**THE EFFECT OF TWO COMMERCIAL ANTIFUNGAL FORMULATIONS ON
THE METABOLISM OF A COMMERCIAL *SACCHAROMYCES CEREVISIAE*
STRAIN AND THEIR REPERCUSSION ON FERMENTATION EVOLUTION
AND PHENYLALANINE CATABOLISM**

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ABSTRACT

The effect of two commercial formulations (incorporating mepanipyrim and tetraconazole as active substances) on the metabolism of *Saccharomyces cerevisiae* Lalvin T73™, growing on a synthetic grape must, and their influence on the alcoholic fermentation course and the biosynthesis of volatiles derived from phenylalanine catabolism was studied. No essential effects were observed for mepanipyrim except for glycerol production. On the contrary, in the presence of tetraconazole many genes and some proteins related to cell cycle progression and mitosis were repressed. This fact could explain the lower biomass concentration and the lower sugar consumption registered for tetraconazole at the end of the study. However, the biomass-to-ethanol yield was higher in connection with the overexpression of the *ADH1* gene. The presence of tetraconazole residues seems to accelerate the Ehrlich pathway. These results agree with the overexpression of several genes (*BAT1*, *PDC1*, *PDC5*, *ADH1*, *SFA1*, *ATF2*, *PFK1*, *PFK2* and *ARO3*) and two proteins (Gap1p and Atf2p) involved in this metabolic pathway.

Keywords: fungicides, cell cycle, Ehrlich pathway, aromatic compounds, proteomics, transcriptomics.

1. INTRODUCTION

Fungicide residues can modify the structure of the cellular membranes of yeasts, causing sluggish and stuck fermentations (Calhelha et al., 2006; Russo et al., 2019). In this sense, Doignon & Couty, (1992) observed sluggish fermentations, lower ethanol production and an increment of the frequency of stuck fermentations in the presence of triadimephon, flusilazol and triadimenol. Sluggish fermentations were also obtained in the presence of benomyl (Calhelha et al., 2006). A reduction in the sugars consumption during the first fermentation days was observed after the application of pyrimethanil in vineyards (Čuš & Raspor, 2008) and also lower specific growth rates were obtained in laboratory scale assays using synthetic must (Gil et al., 2014). Quinoxifen decreased the ethanol production rate and the sugars-to-ethanol yield (Saaris et al., 2009) while pyraclostrobin increased the ethanol and the biomass production (González-Rodríguez et al., 2011). The sugars-to-ethanol yield was also altered in the presence of ametoctradin, dimethomorph and mepanipyrim (Noguerol-Pato et al., 2014b).

Besides, the presence of fungicide residues in grapes were associated with changes in the aromatic profile of wines (González-Álvarez et al., 2012a, 2012b; Kosel et al., 2018; Noguerol-Pato et al., 2014a, 2015; Oliva et al., 2015). Previous results demonstrated the influence of mepanipyrim and tetraconazole residues on the biosynthesis of higher alcohols and acetates (Sieiro-Sampedro et al., 2019b, 2019a, 2020). Two volatile aromatic compounds desired in wines are 2-phenylethanol and 2-phenylacetate since they have a positive contribution to wine aroma with rose nuances (Swiegers et al., 2005). 2-Phenylethanol is generated by yeast from its amino acid precursor –phenylalanine– through the Ehrlich pathway during the exponential growth phase and later, this compound is oxidised to 2-phenylacetate (Dzialo et al., 2017). These changes being dependent on the fungicide applied and its concentration, the grape variety and the yeast

strain used during the fermentation process. However, mechanisms involved in these changes remain quite unexplored.

Articles focused on the study of the impact of fungicides on *Saccharomyces* yeast metabolism from a proteomic or a transcriptomic point of view are yet scarce on the literature. Exposure to thiuram induced some genes involved in DNA repair, folding and proteolysis, response to oxidative stress and carbohydrate metabolism (Kitagawa et al., 2002). Pyrimethanil modified the expression of 1667 genes of the *S. cerevisiae* yeast, showing a substantial effect on those genes involved in amino acids metabolism, energy conservation, antioxidant response, and multidrug transport processes (Gil et al., 2014). Mancozeb increased the abundance of antioxidant enzymes, and also the expression of some proteins involved in the carbohydrate metabolism, protein synthesis, and protein degradation (Santos et al., 2009). *FLR1* gene was identified as a determinant of yeast resistance to mancozeb (Teixeira et al., 2008).

