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## CONCENTRATION-DEPENDENT EFFECTS OF *RHODIOLA ROSEA* ON LONG-TERM SURVIVAL AND STRESS RESISTANCE OF YEAST *SACCHAROMYCES CEREVISIAE*: THE INVOLVEMENT OF YAP 1 AND MSN2/4 REGULATORY PROTEINS

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□ Concentration-dependent effects of aqueous extract from *R. rosea* root on long-term survival and stress resistance of budding yeast *Saccharomyces cerevisiae* were studied. At low concentrations, *R. rosea* aqueous extract extended yeast chronological lifespan, enhanced oxidative stress resistance of stationary-phase cells and resistance to number stressors in exponentially growing cultures. At high concentrations, *R. rosea* extract sensitized yeast cells to stresses and shortened yeast lifespan. These biphasic concentration-responses describe a common hormetic phenomenon characterized by a low-dose stimulation and a high-dose inhibition. Yeast pretreatment with low doses of *R. rosea* extract enhanced yeast survival and prevented protein oxidation under H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. Positive effect of *R. rosea* extract on yeast survival under heat shock exposure was not accompanied with changes in antioxidant enzyme activities and levels of oxidized proteins. The deficiency in transcriptional regulators, Msn2/Msn4 and Yap1, abolished the positive effect of low doses of *R. rosea* extract on yeast viability under stress challenges. Potential involvement of Msn2/Msn4 and Yap1 regulatory proteins in realization of *R. rosea* beneficial effects is discussed.

*Key words:* *Saccharomyces cerevisiae*; *Rhodiola rosea*; *lifespan*; *stress resistance*; *Yap1p*; *Msn2/Msn4p*.

### INTRODUCTION

The yeast *Saccharomyces cerevisiae* is a widely used model organism to study cell biology, metabolism, cell cycle and signal transduction. The availability of different yeast mutants makes possible evaluation of the role for any gene in cell metabolism and adaptation to environmental changes (Longo 1999; Fabrizio and Longo 2003; Semchyshyn 2009; Lushchak 2006; 2010; 2011). Some studies have reported that bakery's yeast, similarly to nematode *Caenorhabditis elegans* and fruit fly *Drosophila melanogaster*, is a good model system for development of anti-aging approaches. Life-extending effect of some extracts or purified compounds from plants, such as resveratrol from grape skin (Howitz *et al.* 2003), quercetin from

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M. M. Bayliak and others

oak bark (Belinha *et al.* 2007) and *Rhodiola rosea* extract (Bayliak and Lushchak 2011) was demonstrated for *S. cerevisiae* cells.

*Rhodiola rosea* is a well-known plant with adaptogenic properties widely used in folk medicine in Eastern Europe and Asia, particularly in Western Ukraine. The extract from *R. rosea* rhizome was reported to have numerous health benefits in humans and animals, including adaptogenic, antioxidant, cardioprotective, anticancer and anti-aging activities, stimulation of central nervous system and enhancement of work performance (Jafari *et al.* 2007; Wiegant *et al.* 2008; Panossian *et al.* 2010; Gospodaryov *et al.* 2013). Despite documentation of a variety of beneficial effects, molecular mechanisms of *R. rosea* action are not clear yet. Some authors consider effects of *R. rosea* on organisms are partly attributed to antioxidant properties of its phenolic compounds (Lee *et al.* 2004; Kim *et al.* 2006b; Schriener *et al.* 2009b). However these compounds are not only antioxidants, but may be suggested to also stimulate antioxidant system of organisms. These both properties are considered to be one of the mechanisms for lifespan extension along with enhancement of stress resistance (Boon-Niermeijer *et al.* 2000; Calcabrini *et al.* 2010; Mao *et al.* 2010). However, stress-protective effects of *R. rosea* on cell viability without activation of antioxidant defense were also demonstrated (Schriener *et al.* 2009a).

The beneficial effects of *R. rosea* extract depended on its concentration and physiological state of organisms. Being beneficial at low concentrations, *R. rosea* may become toxic at higher levels (Wiegant *et al.* 2009; Schriener *et al.* 2009b; Bayliak and Lushchak 2011; Gospodaryov *et al.* 2013). This phenomenon has been widely observed in toxicology and medicine, and was termed “hormesis” (Wiegant *et al.* 2008; Calabrese and Mattson 2011). In this respect, phytochemicals are considered as hormesis-providing compounds, that induce an adaptive stress response making organisms resistant not only to high (and normally harmful) doses of the same agent, but also against other stressors including oxidants, nutrients and heat (Son *et al.* 2008). In previous study we found that *R. rosea* rhizome extract at concentrations extending *S. cerevisiae* lifespan did not demonstrate protective effect against oxidative stress induced by H<sub>2</sub>O<sub>2</sub> and significantly increased sensitivity of “young” exponentially growing cells to this oxidant (Bayliak and Lushchak 2011).

