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IMPACT OF PESTICIDES USED IN THE CULTURE OF THE VINE ON THE VIABILITY OF THE YEAST *SACCHAROMYCES CEREVISIAE* WINE IN CHRONOLOGICAL AGING

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ABSTRACT

Pesticides, used in culture, may induce oxidative stress by stimulation of free radicals production, what may result in lipid peroxidation, proteome damage, changes in DNA and RNA structures and disturbance of total antioxidative capacity in organisms' cells. In disturbances caused by increase synthesis ROS (reactive oxygen species) or lack antioxidative defense that is in oxidative stress it is seen one of all causes of aging process. Chronological aging of baker's and wine yeast *Saccharomyces cerevisiae* in liquid stationary culture is used as model of research on the aging process. As a result of aging changes take place in yeast cells which have physiological, genetic, metabolic and morphological character, what cause their death in consequence. Some scientists treat chronological yeast aging as analogy of fibroblasts aging of multicellular organisms, skeletal muscles or nerve cells. The aim of the experiment was to obtain the answer on question connected with toxicity effect two widely available pesticides in shape of trade preparation, used among other things in culture of grapevine Miedzian 50 WP (Cu 50WP) and Siarkol Extra 80 WP (S 80WP) on vitality of wine yeast in chronological aging. During research cells of wine yeast Tokay, which are used in production of white wines, and cells of Malaga strain, used in production of white and red wines, were applied. Yeast culture with pesticides supplementation in determined concentrations was conducted through seven days in YPG medium. At that time vitality of yeast cells was determined by the percentage of cells surviving, percentage of dead cells and culture density. Considerable influence on decreasing vitality of yeast cells in the process of aging showed S 80WP, what correlate with the increase of applied concentration in both example of Malaga and Tokay. Obtained results in application of Cu 50WP indicated lower toxicity in culture of both studied strains in comparison to the control. Our studies suggest that studied pesticides may cause the intensity of processes connected with cells aging.

Keywords: Pesticides, wine yeast, chronological aging, toxicity

INTRODUCTION

Pesticides are applied around the world to chemical protection of plants in order to reduce damages from the existence of both vermin and weeds and to increase the harvest of cultivated plants **(Wrzosek *et al.* 2009).** Despite that toxic actions of pesticides are directed on exact species there is a possibility, that their remains have disadvantageous health effects on both people and animals **(Weiss *et al.* 2004)**.
The mechanism of pesticides acting on cellular level, depending on the organism, is very differential and not fully elucidated. For example glyphosate (N-phosphonomethylglycine), active component, popularly used in herbicidal preparations, reduces the tissue, which assimilates CO_2 in green leaves and as a result is stops the process of photosynthesis. In the last years it was shown that glyphosate does not give in to fast biodegradation, it stays in soil and it may reach to earth water. It was demonstrated that glyphosate with low concentration is detected in blood serum of people **(Kwiatkowska *et al.* 2013)**. It may induce cancerous changes and affect on hormonal ones. Also it stimulates forming reactive forms of oxygen and it influence on the change in system of potential membrane of mitochondrion, what results in necrosis and apoptosis of cells of different types **(Banerjee *et al.* 2001, Heu *et al.* 2012)**. On the other hand, fungicides are directed on disturbances of integrity of cellular membrane or cell wall of fungi on the whole. Despite directed fungicidal application those chemical means to curing plant diseases have also others mechanisms of acting, including that they stop biosynthesis of sterols and polymerization of microtubules and damages mitochondrial respiratory chain in others than exact organisms. Toxic effect of fungicides may result in instability of cell wall, changes of osmolarity and production of reactive oxygen species (ROS) even within people **(Hayes *et al.* 2014)**. Therefore pesticides used in culture may induce oxidative stress by stimulation of production of free oxygen radicals **(Grosicka-Maciąg 2011)**. It is true that water radicals in physiological concentrations are essential in cell, for example H_2O_2 acts like signal molecule, but their excess is harmful and it may result in peroxidation of lipids, damage of proteome, changes in DNA and RNA structure and to disturbance of total antioxidative capacity, and even in death of cell **(Braconi *et al.* 2009, Gough and Cotter 2011)**. Fortunately, organisms are not defenseless against oxidative effect of these oxygen radicals. During course of evolution, with adaptation to oxygen conditions and reactive forms of oxygen, organisms also developed different defense systems against ROS. However, they are not able to eliminate damages caused by those factors completely. The result of it is aging of organisms **(Harman 1956, Passos and von Zglinicki 2005, Okusaga 2013)**. So that we decided to ask whether popular pesticides Cu 50WP and S 80WP have an influence on processes, connected with aging in model of long-term stationary culture of yeast *Saccharomyces cerevisiae*, which according to many scientists can be concerned as analogy to state of non-proliferating cells of human: fibroblasts, skeletal muscles and nerve cells **(Longo *et al.* 1996, Chen and Runge 2009, Balazsi 2010)**. Chronological aging of yeast is commonly used in studying of aging processes **(Grey *et al.* 2004, Longo *et al.* 2012)** (figure1).

