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Effects of penconazole on two yeast strains: Growth kinetics and molecular studies

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The aim of this study consisted to evaluate the impact of a pesticide (penconazole) on the growth kinetics and genotoxicity on two yeast strains (*Saccharomyces cerevisiae* and *Metschnikowia pulcherrima*). When the penconazole was added at different phases of the growth of *M. pulcherrima*, no effect was noticed on the kinetics of yeast growth but DNA adducts were observed when penconazole was added in the exponential phase. Increasing doses (1 – 15 maximum residue limit) of the pesticide added at the beginning of the fermentation did not induce DNA adducts while kinetics were affected.

Keywords: Growth kinetics / *Metschnikowia pulcherrima* / Penconazole / *Saccharomyces cerevisiae* / Yeast

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1 Introduction

Pesticides are widely used throughout the world to control pests and enhance agricultural production. Many of them are highly toxic and they are one of the main classes of chemical environmental pollutants which can contaminate the ecosystem. Their accumulation in living organisms can be the cause of serious diseases [1 – 4]. In spite of the efforts to solve this common problem by implementing the integrated crop management system, developing new analytical procedures to control pesticide residues, and creating very strict regulations, toxic residues still occur in food products, water, air, and soil [5 – 8]. Besides their impact on food hygiene, these residues have the potential to inhibit yeast fermentations, should there be residues on fruits at harvest [9 – 13]. A previous study [14] established clearly the interaction between six pesticides and two yeast strains frequently used in fermentation industries: *Saccharomyces cerevisiae* and *Metschnikowia pulcherrima*. Among the molecules tested, Penconazole (Fig. 1) was shown to be the most toxic for both strains as it affected growth and fermentation kinetics at all concentrations tested, starting at 0.2 mg/L, its maximum residue limit (MRL) and depending on the cultural conditions (aerobiosis and anaerobiosis). Furthermore, the two yeast strains responded differently to the presence of penconazole in the culture medium, *S. cerevisiae* appeared to be more resistant as normal fermentations were observed even in presence of ten MRL of the fungicide. Penconazole is a triazolyl fungicide, it is known to exert its toxicity by inhibiting ergosterol biosynthesis causing membranes dysfunction and disability to ensure substrates intake [15, 16]. On the other hand, numerous pesticides may be metabolically activated to electrophilic compounds able to interact with DNA and to form DNA adducts [2, 17 – 19]; genotoxicity resulting from such covalent binding of a chemical to DNA is already well established [20, 21]. In this work we have evaluated the cytotoxicity and genotoxicity of penconazole by studying its impact on growth kinetics, and investigating its ability to induce DNA adducts, genotoxicity biomarkers, for the two yeast strains at various concentrations and different stages of yeast growth.

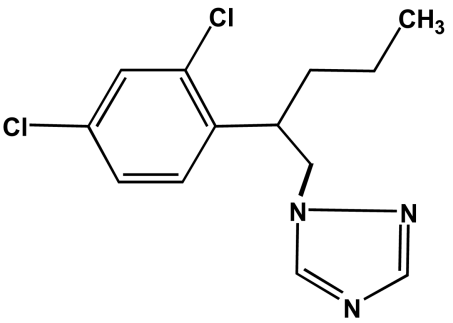

Figure 1. Chemical structure of penconazole.

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Abbreviation: MRL, maximum residue limit

