REVIEW OF THE SCIENTIFIC AND TECHNOLOGICAL LITERATURE OF FUNGICIDES IN TANNERY INDUSTRY: REDUCING THE USE AND INCREASING THE EFFICIENCY OF FUNGICIDES IN THE LEATHER INDUSTRY

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Abstract. One of the main challenges of the tannery industry chain is to reduce the use of biocides and restricted substances and at the same time increase efficiency with the available products. Such conduct must permeate suppliers and the tanneries in order to obtain better results, diminish the biocide resistance dissemination, optimize costs and be ecologically friendly. In this sense, we present herein an updated review and discussion of the scientific and technological literature on the aspects involving the action of fungicides in tannery industry and how the application of this knowledge can reduce application of biocides and restricted substances in the tanning process. We have organized a review by consulting the databases PUBMED, Web of Science, Science direct, and all literature with excellence scientific support available. The review focused on: (i) Fungal diversity involved in wet-blue biodeterioration; (ii) Mechanisms of action of fungicides; (iii) Fungicide combinations to enhance activity; (iv) Fungal mechanisms of resistance and the known causes of resistance emergence. As a result of this study we are able to track the fungal phylogenetics (and relationship) responsible for leather biodeterioration enabling a guiding strategy for fungal biocide application. Moreover, understanding of the mechanisms of action and interaction between molecules can determines the extent of the biocides inhibitory effect in different fungal species. Fungicide effect could vary, and such information corroborates with the idea that even in the same species the interaction of the different molecules may vary, possibly due to variation in cytochrome protein. For example, the most accepted mechanism of action of *azoles* is the inhibition of synthesis of or direct interaction with ergosterol (present in all fungi). Considering that the target is always the same, a question arise, how do the distinct *azoles* present different activities upon fungal strains? As result of this study we show that structural differences will influence the higher or lower interaction of the azole functional group and consequently the activity. The appropriated knowledge of the mechanisms by which microbial cells might develop resistance, highlights the need for an improved understanding of the reasons for their emergence and greater attention to methods that can be used to prevent and control them. In this sense, a successful combination of biocide molecules enhances a synergetic effect, avoiding fungal mechanisms of resistance and reduces dosage of each compound, being effective against a variety of fungi.

1 Introduction

Leather is a biological product suitable to microbial growth due to the presence of proteins and lipids. Additionally, tanneries provide an interesting environment for microbiological development, since there are sources of nutrients and water along the complete process (**Fig. 1**). Particularly pickled pelts, wet-blues and vegetable tanned moist leathers are prone to microbial attack, even when stored or shipped[1,2].

After chrome or vegetable tanning, leathers and finished leathers contain several compounds, such as ammonium salts, phosphates, surfactants, fat liquoring agents and other organic agents enabling microbial - especially fungal growth causing damages on leather matter.

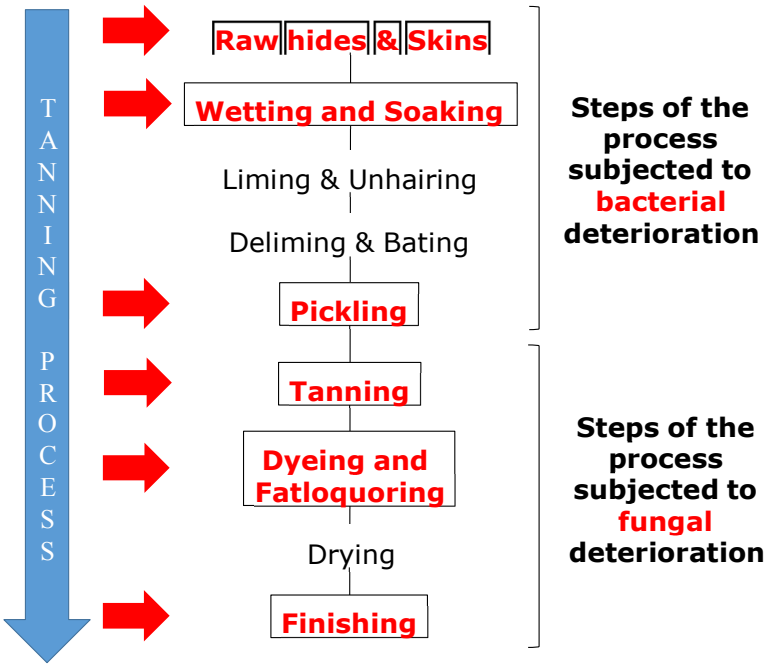

Fig. 1. General tanning process fluxogram. In red all steps in which is possible to use biocides.

2 Methods

The review presented herein was organized by consulting the databases PUBMED (PubMed comprises more than 29 million citations for biomedical literature from MEDLINE, life science journals, and online books), Web of Science, (a platform with more than 9,200 titles journals) and ScienceDirect (the world's leading source for scientific, technical, and medical research), and all literature with excellence scientific support available in books and internet.

3 Results

3.1 Environmental Conditions for Fungal Proliferation in Tanneries and Fungal Diversity Involved in Wet-blue Biodeterioration

Besides the fact that hides and leather are suitable substracts for microbial proliferation, other variables may directly influence the growth of fungi as well as bacteria. In this sense, some steps of the process could be cited as major problems which favor fungal growth:

- Tanning processes with quality deviations mainly related to insufficient degreasing and the presence of reducers compounds;
- Longer storage time than the half live of most of the fungicides;
- Poor storage conditions, with non-appropriated cleaning disinfection routines;
- Poorly controlled drying operations, where the humidity remains high or the drying process takes too long;
- Poor airflow;
- High temperatures inside the drying rooms.

Environmental conditions also have an influence on fungal diversity in leather contamination. Temperature, for example, directly impacts fungal growth. As we can observe in **Fig. 2** *Penicillium* sp. and *Scopulariopsis* sp. hardly grow in temperatures above 28 °C and most of fungi are not able to grow appropriately in temperatures around 45 °C[2]. In this sense, it is important to highlight that at 45 °C fungi are not necessarily dead, even though there is no colony growth. In this case (and on other stressful conditions) most fungi are able to generate spores, which are the most resistant form of fungi in nature. Then, when environmental conditions are favorable again, they can grow as usual.

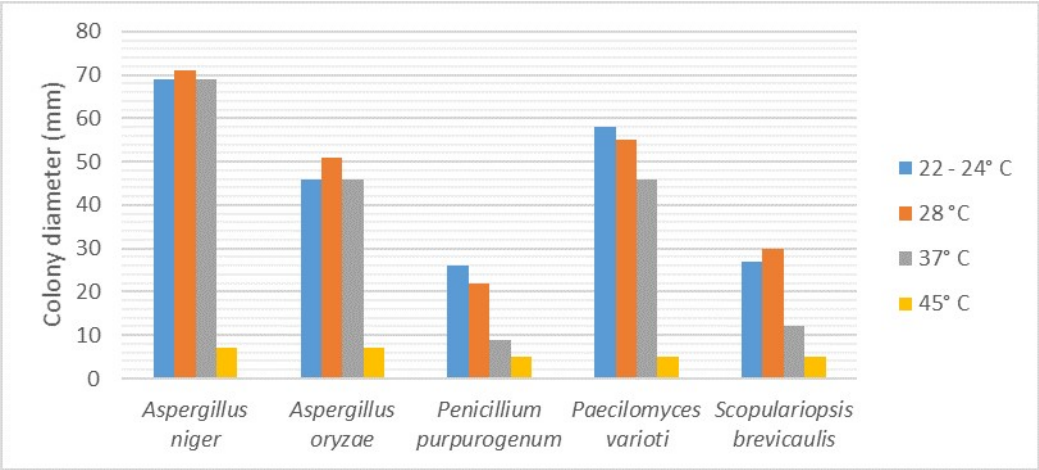

Fig. 2. Temperature influence in fungal growth. Adapted from 2.

