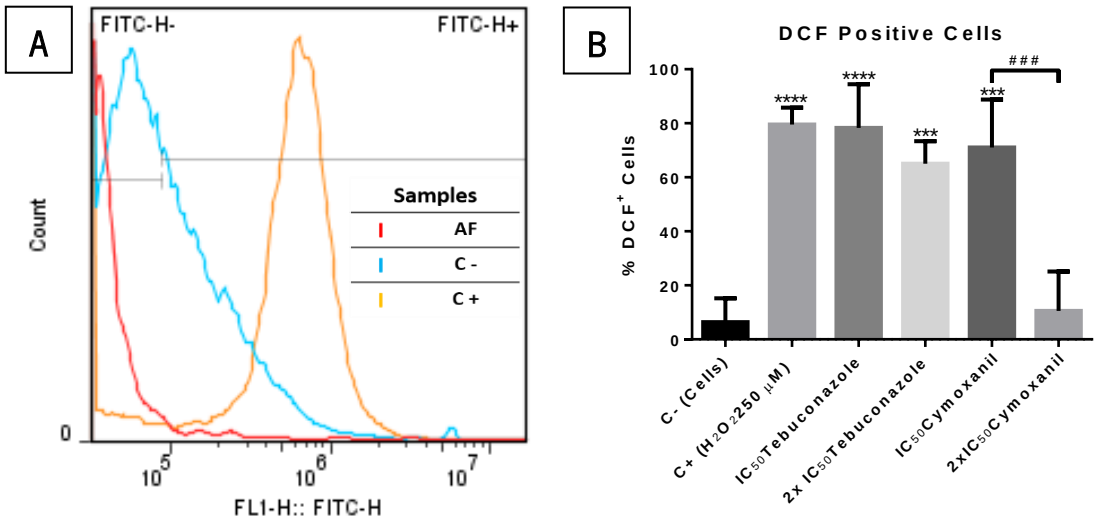


Figure 14. All the tested conditions but the highest concentration of cymoxanil induced an increased population of NCM460p cells producing reactive oxygen species (ROS). (A) Set the marker to define positivity. **(B)** Analysis on the percentage of positive cells for DCF signal of 3 different experiments in NCM460p cells. After being exposed to tebuconazole or cymoxanil for 48 hours, cells were incubated with H_2DCFDA to assess oxidative stress. ### P≤ 0.001 comparing two different concentrations of the same fungicide.

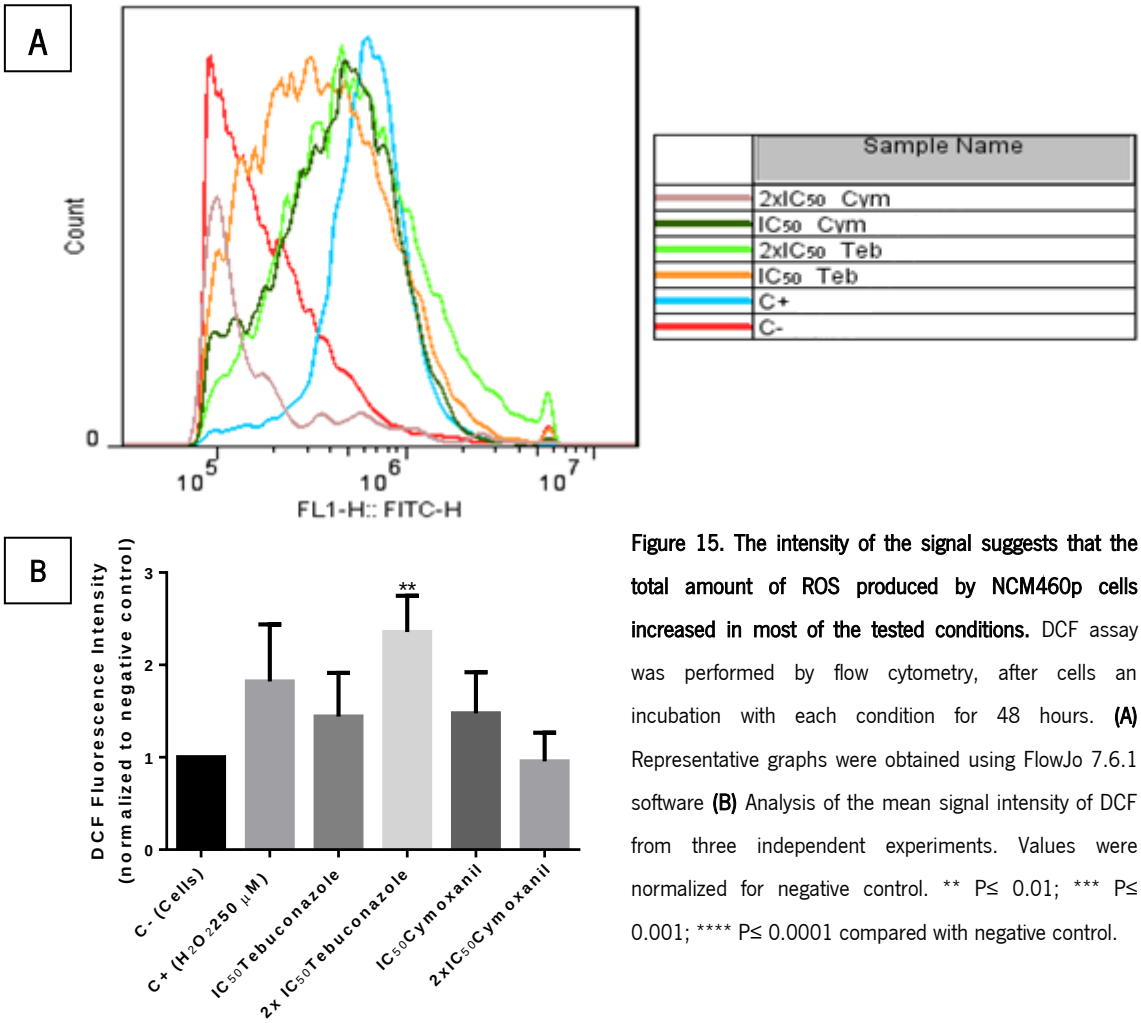


Figure 15. The intensity of the signal suggests that the total amount of ROS produced by NCM460p cells increased in most of the tested conditions. DCF assay was performed by flow cytometry, after cells an incubation with each condition for 48 hours. **(A)** Representative graphs were obtained using FlowJo 7.6.1 software **(B)** Analysis of the mean signal intensity of DCF from three independent experiments. Values were normalized for negative control. ** P≤ 0.01; *** P≤ 0.001; **** P≤ 0.0001 compared with negative control.