In animal studies, some mechanisms of protective activity of *R. rosea* have been identified. There were a regulation of chaperone biosynthesis (Panossian *et al.* 2009) and influence on insulin/IGF-1-like/daf-2 pathway by activating DAF-16, a FOXO-family transcription factor, that controls the synthesis of proteins involved in stress resistance and thereby declines the rate of aging in *C. elegans* (Wiegant *et al.* 2009). In studies performed on cell culture, *R. rosea* increased expression of heme-oxygenase-1 (HO-1), which can be activated via the antioxidant-responsive element (ARE) in response to oxidative challenge (Wiegant *et al.* 2008). In *S. cerevisiae*

*Effect of Rhodiola rosea on yeast stress resistance*

Ras2/Cyr1/cAMP/PKA and the Sch9 pathways are similar to that identified in worms (insulin /IGF-1-like/daf-2 pathway) and regulate stress resistance and longevity by modulating activity of transcription factors Msn2/Msn4, which induce the expression of similar proteins (heat shock proteins, catalase, Mn-SOD and other housekeeping proteins) (Fabrizio and Longo 2003; Lushchak 2010). Msn2/Msn4p proteins are considered to be components of general stress response activated under different stress conditions (Costa and Moradas-Ferreira 2001; Erkina *et al.* 2009). Another transcriptional factor, Yap1p, is a key player in *S. cerevisiae* resistance to oxidative stress and many cytotoxic agents (Lushchak 2010; 2011), and some plant polyphenols seems realize their action in yeast via Yap1p (Maeta *et al.* 2007; Escoté *et al.* 2012).

In the present study, we estimated concentration-dependent effects of aqueous extracts from *R. rosea* rhizome on nonspecific stress resistance of “young” exponentially growing *S. cerevisiae* cells, which are somewhat relevant to proliferating cells of metazoas, and lifespan of “old” stationary yeast cultures, which are considered as a model of ageing of postmitotic somatic cells of higher eukaryotes. The involvement of antioxidant enzymes and transcriptional factors Msn2/Msn4p and Yap1p in stress-protective action of *R. rosea* aqueous extract was also examined.

## Materials and Methods

### Strains and growth conditions

The *S. cerevisiae* strains used in this study were as it follows: YPH250 (*MATa trp1-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52*) and YPH250 (*Δyap1*) (*MATa trp1-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52 yap1::HIS3*) kindly provided by Dr. Y. Inoue (Kyoto University, Japan); W303-1A (*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) and Wmsn2msn4 (*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 msn2::HIS3 msn4::TRP1*) kindly provided by Dr K. Küchler (Vienna University, Austria). Cells were grown at 28°C with shaking at 175 rpm in liquid medium containing 1% yeast extract, 2% peptone, 2% glucose (YPD). The starting cell concentration in medium was about  $0.3 \times 10^6$  cells ml<sup>-1</sup>. Exponential-phase and stationary-phase cells were harvested after cultivation for 15 h and 72 h, respectively.

### Preparation of *R. rosea* aqueous extracts and determination of salidroside concentration

Dried roots of *R. rosea*, collected at high altitudes in Ukrainian Carpathians, were used in this work. The rhizomes were comminuted to obtain particle fraction of 1-2 mm in size. The aqueous extraction was performed on the boiled water-bath in the ratio of 1:20 (comminuted dried rhizome material:distilled water) for 30 min. All liquid extracts

M. M. Bayliak and others

were filtered, sterilized by boiling for 20 min, and kept at 4°C for 2 days. The content of salidroside in aqueous extracts and in dried rhizome material was estimated spectrophotometrically at 486 nm according to method approved by the Soviet Pharmacopoeia (National Pharmacopoeia of the USSR 1990). The content of salidroside in dried rhizomes of *R. rosea* was  $17.7 \pm 0.5$  mg/g, and in prepared aqueous extract from *R. rosea* rhizomes the concentration of salidroside was  $0.080 \pm 0.009$  mg/ml.

### **Stationary-phase survival**

After 72 h of growth, the yeast cells were washed twice and then resuspended in sterile distilled water. The appropriate amounts of aqueous extracts from *R. rosea* root were added to cell suspensions, followed by incubation at regimen described above. The cells were washed twice every 2 days to remove all nutrients released by dead yeast, and resuspended in sterile water with addition of *R. rosea* preparations. To determine the number of viable yeast cells, starting at day 2 of incubation, every 2 days the aliquots were taken from each flask, serially diluted and plated. The viability was defined as the ability of an individual yeast cell to reproduce and form a colony within 72 h (colony forming unit, or CFU) on YPD agar plates (Fabrizio and Longo 2003). The amount of dead cells in yeast culture was measured by methylene blue staining (Smart *et al.* 1999).