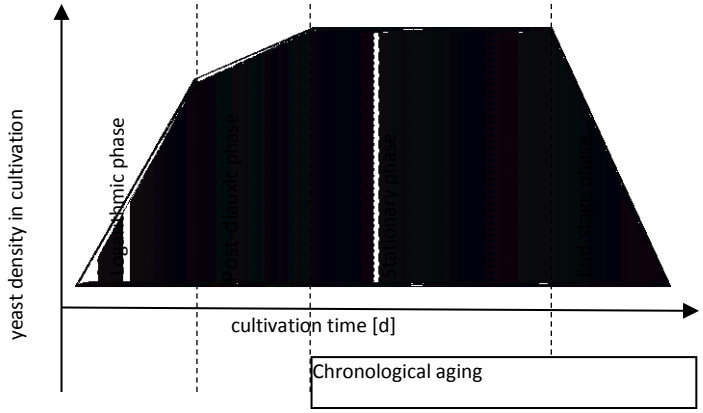

Figure 1 The dependence of the density and the metabolic state of the cells in YPD liquid culture on time (measured in days).

MATERIALS AND METHODS

For the experiment we used two, generally accessible, pesticides in the form of commercial preparations: Miedzian 50 WP authorization holder: Chemical Plant "Organika-Nitrogen" SA, ul. Chopena 94, 43-600 Jaworzno, e-mail: rozwoj@azot.pl, packer: Agrecol Company Ltd. 98-400 Wieruszów, Mesznary 2; email: agrecol@agrecol.pl; The active ingredient content: copper oxychloride as copper- 50%. Siarkol 80WP entity which is authorized: Chemical Plant "Organika-Sarzyna" SA, ul Chemists 1, 37-310 Nowa Sarzyna, e-mail: zch@zch.sarzyna.pl, packer „Nobila Sp. z o.o."Sp.K. ul. New 32, 37-400 Nisko, www.nobilapoland.com, The content of the active ingredient sulfur - 80%
The suspension pesticide prepared in sterile water in an amount to give a final concentration of pesticide in culture: 0.5 mM, 2 mM, 5 mM.
In this paper two strains in the form of commercial preparations wine yeast Saccharomyces cerevisiae has been used to Tokay and Malaga. Tokay is used for the production of white wines and cells of a strain Malaga for the production of white and red wines. The manufacturer of liquid culture of wine yeast is BIOWINE.K. 93-373 Łódź, ul. Pryncypalna 129/141, www. biowin.pl
Experiments were carried out in the tubes with a volume of 10 cm^3 in temperature of 28°C with shaking of 250 turns per minute, in 3 cm^3 of yeast medium . Inoculum was prepared in sterile liquid medium YPD (2% of glucose, 1% yeast peptone, 1% yeast extract, water) in all night long culture. Appropriated and long-term cultures were grafted by suspension of cells with density of $5 \cdot 10^5$ cells$\cdot cm^{-3}$. 24 hours after inoculation culture added a pesticide in the form of suspension to final concentrations of 0.5 mM, 2 mM, 5 mM. Measurements were started after one day the addition of pesticides. The same measurement was performed seven days later. Density of culture was determined by counting cells in Malasseza's chamber under the microscope, and next the culture was diluted by sterile water to density of $10^3 \cdot cm^{-3}$.
The term "vitality of cells" was proposed for work needs, which indicate metabolic state of cells in stationary phase, which allow taking reproduction by budding, checked by pan test on constant medium YPD and by measurement of culture density. Simultaneously vital cells may neither bud, nor stay alive. Then they do not colour by methylene blue.
In order to count percentage of cells' surviving (that is percentage of cell, capable to making colonies) in separate days of culture planted determined capacity of culture to known density so as to consist of 150 cells, on constant medium of YPD on polystyrene Petri dishes about 90 mm diameter and 16 mm height. Growth was assessed after 48 hours of culture in the temperature of 28°C and the percentage of survival was counted in comparison to planted amount of cells.
Percentage of dead cells was determined with application of dye (1 mg$\cdot cm^{-3}$ methylene blue in 2% of sodium citrate) through two minutes incubation in room temperature of water suspension of yeast with density of $1 \cdot 10^6 \cdot cm^{-3}$ with the same capacity of dye. Percentage of blue dyed cells (dead) was counted in Malasseza's chamber **(Kocwowa 1981)**.
All experiments were carried out in three independent repeats.