Regarding the prejudices caused by fungal contamination diversity, the literature converges always to the same genera: Alternaria, Aspergillus, Mucor, Rhizopus, Paecilomyces, Penicillium and Trichoderma. Among them, many distinct species may appear, as indicated by different authors in the **Table 1**. On the other hand, no convergence is found on which is the most common fungi in general leather contamination. Some authors point out *Trichoderma* spp. as the most frequent[3] while others indicate *Aspergillus niger* as well as various species of Penicillium[4]. Other genera also are cited, such as *Cephalosporium, Cladosporium, Curvularia, Fusarium, Scopulariopsis and Verticillium*, but they are not commonly found on all types of leather samples [1,5,6] .

Table 1. List of fungi reported by distinct authors as source of damage in wet-blue leathers.

Most common fungi reported as agents of leather damage		Reference
Aspergillus sp. *Mucor* sp. *Paecilomyces variotii*	*Penicillium* sp. *Rhizopus nigricans* *Trichoderma viride*	3
Aspergillus niger *Aspergillus terreus* *Paecylomyces nivea*	*Penicillium sp.* *Trichoderma atroviride* *Trichoderma harzianum*	7
Alternaria alternata *Aspergillus niger* *Aspergillus oryzae* *Aspergillus wentii* *Aspergillus versicolor* *Aureobasidium pullulans* *Chaetomium globosum* *Cladosporium herbarum* *Cladosporium sphaerospermum*	*Paecilomyces variotii* *Penicillium commune Penicillium duclauxii* *Penicillium glabrum* *Penicillium ochrochloron* *Penicillium purpurogenum* *Penicillium rubrum* *Penicillium verrucosum* *Scopulariopsis brevicaulis*	2

Fusarium chlamydosporum *Myrothecium verrucaria*	*Trichoderma viride Verticillium tenerum*	
Alternaria geophila *Aspergillus niger* *Aspergillus chevalieri* *Aspergillus fumigatus* *Aspergillus conicus* *Aspergillus flavus* *Aspergillus terreus* *Aspergillus repens* *Aspergillus sulphureus* *Aspergillus tamari* *Aspergillus luchuensis* *Aspergillus amstelodami* *Aspergillus sydowii* *Botrytis cinarea* *Cladosporium herbarum* *Chaetomium globosum*	*Curvularia luneta Drechslera papendorfii* *Fusarium* sp. *Helminthosporium* sp. *Mucor ambiguus* *Paecilomyces varioti* *Penicillium asperum* *Penicillium camemberti* *Penicillium citrinum* *Penicillium funiculosum* *Penicillium oxalicum* *Penicillium purpurogenum* *Penicillium stipitatum* *Rhizopus nigricans* *Rhizopus oryzae* *Trichoderma lignorum*	4
Aspergillus niger *Aureobasidium pullulans* *Chaetomium globosum* *Cladosporium sp.* *Fusarium sp.Mucor sp.*	*Paecilomyces sp.* *Penicillium funiculosum* *Rhizopus stolonifer* *Trichoderma viride*	8
Aspergillus sp. *Mucor sp.* *Paecilomyces variotii Penicillium sp.*	*Rhizopus nigricans* *Trichoderma viride*	9

3.2 Mechanisms of Action of Fungicides

The tannery industry applies biocides along its production process chain in order to avoid leather damage due to fungal contamination. In summary, **Fig. 3** schematically represents the mechanisms of action of the fungicides most commonly used in this industry sector.

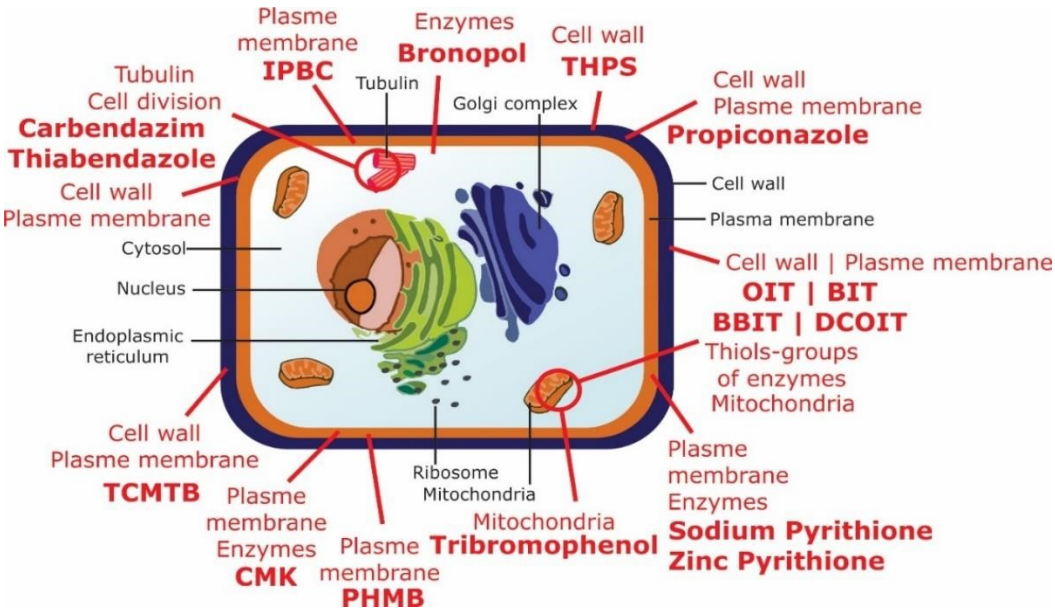

Fig. 3. Summary of the mechanisms of action of the main fungicides used in tannery industry.

In order to clarify the mechanisms of action of fungicides mentioned in **Fig. 03**, we present below an overview of the known fungal growth inhibition pathways may responsible for the biological activity of these biocides.

3.2.1 Azole compounds – mechanism of action

The use of AZOLE compounds as fungicides appeared as an important alternative to the use of amphotericin B, in the 1980`s. Azoles, as well as polyenes and allylamine/thiocarbamates, are nowadays the three major groups of antifungal agents in use.

The most accepted mechanism of action of AZOLES, (including TCMTB, propiconazole, carbendazim, OIT, BIT, BBIT, DCOIT and also thiabendazole) is based on the inhibition of ergosterol biosynthesis or on the direct interaction of azole compounds with it. Ergosterol is a key molecule in fungi, since it serves as a bioregulator of membrane fluidity and integrity.(**Fig. 04**). Importantly, even though cell wall components may drastically vary among species, ergosterol is a molecule found on all fungi cell membranes, despite fungal genus (**Fig. 05**). That is why azole compounds usually have a broad spectrum of activity.