### **Stress treatment**

Exponential-phase cells were harvested and resuspended in fresh medium, and incubated with different concentrations of *R. rosea* aqueous extract during 2 h. Then the cells were harvested, washed and resuspended in equal volume of 50 mM potassium phosphate buffer (pH 7.0). Aliquots of the experimental cultures were exposed to different stressors during 1 h at 28°C. To test stress resistance in stationary-phase cells, aliquots were removed from experimental cultures incubated in water for 2 days, and challenged with hydrogen peroxide for 1 h at 28°C. Cell survival after stress exposure was monitored measuring a number of colony-forming units' as described above.

### **Preparation of cell-free extracts, assay of enzyme activities, protein carbonyls, and protein concentration**

The preparation of cells extracts, measurement of activities of superoxide dismutase (SOD) and catalase, and determination of the content of carbonyl groups in proteins were conducted as described earlier (Lushchak *et al.* 2005). Protein concentration was determined by the Coomassie brilliant blue G-250 dye-binding method (Bradford 1976) with bovine serum albumin as a standard.

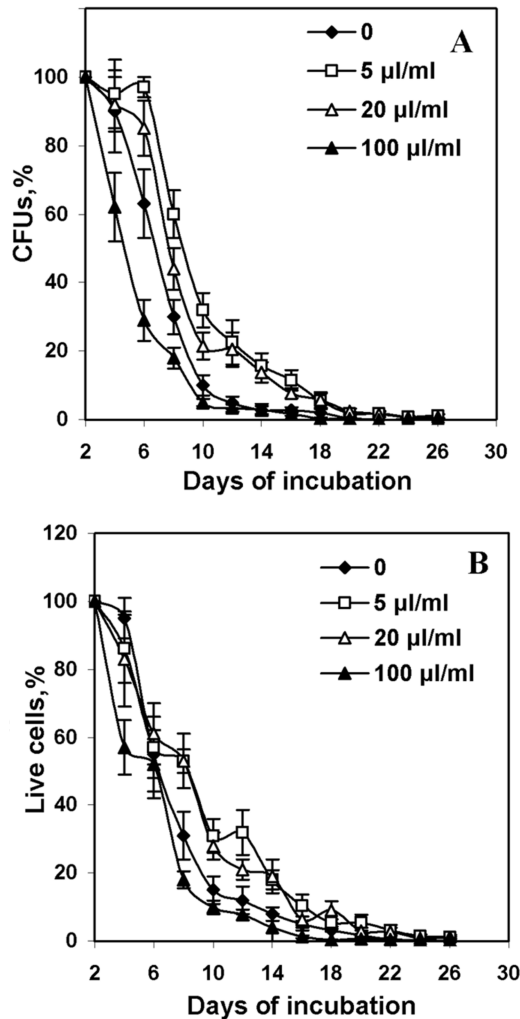
*Effect of Rhodiola rosea on yeast stress resistance***Statistical analysis**

Experimental data are expressed as the mean value of 4-6 independent experiments  $\pm$  the standard error of the mean (SEM), and statistical analysis used Student's t-test.

**RESULTS AND DISCUSSION****Stationary-phase survival**

Recently we demonstrated that supplementation of YPD medium with aqueous *R. rosea* extract at the concentration of 20  $\mu\text{l/ml}$ , which corresponded to the final concentration of salidroside of 16  $\mu\text{g/ml}$ , increased viability and reproduction ability of yeast cells at stationary phase (Bayliak and Lushchak 2011). In YPD medium yeast cells are actively budding and produced large amount of young cells. So, the population contains every time a mixture of young and old cells. It makes difficult accurate estimation of maximal yeast lifespan. Growth would create a mixed population containing both young and old organisms, which would invalidate the survival studies (Fabrizio and Longo 2003). The incubation of yeast cells in water is another approach used to investigate chronological yeast lifespan. Yeasts incubated in water have low respiration, enter a non-dividing hypometabolic stationary phase in which organisms are highly resistant to multiple stresses and can survive for up to 3 months (Longo 1999; Fabrizio and Longo 2003). We used this approach, i.e. yeast incubation in distilled water, to determine the effect of *R. rosea* aqueous extract at different concentrations on lifespan of *S. cerevisiae* strain YPH250. During incubation, the cells were washed every 2 days to remove all nutrients released by dead yeasts and re-suspended them in sterile water. Incubation in water and the removal of nutrients released by dead organisms minimizes the chance of growth during long-term survival in stationary phase. Figure 1A demonstrates the effect of *R. rosea* extract on the yeast viability evaluated by monitoring cell ability to form colonies (colony forming units – CFUs) on complete medium. CFUs were monitored until 99.5% of the population died. The number of viable cells which could reproduce and form colonies decreased in cultures with and without *R. rosea* extracts over time. A concentration-dependent modulation of yeast reproductive capability by the extracts was observed. Low concentrations of roseroot extract (5 and 20  $\mu\text{l/ml}$ ) increased CFUs by 20-25% at days 6-12 and by 5-10% ( $P < 0.05$ ) at days 14-18. Significant difference in yeast viability between experimental and control cultures was not observed after day 12. The highest concentration (100  $\mu\text{l/ml}$ ) of *R. rosea* preparation significantly decreased viability at first 8 days of incubation. Particularly, the amount of CFUs in cultures with 100  $\mu\text{l/ml}$  of *R. rosea* extract was lower by 40% than in control cultures at day 6. At further