4.4. Fungicides might inhibit cell proliferation

We used the colony formation assay in order to test if the studied pesticides were able to inhibit cell proliferation in normal colon NCM460 cells. This technique requires isolated colonies of cells in order to be possible to count the final colonies by the naked eye. The number of plated cells is generally much lower for this technique than concentrations generally used in most assays. NCM460 cells are not easy to form isolated colonies because this cell line is more dependent on the cell-cell contact in order to grow and divide faster. We needed isolated colonies, resulting from successive divisions of a single cell. Different cell densities were tested and determined that plating 300 cells/ml was the cell density that allowed obtaining visible colonies after 14 to 15 days. Due to the reduced size of the resulting colonies (**Figure 16 (A)**), we took photographs of the individual wells and counted the colonies using image J software with a manual cell counter.

In this assay, we had to adjust the tested concentrations because most drugs started affecting cell proliferation at lower concentrations, in comparison with concentrations required to induce cell death. Moreover, due to the lower number of cells plated in this assay, the cells became more sensitive, as a result of a higher ratio of fungicide per cell. We decided to adjust the tebuconazole concentration to the maximum allowed concentration in most vegetables, which corresponds to approximately 10% of our calculated IC_{50}, 5 μg/ml. We also used 10% of the cymoxanil IC_{50}, corresponding to 12 μg/ml. We also tested 50% of the IC_{50} of each fungicide, approximately 25 μg/ml and 58 μg/ml for tebuconazole and cymoxanil, respectively.

Regarding the results, cymoxanil decreased the number of colonies in all experiments at both tested concentrations, while the results of tebuconazole were not so clear in all experiments. In some wells, cells were able to form more colonies after exposure to the lower concentration of tebuconazole, but in the remaining conditions, there was always a decrease in the number of colonies. Overall, with the IC_{50} of tebuconazole, we could observe a slight decrease in the number of final colonies, but this difference was not statistically significant. At the concentration of 25 μg/ml, the number of colonies decreased to values corresponding to nearly 50% of the number of colonies observed in the negative control, suggesting tebuconazole affects cell proliferation at this concentration (**Figure 16 (B)**).

In wells where cells were treated with cymoxanil, the changes were more evident. At the lowest tested concentration of cymoxanil, the average number of colonies per well was near the

number of colonies obtained in wells treated with 25 µg/ml of tebuconazole. These results suggest that the effect of cymoxanil on the inhibition of proliferation of normal colon cells is higher than that of tebuconazole (**Figure 16 (B)**). On cells treated with 58 µg/ml of cymoxanil, in all the 6 wells from three independent experiments only 2 colonies were counted. By the fact that, at 24 hours and 48 hours after exposing the cells to fungicides, a great number of them was seen floating on the medium, it suggests that cymoxanil was affecting the colony formation by inducing cell death during the 48 hours of exposure and not by inhibiting proliferation.

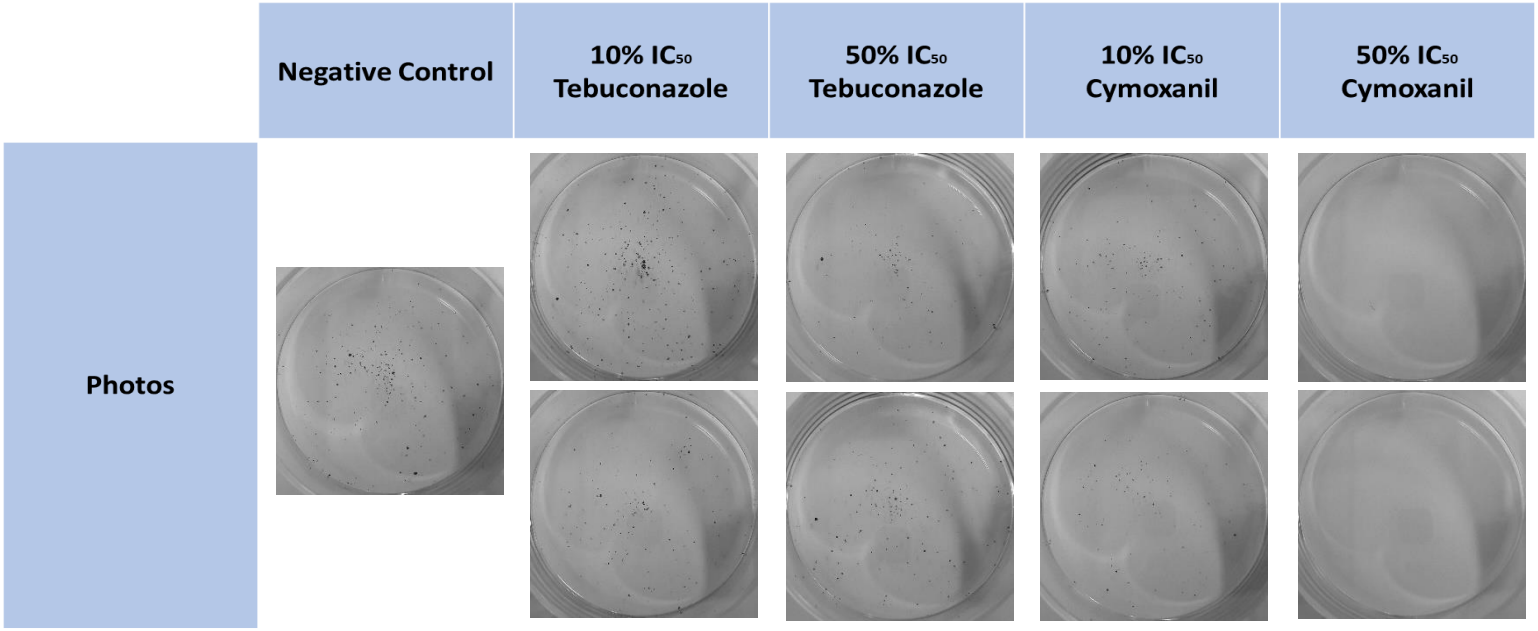
To confirm these results, we tried another assay, which merely assesses the number of cell divisions since the test is started until cells are collected. It is called carboxyfluorescein diacetate succinimidyl ester (CFSE) assay. This assay requires a larger number of cells, so we plated the same cells at the same density as used for annexin V/PI or DCF assays. Because of this, the concentrations tested for this assay were the same that we previously tested, i.e. the IC_{50} and $2xIC_{50}$ of both fungicides. Although some adjustments were required after the first tested protocol, which could still be improved (see discussion), it was possible to perform the experiment once.

For this assay, it was decided to test two different concentrations of etoposide in order to establish the proper positive control, since it was not found any published data regarding the best concentration to inhibit the proliferation of NCM cells. Through this experiment, we concluded that 60 µM etoposide was enough to inhibit cell division.

Regarding fungicides, with the IC_{50} of both compounds, cell proliferation was inhibited within similar levels to those obtained with positive controls, while with $2xIC_{50}$ of cymoxanil it seems that cells divided more. Cells treated with $2xIC_{50}$ showed an increased proliferation compared with the IC_{50}, although this difference was not so evident as it was with tebuconazole. These differences from the IC_{50} to the $2xIC_{50}$ of both fungicides may be associated with the increase in cell death that was previously verified (**Figure 17**).

