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Resistance of phylloplane-inhabiting yeast to fungicides[1]

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ABSTRACT

Yeasts colonize the surface of plants and act as natural biological control agents, reducing the incidence and severity of diseases. However, fungicide applications can lead to the reduction and/or inhibition of these species in the phylloplane. The objective was to evaluate the sensitivity of yeast strains from the phylloplane against fungicidal products used in agriculture for the control of plant diseases. Thus, strains of different species of yeast from the leaf surface of plants were evaluated in vitro for sensitivity to fungicides, measuring the inhibition zone radius for each yeast strain exposed to fungicidal products. The yeast *Zygoascus hellenicus*, *Rhodotorula aurantiaca*, *Pichia* spp., and *Sporobolomyces roseus* were insensitive to most of the fungicidal. Regarding the products, those composed of a mixture strobilurins and carboxamides and the multisites were shown to be less toxic to yeast, whereas the compounds chemicals containing the active ingredient of the triazole group were shown to be more toxic. The products acting on only one mechanism of action, the inhibition of respiration, proved to be more selective to yeast. The yeast has natural resistance to most of the fungicidal products; however, some species show significant sensitivity to compounds containing the active principle of the triazole group.

Keywords: bioprotectors, inhibition zone, insensitivity, mechanism of action, synthetic fungicides.

INTRODUCTION

The phylloplane describes the leaf surface, which houses diverse microbial communities, including bacteria, fungi, algae, and yeast, making it an ecologically important ecosystem. The leaf surface has an ideal environment for these microorganisms in terms of nutrition, humidity, pH, and temperature for their survival.[1] These microorganisms are resistant to different conditions, as they are subject to varying temperature, humidity, and solar radiation throughout the day and night.[2]

Yeasts, which are natural inhabitants of ecosystems, colonize the surface of plant structures and act as natural biological control agents by reducing the incidence and/or severity of plant diseases. They play a role in resistance induction, growth promotion, competition for space and nutrients, antibiosis, and parasitism.[3]

Perreault & Laforest-Lapointe[4] reported that leaf-associated microorganisms influence host fitness and growth, abiotic stress resilience, and pathogen resistance. Phylloplane microorganisms have been studied as bioprotectors and growth stimulators of plants because of the numerous benefits they bestow on hosts.

Currently, due to the excessive demand for food and risks due to pests and diseases, pesticides have been used exponentially in agricultural systems to solve phytosanitary problems, provide improved conditions for crops, and avoid economic losses. One of the undesirable environmental effects of pesticides is the contamination of species that do not negatively affect the production process, which are referred to as non-target species. Among pesticides, fungicides are normally used to control diseases, and although their main objective is the control of phytopathogenic organisms, they have also been observed to affect other microorganisms in the phylloplane.[5]

According to Gonçalves et al.[6], many fungicides decrease the antagonistic activity of some microorganisms in the phylloplane, while others increase this activity. These changes affect the dynamic balance of the phylloplane, which can lead to the development of secondary plant diseases or the intensification of existing diseases. Kucharska, Wachowska & Czaplicki[7] reported that fungicides can act selectively on several species of yeast, which can lead to complete inhibition for some species, with no effect on others, causing an imbalance in microbiota.

The challenge is to expand the knowledge on the behavior and sensitivity of yeast strains from the phylloplane of different plant species when subjected to applications of fungicidal products routinely used in agriculture.

MATERIALS AND METHODS

The experiment was conducted in a completely randomized design using a factorial scheme with eight treatments (seven fungicidal products and one control), 37 yeast strains, and four replicates.

Yeast strains were obtained from leaf and flower phylloplanes of different plant species stored in the yeast collection of the Phytopathology Laboratory of the State University of Western Paraná. They were preserved in sterile mineral oil and stored in a refrigerator.[8] The species used were *Candida albicans*, *Cryptococcus laurentii*, *Pichia guilliermondii*, *Pichia pini*, *Rhodotorula aurantiaca*, *Rhodotorula glutinis*, *Sporidiobolus johnsonii*, *Sporobolomyces roseus,* and *Zygoascus hellenicus*. These yeasts are non-target organisms for fungicide products and, by colonizing the plant surface, can bring several benefits to crops, therefore they were selected for the study. Strains were removed from the stock and cultivated in Petri dishes containing YEPG-agar medium (20 g glucose, 10 g peptone, 5 g yeast extract, 20 g agar, and 1000 mL distilled water) for use in the assay.