2 Materials and methods

2.1 Chemicals

The enzymes were purchased as follows: proteinase K (used as received), RNase A. RNase T1 (boiled for 10 min at 100°C to destroy DNases), and micrococcal nuclease (dialyzed against deionized water) were from Sigma (Saint Quentin Fallavier, France); spleen phosphodiesterase (centrifuged before use) was from Calbiochem (VWR, France); and nuclease P1 and T4 polynucleotide kinase were from Roche diagnostics (Meylan, France). [$^{\gamma 32}$P-ATP] (444 Tbq/mmol, 6000 Ci/mmol) was from Amersham (Les Ullis, France); rotiphenol (phenol saturated with Tris-HCI, pH 8) was from Rothsichel (Lauterbourg, France); cellulose MN 301 was from Macherey Nagel (Düren, Germany); polyethyleneimine (PEI) was from Corcat (Virginia Chemicals, Portsmouth, VA); Whatman no. 1 paper was from VWR; and PEI/cellulose TLC plates used for ^{32}P-postlabeling analyses were prepared in the laboratory. Deionized water from a Milli-Q system (Millipore) was used for preparation of all aqueous solutions. Penconazole was a Riedel De Haën product (Germany) guaranteed 99% pure. Stock solution (≈500 mg/L) and working solutions were prepared in pure ethanol. At first, different doses (1 – 15 MRL) of the fungicide were added to the fermentation media before inoculation, and in the second test, ten MRL of penconazole were added at different stages during yeast growth: exponential growth phase, decreasing growth rate phase, and stationary phase.

2.2 Yeasts

The yeast strains used were *S. cerevisiae* MUCL 31497 and *M. pulcherrima* MUCL 29874, both obtained from the Fungi collection of Catholic University of Louvain in Belgium.

2.3 Cultures

Precultures and fermentative cultures were prepared in a semidefined medium [22]. Preculture was grown in two steps to ensure 3×10^6 cells/mL in each of the culture media. A regular evaluation of yeast population was carried out during fermentation by microscopic count (yeast cells *per* milliliter). Samples collected for chemical and molecular analysis were stored at −20°C.

2.4 Isolation of DNA

DNA was extracted and purified as previously described [23] with a minor modification described below. Yeast pellets were homogenized with 0.7 mL of a SET solution containing NaCl (0.1 M), EDTA (20 mM), and Tris-HCl pH 8 (50 mM) and were agitated by vortex for 6 min in presence of glass beads (0.5 μm). This modification was introduced because yeast walls are difficult to break in comparision with animal cells.

2.5 ^{32}P-postlabeling analysis of DNA adducts

The method used for ^{32}P-postlabeling was that initially described by Reddy and Randerath [24], with minor modifications fully described in Faucet *et al.* [25].

3 Results

3.1 Kinetics

3.1.1 Addition of various concentrations of penconazole before inoculation

Different concentrations (1 – 15 MRL) of penconazole were added in the culture medium before inoculation. Figure 2 shows the negative effect of the pesticide on the growth kinetics of the two yeast strains. *M. pulcherrima* seems to be more sensitive than the other strain. Indeed, it was affected even by small concentrations of this toxic, whereas *S. cerevisiae*, more resistant, showed only altered growth kinetics for concentrations higher than two MRL of penconazole.

To better compare the different behavior obtained by the two yeasts, other criteria might be used, *i. e.*, final biomass produced (ΔX max) and average growth rate (r_x). Figure 3 shows the evolution of these criteria in function of the toxic doses used.

3.1.2 Addition of penconazole during yeast growth

S. cerevisiae being more resistant than *M. pulcherrima* in the experiment described before (see Section 3.1.1), we tested first for the effect of penconazole added at different growth stages of *M. pulcherrima*, the sensitive yeast. The presence of the fungicide at ten MRL did not affect in any way the growth kinetics of this yeast; the same normal growth pattern was obtained in the cultures contaminated at different times after the beginning of the fermentations (4, 12, and 24 h) and in the control free of the fungicide (Fig. 4). This result may lead us to suppose that *S. cerevisiae* will not be affected, but the confirmation test should be done in the near future.