Based on the occurrence of cell death with these conditions, we cannot make conclusions from these preliminary results. Besides that, there are some changes that could be implemented in this assay in order to make it easier to plan an appropriate experimental design and make conclusions from it such as testing lower concentrations and use propidium iodine (PI) to exclude cells PI positive cells, whose membrane integrity was compromised consequently probably dead.

A



B

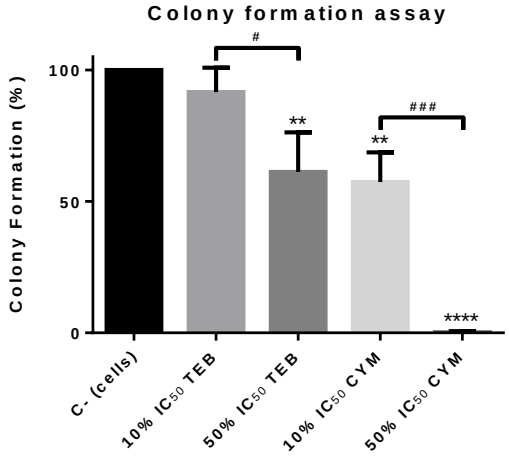


Figure 16. Effects of tebuconazole and cymoxanil on the capacity of NCM460 cells to form colonies. 600 cells per well were exposed to two different and low concentrations of tebuconazole and cymoxanil for 48 hours and left growing for the next 15 days after the first change of medium. The medium was changed 2 times a week. **(A)** Representative images of little NCM460 cell colonies formed 15 days after being exposed to pesticides. **(B)** Analysis of colony formation assay in NCM460 cells. Values represent mean ± SD of three independent experiments. ** P≤ 0.01; **** P≤ 0.0001 compared with negative control. # P≤ 0.1; ### P≤ 0.001 comparing two different concentrations of the same pesticide.

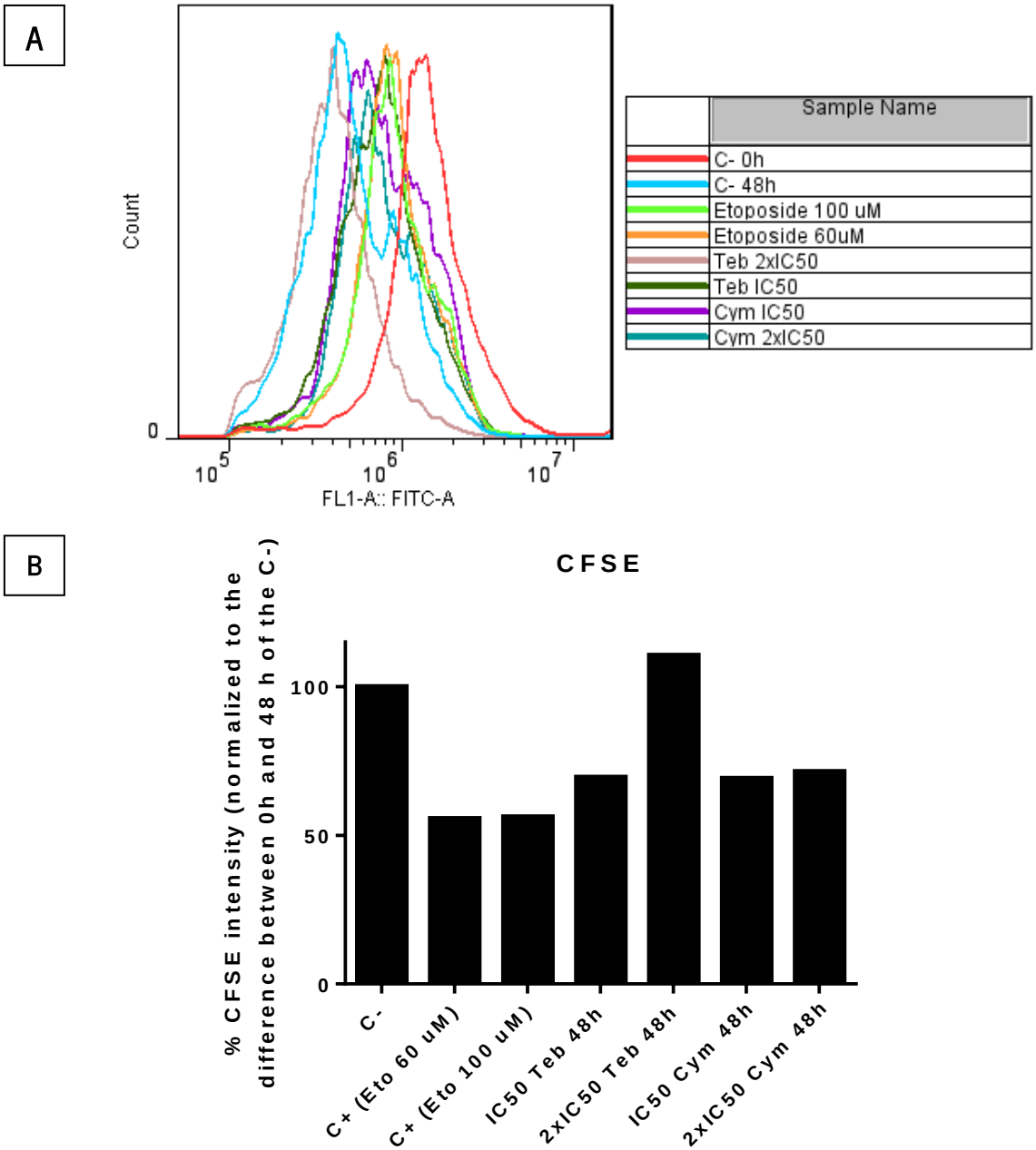


Figure 17. Effects of tebuconazole and cymoxanil on cell proliferation of NCM460 acssessed by CFSE assay. **(A)** Picks from one experiment were obtained using FlowJo 7.6.1 software. **(B)** Analysis of CFSE assay, representing the proliferation of cells on different conditions, comparing the difference of picks from the pick obtained at 0 hours, to 48 hours after exposition. All values were normalized to the negative control, assuming it represents a normal proliferation rate of the cell line.

4.5. Effect of tebuconazole in genomic instability

Another objective of this work was to study the mutagenicity of tebuconazole and cymoxanil. Yeasts and humans share many genes and proteins whose function is to detect and recover from genome damage, either caused by external factors or occurring naturally. This makes yeasts a powerful and more affordable model to study the effects of several conditions on DNA damage (Ribeiro *et al.*, 2000; Richardson *et al.*, 2007; Klionsky *et al.*, 2012; Mattiazzi, Petrovič and Križaj, 2012; Carmona-gutierrez *et al.*, 2018). For this purpose, we used the *Saccharomyces cerevisiae* model system, and the RDKY3615 reporter strain to assess whether the drugs induce genomic instability by fluctuation assay. This strain carries specific mutations that facilitate quantification of mutation rates. One of those is *hom3-10*, a point mutation on the *HOM3* gene (Erdeniz *et al.*, 2005).