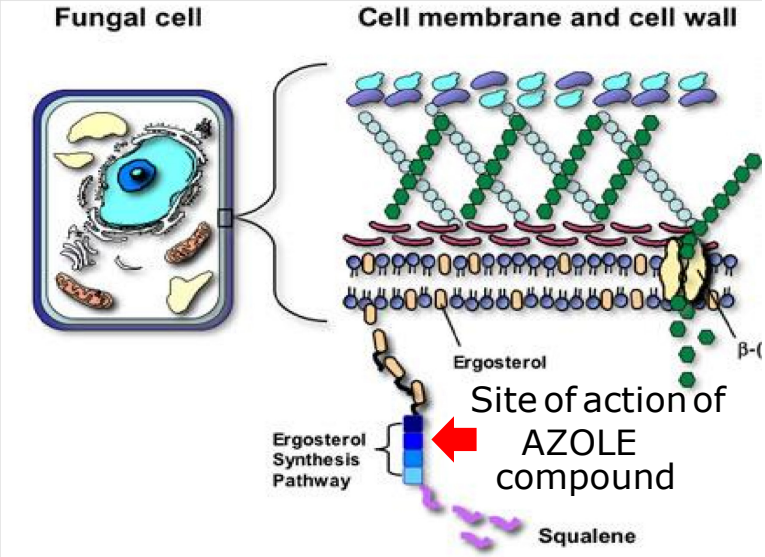

Fig. 4. Schematic representation of the mechanism of action of azole compounds. Red arrow indicates the target of azole compounds (adapted from 10).

However, considering that the target is always the same, a question may arise: *how do the distinct AZOLES exert different activities upon fungal strains?*

The primary target of azoles is the *heme* protein, which cocatalyzes cytochrome P-450dependent 14α-demethylation of lanosterol (**Fig. 06**). Inhibition of 14α-demethylase leads to depletion of ergosterol and accumulation of sterol precursors, including 14a-methylated sterols (lanosterol, 4,14-dimethylzymosterol, and 24-methylenedihydrolanosterol), resulting in the formation of a membrane with altered structure and function.

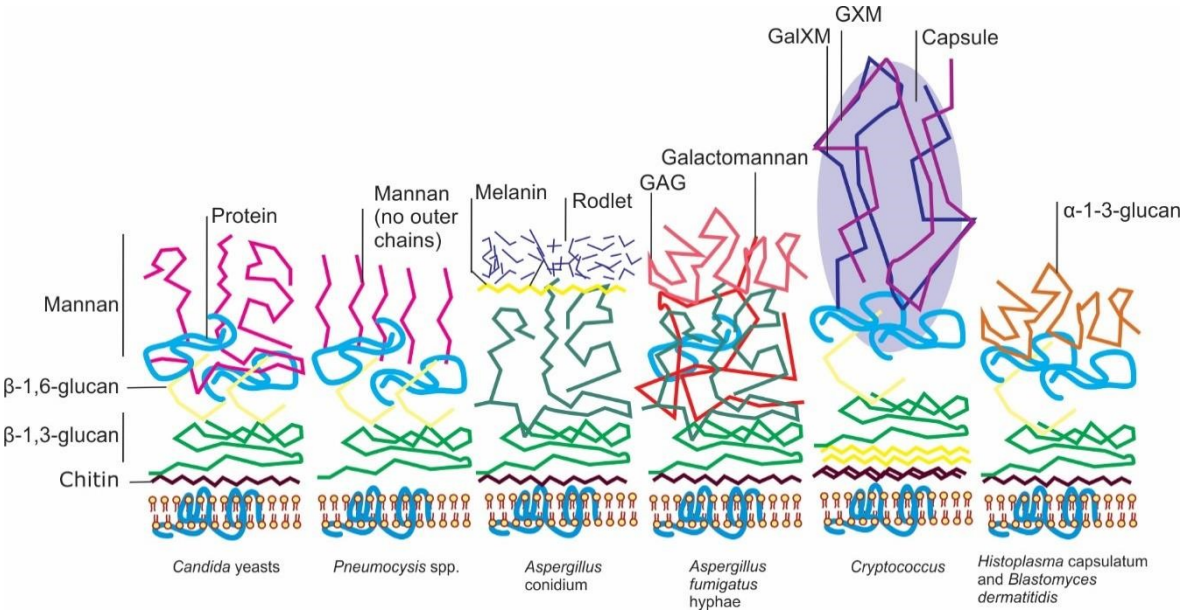

Fig. 5. Representation of the possible variation of the fungal cell wall in distinct genera (adapted from 11).

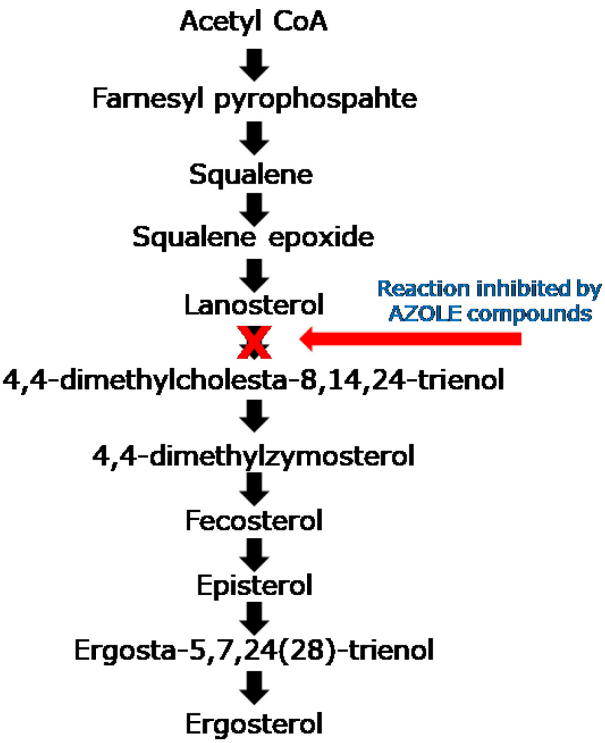

Fig. 6. Synthesis of ergosterol. Red arrow indicates the step which azole compounds are capable to inhibit.

Although contemporary azole antifungals are 14α-demethylase inhibitors, there is a heterogeneity of action among them. The main azole target, *cytochrome P450*, catalyses the oxidative removal of the 14α-methyl group of lanosterol and/or eburicol in fungi by a typical P450 mono-oxygenase activity. This protein contains an iron protoporphyrin moiety located at the active site, and the antifungal azoles bind to the iron atom via a nitrogen atom. Therefore, the azole molecule binds to the protein in a manner dependent on the individual azole's structure (**Fig. 07**).

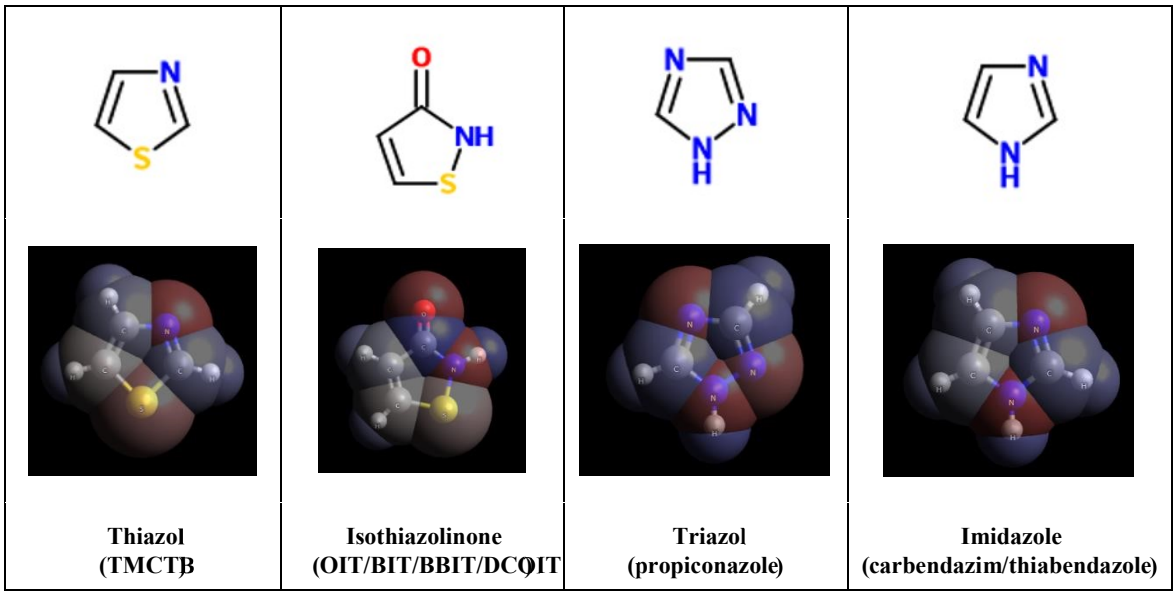

Fig. 7. Synthesis of ergosterol. Red arrow indicates the step which azole compounds are capable to inhibit.