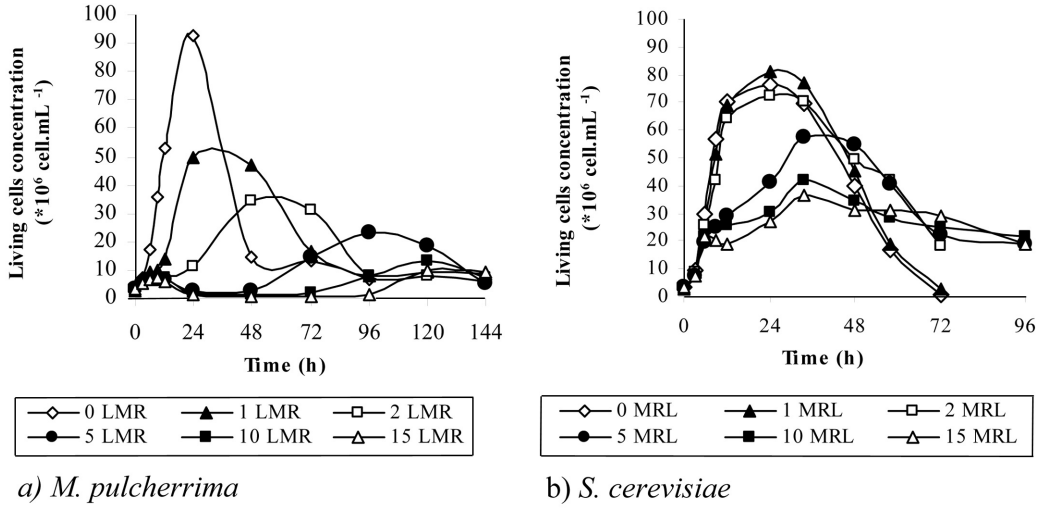

Figure 2. Effect of various doses of penconazole in addition before inoculation on growth kinetics of the two yeasts (a) *M. pulcherrima*, (b) *S. cerevisiae*.

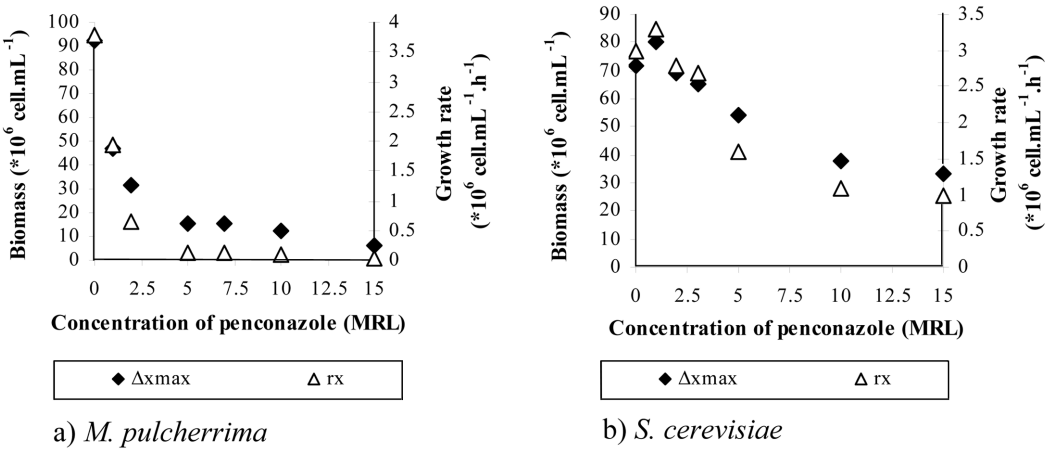

Figure 3. Effect of different doses of penconazole in addition before inoculation on the final biomass produced and the average growth rate of the two yeasts.