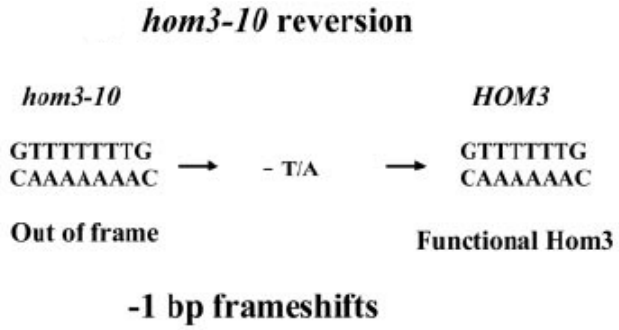


Figure 18. Representative reversion of the *hom3-10* mutation. To grow on CSM medium lacking threonine, RDKY cells need to do this revertion to make the HOM3 gene functional. Adapted from Erdeniz et al., 2005.

HOM3 encodes aspartate kinase (L-aspartate 4-P-transferase), a cytoplasmic enzyme necessary for threonine biosynthesis. If yeast *hom3-10* mutants are grown on a medium lacking threonine, cells will die unless they revert the mutation. This process can occur naturally in the whole genome, however specific mutations or exposure to mutagenic drugs or general cell stressors may increase its frequency. For instance, mutation of the *MLH1* gene, which plays a role in DNA repair, highly increases mutation frequencies. Because mutations occur in wild-type cells with very low frequencies often difficult to quantify, we used both RDKY3615 and an *mlh1* mutant for these assays.

First, we sought to determine the concentrations at which the yeast growth was affected by fungicides but without a complete loss of cell viability. We assessed growth curves of yeast cells exposed to 5 µg/ml and 20 µg/ml tebuconazole (based on results on another yeast strain background in another work within the EcoAgriFood project (C. Carvalho, 2018). In the end of the

experiment, aliquots of the cells were washed, diluted in 10x dilutions to a concentration corresponding to 10^{-4} of the original and plated on rich media before and after 24 hours of exposure to tebuconazole. Both strains were almost equally affected by the two tested concentrations, i.e. growth was slower after 3-4h of incubation and cells almost stopped growing between 7-8h (**Figure 19 (A) and Figure 20 (A)**). However, cells maintained high viability, as they were still capable of forming colonies when plated in media without tebuconazole, as seen in the spot assay (**Figure 19 (B) and Figure 20 (B)**).

With the fluctuation assay, our results were not conclusive. Although there was a tendency of a higher mutation rate when RDKY3615$_{wt}$ cells were exposed to 20 µg/ml tebuconazole compared to the negative control and to the cells exposed to 5 µg/ml tebuconazole, there was high variation in the results (**Table 2**). These differences resulted in a large standard deviation, which may have been the reason we could not calculate any statistically significant differences.

With the RDKY3615$_{\Delta mlh1}$ mutant strain, we were able to calculate mutation rates for all conditions, as expected, as the basal rates were higher. For that strain, statistically significant differences were observed between the lowest tested tebuconazole concentration (5 µg/ml) and the negative control, although this difference was low ($P \leq 0.1$). In this case, although higher mutation rates were always calculated relative to those calculated for the negative control, differences between the three replicas were generally low. At the higher concentration (20 µg/ml), even higher mutation rates were generally obtained, except for one of the assays where the mutation rate was within the range obtained with 5 µg/ml (**Table 3**). However, as observed with the wild-type strain, at this concentration there was a greater variation of the mutation rates, which makes it difficult to obtain statistically significant differences. Nonetheless, calculated values were higher than those for the lowest concentration tested and the negative control. This suggests that tebuconazole may be genotoxic in a dose-dependent manner. A greater number of trials would be necessary to lower the standard deviations for a definitive conclusion. Similar experiments with cymoxanil are planned.