The yeast suspensions were prepared from Petri dishes when the yeast were seven days old, by diluting aliquots of the colonies in microtubes containing 2 mL saline solution sterile (NaCl 0.85%) and slightly shaking the tubes for complete homogenization of the suspensions. Subsequently, suspensions of each yeast were prepared, counted in a Neubauer chamber, and adjusted to 1×10^7 colony-forming units (CFU) mL^{-1}. Preparations were performed in an aseptic room.

The treatments were control, lime sulfur, bordeaux mixture, copper oxychloride, trifloxystrobin + tebuconazole, bixafen + prothioconazole + trifloxystrobin, fluxapyroxad + pyraclostrobin, azoxystrobin + benzovindiflupyr (Table 1). The fungicidal products were prepared according to the manufacturer's recommendations. The lime sulfur was prepared at a concentration of 0.3 °Baumé, which is suitable for application in the vegetative phase and equivalent to diluting 1 L of pure solution and 129 L of water for a solution of 30 °Baumé. The Bordeaux mixture was prepared with 10 g L^{-1} of quicklime and 10 g L^{-1} of copper sulfate, which was used pure, equivalent to a concentration of 1%. Sterilized distilled water was used as the control.

Table 1. Fungicide treatments and concentrations used for diffusion tests on yeast strains

Treatment[1]	Mechanism of Action[2]	Active ingredient	Concentration	Volume of syrup
Control	-	-	-	-
Lime sulfur	M	-	0.3 ºBaumé	-
Bordeaux mixture	M	-	1%	-
Copper oxychloride	M	588 g/L	1.5 L/ha	200 L/ha
Trifloxystrobin + tebuconazole	C + G	100 g/L + 200 g/L	0.6 L/ha	100 L/ha
Bixafen + prothioconazole + trifloxystrobin	C + G + C	125 g/L + 175 g/L + 150 g/L	0.5 L/ha	100 L/ha
Fluxapyroxad + pyraclostrobin	C + C	167 g/L + 333 g/L	0.35 L/ha	100 L/ha
Azoxystrobin + benzovindiflupyr	C + C	300 g/kg + 150 g/kg	300 g/ha	200 L/ha

[1] The treatments include different commercial fungicides and specific combinations of active ingredients used in agriculture. Each concentration was adjusted according to the manufacturer's recommendations. [2] Mechanisms of action of fungicidal products according to the FRAC 2024 classification. M: chemicals with multi-site activity, C: respiration, G: sterol biosynthesis in membranes.

In Petri dishes containing sterile YEPG-agar culture medium, 100 µL of the yeast suspension was dispensed onto the medium and spread over the entire surface using a Drigalski strap. Filter paper disks (8 mm in diameter) soaked with the fungicide solutions under study were deposited and distributed on the circular edge of the plates, with the control disk (distilled water) in the center. The Petri dishes were sealed with plastic PVC film and incubated in an incubator chamber (BOD type) at 25 °C for 48 h.

After the incubation time (48 h), the zone of inhibition formed on each paper disk was evaluated with the aid of a caliper and measured from the edge of the disk to the end of the zone of inhibition. The radius of the zone of inhibition for each product with each yeast strain was tabulated in a spreadsheet, averaged, and classified according to the degree of sensitivity proposed in Table 2. Subsequently, the yeast strains were grouped according to their respective classification.

Data were subjected to analysis of variance using the F test, and the means, when significant, were grouped using the Scott-Knott test with 5% significance. Statistical analyses were performed using SISVAR software.[9]

Table 2. Classification of degree of sensitivity according to the radius of the zone of inhibition formed[1]

Degree of sensitivity	Inhibition zone radius
Insensitive (I)	0 to 1 mm
Low Sensitivity (LS)	1 to 4 mm
Sensitive (S)	4 to 16 mm
High Sensitivity (HS)	higher than 16 mm

[1] Classification is based on the inhibition radius in mm.

RESULTS

To characterize the effects of fungicides widely used in agriculture on populations of phylloplane-inhabiting yeasts, the studied strains were grouped into the following categories: insensitive, low sensitivity, sensitive, and high sensitivity. The number of yeast strains, according to the proposed degree of sensitivity, is presented in Table 3. The yeast strains had a significant effect ($p < 0.05$) on the size of the zone of inhibition for each antimicrobial product (Figure 1).