Statistical analysis

All data are expressed as the mean±standard deviation (SD). The Mann–Withney U test was used for analyzing the results. We used the following software for statistical analyses: STATISTICA – StatSoft Polska sp. z o. o.(version 10.0 for Windows), (p<0.05).

RESULTS

In presented study on the pesticide influences on process of yeast aging culture we checked the aging indicators, such as density culture, the viability of cells and the number of dead cells. Results are shown in figures 2 - 7.
During the first day of culture significant drop of culture density of Malaga in supplementation of Cu 50WP and S 80WP was observed in comparison to control. Especially addition S 80WP decreased density about 30% in comparison to control. Also toxic result of S 80WP influenced Tokay cells. Whereas Cu 50WP indicated differentiated effect on level of culture density of Tokay and with concentration of 2 mM significant increase of culture density was noted in comparison to control (figure 2).

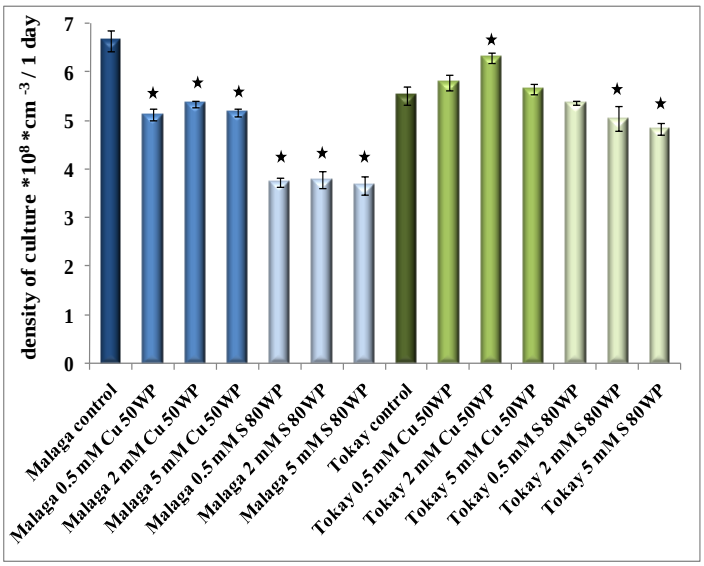

Figure 2 The comparison of the density of culture *Saccharomyces cerevisiae* strains Malaga and Tokay after 1 day of culture after the addition of pesticides Cu 50WP and S 80WP in long-term cultures in liquid medium YPG.

After seven days of culture pesticides Cu 50WP and S 80WP decreased culture density in both studied strains significantly in comparison to control. Especially S 80WP turned out to be toxic for both Malaga and Tokay (figure 3).