M. M. Bayliak and others



**FIGURE 1.** Viability of *S. cerevisiae* YPH250 incubated in water at the presence of *R. rosea* aqueous extracts. Yeast viability was determined by counting the number of colony-forming units (CFUs) (A) and by staining of dead cells with methylene blue (B). CFU was calculated as the ratio between the numbers of colonies formed to the total number of cells plated. As 100% accepted number of viable cells at day 2. (n=4-6).

cultivation, the percentage of cells which kept reproductive capability was similar in cultures with roseroot preparations and in control.

The yeast viability was also evaluated by counting dead cells after methylene blue staining. The age-dependent decrease in the amount of live cells in cultures was observed (Fig. 1B). The results were similar to those obtained by CFU method. In cultures supplemented with 5 and 20 µl/ml of *R. rosea* aqueous extract percentage of live cells was higher by 15-20% on days 8-14, than control ones. The fastest death of yeast cells was observed for the cultures incubated with 100 µl/ml *R. rosea* extract. Any

*Effect of Rhodiola rosea on yeast stress resistance*

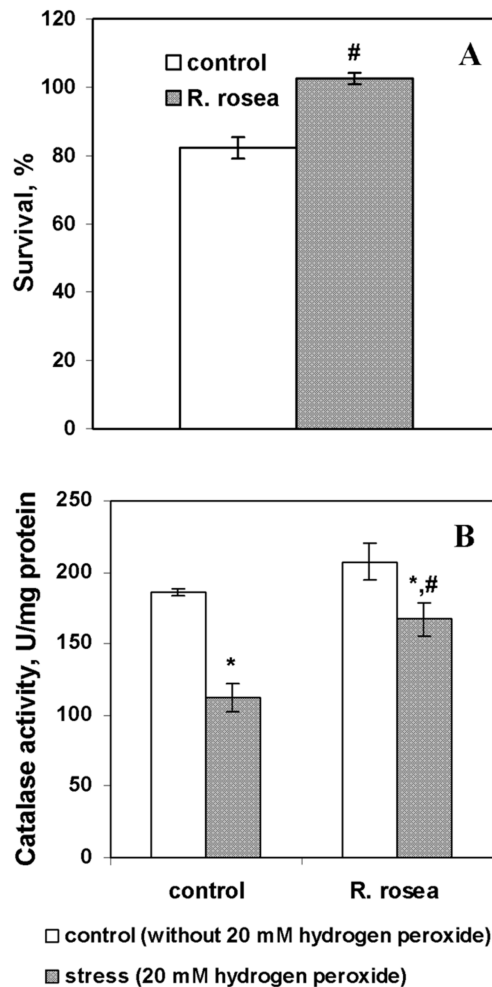
of *R. rosea* extract concentrations did not influence maximal lifespan of yeast cultures. A strong positive correlation was found between CFUs and a number of alive cells ( $R^2 = 0.952$ ). It is consistent with previous studies reported that the loss of the ability to reproduction was closely related with yeast cell death, but not with loss of ability to produce new buds (Fabrizio and Longo 2003; Bayliak and Lushchak 2011). These results confirm that *R. rosea* aqueous extract affects long-term viability of yeast cells in concentration-dependent manner. Low concentrations of *R. rosea* extract prevented cell death and increased the reproductive potential in population of aged yeast cells, while high concentrations of *R. rosea* extract decreased cell viability. This is in the line with studies demonstrated the similar dose-dependent action of *R. rosea* extract on lifespan of fruit flies (Jafari *et al.* 2007; Schriener *et al.* 2009a; Gospodaryov *et al.* 2013) and nematodes (Wiegant *et al.* 2009). It was proposed that at low concentrations *R. rosea* may act as a mild stressor, inducing adaptive response, which leads to an increase in cellular resistance, and thereby increasing maintenance and extending yeast lifespan. Higher *R. rosea* extract concentrations may be toxic inducing yeast cell death.