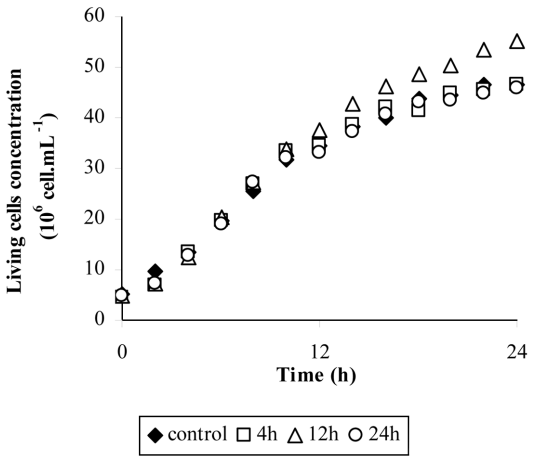

Figure 4. Effect of ten MRL of penconazole in addition during growth of *M. pulcherrima*, after 4, 12, and 24 h of the beginning of fermentation.

3.2 Molecular analysis

3.2.1 *M. pulcherrima*

DNA adducts analysis were carried out for all the culture experiments. The penconazole showed genotoxic effect only when added 4 h after the beginning of fermentation (during exponential growth phase). Figure 5 shows some autoradiograms obtained for different contact times yeast-fungicide at ten MRL. The DNA adducts were detected after 4 h of contact, they increased until 16 h and then disappeared after 24 h.

The addition of different doses of penconazole before inoculation did not induce any DNA adducts formation for any concentration (Fig. 6).

3.2.2 *S. cerevisiae*

Penconazole was not genotoxic for *S. cerevisiae* in the conditions tested, as no DNA adducts were obtained at any of

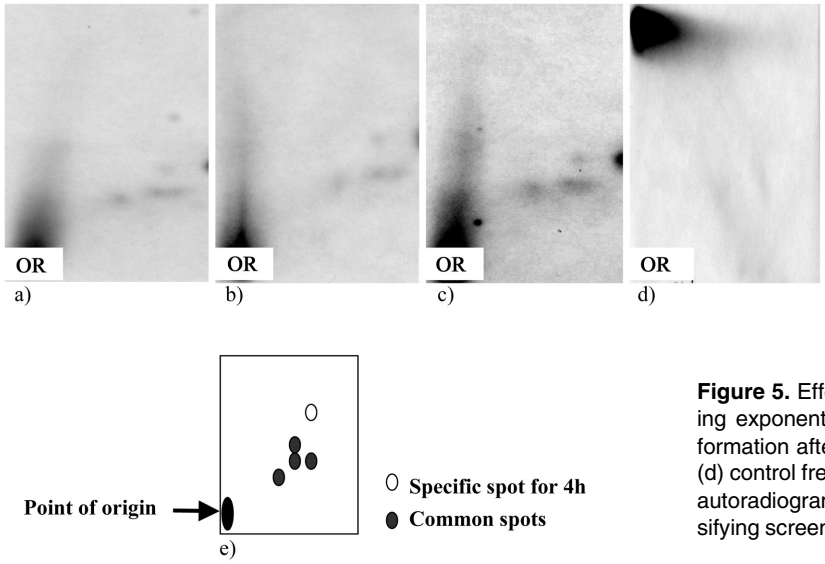

Figure 5. Effect of ten MRL of penconazole in addition during exponential phase of *M. pulcherrima* on DNA adducts formation after different contact times: (a) 4, (b) 8, (c) 12 h, (d) control free of pesticide, and (e) schematic profile. All the autoradiograms were exposed for 48 h at −80°C with intensifying screens.

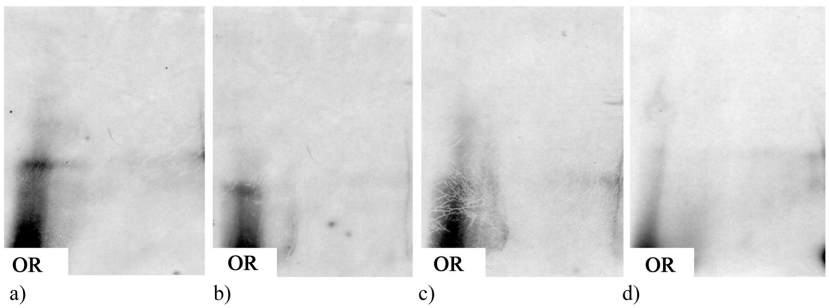

Figure 6. Effect of ten MRL of penconazole in addition before inoculation of *M. pulcherrima* on DNA adducts formation after different contact times: (a) 3, (b) 12, (c) 24 h, and (d) control free of pesticide. All the autoradiograms were exposed for 48 h at −80°C with intensifying screens.