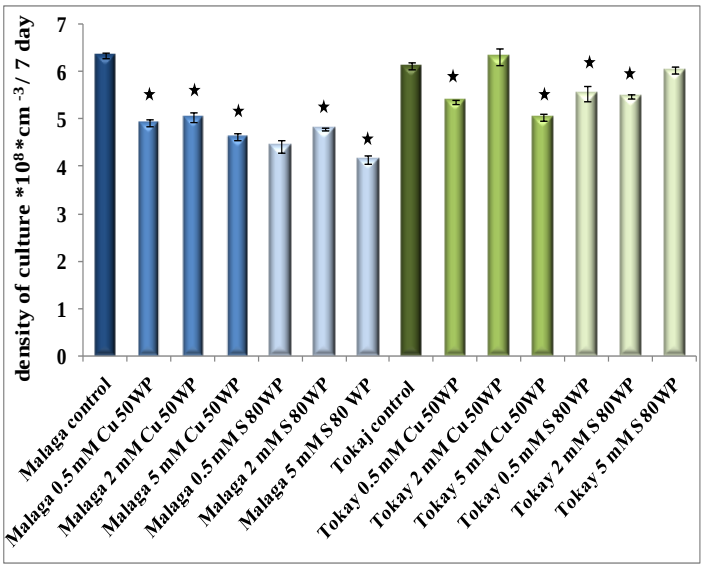

Figure 3 The comparison of the density of culture *Saccharomyces cerevisiae* strains Malaga and Tokay after 7 day of culture after the addition of pesticides Cu 50WP and S 80WP in long-term cultures in liquid medium YPG.

As early as after 1 day in culture both tested pesticides Cu 50WP and S 80WP significantly reduced the survival level in yeast strands Malaga and Tokay (figure 4). There was about fivefold reduction in comparison to the control level

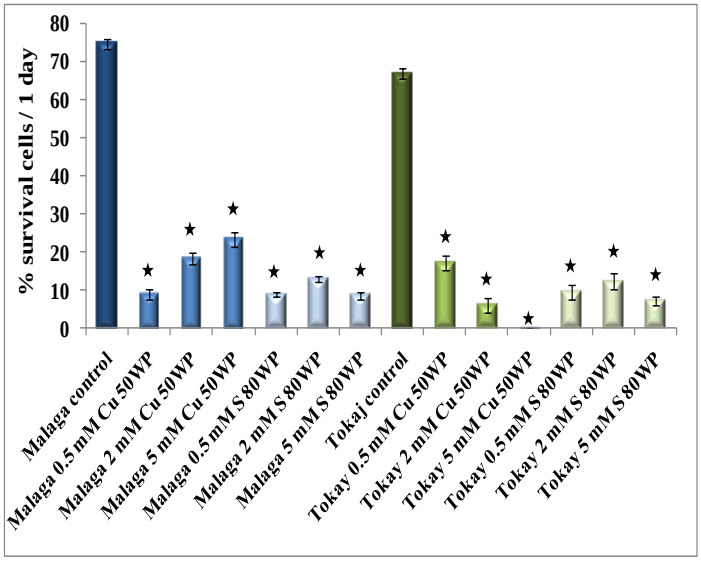

Figure 4 The comparison of the cell survival of *Saccharomyces cerevisiae* strains Malaga and Tokay after 1 day of culture due to pesticides Cu 50WP and S 80WP treatment.

After 7 day in culture there was twice decrease of survival due to Cu 50WP in Malaga strain (figure 5). Observed decrease was dosage independent. In Tokay strain decrease of survival also was observed, however it was dosage dependent and 0.5 mM Cu 50WP induced only 15% downregulation, 2 mM and 5 mM Cu 50WP caused 40 % and almost 100% decrease, respectively. S 80WP in 7 days time point severely reduced the survival level in both yeast strands and in all tested concentrations.