Considering the unavoidable use of fungicides to combat fungal disease on vineyards and their repercussions on the fermentation course and the aromatic profile of wines, a better knowledge of the interactions among nitrogen substrate, yeasts, and fungicides during the alcoholic fermentation is mandatory. Therefore, this work aimed to evaluate the effect of two commercial antifungal formulations (Frupica[®] and Domark[®] Evo) on the growth of Lalvin T73[™] yeasts and the biosynthesis of some aromatic compounds derived from the phenylalanine catabolism (phenylacetaldehyde, 2-phenylethanol and 2-phenylacetate). In this way, laboratory-scale assays could be a useful tool to study the effect of these fungicides. Moreover, a synthetic must supplement with phenylalanine as the unique nitrogen source was used for a better understanding of their catabolism. In order to explain the obtained results, proteomic and transcriptomic analysis was done. Additionally, the

effect of both fungicides on other fermentative parameters (such as sugars consumption and ethanol production) and phenylalanine consumption was evaluated.

2. MATERIALS AND METHODS

2.1. Analytical standards, solvents and reagents

Phenylacetaldehyde ($\geq 90\%$), 2-phenylethanol ($\geq 99\%$) and 2-phenylacetate ($\geq 97\%$) analytical standards were purchased from Sigma-Aldrich (Madrid, Spain). L-phenylalanine (98.5 – 101.0 %), DL-malic acid Plus[®] (99 %), potassium bisulfide Bioultra ($\geq 97.0\%$), Tween[®] 80, glycerol ACS ($\geq 99.5\%$), acetic acid, sodium azide Plus[®] ($\geq 99.5\%$), diethyl etoximethylenmalonate (DEEMM, 99 %), potassium hydroxide ($\geq 85\%$), sulfuric acid (95 – 97 %), β -mercaptoethanol ($\geq 98\%$), sodium dodecyl sulfate (SDS, suitable for electrophoresis $\geq 98.5\%$), protease inhibitor cocktail, Bradford reagent, Coomassie Brilliant Blue R, DL-Dithiothreitol (DTT, $\geq 99\%$), iodoacetamide (IAA, $\geq 99\%$), trifluoroacetic acid (99 %) and trypsin from porcine pancreas (Proteomics grade) were also from Sigma-Aldrich. D(+)-glucose anhydrous, D(-)-fructose extrapure Pharmpur[®] and ammonium hydrogen carbonate (reagent grade) were from Scharlab S.L. (Barcelona, Spain). Citric acid monohydrate, sodium hydroxide, boric acid, calcium carbonate, ethylenediaminetetraacetic acid, disodium salt (EDTA, $\geq 99\%$), sodium hydroxide, pellets (98 %) and yeast extract for microbiology from Panreac S.A. (Barcelona, Spain). BD Difco[™] dehydrated culture media from Becton, Dickinson and Company (Nueva Jersey, EE.UU). Yeast nitrogen base (YNB) without amino acids and ammonium sulphate, ergosterol (98 %) and sodium oleate (65 – 90 %) from ACROS Organics[™] Fisher Scientific (Pittsburgh, EE.UU.). 30 % acrylamide/Bis Solution, 37.5:1 was purchased from Bio-Rad (Hercules, Ca, USA).

Hydrochloric acid (37 %), absolute ethanol Multisolv[®] and water for HPLC were from Scharlab S.L. (Barcelona, Spain); methanol, chloroform and dichloromethane Chromasolv[™] for HPLC (≥ 99.9 %) from Sigma-Aldrich SL and acetonitrile Chromasolv[™] for HPLC (≥ 99.9 %) from Honeywell Riedel de Haën[™] of Fisher Scientific.