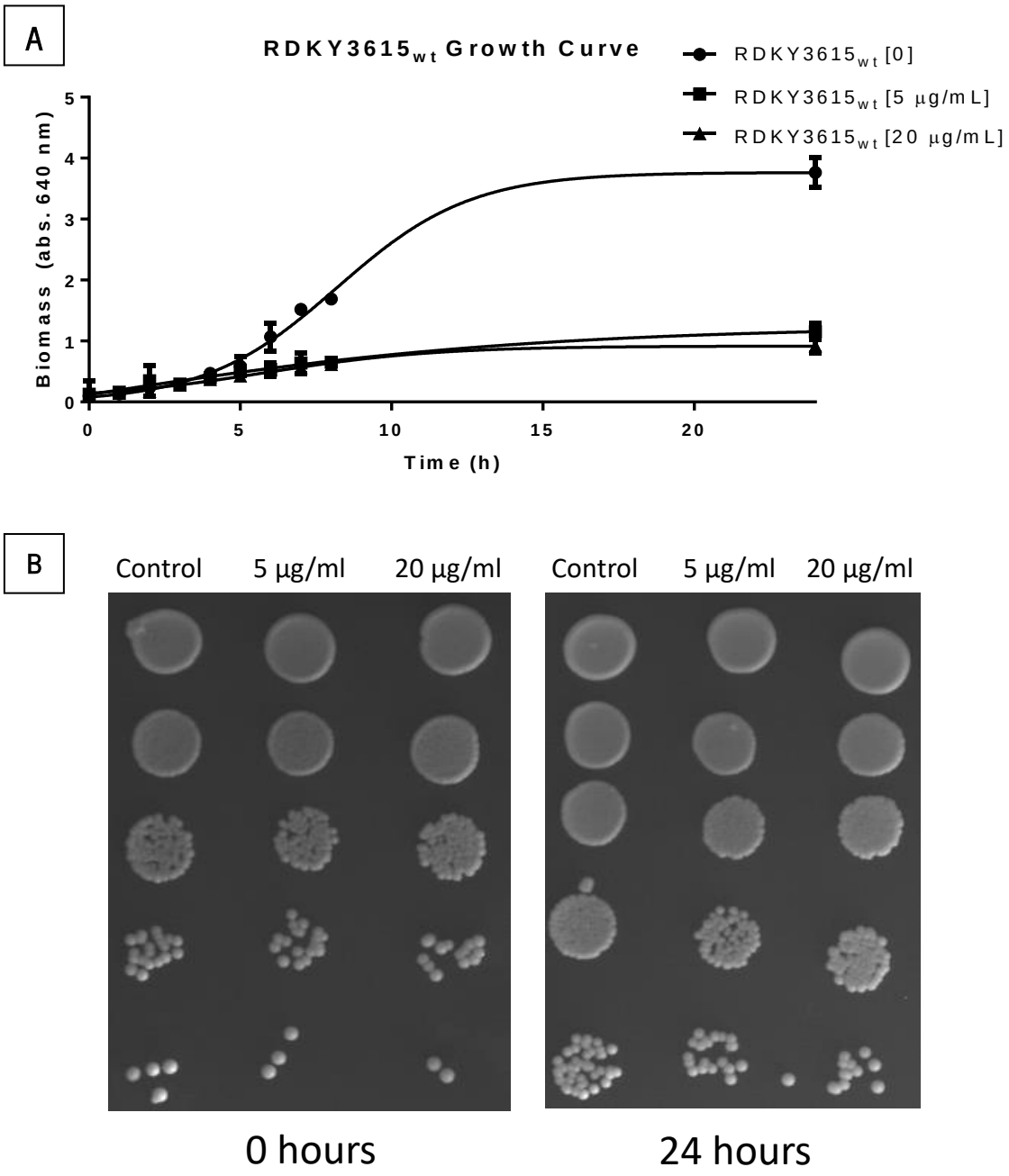


Figure 19. Response of *S. cerevisiae* wild type RKDY3615 to 5 and 20 µg/ml of tebuconazole in YPD. (A) Cells were diluted to an OD (640nm) = 0.1 and exposed to 5 and 20 µg/mL of tebuconazole for 24 hours in YPD. OD640nm was read every hour for 8 hours and at time point 24h. Values are mean ± SD of three independent experiments. **(B)** Viability of cells from **(A)** was assessed by spot assay at 0 hours and at 24 hours after exposure.

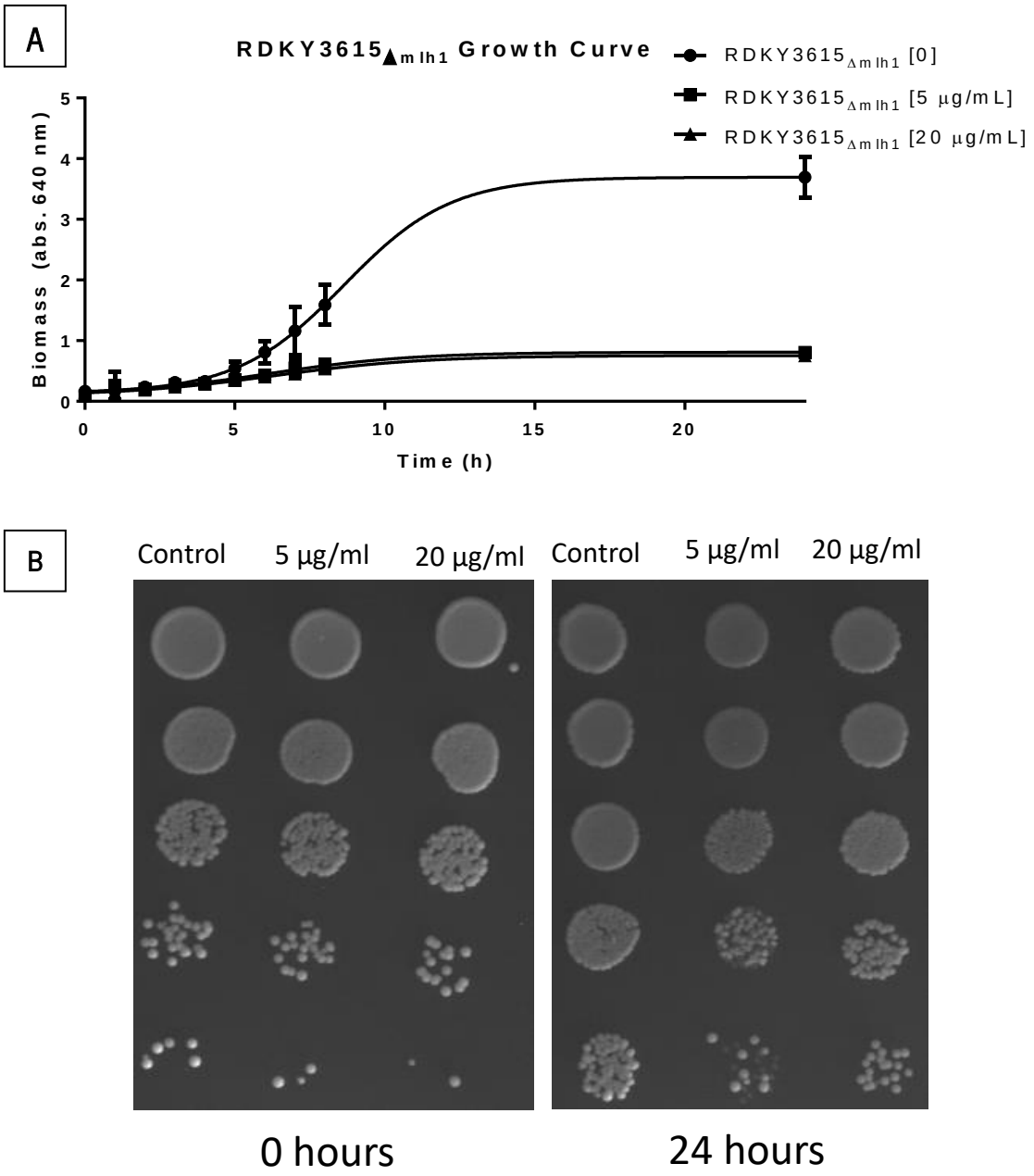


Figure 20. Response of *S. cerevisiae* mutated *mlh1* RKDY3615 to 5 and 20 µg/ml of tebuconazole in YPD. (A) Cells were diluted to an OD (640nm) = 0.1 and exposed to 5 and 20 µg/mL of tebuconazole for 24 hours in YPD. OD640nm was read every hour for 8 hours and at time point 24h. Values are mean ± SD of three independent experiments. **(B)** Viability of cells from **(A)** was assessed by spot assay at 0 hours and at 24 hours after exposure.

Table 2. Mutator phenotype of wild type RDKY3615 cells (mutations/cell)

Treatments	*hom3-10* (x10^{-10}) [a]	S.D. (x10^{-10})
DMSO	4.97	8,61
5 µg/ml	8.65	12,2
20 µg/ml	74.5	117

[a] Mean difference of three cultures. Values not statistically significant (One-way ANOVA, p>0.05)

Table 3. Mutator phenotype of mlh1Δ RDKY3615 (mutations/cell)

Treatments	*hom3-10* (x10^{-8})	S.D. (x10^{-8})
DMSO	1.67	0.52
5 µg/ml	2.75 [b]	0.57
20 µg/ml	4.81 [a]	2.2

Mean differences of three cultures. [a] Values not statistically significant (One-way ANOVA, p>0,05). [b] Value statistically significant (One-way ANOVA, p<0,1 comparing to control with DMSO)

5. Discussion

Pesticides are still the most used and perhaps the easiest tool used worldwide to control plagues and diseases in agriculture in order to decrease the loss of crops. The introduction of pesticides is considered a better way to increase food production, rather than exploit more land reserved for food production on a global scale (Forget, 1994; Popp, Pet and Nagy, 2013). Several agencies are responsible for regulating the use of pesticides and despite the published data and the results obtained on the agencies research, new pesticides were developed, and its use was allowed for the agri-food sector even before their potential effects were tested or investigated. As a consequence of the use of these new pesticides, several side effects may occur like affecting non-target species, including human well-being (Alavanja, Hoppin and Kamel, 2004; Sanderson, 2006; Popp, Pet and Nagy, 2013). Another risk associated with the use of pesticides is the resistance that many pathogens can develop, which ultimately reduces the effectiveness of these pesticides, forcing farmers to use higher concentrations of pesticides or the industry to design and introduce other pesticides in the market (Gullino, Mescalchin and Mezzalama, 1997; Tilman *et al.*, 2002). This creates a problem not only for human health in a direct way, but also for the environment and for the fresh waters near fields where these products are used, that will potentially affect the human well-being indirectly (Zimmer and Renault, 2003).