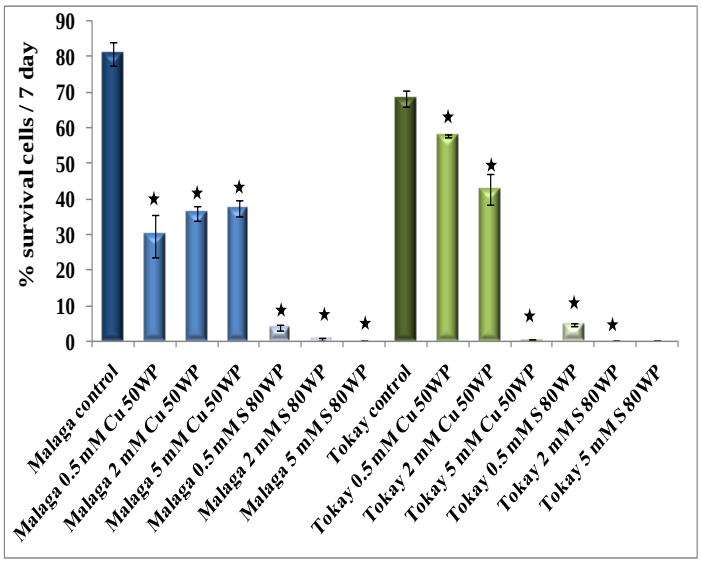

Figure 5 The comparison of the cell percentage survival of *Saccharomyces cerevisiae* strains Malaga and Tokay after 7 day of culture after the addition of pesticides Cu 50WP and S 80WP in long-term cultures in liquid medium YPG.

On the first day of culture it was observed increase of percentage of dead cells in culture subjected to pesticides effect in comparison to monitoring cultures in both studied yeast strains. Cultures of Malaga yeast indicated significant increase of percentage of dead cells, caused by pesticides Cu with concentration of 2 mM in all applied concentrations (0.5 mM, 2 mM, 5 mM) of S 80WP. Cultures of Tokay yeast indicated significantly, even to 50%, higher percentage of dead cells under the influence of pesticide Cu 50WP (2 mM and 5 mM) and up to 60% under the influence of pesticide S 80WP in all applied concentrations in comparison to control (figure 6).

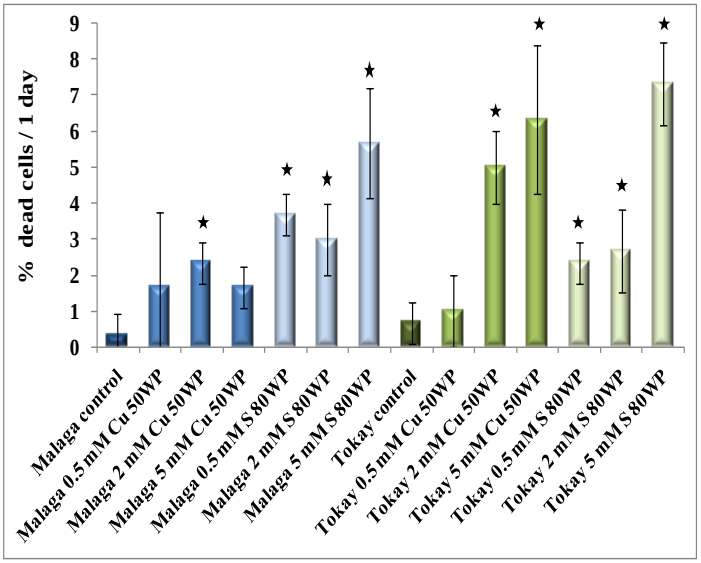

Figure 6 The comparison of the dead cells percentage of *Saccharomyces cerevisiae* strains Malaga and Tokay after 1 day of culture after the addition of pesticides Cu 50WP and S 80WP in long-term cultures in liquid medium YPG.

After 7 days of culture significantly it was seen increase of percentage of dead cells of Malaga strain statistically under the influence of both applied pesticides in all concentrations. Especially S 80WP with doses of 2 mM and 5 mM increased mortality of cells one after the other about 40% and 80% in comparison to control. On the other hand, Tokay cells indicated increased mortality under the influence of Cu 50WP than S 80WP. With concentrations Cu 0,5 mM it was observed about 20% in 2 mM- about 60%, and in 5 mM- about 70% more dead cells in comparison to control. Tokay cells were less sensitive to S 80WP and in concentrations of pesticide 2 mM and 5 mM indicated increase of cells mortality one after the other about 15% and 30% in comparison to control (figure 7).