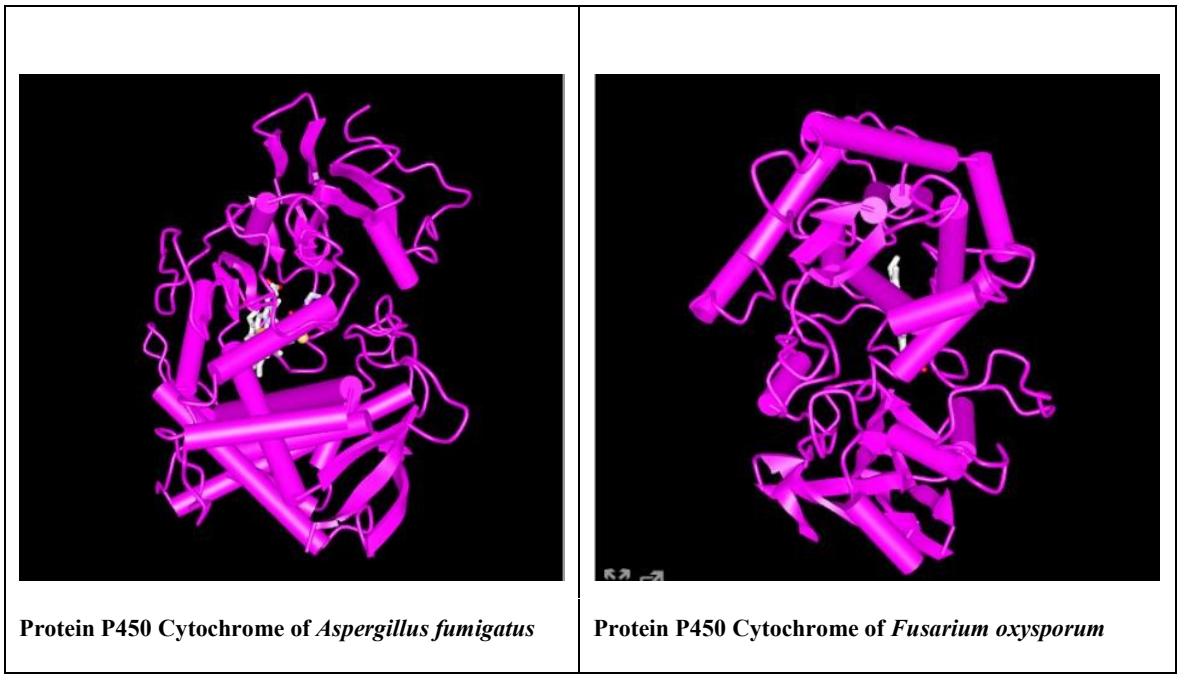

Fig. 8. Representation of the P450 Cytochrome protein of two fungal species, possessing remarkable differences which directs interferes in the fungicide activity [12].

The exact conformation of the active site differs between fungal species and among the many mammalian P450 mono-oxygenases. Above, it is possible to observe the structural differences between the two fungal P450 cytochorme protein (Fig. 08). These differences will influence the higher or lower interaction of the azole functional group and consequently its activity. In conclusion, the precise nature of the interaction between each azole molecule and each kind of P450 determines the extent of the azole's inhibitory effect in different fungal species.

3.2.2 Isothiazolinone compounds – mechanism of action

The ISOTHIAZOLINONE chemical group comprises OIT, BIT, BBIT, DCOIT and correlated compounds that exhibit the capacity to bind thiol-groups of fungal proteins, besides the previously described ability to inhibit ergosterol biosynthesis. Most of the scientific reports mention that isothiazolinone chemical group acts on distinct proteins related to crucial Krebs cycle pathways. The Krebs cycle (or citric acid cycle) is a part of cellular respiration (**Fig. 09**). All these reactions occur inside mitochondria of all aerobic organisms.

Isothiazolone biocides inhibit specifically sulfur-containing dehydrogenase enzymes related to crucial Krebs cycle pathways, including pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase, succinate dehydrogenase, NADH dehydrogenase, lactate dehydrogenase, and alcohol dehydrogenase. The disablement of these important enzymes can result in complete inhibition of critical metabolic functions concerned with energy generation and cell growth. Moreover, isothiazolone biocides are known to react with nucleophilic materials and proteins thiols, inactivating them. Thiol-active sites are commom to dehydrogenase enzymes and other proteins[13,16].

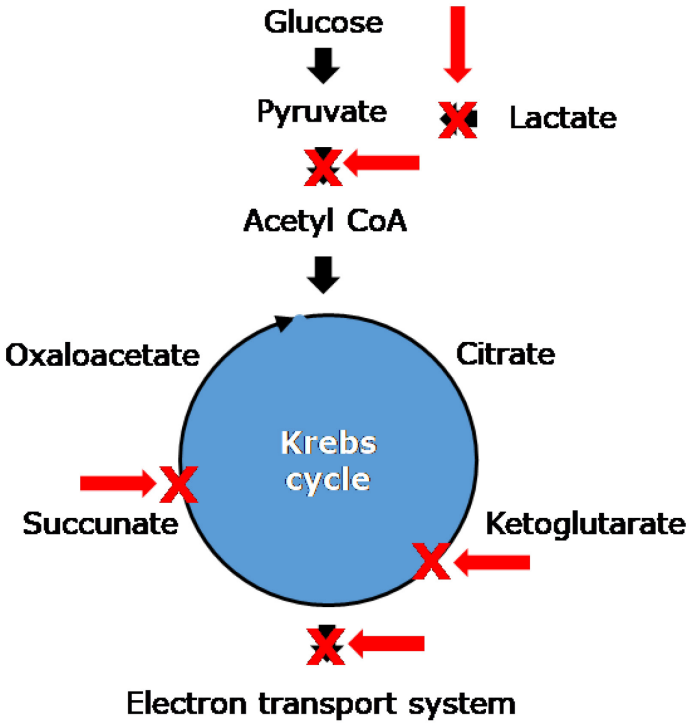

Fig. 9. Representative scheme of action of OIT upon Krebs cycle pathways.

3.2.3 Imidazole compounds – mechanism of action

Carbendazim and thiabendazole exert their biological activity by binding to tubulin proteins. Tubulin is a major component of the eukaryotic cytoskeleton which is involved in structural support, intracellular transport and DNA segregation. The action of imidazole compounds disrupts microtubule assembly, preventing appropriated cell division, and thus resulting in the malsegregation of chromosomes during cell division (**Fig. 10**).