2.2. Yeast strain and medium

Commercial *S. cerevisiae* Lalvin T73[™] yeast (hereafter T73) was purchased from Lallemand Inc (Montreal, Canada). Dried yeast (5 g) was rehydrated in water (50 mL) and cultured during 24 h at 30 °C in a YPD-agar medium (15 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 15 g/L agar). Afterwards, cells were cultured overnight at 30 °C, with orbital agitation (120 rpm), in a YPD medium (15 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose).

A synthetic must frequently use in microvinification experiments (Martínez-Moreno et al., 2012) with slight modifications was prepared as follows: 120 g/L of glucose, 120 g/L of fructose, 0.6 % citric acid, 0.6 % malic acid, 0.17 % of YNB without amino acids and ammonium sulphate, 15 mg/L of ergosterol, 5 mg/L of sodium oleate, 0.5 mL/L of Tween 80 and 60 mg/L of potassium disulphide. Phenylalanine at 160 mg/L was used as the unique nitrogen source. The medium was filtered through nitrocellulose filters (0.22 μ m) and the pH adjusted to 3.5 with 10 N KOH.

2.3. Alcoholic fermentation

Synthetic must was fermented under semi-anaerobic conditions, in the absence and the presence of the target fungicides (mepanipyrim and tetraconazole), during 9 days at 21

140 °C with orbital agitation (120 rpm). Triplicate fermentations were carried out using 400
141 mL of the synthetic must.
142 Frupica® (50 % w/w of mepanipyrin) and Domark® Evo (12.5 % w/v of tetraconazole)
143 commercial formulations obtained from Sipcam Iberia (Valencia, Spain) were dissolved
144 in ethanol (1000 µg/mL) and separately added to the fermentation medium. To a better
145 visualisation of the effects, a fungicide concentration level of twice their corresponding
146 maximum residual level (MRL) on wine grapes (4 and 1 mg/kg for mepanipyrin and
147 tetraconazole, respectively, according to Regulation 396/2005) was used. Before yeast
148 inoculation (10^6 cells/mL), ethanol concentration was standardised to values of 0.4 % in
149 all fermentations, and the fungicide concentration in the medium was confirmed by GC-
150 MS analysis (González-Rodríguez et al., 2009).

152 **2.4. Analytical determinations**

153 ***2.4.1. Fermentation parameters***

154 Along the fermentation process, aliquots (1 mL) of the liquid medium were taken to
155 monitor the fermentation course. Growth curves were followed by measuring culture OD
156 at 650 nm ($OD_{650\text{ nm}}$) in a Genova UV-Vis spectrophotometer of Jenway (Essex, England)
157 and results transformed in concentration using a calibration curve. The calibration curve
158 of yeast strain T73 was obtained from successive dilutions of a yeast extract with $OD_{650\text{ nm}} = 1$
159 by representing the $OD_{650\text{ nm}}$ at each dilution point vs the concentration of yeasts
160 (cells/mL) determined by plate counting at the same point. In order to confirm the validity
161 of the calibration curve for all the studied conditions, determinations of the concentration
162 of yeast by plate counting were also performed periodically. No interference of
163 ingredients of the antifungal formulation or the secondary metabolites were observed.

In addition, the content of reducing sugars, ethanol, glycerol and acetic acid was determined in a high-performance liquid chromatography (HPLC) model 1200 of Agilent Technologies (Santa Clara, California, USA) equipped with a refraction index detector (RI) and an ICSep Corregel-87H1 (300 x 7.8 mm i.d.) column of Transgenomic (San Jose, California, USA) following the chromatographic conditions described on González-Rodríguez et al. (2011). For a better monitoring of the fermentation course, other fermentation parameters (maximum specific growth rate ($\mu_{\max}$), sugar consumption rate (r_s), ethanol production rate (r_e), biomass-to-ethanol yield ($Y_{E/B}$), and sugar-to-ethanol yield ($Y_{E/S}$)) were calculated as described on González-Rodríguez et al., (2011) and Sieiro-Sampedro et al., (2019b).