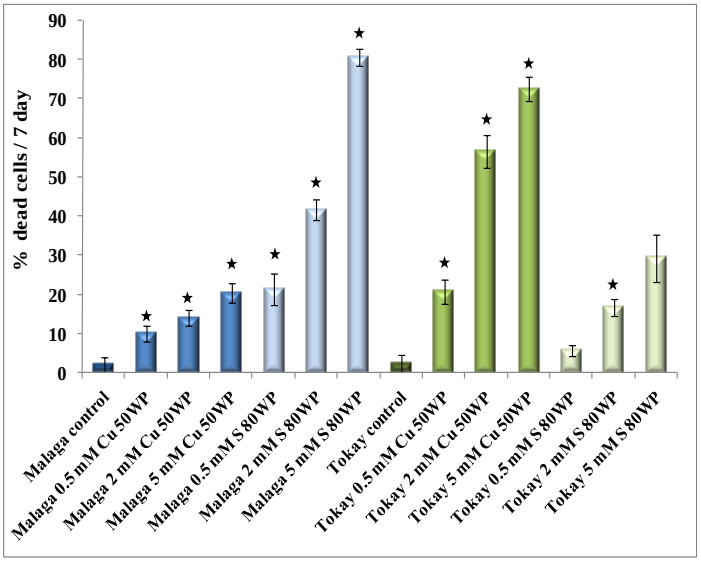

Figure 7 The comparison of the dead cells percentage of *Saccharomyces cerevisiae* strains Malaga and Tokay after 1 day of culture after the addition of pesticides Cu 50WP and S 80WP in long-term cultures in liquid medium YPG.

CONCLUSIONS AND DISCUSSION

The addition of pesticides Cu 50WP and S 80WP in proposed concentrations of culture of wine yeast Malaga and Tokaj significantly decreased cells vitality culture. It was observed that toxic influence of Miedzian was a little lower in cultures of both studied strains in comparison to S 80WP with reference to control. That result can explain that effect of sulphur is bipolar. Sulphur, as active substance S 80WP, gets in to fungi cell due to its solubility in fats, disintegrates cellular membrane and causes water flow from the cell, which results in death of fungi **(Ciesielska *et al.* 2011)**. Also sulphur indicated effect on level of respiratory system (cytochrome b), which comes in place of oxygen as acceptor of electrons. As a result of sulphur reduction hydrogen sulphide comes into being, which prevents ATP form coming into existence and causes significant loss of energy on cellular level **(Ciesielska *et al.* 2011)**. Wheras copper contained in Cu 50WP effects on superficial mainly. Copper ions gets in to semipermeable membrane and chitinous wall of fungi scores, driving out ions of hydrogen,

calcium and magnesium. Then inactivation of structural and enzymatic proteins of cellular membrane takes place **(Ciesielska *et al.* 2011)**. Copper compounds in shape of oxychloride also participate in inhibition of energetic processes and respiratory enzymes in sprouting mushrooms spore **(Meszka 2011)**. Hypothesis can be taken into consideration that both pesticides Cu 50WP and S 80WP may also cause to appearance of free spores in yeast cells. This may result in increased chronological aging of yeast cells **(Longo *et al.* 2012)**. As a result of accumulation of oxidative damages and high level of superoxide anion yeast cells lose ability of budding in chronological aging **(Demir and Koc 2010)**. Whereas increase of surviving yeast cells in both studied strains on the seventh day of stationary culture in presence of Miedzian in comparison to the first day of culture may prove about cells adaptation to oxidative stress known as mitohormesis **(Kharade *et al.* 2005, Piper *et al.* 2006, Ristow and Zarse 2010)**. In the presence of sulphur in Malaga culture shown increased sensitivity on that pesticide. Our studies suggest, that studied pesticides may cause to increase processes, connected with cells aging. In the next stage of research we are planning to check the level of total antioxidative ability and formation of superoxide anion in those aging cultures.

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