Various studies point to the possibility that the increased number of diagnostics of some diseases seems to be related the increased use of pesticides. Depending on the pesticide, dose, type or route, time and frequency of the exposure, pathologies like Alzheimer among other kinds of dementia, hormonal changes, infertility, physical and neurological development problem, whereas autism is included, chronic inflammation and many kinds of cancer are just some examples of pathologies that have been associated with exposure to pesticides all over the world (Betarbet *et al.*, 2000; Gauthier *et al.*, 2001; Cocco, 2002; Alavanja, Hoppin and Kamel, 2004; Roberts *et al.*, 2007; Koutros *et al.*, 2009; Remor *et al.*, 2009; Zaganas *et al.*, 2013; Zhang *et al.*, 2013).

European Union policy is directed towards significant reductions in pesticide use in the short to medium term and start adopting alternatives, claiming that the registration, that is mostly based on risk assessment for the environment, should also consider whether these products affect human health (Hillocks, 2012).

As an easy and frequent place where the exposure to pesticides takes place, the lower gastrointestinal tract is potentially one of the most affected regions in humans and mammals in general by the misuse of pesticides. A growing number of cases diagnosed with cancer in the gastrointestinal tract is a reality, within which the colon appears to be one of the most affected areas. An estimate points out that 75% or more of the risks of developing colon cancer can be attributed to diet (Wu *et al.*, 2015). Furthermore, cases of food poisoning are still occurring nowadays, within alarming numbers, particularly within the population that works directly in the handling and application of pesticides, particularly in developing countries. (Lee *et al.*, 2007; Macfarlane, Benke and Sim, 2010; Blair, Wesseling and Freeman, 2015). It is important to understand how the use of some of the most used pesticides, in Portugal and in the world, may be linked to some of these human health problems, thus in this work we aimed to study the possible effects of the exposure to two of the most used fungicides (tebuconazole and cymoxanil) in the human colon.

Analysis of the effects of tebuconazole on human health

There is a lack of studies regarding the concentrations at which tebuconazole can be present in different parts of the body, including small intestine, through dietary intake. Only one independent study regarding dermal exposure and inhalation faced by farmers is found in the literature (Fustinoni *et al.*, 2014). Fustinoni and colleagues determined that the maximum body exposure to tebuconazole should be 21500 µg in the whole body and concluded that the absorption of tebuconazole in winegrowers primarily occurs through skin absorption, which results in a slower and more persistent absorption over time, followed by inhalation (Fustinoni *et al.*, 2014). As far as we are aware, exposure through ingestion was not reported in this work or in any other article in the literature. This makes it difficult to predict concentrations at which tebuconazole might be present in the human colon, either by occupational or acute exposure. Due to the lack of this information, we started by assessing the concentrations at which this fungicide affects the overall cell growth of colon-derived cells. Our results showed that tebuconazole started to have an effect at concentrations of 30 µg/ml and above, and the IC_{50} in "normal" colon-derived cells NCM460 is 50.72 µg/ml. Tebuconazole is usually available on the market in solutions whose concentrations vary between 200 µg/ml and 250 µg/ml (Mesnage *et al.*, 2014) and is used in concentrations around 10 g/hl (100 µg/ml). *In vivo* test concluded that up to 98 % of tebuconazole is absorbed after oral administration within 48 hours (EFSA, 2008a), taking into account our data on the IC_{50} on human colon cells led us to conclude that

inappropriate working practices may put workers under risk from suffering from acute pesticide poisoning (Thundiyil, Stober and Pronczuk, 2008; Seidle, Prieto and Bulgheroni, 2011).

In our work, tebuconazole did not induce a significant increase in cell death at lower concentrations (50.72 µg/ml), however, it increased ROS formation and affected cell proliferation. Additionally, cell death was highly increased on cells exposed to higher concentrations of tebuconazole (101.44 µg/ml), having similar effects on ROS production and cell proliferation as those observed with the lowest concentration. Bcl-2 down-regulation and Bax overexpression have been recently reported to be correlated to the slightly increase of cell death in human placental trophoblasts (HTR-8) exposed to tebuconazole concentrations below those tested in our work (6.16 µg/ml – 24.63 µg/ml *vs* 50.72 µg/ml and 101.44 µg/ml) in which cell proliferation and changes the cell cycle distribution of G1 and G2 phases were observed (Zhou *et al.*, 2016). In the mitochondrial pathway, the caspases cascade is activated after a Bcl-2 downregulation, whereas accumulation of ROS is generally associated with the mitochondrial dysfunction itself (Moungjaroen *et al.*, 2006). We hypothesize that, at lower concentrations tebuconazole induces apoptosis through the mitochondrial pathway, started by an intercellular accumulation of ROS that leads to mitochondrial disruption, what might result in downregulation of Bcl-2. Since Bcl-2 is required to inhibit apoptosis in the mitochondrial outer-membrane, its downregulation triggers the caspase cascade through cytochrome *c* and therefore the apoptosis is triggered. To confirm this hypothesis, and because these other tests were performed in different cell lines and with different concentrations, it would be necessary to study the Bcl-2 expression and/or localization, as well as caspase activation under the conditions we tested. Western blot is an easy way to assess protein expression (Li *et al.*, 1999), while Bcl-2 localization could be assessed by fluorescent microscopy, which could be planned so that the mitochondrial disruption would be studied at the same time through a marker that is frequently used for that purpose – MitoTracker green. Using flow cytometry, this second marker could also be used along with PI in order to trace a correlation between membrane permeabilization (associated with cell death and assessed by PI) and mitochondrial disruption (Riccardi and Nicoletti, 2006; Fukumori *et al.*, 2013).

Regarding cell proliferation, Jinghua Zhou and co-workers have found that 24.63 µg/ml (80 µM) of tebuconazole increases the percentage of cells (human placental trophoblast - HTR-8) in G0/G1 phase and G2/M phase and decreased the percentage of cells in S phase (Zhou *et al.*, 2016). Increases in G1 and G2 phases are generally associated to DNA damage since in these

phases the cell passes through checkpoint control mechanisms to ensure everything is alright to start DNA duplication and mitosis, respectively (Cooper, 2000; Tanaka *et al.*, 2000). This led us to hypothesize that tebuconazole can induce genotoxicity in a ROS-dependent manner since at lower concentrations of tebuconazole a large amount of ROS is being produced, however not inducing cell death. As mentioned in the introduction, the excess of ROS production damages several cellular structures, including DNA (Fulda *et al.*, 2010).The inability to recover from damage in the DNA might result in permanent mutations, which is associated with several diseases, such as cancer and type II diabetes (Apel and Hirt, 2004; Ott *et al.*, 2007).