2.4.2. Phenylalanine concentration

In order to determine the amino acid consumption rate, liquid samples were taken at different times along the fermentation process. Before chromatographic analysis, samples (1 mL) were centrifuged (5 min at 4000 rpm) and derivatised with DEEMM in a borate medium (pH 9) for 30 min using a Cleanosonic model 3510 ultrasonic bath of Branson (Wareham, Massachusetts, USA). The excess of DEEMM was removed by heating the samples at 70-80 °C for two hours (Gómez-Alonso et al., 2007).

Phenylalanine concentration was determined in a liquid chromatograph from Thermo Separation-Products (Massachusetts, USA) equipped with an SCM1000 vacuum membrane degasser, a P4000 quaternary pump, a TSP AS3000 autosampler, and a Surveyor PDA plus detector linked to a computer running the Xcalibur version 2.2 software program (Thermo Fisher Scientific). An Hypersil Gold C18 (100 x 4.6 mm i.d., 5 μ m) analytical column with a Pelliguard LC-18 (50 x 4.6 mm i.d.) guard column of Supelco (Bellefonte, Pennsylvania, USA) was used with a flow rate of 1 mL/min. The

mobile phase was 25 mM acetate buffer (pH 5.8) with 0.4 g of sodium azide (A) and a mixture 80:20 (v:v) of acetonitrile and methanol (B) with the following gradient conditions: 10 % of B for 2 min, from 10 to 90 % of B in 8 min, from 90 to 10 % in 2 min and keep the initial conditions for 10 min for reconditioning the column.

2.4.3. Volatile compounds

The concentration of 2-phenylethanol was determined by direct injection following the method of Peinado et al. (2004) with slight modifications. Samples were injected in the split mode (1:10) in a gas chromatograph equipped with a flame ionisation detector (GC-FID) from Thermo Fisher Scientific. Concentrations of phenylacetaldehyde and 2-phenylacetate were also determined by GC-FID after liquid-liquid extraction of the samples according to the protocol described by Ortega et al. (2001). Volatile compounds were separated in an HP-INNOWAX (60 m x 0.25 mm i.d.; 0.25 µm) analytical column from Agilent Technologies using the temperature program described by González-Álvarez et al. (2012). Helium was used as carrier gas (1.2 mL/min) and an air/hydrogen ratio of 10/1 (350 and 35 mL/min). Injector and detector temperatures were 250 °C.

2.4.4. Statistical analysis

All results were expressed as means $\pm$ standard deviations (n=3). The Statgraphics Centurion XV 15.2.05 Software (Statpoint Technologies, Inc., Warrenton, VA, USA) was used to finding statistically significant differences. Homogeneity of variances test was initially applied (Levene test), followed by an analysis of variance (ANOVA) and a Tukey's honestly significant differences test.

2.5. Transcriptomic analysis

Yeast cells were collected at three different fermentation times (10 h, 36 h, and 48 h). Differences in detected metabolites synthesised by yeast are expected to be caused by previous transcriptomics response to fungicides that would affect metabolism; therefore, early time points of fermentation were selected. Yeasts were centrifuged, snap-frozen in liquid nitrogen, and stored at -80 °C until use. RNA isolation was performed as described in (Alonso-del-Real et al., 2019) with slight modifications. Despite the kit recommendation to digest the yeast cell wall by the enzymatic reaction, a mechanic procedure was followed instead. It consisted of 3 x 30 seconds bead-beating steps using acid-washed glass beads, alternating with 1 minute resting on ice. RNAseq libraries were generated using the TruSeq Stranded mRNA Library Preparation Kit (Illumina, California, USA) and sequenced on a NextSeq Sequencing System from Illumina (2 x 75 bp). Raw files were deposited under the BioProject.

Sequence reads were mapped to the *S. cerevisiae* T73 genome using Bowtie2 v. 2.2.9 (Langmead and Salzberg, 2012) and the read counts for each gene were obtained with the HTSeq-Count (HTSeq-0.6.1p1, -m intersection-nonempty) (Anders et al., 2015). An average of 2.9 million reads per sample and a median of 245 reads per gene were obtained. Differential gene expression analysis was performed using the R package *DESeq2* (Love et al., 2014) and the Wald tests as previously described on (Alonso-del-Real et al., 2019). For this, every fungicide treatment at every time point was compared with the control at the same time point.