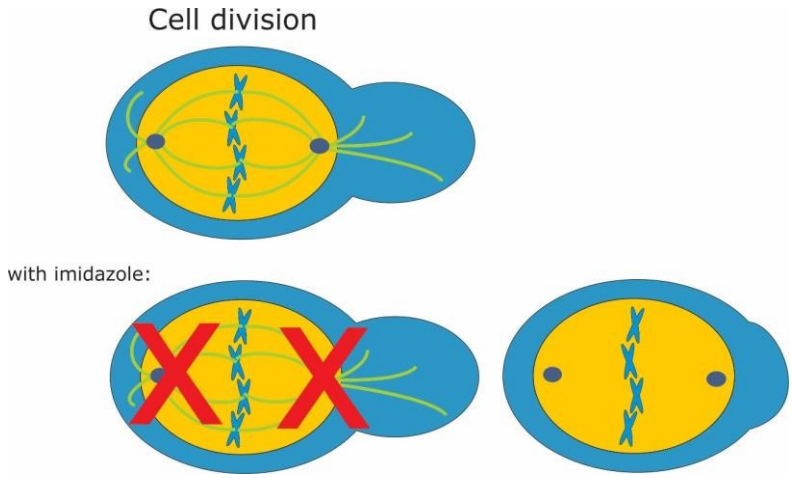

Fig. 10. Fungal cell division process. Up is the normal process and bottom the process being interfered by the fungicide carbendazim.

3.2.4 CMK – mechanism of action

CMK (4-chloro-3-cresol) acts specifically on the cell membrane and inactivates intracytoplasm enzymes by forming unstable complexes. The lipophilic molecules are trapped by the membrane phospholipids. The following processes are involved (**Fig. 11**):

- If CMK concentration is low, the cell constituents (nucleic acids, glutamic acid) are liberated in the external media.
- If CMK concentration is high, the disinfectants inhibit permeases, thus causing denaturation of the proteins and lysis of the cell membrane[14].

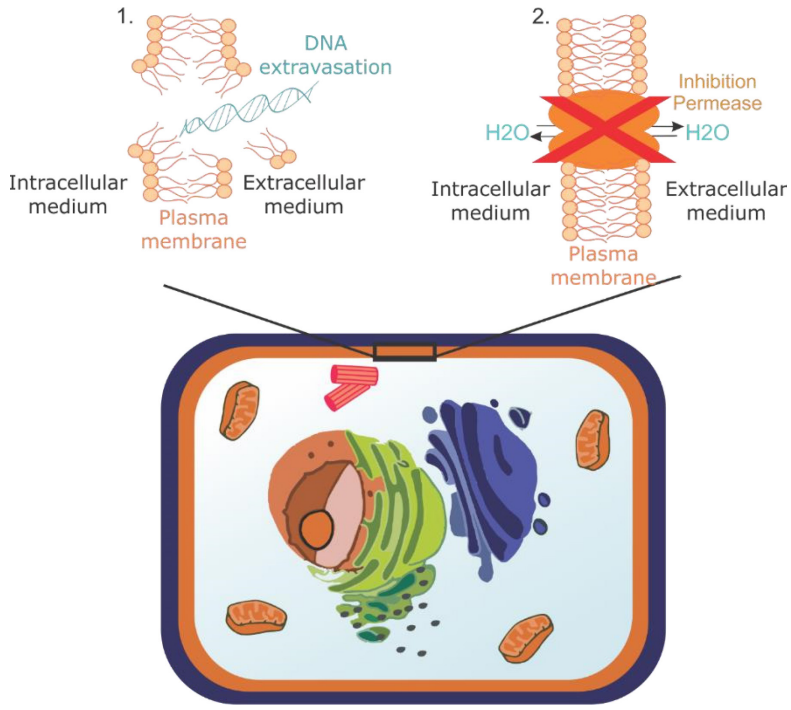

Fig. 11. Representation of the mechanism of action of CMK fungicide.

3.2.5 IPBC – mechanism of action

Carbamate fungicides, such as 3-iodo-2-propynyl butylcarbamate (IPBC), disrupt the formation of fungal cell walls by interfering with synthesis of phospholipids and fatty acids, as demonstrated in **Fig. 12**. They also affect mycelial growth, spore production and germination. Also, IPBC is considered an iodophore, since it is composed of iodine complexed with solubilizer, acting as "free" iodine. Therefone, this compound is corrosive to exposed membranes.

3.2.6 Pyrithione compounds – mechanism of action

Sodium Pyrithione and Zinc Pyrithione are inhibitors of fungal membrane transporters. Incubation of these compounds with Penicillium sp. resulted in decreased activity of transport systems, including those for inorganic sulfate, inorganic phosphate, glucose, L-methionine, among others. It has also been reported that pyrithione biocides are able to reduce ATP levels in fungi along with membrane depolarization (**Fig. 13**).

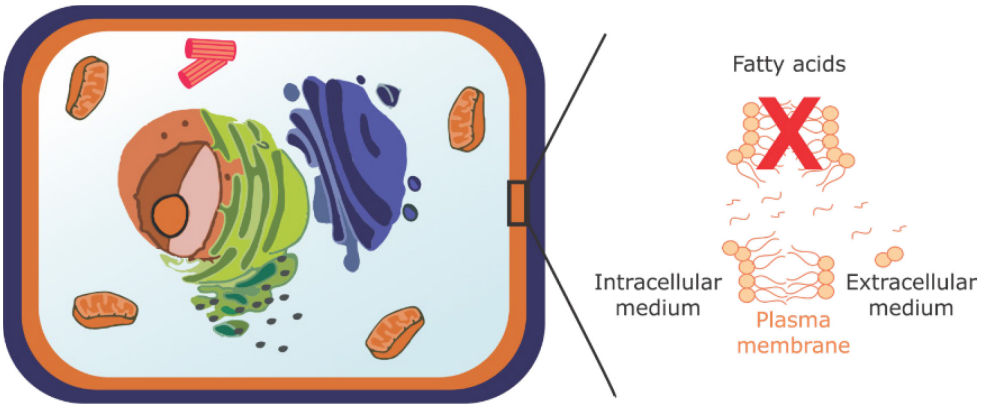

Fig. 12. Representation of the mechanism of action of IPBC fungicide.

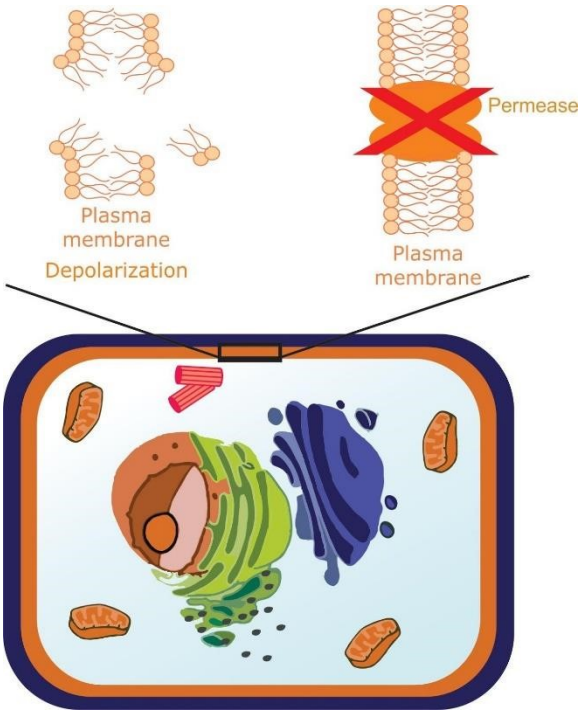

Fig. 13. Representation of Pyrithione compounds mechanism of action on fungal plasma membrane.