As a first strategy to easily quantify mutagenesis, we used a well-established yeast model, using *S. cerevisiae* reporter strains to perform fluctuation analysis: a wild-type and a *mlh1* mutant strain used to increase the sensitivity of this method by decreasing the effectiveness of the DNA mismatch repair mechanism. In addition, this strain reflects the increased predisposition that some people have to develop several kinds of cancer, namely people with Lynch syndrome or Muir-Torre syndrome, which are disorders that might be caused by one or two dysfunctional *mlh1* genes, respectively, or other genes involved in DNA repair when it is duplicating, which leads to microsatellite instability (Mathiak *et al.*, 2002; Martinez and Kolodner, 2010; Veloso *et al.*, 2010; Peltomaki, 2016).

We used cells exposed to 5 μg/ml and 20 μg/ml of tebuconazole, which inhibited growth but did not greatly affect viability (confirmed by spot assay). As expected, *S. cerevisiae* was more sensitive to tebuconazole than the cell line tested, as its main target is ergosterol biosynthesis, absent from mammals. However, it seems to inhibit the ergosterol synthesis by affecting the activity of cyp-51 of yeasts and the animal homologous protein is required on the cholesterol synthesis (Lamb, Kelly and Kelly, 1998), which could be one reason why tebuconazole decreased the cell proliferation in our experiments. By decreasing the cholesterol concentration in cellular membranes over some generations, membrane permeability and stability (mitochondrial membrane included) will be affected and thus this can be the event that starts mitochondrial disruption. Since yeasts proliferate much faster than mammalian cells, it is natural that the fungicide effects are felt sooner or at lower concentrations.

Regarding cell lines, studying the levels of cholesterol per cell could also be a way to test this hypothesis. The final amount of cholesterol per sample (cells exposed to the fungicide) could be assessed by a colorimetric enzymatic assay (Cholesterol Quantitation Kit, Sigma-Aldrich®), making it possible to calculate the amount of cholesterol per cell. For this test, using lower

concentrations (such as those tested in our colony formation assay) for a longer period would increase its relevance, since it would be possible to evaluate the effects of the maximum allowed concentration of tebuconazole in most fruits and vegetables, such as grapes, in cholesterol synthesis and therefore evaluate the effects of its dietary intake.

We observed that tebuconazole tended to increase the rate of mutations in yeasts in a dose-dependent manner. This assay requires a great number of repetitions in order to calculate statistically significant differences, and since we only performed 3 replicas, differences were not statistically significant. However, both mean and median reflect the differences that were generally observed among the 3 replicas in both tested strains. Although yeasts are eukaryotes, we cannot conclude that tebuconazole is a mutagenic fungicide for higher organisms, such as humans, at least with the same concentrations. For that, other kinds of studies are necessary, either *in vivo* or with cell lines. Microsatellite instability can be assessed through PCR followed by capillary electrophoresis and analysis of allelic profiles of microsatellite markers in cell lines (Li *et al.*, 2004).

Since the target of this fungicide is not the DNA, we hypothesize that these effects can have at least two different origins. On one hand, as an alternative mode of action, this fungicide can affect DNA stability. By inhibiting ergosterol synthesis, it alters the stability and permeability of cellular membranes, thus increasing the exposure of the nucleus, and therefore the DNA itself, to components that otherwise would never cross the nuclear membrane. These elements, including tebuconazole itself or any of its metabolites, might form adducts on DNA (Li *et al.*, 2004). Curiously, vinyl chloride (known to cause DNA adducts) is used to synthesize organochlorines such as tebuconazole, thus being one of the possible products of its degradation (Keppler *et al.*, 2002; Bartsch, Arab and Nair, 2009). On the other hand, if tebuconazole also induces apoptosis through the mitochondrial pathway in yeast, as we have seen in human cells, then there is a strong probability that the damage on DNA is a consequence of ROS overproduction, which directly damages the DNA and can damage several proteins involved either in its replication and in its repair (Ott *et al.*, 2007; Fulda *et al.*, 2010).

Analysis of the effects of cymoxanil on human health

There is also a lack of studies regarding the distribution of cymoxanil along the digestive tract or where the higher concentrations can be found after oral exposure. To the best of our knowledge, the cymoxanil mode of action is still unknown.

Cymoxanil is sold in powder at concentrations ranging between 4.5 % and 6.6 % w/w (weight/weight). It is further diluted in water, being subsequently applied, usually several times depending on several factors, at a recommended concentration of 12 g/hl (120 µg/ml) or above, because the occurrence of resistant fungi was reported after application of lower concentrations (Gullino, Mescalchin and Mezzalama, 1997).

We determined that its IC_{50} on normal colon cells is 115.6 µg/ml, only observing effects at concentrations above 100 µg/ml. Although these concentrations are close to recommended concentrations on the field, no independent studies can be found regarding where the major absorption of cymoxanil occurs or regarding its distribution along the digestive tract after oral administration. It seems possible that cymoxanil might represent a risk for workers who are not carefully protected when applying it. On the other hand, the IC_{50} that we determined is much higher than the maximum allowed concentration for grapes (0.1 µg/ml) which, considering its short persistence, suggests that its maximum allowed concentration is safe for consumers.

In yeasts, cymoxanil has been reported to affect the respiration rate (Ribeiro *et al.*, 2000) which, since this process occurs in the mitochondria, suggests that cymoxanil induces cell death through a mitochondrial dysregulation. Other possibilities are the inhibition of sphingolipid synthesis, which occurs in the ER in mammalian cells, or the pyrimidines biosynthesis by affecting the dihydroorotate dehydrogenase activity. This enzyme is present in yeast cytosol, and the human ortholog is located in mitochondria, where it also participates in the electron carbon chain and is associated with the overproduction of ROS and induction of apoptosis in colon carcinoma cell lines (Nagiec *et al.*, 1994; Vorísek *et al.*, 2002; Yasuda, Nishijima and Hanada, 2003; Lum *et al.*, 2004; Hail *et al.*, 2010). This suggests that, in mammalian cells, the mitochondria might be the most affected organelle and that it will affect cells in a ROS-dependent manner, either by increasing its production affecting the mitochondria in a non-specific manner or decreasing the ROS production consequent of the decrease in dihydroorotate dehydrogenase activity.