The majority of the yeast strains showed low sensitivity or were insensitive to the antifungal products tested (Table 3). However, certain strains revealed sensitivity to products composed of trifloxystrobin + tebuconazole, bixafem + prothioconazole + trifloxystrobin, and azoxystrobin + benzovindiflupyr. Only the trifloxystrobin + tebuconazole product produced strains with high sensitivity.

In Figures 1A and 1B, this inhibitory effect on some yeast strains can be observed, where 60% and 100% of the *C. albicans*, 16.6% and 33.3% of the *R. glutinis*, and 25% and 50% of the *S. johnsonii* strains were inhibited by copper oxychloride and lime sulfur, respectively. In addition, 50% of the *C. laurentii* strains showed some degree of inhibition for both products, and the *S. roseus* strain was inhibited only by copper oxychloride. However, even though the size of inhibition differed among the copper oxychloride and lime sulfur treatments, all yeast strains showed zone of inhibition of no more than 4 mm.

The product composed of trifloxystrobin + tebuconazole was considered the least selective among those tested, presenting eight strains (21.6%) with high sensitivity to the chemical and elevated growth inhibition. Four (10.8%) of the strains were sensitive and seven (18.9%) had low sen-

sitivity (Table 2). Figure 1C shows the effect of the product on each strain, whereby 12 strains had a higher inhibitory effect, and seven strains had an intermediate effect, according to the Scott-Knott test of averages. Considering the strains with superior and intermediate inhibitory effects, all the *C. albicans* and *P. guilliermondii,* 66.6% of the *C. laurentii*, 50% of the *S. johnsonii* and *Z. hellenicus*, 25% of *R. aurantiaca*, and 16.6% of *R. glutinis* strains showed some degree of inhibition by the chemical product, thereby verifying its low selectivity to non-target organisms.