**Susceptibility to H<sub>2</sub>O<sub>2</sub> of stationary-phase cells**

It is known, that yeast cells in stationary phase acquire resistance to different stresses, such as oxidative, heat and osmotic stresses, where the boost in antioxidant defense plays an important role (Lushchak 2010; Semchyshyn *et al.* 2011). It is supported by multiple reports demonstrating that starving yeast cells were capable to withstand additional environmental oxidative stress. For example, the expression of genes encoding antioxidant enzymes, superoxide dismutases and glutathione reductase, was increased when yeast cells at stationary stage were exposed to menadione, a compound producing superoxide anion at autoxidation (Cyrne *et al.* 2003). Catalase activity was also increased in starving cells upon treatment with low concentrations of hydrogen peroxide (Bayliak *et al.* 2005). The ability of *R. rosea* to increase resistance against different stresses in a variety of model systems was shown (Boon-Niermeijer *et al.* 2000; De Sanctis 2004; Wiegant *et al.* 2009; Calcabrini *et al.* 2010; Mao *et al.* 2010). Therefore, we addressed the question whether incubation with *R. rosea* preparations could influence an acquirement of stationary yeast cells to additional resistance to oxidative challenge with H<sub>2</sub>O<sub>2</sub>. The concentration of *R. rosea* extract (5 µl/ml) that showed a strong positive effect on yeast viability was chosen to check the ability to provide resistance to hydrogen peroxide. Cell survival upon treatment with 20 mM H<sub>2</sub>O<sub>2</sub> decreased in control cultures by 20%, but was not changed in cells, preincubated with 5 µl/ml of *R. rosea* aqueous extract (Fig. 2A). The activity of catalase, a major H<sub>2</sub>O<sub>2</sub>-detoxifying enzyme in yeast, was similar in cells from control and roseroot-supplemented cultures (Fig. 2B). Exposure to 20 mM H<sub>2</sub>O<sub>2</sub> decreased catalase activity in con-



M. M. Bayliak and others



**FIGURE 2.** Effect of exposure to 20 mM H<sub>2</sub>O<sub>2</sub> for 1 h on cell survival (A) and catalase activity (B) in *S. cerevisiae* cells YPH250 incubated in water with 5 µl/ml of *R. rosea* aqueous extract during 2 days (n=4-6). \*Significantly different from respective values of untreated cells (without H<sub>2</sub>O<sub>2</sub>) with  $P < 0.05$ . #Significantly different from respective values in control cultures with  $P < 0.05$ . (n=4-6).

control cells by 40% and did not affect the enzyme activity in cells, incubated with *R. rosea* extract (Fig. 2B). The reduced catalase activity in control cells incubated with 20 mM H<sub>2</sub>O<sub>2</sub> could result from oxidative inactivation of this enzyme as it was observed earlier in yeasts (Bayliak *et al.* 2006; Semchyshyn and Lozinska 2012) and bacteria (Semchyshyn *et al.* 2001). Hence, incubation with *R. rosea* extract at the concentration 5 µl/ml enhanced viability of stationary-phase yeast cells by possible prevention of oxidative inactivation of catalase under H<sub>2</sub>O<sub>2</sub>-induced stress. The strong positive relationship ( $R^2 = 0.75$ ) between catalase activity and yeast surviving under H<sub>2</sub>O<sub>2</sub>-induced stress was described by us earlier (Bayliak *et al.* 2006).

*Effect of Rhodiola rosea on yeast stress resistance***Stress resistance of exponential-phase cells**

In the experiments described above we observed that yeast incubation with 5  $\mu\text{l}/\text{ml}$  of *R. rosea* aqueous extract enhanced the resistance of yeast cells in stationary phase to  $\text{H}_2\text{O}_2$ . Recently we found that yeast growth in the presence of *R. rosea* aqueous extract at concentration of 20  $\mu\text{l}/\text{ml}$  resulted in sensitizing of exponential-phase yeast cells to  $\text{H}_2\text{O}_2$  (Bayliak and Lushchak 2011). The difference in the results can reflect a concentration-dependent action of *R. rosea* observed above in yeast lifespan experiments. In addition, the difference in yeast physiological state at stationary and exponential culture phases could be important. To elucidate this situation, we examined the effect of pre-incubation with different concentrations of *R. rosea* aqueous extract on resistance of the exponentially growing *S. cerevisiae* cells to a number of stressors, which are known to have adverse effects on yeast viability. Table 1 presents the results of pre-incubation with *R. rosea* extract during 2 h on yeast survival under followed exposure to different stress factors: 10 mM  $\text{H}_2\text{O}_2$ , 2 mM  $\text{Cu}^{2+}$ , 2 mM  $\text{Cu}^{2+}/4$  mM  $\text{H}_2\text{O}_2$ , 20%  $\text{C}_2\text{H}_5\text{OH}$ , 200 mM  $\text{CH}_3\text{COOH}$ , and heat shock (40°C). It was shown that survival of *S. cerevisiae* YPH250 cells in control cultures without *R. rosea* extract was substantially reduced by treatment with these stressors. Pre-incubation with *R. rosea* extract at all used concentrations had no effect on yeast resistance to 20% of ethanol. In the case of hydrogen peroxide, effect of *R. rosea* extract was concentration-dependent. Pre-treatment with 1  $\mu\text{l}/\text{ml}$  of *R. rosea* extract resulted enhanced cell survival upon next exposure to 10 mM  $\text{H}_2\text{O}_2$  by about 25% compared to