2.6. Proteomic analysis

Sample collection for proteomic analysis was made at the same times and in the same way described above for the gene expression analysis. Soluble protein extracts were obtained following the extraction method proposed by Von der Haar (2007). Then, 20 µg

of total protein per sample was loaded on a sodium dodecyl sulphate polyacrylamide gel (12 %) and concentrated in a small piece of gel using a Mini-PROTEAN® Tetra vertical electrophoresis cell from Bio-Rad (Hercules, California, USA). Protein bands were excised and subjected to an overnight in-gel digestion protocol with trypsin (40 ng) after a reduction (10 mM dithiothreitol at 56 °C) and alkylation (55 mM iodoacetamide at room temperature) step. Tryptic peptides were extracted from the gel matrix once with 0.5 % trifluoroacetic acid and twice with acetonitrile. Before LC-MS/MS analysis, samples were evaporated to dryness, resuspended in LC/MS-grade water (24 µL) containing 0.1 % (v/v) of formic acid and centrifuged (14000 rpm, 5 min).

Tryptic digests (12 µL) were separated on a PepMap® RSCL C18 reverse-phase column (75 µm x 50 cm, 2 µm, 100 Å) using a Proxeon Easy-nLC 1000 UHPLC system (Thermo Fisher Scientific, Waltham, MA USA). Acidified water (0.1 % of formic acid) and acidified acetonitrile (0.1 % of formic acid) were used as mobile phase A and B, respectively, increasing the percentage of B from 5 to 30 % in 240 min at a flow rate of 5 µL/min. Elutes were analysed by electrospray ionisation tandem mass spectrometry on a hybrid high-resolution LTQ-Orbitrap Elite Mass Spectrometer from Thermo Fisher Scientific using the following parameters: positive ion mode, data-dependent scan, mass range from 380–1600 m/z, 15 - 38 % of the normalised collision energy, dynamic exclusion time of 30 s, minimum signal threshold of 1000, and isolation width of 1.50 Da.

Proteome Discoverer (Thermo Fisher Scientific) software was used to protein identification against NCBIProt database from *S. cerevisiae* and label-free relative quantification, using a decoy sequence database to calculate the false discovery rate (FDR). The following parameters were used for positive protein identification: precursor mass tolerance 8 ppm, fragment mass tolerance 4 Da, number of matched peptide

sequences ≥ 1 , and peptide-spectrum matches (PSMs) < 0.5 % FDR. The QuickGO software from EMBL-European Bioinformatics Institute (<https://www.ebi.ac.uk/QuickGO/>) was used for functional annotation while the Perseus software (<https://maxquant.net/perseus/>) was used to detect statistically significant differences (p -value < 0.01) between treated and control yeasts.

3. RESULTS AND DISCUSSION

3.1. Effect of fungicides on the yeast growth

Under the studied conditions, yeast showed a lag phase lower than 10 h (**Figure 1**). During the exponential growth phase (1-3 days), the presence of fungicides did not affect biomass production, obtaining a $\mu_{\max}$ value around 2.1 days⁻¹ for all experiments. However, the behaviour of both fungicides during the stationary phase was different. Yeast inoculated on a mepanipyrin-enriched medium showed a similar growth trend to those growing in the control medium (without fungicides). Similar results were obtained for other fungicides (González-Rodríguez et al., 2011; Noguerol-Pato et al., 2014b; Saarís et al., 2009), including the fungicide pyrimethanil belonging to the same chemical family that mepanipyrin (Čuš and Raspor, 2008). However, in the presence of tetraconazole residues, a significant reduction of the total biomass concentration during the stationary phase was observed ($112.4 \times 10^6 \pm 3.37$) vs $121.6 \times 10^6 \pm 1.96$ cells/mL). These results agree with those reported for tebuconazole, another triazole fungicide, for which a reduction on the maximum biomass production was observed (González-Rodríguez et al., 2009b). However, no effects on biomass production were observed for the triazole fungicide fluquinconazole (Oliva et al., 2007). These results agree with our transcriptomic and proteomic data. As it can be seen in **Figure 2**, many genes and some proteins related to cell cycle progression and mitosis