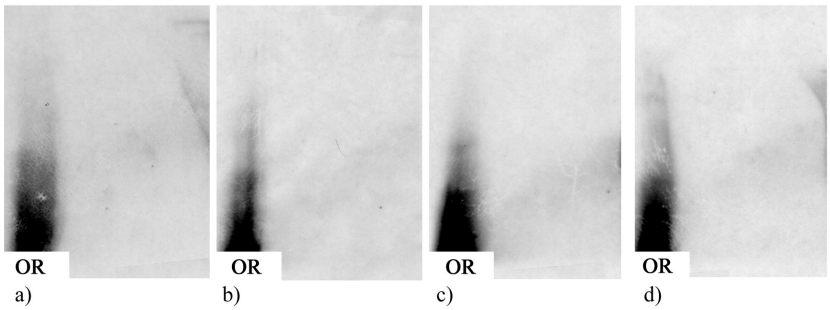

Figure 7. Effect of ten MRL of penconazole in addition before inoculation of *S. cerevisiae* on DNA adducts formation after different contact times: (a) 3, (b) 12, (c) 24 h, and (d) control free of pesticide. All the autoradiograms were exposed for 48 h at −80°C with intensifying screens.

the concentrations of the fungicide added before inoculation (Fig. 7).

4 Discussion

In this study it was shown clearly that penconazole affects the growth kinetics of *M. pulcherrima* and *S. cerevisaie* when present in the culture medium before inoculation. The effect was permanent all the way during the fermentations but the intensity of the response was not the same for the two yeast strains: *M. pulcherrima* exhibited high sensitivity toward the pesticide as its growth was altered even at small concentrations (0.2 mg/L), while *S. cerevisiae* showed more resistance.

This comes as a confirmation to a previous study [14]: a larger number of concentrations of the toxic were tested in a better defined medium (a natural undefined medium was used in the precedent study). The fungicide was further investigated for its effect depending on the physiological condition of *M. pulcherrima*; therefore, a unique dose (ten MRL) was added to the growing cultures of this yeast at three different stages, and no inhibition of growth was obtained at any stage. The addition of the fungicide on active, may be less sensitive, growing populations did not

affect their growth kinetics, this might be due to the fact that, by the time the fungicide was added to the medium, the yeasts population had already grown up to 15×10^6 to 20×10^6 cell/mL, while at the initial time, cells concentration was only around 3×10^6 cell/mL, so the ratio "toxic concentration/cells number" is to be considered. Further work is in progress to clarify these issues.

On the other hand, the molecular study revealed that penconazole, in our experimental conditions, failed to produce DNA adducts in *S. cerevisiae*. In contrast, for *M. pulcherrima*, penconazole was shown to be genotoxic according to the application way of the fungicide. DNA adducts were only detected when the fungicide was added during the exponential phase, while its addition in other phases at the same dose or even in higher doses before inoculation failed to produce DNA adducts. Interestingly, the genotoxicity was revealed in opposite conditions of growth kinetics alteration. This is not surprising because mechanisms involved in cell growth are different of those leading to DNA adduct formation. In general, DNA adduct results of the covalent binding of metabolites of the toxic. As it was possible to observe DNA adduct in the exponential phase of growth, it means that yeast has metabolic capacity to biotransform penconazole into genotoxic compounds. None is known on the metabolic pathway of penconazole in yeast and/or mammalian cells. In the future, we will compare the genotoxicity of this fungicide on mammalian cells.

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