3.2.7 Tribromophenol – mechanism of action

Tribromophenol is the most produced brominated phenol in the world. This compound, as well as its water-soluble sodium tribromofenate salt, began to be used in Brazil approximately in 1985 as an alternative product to other oil-soluble products, such as pentachlorophenol and sodium pentachlorophenate.

The mechanism of action of pentachlorophenol and similar oleosoluble products is based on mitochondrial oxidative phosphorylation, causing acceleration in metabolism and heat production, thus resulting in loss of the membrane electrical resistance (**Fig. 14**).

3.2.8 THPS – mechanism of action

Tetrakis hydroxymethyl phosphonium sulfate (THPS) is a quaternary phosphonium compound. The molecule has a relatively fast mode of action and works well against various organisms, including fungi. The phosphine, THP, is responsible for its biocidal properties as it interferes with disulfide linkages in proteins, causing them to lose catalytic capacitydue to the breakdown of tertiary structure. Besides, THP causes the loss of free thiol groups, leadingto cell destabilization[15]. In addition, the sulfate reduction process within the sulfate reducing bacteria (SRB) is inhibited[16]. (**Fig. 15**).

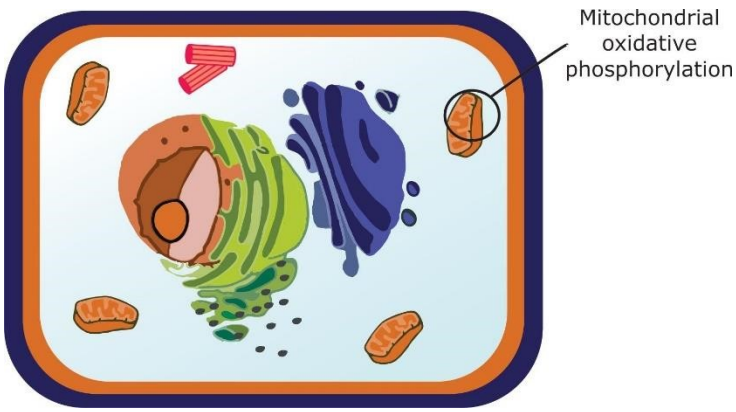

Fig. 14. Schematic representation of Tribromophenol mechanism of action on fungal cells.

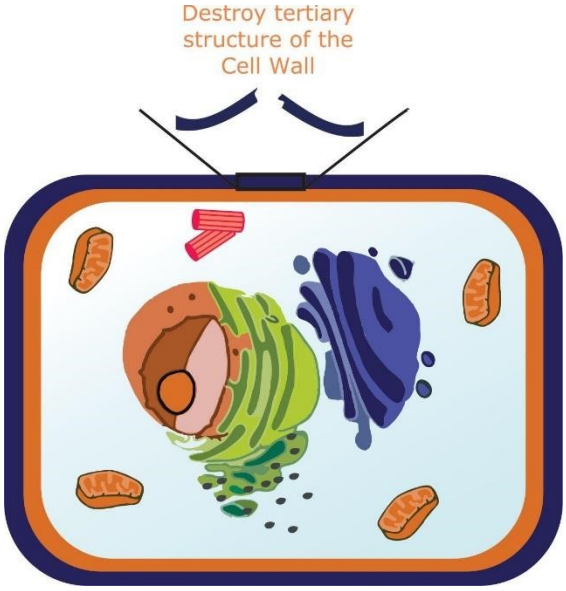

Fig. 15. Schematic representation of THPS mechanism of action on fungal cells.

3.2.9. Bronopol - mechanism of action

Bronopol has a broad spectrum of activity against all groups of bacteria, including the anaerobic sulphate reducing bacteria (SBR). On the other hand, its activity against fungi is more variable and generally higher doses are required to inhibit growth.

Anyway, under aerobic conditions, bronopol catalytically oxidizes intracellular molecules with thiol grouping, such as cysteine, using atmospheric oxygen as the oxidizing agent (**Fig. 16**). As a result, reactive oxygen species (ROS), such as superoxide and peroxide, are generated. These ROS are directly responsible for the bactericidal activity of the compound and for reduced growth rate after the bacteriostatic period.

3.2.10. PHMB – mechanism of action

Polyhexamethylene biguanide (PHMB) is a linear polymer comprised of a hydrophobic backbone with multiple cationic groupings separated by methylene chains. It is widely reported in literature that the lethal action of PHMB is due to membrane disruption and irreversible loss of essential cellular components. The molecule binds to the surface of the bacterial cell membrane and causes reorganization of the membrane in a way that prevents removal of the antimicrobial agent (**Fig. 17**)[17,18,19]. This mode of action makes the development of microbiological resistance very unlikely. However, it has been observed that a range of bacterial species, when treated with PHMB, displayed cell division arrest and chromosome condensation, suggesting DNA binding as an alternative antimicrobial mechanism. A DNA-level mechanism was confirmed by observations that PHMB formed nanoparticles when mixed with isolated bacterial chromosomal DNA[20].

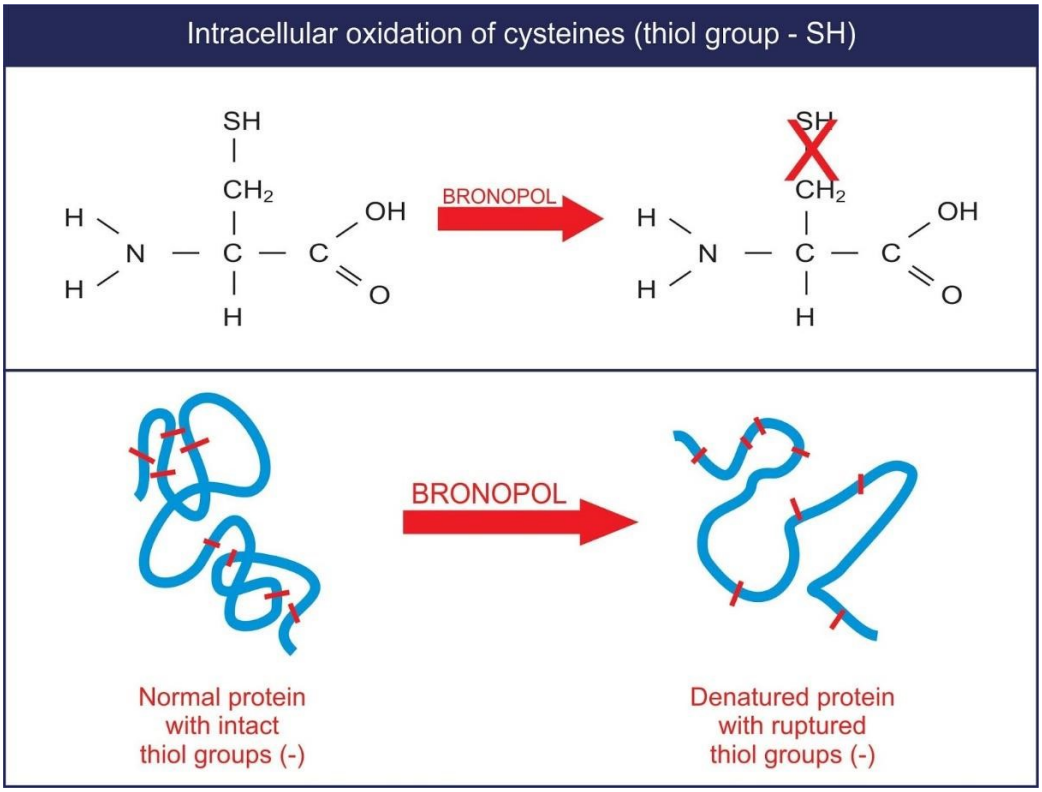

Fig. 16. Representation of Bronopol mechanism of action on intracellular molecules containing thiol grouping.