were repressed in the samples treated with tetraconazole after 48 h of fermentation (**S1** and **S2** of the supplementary material). *Cell cycle* was the main enriched functional category for this group of samples (GO, biological process, p -value = 1.03×10^{-11}), and it was not the only one related to these process. Especially, *mitotic spindle organisation in the nucleus* (5.45×10^{-9}) and *chromosome segregation* (3.76×10^{-8}) seem to be the most repressed steps, with the most important number of genes present. Moreover, essential activators of Cdc28p by phosphorylation such as *CLN1*, *CKS1*, *MIH1*, and *CAK1*, that are key to cell cycle progression regulation were also repressed.

3.2. Effect of fungicides on fermentative parameters

The presence of both fungicides promotes different effects on fermentative parameters. Several indices were calculated to describe the fermentation course in presence and absence of each one of the target fungicides (**Table 1**).

- *Sugar consumption rate* (r_s). Sugars consumption followed a sigmoidal-decay response and no significant effects of fungicides were detected on this parameter during the exponential growth phase (**Table 1**) as previously reported by other authors (Noguerol-Pato et al., 2014b; D. Saaris et al., 2009). At the end of the study (9 days), 20.5 % and 58.6 % of the initial glucose and fructose, respectively, remain in the control medium. However, the final concentration of glucose was significantly higher in the presence of tetraconazole (26.6 % of the initial glucose). This could be related to the already reported repression of cell cycle progression, which would involve less cell density and, consequently, a lower sugar consumption. Moreover, differential expression of several genes encoding for sugar transporter was obtained. High-affinity sugar transporters *HXT2* and *HXT4* were down-regulated and

low-affinity sugar transporters *HXT1* and *HXT3* were overexpressed in the fermentation treated with tetraconazole concerning the control. Sugars concentration in the media is one of the main factor driving the expression of the different sugar transporters in yeast. Low-affinity transporters are more actively expressed at the initial stages of wine fermentation, while high-affinity transporters tend to appear at the end of the process when nearly all sugars have been consumed (Perez et al., 2005). In our case, as we mentioned, tetraconazole treated fermentations present higher levels of sugars than the control, which could be the reason for this set of genes differential expression.

On the contrary, the consumption of sugars in the mepanipyrin-enriched medium was similar to the control. No influence of aniline pyrimidine fungicides on the reducing sugars concentration was previously reported by (Čuš and Raspor, 2008).

- *Ethanol production rate.* While no effects were found for mepanipyrin, a lower ethanol production rate was observed during the exponential growth phase in the tetraconazole-enriched medium in comparison to the control (**Table 1**). However, as it can be seen in **Figure 1**, the maximum ethanol content achieved at the end of the study was similar for all fermentations (around 57.0 - 59.5 mg/L). These results agree with those reported for tebuconazole (González-Rodríguez et al., 2009b). For a better analysis of the effect of fungicides on ethanol production, the $Y_{E/B}$ and the $(Y_{E/S})$ parameters were calculated.

As expected, a higher $Y_{E/B}$ was obtained in the presence of tetraconazole, while no statistically significant differences were obtained for mepanipyrin. $Y_{E/B}$ increment could be related with the overexpression of *ADH1* (an alcohol dehydrogenase involved in the reduction of acetaldehyde to ethanol) registered in the presence of tetraconazole residues after 48 h of fermentation.

For $Y_{E/S}$, no significant effects were observed for anyone of the target fungicides. In previous work, lower ethanol production and lower $Y_{E/B}$ and $Y_{E/S}$ were observed in both a commercial pasteurised must and a Tempranillo red must fermented by *S. cerevisiae* strain VRB in the presence of mepanipyrim (Noguerol-Pato et al., 2014a). The different behaviour between both studies could be attributed to differences in the fermentation media (a more restrictive medium was used in this work) and also to the fungicide concentration assayed (3 mg/kg vs 4 mg/kg in this work).