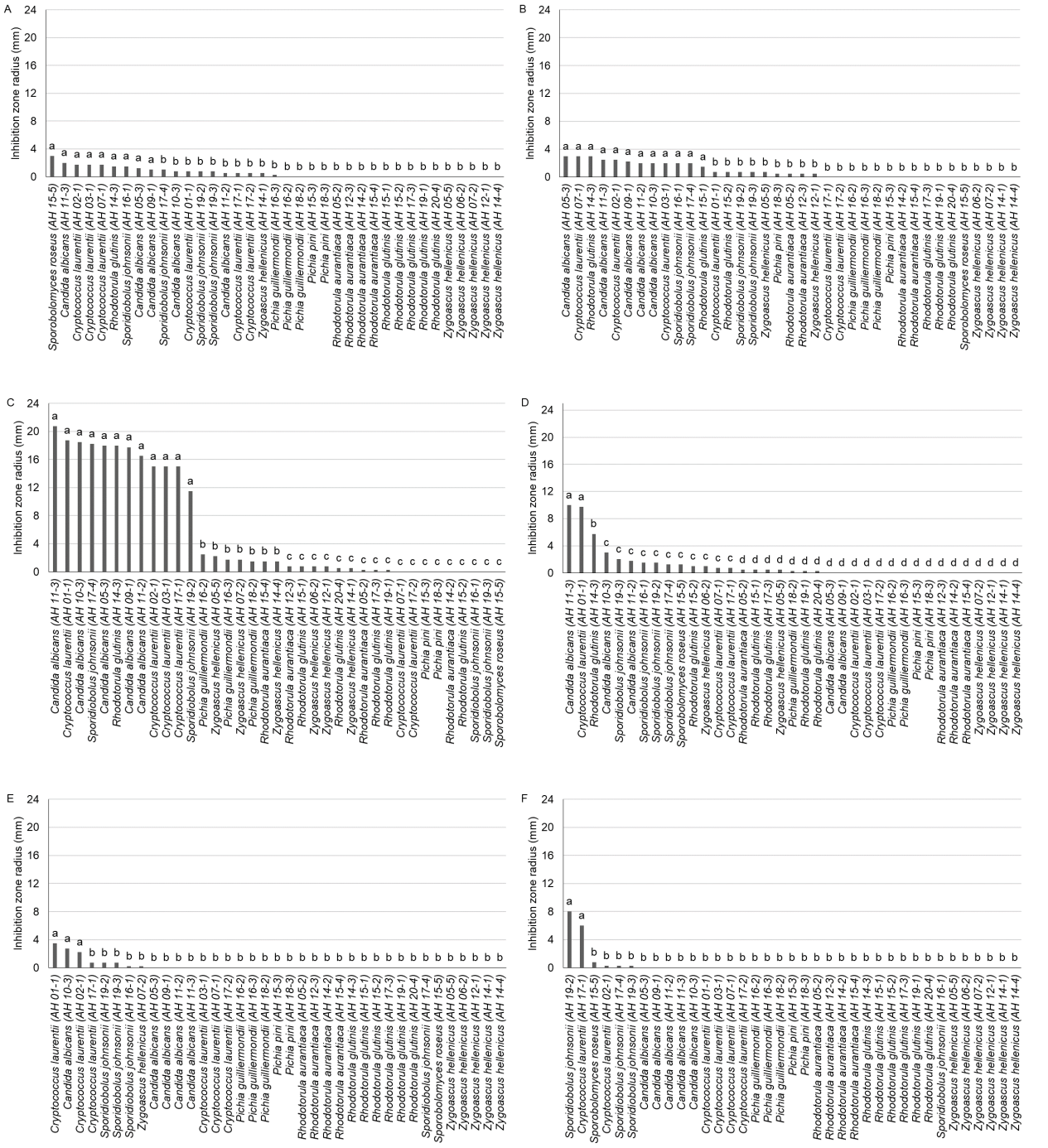

Figure 1. Measurement of the inhibition zone radius (mm) of yeast strains in contact with treatments. A: copper oxychloride*, B: lime sulfur*, C: trifloxystrobin + tebuconazole*, D: bixafem + prothioconazole + trifloxystrobin*, E: fluxapyroxad + pyraclostrobin*, F: azoxystrobin + benzovindiflupyr*. The control and Bordeaux mixture treatments did not show significance between the yeast strains. Data transformed using the equation: $\sqrt{(X + 0.5)}$. Coefficient of variation (CV) = 48.07%. * Yeast strains followed by the same lowercase letter do not differ from each other by the Scott-Knott test at the 5% error probability level.

Table 3. Number of yeast strains in relation of their degree of sensitivity to the antimicrobial products tested

Treatment	Chemical/ biological group[1]	Number of yeast strains[2]			
		I	LS	S	HS
Control	-	37	0	0	0
Lime sulfur	M	25	12	0	0
Bordeaux mixture	M	34	3	0	0
Copper oxychloride	M	29	8	0	0
Trifloxystrobin + tebuconazole	S + T	18	7	4	8
Bixafen + prothioconazole + trifloxystrobin	C + T + S	27	7	3	0
Fluxapyroxad + pyraclostrobin	C + S	34	3	0	0
Azoxystrobin + benzovindiflupyr	S + C	35	0	2	0

[1] M – multisite; S – strobilurin; T – triazole and C – carboxamide. [2] I: insensitive; LS: low sensitivity; S: sensitive; HS: high sensitivity.

The product composed of bixafem + prothioconazole + trifloxystrobin did not present high sensitivity for any strain, although three (8.1%) of the strains were sensitive to the fungicide and seven (18.9%) showed low sensitivity (Table 3). Figure 1D identifies two strains with a higher inhibitory effect and 12 strains with an intermediate effect, according to the Scott-Knott test of averages. Considering the strains with superior and intermediate effects, all the *S. johnsonii* and *S. roseus*, 60% of the *C. albicans*, 50% of the *C. laurentii*, 33.3% of the *R. glutinis*, and 16.6% of the *Z. hellenicus* strains showed some degree of inhibition.

The product fluxapyroxad + pyraclostrobin presented only three strains (8.1%) with low sensitivity, while the remainder were insensitive. In contrast, the fungicide product azoxystrobin + benzovindiflupyr negatively affected only two of the strains (5.4%), which were sensitive to the product, and the rest were shown to be insensitive (Table 3). As shown in Figures 1E and 1F, both the fluxapyroxad + pyraclostrobin and azoxystrobin + benzovindiflupyr products presented three and two strains with superior effects, respectively, according to the Scott-Knott test of averages. These strains corresponded to 33.3% of the *C. laurentii* strains and 20% of *C. albicans* for the fluxapyroxad + pyraclostrobin product, and 16.6% and 25% of the *C. laurentii* and *S. johnsonii* strains for the azoxystrobin + benzovindiflupyr product, respectively. The results demonstrate the high selectivity and low toxicity of these products in non-target organisms, since most yeast species did not show any inhibition.