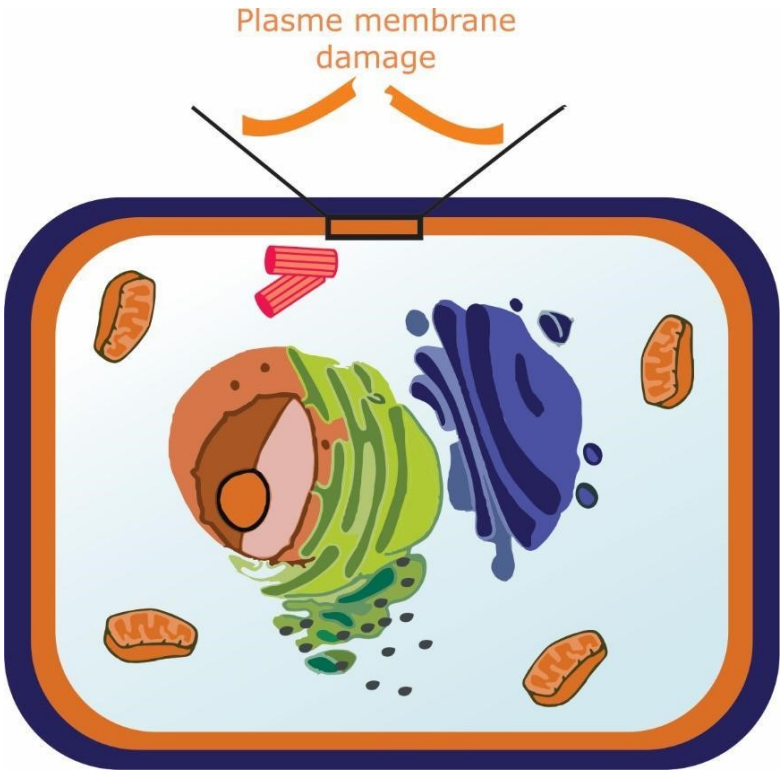

Fig. 17. Representation of PHMB mechanism of action on cellular components.

3.3 Fungal Mechanisms of Resistance and the Known Causes of Resistance Emergence

Fungicide resistance refers to change in the sensitivity of a fungi population to a fungicide. Such phenomenon provokes the failure of biocides in general. Typically, resistance can be developed in situations where the same compounds (or similar) with the same mechanism of action are used uninterruptedly. Resistance might be detected in populations or in single fungi isolates. TCMTB for example is not effective against *Trichoderma viride*, which can be brought into the tannery on the wooden pallets, and *Amorphotheca resinae* are resistant to phenolics (CMK)[9].

In the same way that antibiotics inhibit bacterial growth, antifungal compounds prevent fungal growth. The fact that bacterial antibiotics are no longer effective is well known; however, the antifungal resistance is an emerging phenomenon (**Fig. 18**). This highlights the need for an improved understanding regarding fungal resistance as well as a greater attention to methods that can be used to prevent and control them.

Some species of fungi are naturally resistant to certain types of antifungal agents. Other species may be normally susceptible to a particular type of agent, but develop resistance over time as a result of improper antifungal use - for example, dosages that are too low or treatment courses that are not long enough[22].

As presented in **Fig. 18**, there are two possible ways to induce resistance. In this sense, it important to highlight that sub-lethal fungicide regimen leads to further induction of genes that help resisting subsequent drug treatments. As a consequence, the population is shifted to increasing resistance, and increasing numbers of individuals with higher degrees of resistance are found. In practice, it is very important to apply right dosage indication, specially by teaching technical staff in order to avoid resistance induction.

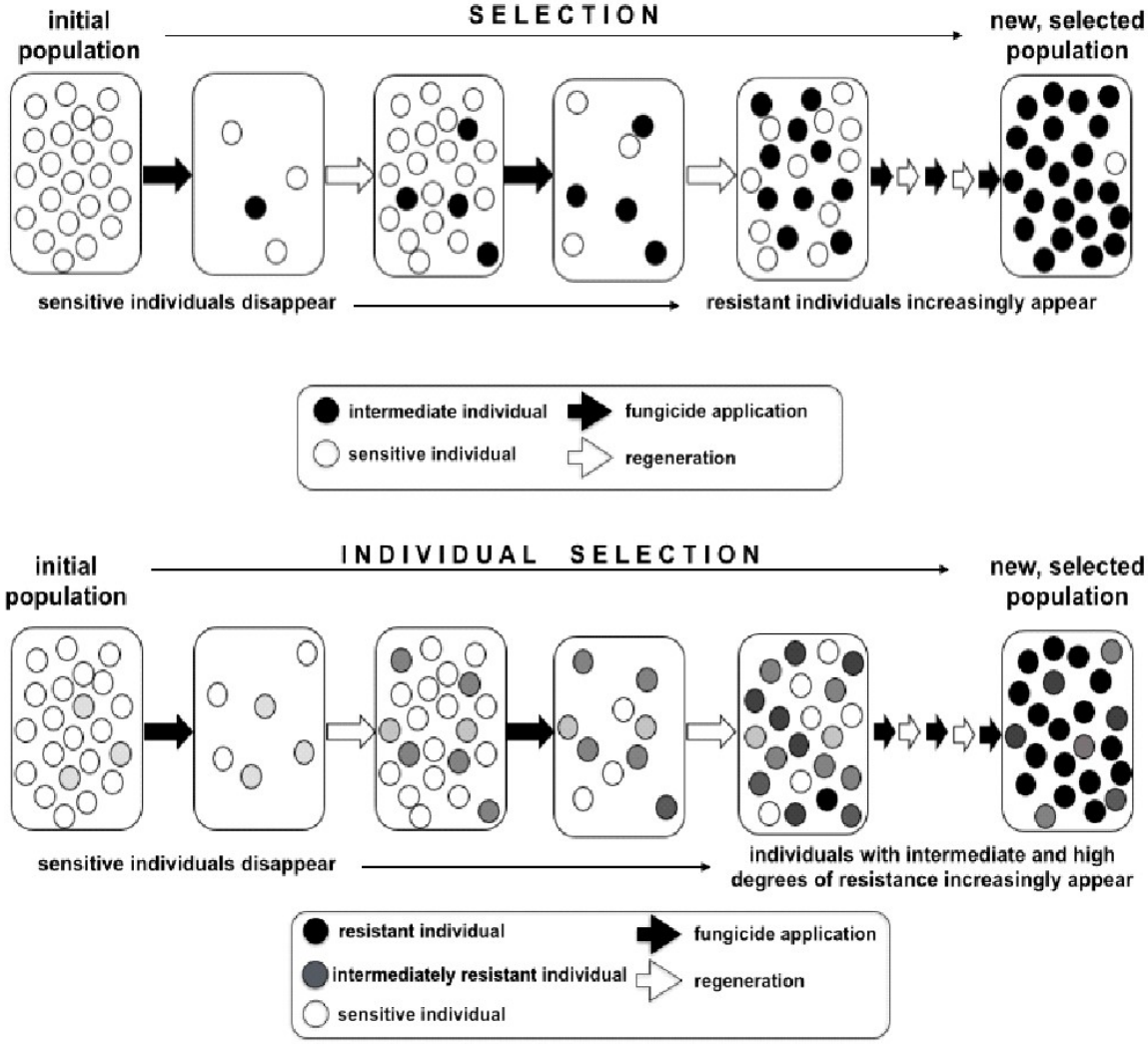

Fig. 18. Development of fungicide resistance is a selection process, with the fungicide as the selecting agent. In qualitative resistance (indicated as "selection"), mutation-based insensitive mutants are selected, and strains are either sensitive or resistant to the drug. In quantitative resistance (indicated as "individual selection") individuals that express genes leading to reduced fungicide sensitivity, are more likely to survive a drug treatment. Adapted from 21.