Our results suggest that, at the lowest tested concentration (115.6 µg/ml), cymoxanil induces cell death in a ROS-dependent manner since when exposed to this concentration for 48 hours, cells tended to be positive for both Annexin V and PI (interpreted as late apoptotic/necrotic cells), even though TUNEL assay was not so sensitive since positive cells were only noticed at the highest concentration (231,2 µg/ml). With the same condition (115.6 µg/ml of cymoxanil, 48 hours), both fluorescence intensity for H_2DCFDA and percentage of ROS positive

cells were increased, suggesting the increase of apoptosis was related to the ROS production, which relates with the lower respiration rates (Ribeiro *et al.*, 2000) found in yeast, but not with the decrease of dihydroorotate dehydrogenase activity, which in this case would theoretically negatively influence the production of ROS. Nevertheless, we cannot exclude the last hypothesis, since at the highest tested concentration a deep decrease on both fluorescence intensity for H_2DCFDA and percentage of ROS positive cells was observed. We initially interpreted this as being a consequence of a major membrane disruption, which would relate with inhibition of sphingolipid synthesis, because sphingolipids play a role on membrane stability and permeability (Pascher, 1976), resulting in the loss of the marker (H_2DCFDA) inside of the cells. The use of PI in this assay would allow us to separate cells whose cell membrane is permeabilized (commonly associated with cell death) from those with a non-permeabilized cell membrane (Riccardi and Nicoletti, 2006) and analyse them separately, which could provide answers in order to understand whether dead cells are still able to produce ROS, although the number of cells to be analyzed had to be greater.

The occurrence of cell death was also a problem in both assays used to determine the effects of cymoxanil on cell proliferation. Detached cells were observed 24 and 48 hours after exposure to cymoxanil and this prevents us from concluding that the loss of colonies is due to inhibition of cell proliferation. We then tried to use the CFSE assay to assess the cellular proliferation, however, this technique is still being implemented in our laboratory. The assay is now being improved and we think that similarly to the DCF assay, the addition of PI could be useful in order to analyze exclusively live cells and therefore easily obtain more conclusive results. For cymoxanil particularly, by decreasing the tested concentrations in both proliferation assays, it would help us to get better results.

Summing up, there is still much work that needs to be done in relation to the risks associated with the use of fungicides, especially exploring possible long-term effects for the human well-being.

6. Conclusions and future perspectives

Although this project is still in the beginning and further studies are needed both with these fungicides and with others, we can raise some conclusions about the risks that exposure to both fungicides may represent for humans.

Regarding the effects of tebuconazole on the human colon, several conclusions can be made:

- The concentrations used in the agriculture in grapevines seem to be safe for humans in terms of acute toxicity. However, we observed a decrease in the levels of cell proliferation (although not statistically significant) and effects on DNA damage that need to be confirmed with other tests and may be associated with long-term effects;
- Studies are needed in order to clarify the distribution and absorption of this pesticide along the digestive tract, but it seems possible that high doses of this pesticide due to the lack of care in handling this fungicide may have implications in terms of acute toxicity, leading to the death of cells from the colon by apoptosis – effects that might be associated to acute pesticide poisoning (APP) (Thundiyil, Stober and Pronczuk, 2008);
- There is an increase in ROS production that seems to be related to induced cell death within cytotoxic concentrations. This data is in accordance with another study that suggests that tebuconazole activates apoptosis via the mitochondrial pathway (Zhou *et al.*, 2016). Although it is not clear whether this is a cause or a consequence of the apoptosis activation, it has been suggested that this has some implication to the cell, namely damaging several structures (Fulda *et al.*, 2010).

Regarding cymoxanil, it is necessary to uncover the mode of action in the target species, as well as the possible effects on other species. With this work, we could reach some conclusions concerning the prediction of its effects in the human colon:

- Since this fungicide has almost no potential to bioaccumulate, and given the difference between values from which this fungicide had effects on the cell line tested, and those who are legally accepted in food, we can suggest that its presence in food does not represent a danger to the human colon in terms of acute toxicity;
- Although less likely in comparison with tebuconazole, based on our results, it is possible that the lack care in handling this fungicide may have implications for people who work directly with it. However, further studies are needed to focus on their distribution and absorption along the digestive tract after ingestion;

- There seems to be a relationship between cell death caused and the increase in ROS production, which is in agreement with other articles suggesting that the action of cymoxanil may interfere with processes occurring in the mitochondria (Ribeiro *et al.*, 2000; Lum *et al.*, 2004);

Briefly, our results suggest that both fungicides, in cytotoxic concentrations, affect cells mainly inducing cell death accompanied by an increase in ROS production. In terms of chronic or long-term effects, at low concentrations, even within legally accepted concentrations in food, they seem to inhibit cell proliferation, although in the case of cymoxanil more studies are needed to confirm it. Moreover, the assays that we performed to evaluate the mutagenic potential of Tebuconazole using the yeast model, point to the possibility of being a mutagenic agent but we still need to perform more replicas. It would be important to also evaluate the possible mutagenic effects of cymoxanil, and confirm those same effects in human cell lines, for which further assays will be required.

Regarding future perspectives, we have other suggestions in addition to the suggestions that we have made on discussion section that were related to the work already performed. Since it is known that there is a strong connection between the cells from the colon epithelium and its microflora, it would be important to study the role of the microbiota and metabolites produced by them on the protection, availability and effect of these fungicides in the colon and how it might have some impact on disease prevention in the colon, such as colorectal cancer (Marques *et al.*, 2013; Oliveira *et al.*, 2015).

It would also be interesting to study how the tebuconazole is distributed and absorbed along the digestive tract through animal experiments. In this case, after oral administration of various concentrations of fungicide, the animals would be euthanized, the various segments of the digestive tract would be removed, separated and homogenized. The fungicide could be quantified, as well as its main metabolites, via liquid chromatography-triple quadrupole mass spectrometry (LC-MS/MS). Silvia Fustinoni *et al* determined the concentrations of this fungicide to which workers can be exposed to. Their protocol could be adapted to study the distribution of this fungicide along the colon (Fustinoni *et al.*, 2014). There are indications that tebuconazole is almost fully absorbed within the first 48 hours, thus we could perform the analysis earlier than that (EFSA, 2008a). Similarly, something similar could be performed with cymoxanil, however, for

this case we did not find any case of monitoring or quantification of this pesticide in animal tissues, so most likely we would have to develop a new protocol.

Lastly, it is known that deregulation of autophagy are associated with some types of cancer and inflammatory diseases (Pattingre *et al.*, 2005; Mostafalou and Abdollahi, 2013; Zhou *et al.*, 2016). Thus, studying the levels of autophagy might be relevant since it has been observed changes at the expression levels of the Bcl-2 protein on cells exposed to tebuconazole which, in addition to inhibiting apoptosis, is important in regulating the levels of autophagy by directly interacting with Beclin 1 (Pattingre *et al.*, 2005; Zhou *et al.*, 2016). By this way, it would be interesting to study the expression of proteins related to apoptosis and autophagy, namely Bcl-2, Beclin1 and Bax, to assess potential changed induced not only by tebuconazole, but also by cymoxanil. Therefore, we could study the expression of such proteins at the IC50s that we have already calculated and lower concentrations (Wei *et al.*, 2001; Pattingre *et al.*, 2005).

7. References

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