Products with more than one mechanism of action that affect different target sites had greater effects on the isolates, suggesting that products with a single mechanism of action on different target sites are more selective, although they are not innocuous. However, in terms of disease control in the field for the main agricultural crops, the best results were found when using composite products with more than one mechanism of action.

In general, all strains of *Z. hellenicus* were insensitive to multisite products and mixtures of carboxamides and strobilurins and were inhibited only by products containing triazoles. The strains of *R. aurantiaca* and *Pichia* spp. were insensitive to the same products, in addition to those that included carboxamide, strobilurin, and triazole. The *S. roseus* strain was insensitive to all products except copper oxychloride. The *R. glutinis* strains were insensitive to both mixtures of carboxamides and strobilurins, but *C. albicans* and *S. johnsonii* were insensitive to only a mixture of carboxamide and strobilurin, with azoxystrobin + benzovindiflupyr for *C. albicans* and fluxapyroxad + pyraclostrobin for *S. johnsonii*. In addition, all strains of all species were considered insensitive to the Bordeaux mixture.

DISCUSSION

The results shown in Table 3 and Figure 1 are similar if the classification system proposed in Table 2 is efficient in measuring the inhibitory effect of fungicide products on yeasts.

The copper oxychloride, lime sulfur, and Bordeaux mixture proved to be highly selective, with all strains classified as insensitive or having low sensitivity; they are considered multisite products that have different mechanisms of action.[10] However, these products cannot be considered innocuous as they influence some strains, indicating that there is an effect on the populations present in the phylloplane, even if this effect is low. These results emphasize the possibility of using these products in organic farming systems, according to Normative Instruction Nº. 46 of MAPA[11], as they are more selective for the natural

enemies present on the surface of plants, leading to less imbalance in agricultural environments.

The synthetic fungicides trifloxystrobin + tebuconazole (strobilurin and triazole) and bixafem + prothioconazole + trifloxystrobin (carboxamide, triazole, and strobilurin) are effective in controlling a broad spectrum of fungal groups through respiration inhibition and sterol biosynthesis in membranes.[10] These chemicals are not selective, as although they present low sensitivity strains, they exert negative effects on many strains and different yeast species.

Furthermore, the synthetic fungicides fluxapyroxad + pyraclostrobin and azoxystrobin + benzovindiflupyr (both composed of carboxamide and strobilurin) can be considered selective due to their low level of damage to the yeast community, although they have negative effects on some strains. The low degree of damage observed can be explained by the unique mechanism of action of the products, which, although they have different active principles, both only cause the inhibition of respiration.[10]

According to Sumby, Caliani & Jiranek[12], when fungi become resistant (or insensitive) to a chemical within a group, they also tend to be resistant to other chemicals in the same group. What is observed in the test, where the fungicides with different active principles that act on the same mechanism and belong to the same group, have similar effects on the number of strains observed. However, yeast strains have different behaviors, whereby the same strain can be highly inhibited for a chemical product and insensitive to another product with the same mechanism of action.

Regarding fungicides, products composed of a mixture of strobilurins and carboxamides are less toxic to yeasts, while products containing the active ingredient of the triazole group showed greater toxicity. Kucharska, Wachowska & Czaplicki[7] evaluated products composed of triazoles, in which those containing triazoles and benzimidazoles had increased toxicity compared to the others sampled, and the mixture of strobilurin and triazole showed the lowest toxicity for yeasts. The greatest toxicity found in both studies occurred with the products of the chemical groups of the triazoles.

The triazole products (tebuconazole and prothioconazole) inhibit sterol synthesis in membranes.[13] Sterols are important regulators of the physical properties of the plasma membrane, such as fluidity and permeability, and ergosterol is responsible for maintaining the structure and function of this membrane.[14] Triazole action on the sterol 14α-demethylase enzyme leads to the inhibition of the formation of ergosterol precursors, leading to a change in permeability and membrane formation, and causing the cells to collapse. Changes in membrane permeability facilitate the entry and exit of water and harmful agents into the cell, which may explain the low selectivity of the products trifloxystrobin + tebuconazole and bixafem + prothioconazole + trifloxystrobin, which contain the active ingredients of triazoles. The inhibition of ergosterol formation may also have facilitated the entry of other fungicidal products present in the mixtures that are able to act on other organelles, such as mitochondria, leading to the inhibition of a greater number of yeast strains.