Fig. 19, below, presents the seven known mechanisms of fungal resistance:

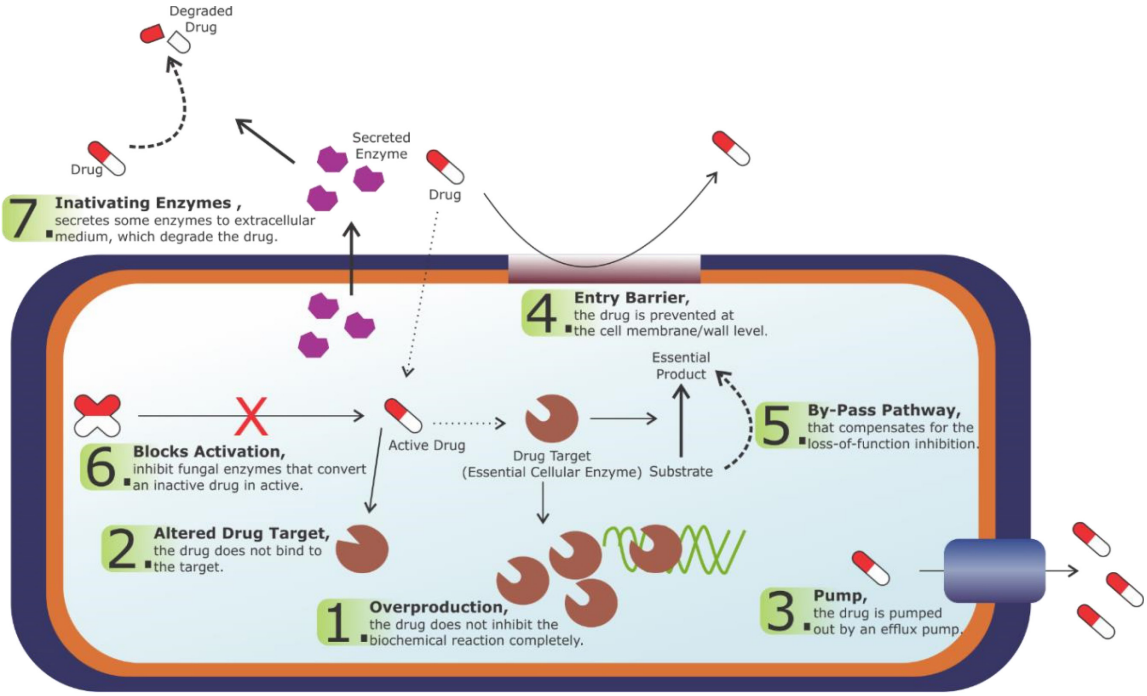

Fig. 19. Mechanisms by which microbial cells might develop resistance. 1: The target enzyme is overproduced, so that the drug does not inhibit the biochemical reaction completely; 2: The drug target is altered so that the drug cannot bind to the target; 3: The drug is pumped out by an efflux pump; 4: The entry of the drug is prevented at the cell membrane/cell wall level; 5: The cell has a bypass pathway that compensates for the loss-of-function inhibition due to the drug activity; 6: Some fungal "enzymes" that convert an inactive drug to its active form are inhibited; 7: The cell secretes some enzymes to the extracellular medium, which degrade the drug (adapted from 23).

3.4 Fungicide Combinations to Enhance Activity

Fungi can develop resistance to biocides, making commercially available fungicides less effective, as mentioned above. Assuming this growing crisis, the need of alternatives to not only diminish such emerging resistance scenario by proper use and regulations, but also new application strategies and search for new molecules is urgent.

In this sense, one of the most used strategy is the combination of distinct biocides – possessing different mechanism of action – in order to overcome resistance. The combination of bioactive compounds is meant to observe a synergetic effect – preferably in lower doses compared to single application – in so called congruous strategy. Additionally, two other strategies might be suitable: (i) syncretic, in which one of the molecules does not act on a essential target for microbe survival and (ii) coalistic, when none of the molecules combined act upon a specific essential target, but together can promote a bioactivity, as presented in **Fig. 20**, below [24].

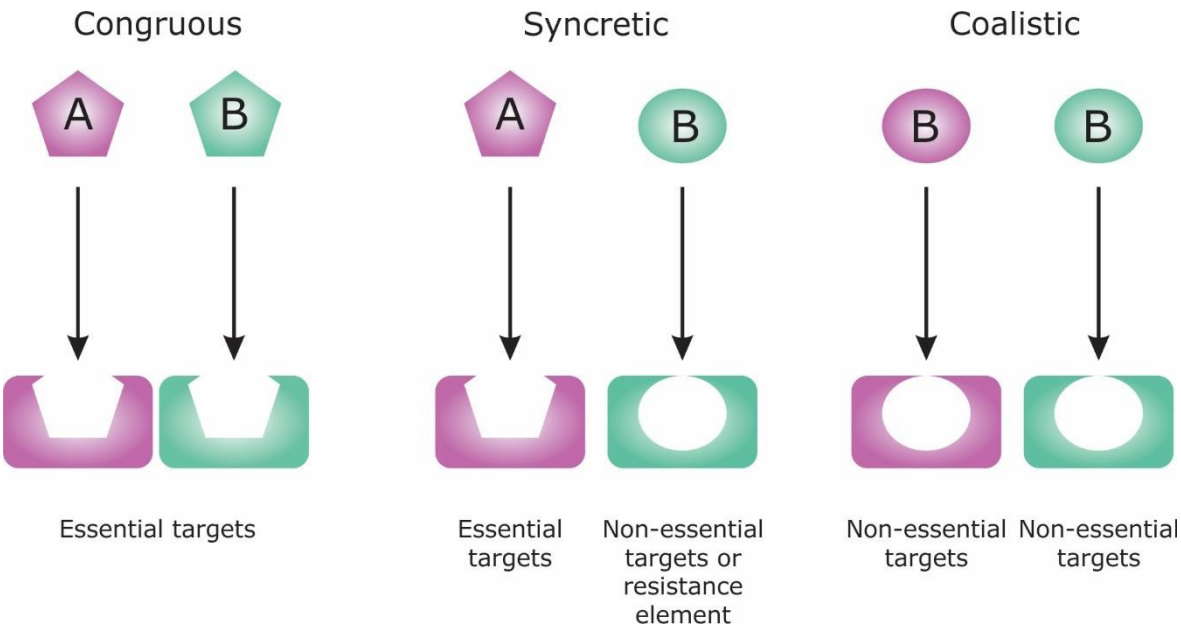

Fig. 20. Possible molecules synergic combinations in order to improve efficacy against fungal resistance.

The synergic effect and non-synergic effect (antagonism) might be calculated, in a so called fractional inhibitory concentration index (FICI). In this sense, combinations of distinct molecules that act (or not) upon a target is the new road to overcome the fungal biocide resistance. Companies are now worried in present solutions to suppress resistance by increasing efficacy. Moreover, investigations on the search for suitable fixed-dose combinations accurately formulated has to be done. Nowadays these frontiers of molecules combinations are only partially explored and it is expected that combinations of three or more molecules will be needed in order to achieve the right doses and duration.

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