**TABLE 1.** Effect of different stressors on survival (%) of exponentially growing *S. cerevisiae* YPH250 (wt) and YPH250 ( $\Delta\text{yap1}$ ) cells after pre-incubation with *R. rosea* aqueous extracts.

| Stressor  | Concentration of <i>R. rosea</i> aqueous extract, $\mu\text{l}/\text{ml}$ medium |             |             |             |
|---|--|-------------|-------------|-------------|
|   | 0  | 1           | 5           | 20          |
| <b>YPH250</b>                                     |  |             |             |             |
| 10 mM $\text{H}_2\text{O}_2$                      | 61 $\pm$ 7   | 81 $\pm$ 7* | 39 $\pm$ 7* | 39 $\pm$ 6* |
| 2 mM $\text{Cu}^{2+}$                             | 27 $\pm$ 6   | 49 $\pm$ 9* | 28 $\pm$ 7  | 22 $\pm$ 6  |
| 2 mM $\text{Cu}^{2+}/4$ mM $\text{H}_2\text{O}_2$ | 10 $\pm$ 4   | 23 $\pm$ 5* | 17 $\pm$ 2  | 16 $\pm$ 3  |
| 20% $\text{C}_2\text{H}_5\text{OH}$               | 14 $\pm$ 4   | 11 $\pm$ 4  | 7 $\pm$ 2   | 10 $\pm$ 2  |
| 200 mM $\text{CH}_3\text{COOH}$                   | 65 $\pm$ 7   | 80 $\pm$ 10 | 78 $\pm$ 12 | 91 $\pm$ 5* |
| Heat shock (40°C)                                 | 54 $\pm$ 5   | 70 $\pm$ 7* | 66 $\pm$ 6  | 57 $\pm$ 9  |
| <b>YPH250 (<math>\Delta\text{yap1}</math>)</b>    |  |             |             |             |
| 10 mM $\text{H}_2\text{O}_2$                      | 39 $\pm$ 6   | 32 $\pm$ 7  | 30 $\pm$ 6  | NS          |
| Heat shock (40°C)                                 | 86 $\pm$ 11  | 94 $\pm$ 3  | 85 $\pm$ 5  | NS          |

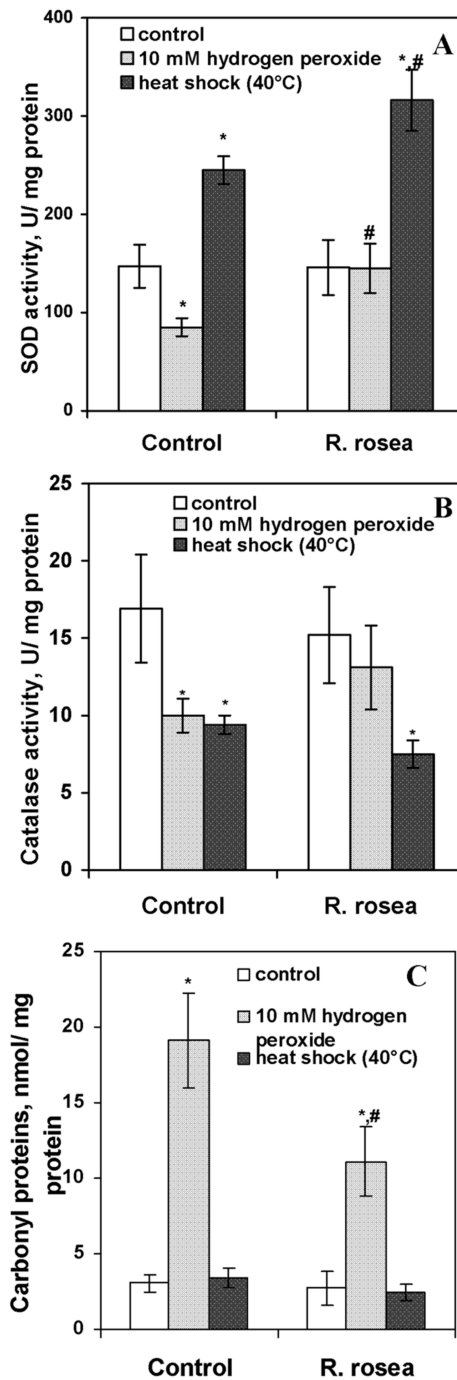
Cells growing exponentially in YPD medium were pre-incubated with *R. rosea* aqueous extracts during 2 h, and then they were collected and resuspended in 50 mM K-phosphate buffer (pH 7.0) and exposed to different stressors for 1 h at 28°C, n=5-6. \*Significantly different from respective control values with  $P<0.01$  using Student's *t*-test. Results are shown as means  $\pm$  SEM (n = 4-6). NS – not studied.