Strobilurins (azoxystrobin, pyraclostrobin, and trifloxystrobin) inhibit the cytochrome bc1 complex (complex III) within the mitochondria, where binding of an inhibitor to the quinone oxidase site blocks electron transfer in complex III. Carboxamides (bixafem, fluxapyroxad, and benzovindiflupyr) act on the succinate dehydrogenase complex (complex II), preventing the oxidation of succinate to fumarate in complex II, which also affects the electron transport chain.[13] Both act on the respiration process in the mitochondria and affect energy synthesis (ATP-adenosine triphosphate) with consequent inhibition leading to cell death.[14] Products composed of strobilurins and carboxamides, which act at the mitochondrial level, are subject to penetration challenges imposed by both cellular and mitochondrial membranes. Receptor components that can identify and/or detoxify these molecules before penetration are also activated, which may have reduced the action of the harmful agents on the yeast strains in the present study.

Cadez *et al*.[15] noted that none of the fungicides from the other chemical groups, such as dicarboximide, phenylpyrrole, and anilinopyrimidine, significantly affected the abundance of different yeast species. Therefore, these fungicides can be considered selective and should be chosen for disease control over products with greater toxicity, such as triazoles.

Wachowska, Irzykowski & Jedryczka[16] studied the effect of agrochemicals on yeasts that colonize wheat grains and observed that the yeast communities present in the grains depended on the number of fungicides used in agricultural activities, which exerted varied inhibitory effects on the strains and reflected on the final grain quality. In an in vitro experiment, Cadez *et al*.[15] also observed that the application of fungicides at concentrations recommended by manufacturers selectively reduced the microbial

communities of grape berries. However, Kosel, Raspor & Cadez[17] concluded that fungicide residues impaired the viability of desirable yeast strains and promoted the growth of various spoilage strains, thereby resulting in a negative impact on wine aroma. The results indicated negative effects on the quality of the products, as these products undergo fermentation processes to obtain bread in the case of wheat and juice and/or wine in the case of grapes. A reduction in the microbial composition negatively affects the fermentation process, reducing the quality of the final product. Thus, it is assumed that changes in the quality of the products may be due to the reduction and/or alteration of the microbial communities that colonize plant tissues due to the action and/or presence of fungicide residues.

Furthermore, the inhibition of a greater number of microorganisms inhabiting the leaf surface can lead to severe microflora imbalance, causing a phytopathogenic agent to have greater space for development and induce disease in plant tissues, especially when the affected microorganisms have become extinct or have slow development, requiring increased time to recolonize the surface. According to Ghini[18], the reduction of saprophytic microflora allows the development of new pathogens or those previously considered secondary. Similarly, pathogens that are resistant to a fungicide will likely benefit from the reduction of the epiphytic microflora, causing increased incidence and/or severity of the disease.

The yeasts used in the test were isolated from the leaves and flowers of several plants by Heling, A. (unpublished data), mainly from an area where there were no records of pesticide use in previous years. Thus, when they were subjected to contact with commonly used fungicides a large portion was insensitive to the action of these products. Therefore, these yeast strains can be important tools for use as biological control agents, especially in conventional systems where biological control has been interspersed with the use of fungicides.

Wachowska *et al.*[16] observed that most yeast strains obtained from plants that were protected with fungicides were resistant to many tested agrochemicals, suggesting that yeasts undergo selection, showing resistant forms when exposed to selection pressure exerted by agrochemicals. However, this was not observed in the present study, as the strains were obtained from environments without previous applications of agrochemicals, which suggests that another mechanism acts on these strains. Kucharska, Wachowska & Czaplicki[7] in their work mention the ability of different yeast species to detoxify environments through the degradation of various chemical compounds. This ability to detoxify fungicidal molecules may also be present in these yeast populations not subjected to selection pressure.

In view of this study, attention should be paid to the use of certain chemical products in agriculture, as it shows results that harm the community of non-target microorganisms, such as yeasts present on the plant surface. These communities must be preserved to maintain the natural biodiversity of ecosystems.

Furthermore, further research should be conducted on the efficiency of yeast strains that are insensitive to fungicides to control diseases in different pathosystems. This information would allow for these strains to be alternatives and/or combined with chemical control in disease management to reduce the application of non-selective chemicals and maintain non-target microorganisms in agricultural systems.

CONCLUSIONS

The yeast has natural resistance to most of the fungicidal products; however, some species show significant sensitivity to fungicidal products containing the active principle of the triazole group.

DATA AVAILABILITY

All of the data that supports the conclusions of this study was used in this article.

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