- *Glycerol production*: a higher glycerol production was observed in the presence of mepanipyrim residues in comparison with the control experiment (7.8 vs 6.7 mg/L, after 9 days of fermentation) while no effects were observed for tetraconazole. Proteomic analysis of yeast growing in the mepanipyrim-enriched medium showed a higher presence of the enzyme Gpd2p, responsible for the formation of glycerol-3-phosphate in the glycerol synthesis pathway. Furthermore, the gene *RHR2*, coding for the enzyme that synthesises glycerol from glycerol-3-phosphate, was overexpressed according to our transcriptomic analysis. Nevertheless, contradictory results were obtained for other genes and proteins involved in glycerol metabolism. Therefore, for a better understanding of the influence of mepanipyrim on glycerol formation, transcriptomic analysis in later stages of fermentation would have been probably needed.

3.3. Effect of fungicides on the phenylalanine consumption

Amino acid concentration decreased exponentially during the first three days of the fermentation process and was practically consumed at this point (Table S3 of the supplementary material). The presence of tetraconazole residues seemed to stimulate

phenylalanine consumption concerning the control experiment, being its consumption statistically different only at 48 h.

3.4. Effect of fungicides on aroma biosynthesis

From a quantitative point of view, higher alcohols are the main aromatic compounds produced by yeast through the Ehrlich pathway (Hazelwood, Daran, Van Maris, Pronk, & Dickinson, 2008). This route is also an alternative mechanism for NADH regeneration and adjusts the redox equilibrium in the cell (Van Dijken and Scheffers, 1986). The production of 2-phenylethanol through this pathway involves three steps: a transamination step to form the α -keto-phenylpyruvate, a decarboxylation step to form phenylacetaldehyde followed by a reduction step to form the higher alcohol. Afterwards, this alcohol can react with acetyl-CoA to form the corresponding ester, 2-phenylacetate (Etschmann et al., 2002; Hazelwood et al., 2008).

Phenylacetaldehyde concentration increased quickly during the first 10 h of fermentation and then decreased to produce 2-phenylethanol (**Figure 3**). As expected, the production of 2-phenylethanol was directly proportional to the phenylalanine consumption; corroborating that the production of this higher alcohol through the Ehrlich pathway was amino acid concentration-dependent (Fleet, 2003). Production of 2-phenylacetate was registered from 36 h of fermentation, increasing its concentration slightly from this point. A similar trend was observed in the presence of the target fungicides. Although no significant differences were observed in the phenylacetaldehyde concentration at each sampling time between treatments and the control experiment, it is essential to remark that its production was higher in the presence of tetraconazole residues (113.2 vs 89.1 mg/L at 10 h) and that it was reduced in a higher proportion during the first day (**Figure 3**). Concerning 2-phenylacetate production, significant differences were observed for

tetraconazole at 48 and 120 h, being its concentration higher than that found in the control experiment (increments between 5-13 %). Slightly higher content of this compound was also found at the final point of this study (216 h), but not statistically significant. In this sense, the presence of tetraconazole residues seems to accelerate the interconversion of phenylacetaldehyde into 2-phenylacetate through the Ehrlich pathway.

Interestingly, as it can be seen in **Figure 4**, analysis of the differentially expressed genes after 48 h of fermentation in the tetraconazole-enriched medium (**Table S1** of the supplementary material) revealed the overexpression of several genes (*BAT1*, *PDC1*, *PDC5*, *ADH1*, and *SFA1*) involved in every step of the Ehrlich pathway from L-phenylalanine (Dickinson et al., 2003), as well as the formation of its corresponding acetate (*ATF2*). Besides, some genes (*PFK1*, *PFK2*, and *ARO3*) involved in phenylpyruvate biosynthesis through the central carbon metabolism were also overexpressed. Moreover, according to our proteomic assays, overexpression of Gap1p (a general amino acid permease) and Atf2p (an alcohol acetyltransferase) was observed at the same fermentation time (**Table S2**). Permease activity of Gap1p could be induced in the presence of a poor nitrogen source (Magasanik and Stanbrough, 1995). Also, the expression levels of *ATF1* and *ATF2* affect the production of volatile esters, including 2-phenylacetate, although Atf2p seemed to play only a minor role compared to Atf1p (Mason & Dufour, 2000; Verstrepen et al. 2003).