M. M. Bayliak and others

viability of H<sub>2</sub>O<sub>2</sub>-treated control culture. In contrast, higher concentrations of *R. rosea* aqueous extract, 5 and 20 µl/ml, enhanced the sensitivity of cells to hydrogen peroxide and reduced survival to 9 and 17% from control viability, respectively. The preparation at 1 µl/ml concentration also enhanced survival of yeast cells under 2 mM Cu<sup>2+</sup>, 2 mM Cu<sup>2+</sup>/4 mM H<sub>2</sub>O<sub>2</sub> and heat shock (40°C) treatments by 22, 13, and 16%, respectively. At higher concentrations of *R. rosea* extract, yeast cells showed virtually the same sensitivity to these stressors as the control cells. However, *R. rosea* extract at the highest concentration used in these experiments, 20 µl/ml, increased by 26% survival of yeast cell culture treated with 200 mM acetic acid while lower concentrations did not affect the resistance of yeast cells to the weak acid stress. Thus, these results demonstrate that *R. rosea* aqueous extract improves adaptive capability in *S. cerevisiae*, enhancing resistance to multiple stressors in dose-dependent manner. Similar results were shown in animal (Schriner *et al.* 2009a) and cell culture (Schriner *et al.* 2009b) studies reporting that *R. rosea* extract being beneficial at low doses, at high doses facilitated the toxic action of stressors.

The mechanisms of action of stressors used in this study are different, but they have one common feature. All of them are accompanied by development of oxidative stress, the situation, when the rate of reactive oxygen species (ROS) production exceeds the rate of their elimination by cellular defense mechanisms, leading to intensification of oxidation of biomolecules (Lushchak 2010; 2011). The rhizome of *R. rosea* contains a range of biologically active substances including several polyphenols, which were proposed to function as antioxidants (Panossian *et al.* 2010). In particular, it was shown, that *R. rosea* extract protected snail larvae against menadione (Boon-Niermeijer *et al.* 2000) and human erythrocytes against hypochlorous acid through antioxidant mechanism (De Sanctis *et al.* 2004). In order to determine whether in our experiments the positive effect of *R. rosea* extract at low concentrations on the stress resistance of *S. cerevisiae* was associated with the modulation of oxidative processes in yeast cells, we measured the activity of major antioxidant enzymes and the level of oxidized proteins. The content of carbonyl groups in proteins was taken as a marker of ROS-induced protein oxidation. Free radical oxidation of several amino acids, namely cysteine, histidine and some others, results in the formation of additional carbonyl groups in proteins. Due to many reasons, carbonyl groups are used commonly to evaluate the intensity of ROS attack to proteins (Stadtman and Levine 2000; Lushchak 2006; 2007). Figure 3 demonstrates the effects of pre-incubation with 1 µl/ml of *R. rosea* extract on the activities of SOD and catalase and level of carbonyl proteins in YPH250 cells treated with 10 mM H<sub>2</sub>O<sub>2</sub> and heat shock (40°C). The pre-incubation itself did not influence neither activities of catalase and SOD, nor level of carbonyl proteins in yeast cells (Fig. 3A-C). At the same time, these parameters were

Effect of Rhodiola rosea on yeast stress resistance



**FIGURE 3.** Effect of exposure by 10 mM H<sub>2</sub>O<sub>2</sub> and heat shock for 1 h on SOD activity (A), catalase activity (B), and level of carbonyl proteins (C) in exponentially growing *S. cerevisiae* YPH250 cells after pre-incubation with *R. rosea* aqueous extracts during 2 h. \*Significantly different from respective values of untreated cells (without H<sub>2</sub>O<sub>2</sub>) with  $P < 0.05$ . #Significantly different from respective values in control cultures with  $P < 0.05$ . (n=4-6).

M. M. Bayliak and others

different in the cells upon exposure to oxidative and heat stresses. The data presented in Fig. 3A-B show that exposure of YPH250 yeast cells to  $H_2O_2$  decreased SOD and catalase activities by 50 % and 70% in control cells, respectively, but roseroot extract efficiently prevented these changes. Figure 3C demonstrates the results of carbonyl proteins measurement in cells treated with different stressors. Upon exposure to 10 mM  $H_2O_2$ , the content of carbonyl groups in proteins increased in cells from both, control and experimental, cultures. However, in cells pre-treated with *R. rosea* extract level of CP consisted of 58% of ones not preincubated with the extract.