4. CONCLUSIONS

Proteomic and transcriptomic assays show that tetraconazole residues have a significant impact on cell cycle progression and mitosis, especially after 48 h of fermentation. Mitotic spindle organisation in nucleus and chromosome segregation seem to be the most repressed steps. Moreover, essential activators of Cdc28p were down-regulated. Consequently, lower biomass production during the stationary phase was observed.

Furthermore, tetraconazole residues promoted overexpression of several genes and proteins involved in the biosynthesis of aromatic compounds derived from the Ehrlich pathway (phenylacetaldehyde, 2-phenylethanol, and 2-phenylacetate). However, at the end of the study, no significant differences in their concentration were found between the fermented control must (without fungicides) and the tetraconazole fermented must. On the contrary, no significant influence of mepanipyrim on *S. cerevisiae* T73 cell cycle progression, the fermentation parameters, and the biosynthesis of aromatic compounds derived from the Ehrlich pathway was observed.

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Conflict of interest

The authors declare that they have no conflict of interest.

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FIGURE CAPTIONS:

Figure 1: Evolution of sugars, ethanol, glycerol and biomass content in the absence (A) or presence of residues of mepanipyrin (B) and tetraconazole (C) commercial formulations.

Figure 2: Genes and proteins related to cell cycle repressed in the fermentations with tetraconazole after 48 hours. Hits observed in the transcriptomic analysis are highlighted in green, and those obtained from the proteomics analysis are highlighted in blue. The different cell cycle phases are depicted in the inner circle, and the main events taking part in every phase are represented in the outer circumference. Blue lines have been included to classify the genes and proteins found according to the process in which they are involved. The master regulator of the cell cycle in *S. cerevisiae*, Cdc28p, is represented at the centre of the inner circumference, with different green arrows pointing at it, meaning the regulation activity of proteins encoded by genes found in the analysis over Cdc28p.

Figure 3: Biosynthesis of aromatic compounds in the absence (A) or presence of residues of mepanipyrin (B) and tetraconazole (C) commercial formulations.

Figure 4: Genes and proteins involved in the Ehrlich and volatile ester formation pathways overexpressed in the presence of tetraconazole after 48 h of fermentation. Hits observed in the transcriptomic analysis are highlighted with a green frame, and those obtained from the proteomics analysis are highlighted in blue. The differential expression

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621 of each gene in the presence (green line) and absence (blue line) of tetraconazole with
622 time were also included.

Table 1. Indices used to describe the fermentation course in presence and absence of fungicides.

	Exponential Growth Phase									Stationary Phase					
	Glucose and Fructose consumption (r_s)			Maximum specific growth rate (μ_{max})			Ethanol production (r_e)			Biomass to Ethanol Yield ($Y_{E/B}$)			Sugars to Ethanol Yield ($Y_{E/S}$)		
	Mean	$\pm$	SD	Mean	$\pm$	SD	Mean	$\pm$	SD	Mean	$\pm$	SD	Mean	$\pm$	SD
Control	32.40 ^a	$\pm$	6.34	2.07 ^a	$\pm$	0.06	14.42 ^b	$\pm$	0.12	5.40 ^a	$\pm$	0.27	0.37 ^a	$\pm$	0.01
Mepanipirim	19.14 ^a	$\pm$	3.11	2.05 ^a	$\pm$	0.11	15.00 ^b	$\pm$	1.44	5.59 ^{ab}	$\pm$	0.41	0.37 ^a	$\pm$	0.01
Tetraconazole	29.83 ^a	$\pm$	1.55	2.15 ^a	$\pm$	0.02	12.42 ^a	$\pm$	0.59	5.93 ^b	$\pm$	0.26	0.40 ^a	$\pm$	0.01

Figure 1

Figure 1: Evolution of sugars, ethanol, glycerol and biomass content in the absence (A) or presence of residues of mepanipyrim (B) and tetraconazole (C) commercial formulations.

Figure 2

Figure 3

Figure 3: Biosynthesis of aromatic compounds in the absence (A) or presence of residues of mepaniprym (B) and tetraconazole (C) commercial formulations.

Figure 4