In our case, hydrogen peroxide treatment was probably too rigorous for cells, leading to possible oxidative inactivation of sensitive enzymes, including antioxidant ones. Increase in ROS-induced protein oxidation is also confirmed by the elevated level of protein carbonyls. The possibility of inactivation of antioxidant enzymes due to oxidative modification was already shown in our previous works and in studies of other authors (Costa *et al.* 2002; Bayliak *et al.* 2006; Lushchak 2006, Semchyshyn and Lozinska 2012). In addition, lower activities of antioxidant enzymes may be due down-regulation of defense mechanisms under lethal levels of stressors. In the cells pre-incubated with *R. rosea* aqueous extract hydrogen peroxide did not affect activities of SOD and catalase and resulted in significantly lower CP level compared with control. In line, yeast viability was higher than that in control. These results suggested that *R. rosea* aqueous extract in low concentrations could enhance the resistance of exponentially growing yeast cells to hydrogen peroxide preventing inactivation of antioxidant enzymes and oxidation of proteins. The same situation might take place during the stationary phase. As it was noted above, the *R. rosea* aqueous extract at low concentrations enhanced survival of stationary-phase yeast cells and prevented inactivation of catalase upon hydrogen peroxide exposure.

In the case of heat shock resistance, the situation was different from  $H_2O_2$  treatment. Heat stress increased SOD activity by 1.7-fold and 2.1-fold in control cells and cells pre-treated with *R. rosea* aqueous extract, respectively (Fig. 3A). Catalase activity decreased in similar manner in cells from both cultures under heat shock exposure (Fig. 3B). At the same time, no effect on carbonyl protein level in yeast cells was observed (Fig. 3C). Earlier it was found in *S. cerevisiae* increase in temperature elevated ROS production and antioxidant defense (Pereira *et al.* 2001; Kim *et al.* 2006a). Taking it into account, the increase in SOD activity in cells exposed to 40°C may be an adaptive response to increased ROS level, probably superoxide anion one. Superoxide anion at high concentrations may inactivate some enzymes, particularly catalase (Kono and Fridovich 1982). It may explain the lower activity of catalase in cells exposed to heat shock. It cannot be excluded that decrease in catalase

*Effect of Rhodiola rosea on yeast stress resistance*

activity could result also from down regulation. As in the case with hydrogen peroxide, *R. rosea* extract showed protective effect on yeast resistance against heat stress without activation of antioxidant defense. Similar results were shown in studies with animals (Schringer *et al.* 2009a) and cell cultures (Schringer *et al.* 2009b). Benefit to yeast cells can be conferred by antioxidant properties of *R. rosea* active compounds (Calcabrini *et al.* 2010; Panossian *et al.* 2010). However, yeast cells were pre-incubated with very low concentrations of aqueous *R. rosea* extract, and unlikely, that the cells accumulated the phytochemicals in amounts sufficient to scavenge H<sub>2</sub>O<sub>2</sub> or products of its metabolisms at high concentrations. At the same time, there is emerging evidence suggesting that hormetic mechanisms of action may account for many of the health benefits of different phytochemicals (Mattson 2008; Calabrese and Mattson 2011). This explanation be applicable to our experiments, as it was mentioned above with lifespan experiments. In this case *R. rosea* extract at low concentrations acts as a mild stressor, which activates defense mechanisms, providing resistance to followed lethal stress. It can be supposed, that components of *R. rosea* preparation may stabilize DNA or interact with mediators of signaling pathways that up-regulate resistance/defense mechanisms.

#### **Yap1 and Msn2/Msn4 proteins may modulate adaptogenic effect of *R. rosea* extract**

In *S. cerevisiae*, several signalling pathways were identified to play a role in regulation of stress resistance and yeast longevity. The transcriptional factors Msn2/4p and Yap1p are involved in these pathways. The synthesis of glutathione, heat shock and other stress proteins are under their control (Erkina *et al.* 2009; Lushchak 2010; 2011). To establish the involvement of these transcription regulators in stress-protective effects of *R. rosea*, we used yeast strains, defective for genes *MSN2/4* and *YAP1*. The strain  $\Delta yap1$  is isogenic derivative strain of YPH250, for which we have observed the positive effects of *R. rosea* described above. The sensitivity of  $\Delta yap1$  mutant to hydrogen peroxide was higher, than parental strain YPH250 (Table 1). In addition,  $\Delta yap1$  cells were more sensitive to hydrogen peroxide than to heat shock under selected conditions, which could be expected because protein Yap1 regulates yeast resistance to hydrogen peroxide (Lushchak 2010). Pre-incubation with *R. rosea* extract at low concentrations did not affect yeast resistance to oxidative and heat stresses.

The strain  $\Delta msn2\Delta msn4$  was the derivative of wild-type *S. cerevisiae* strain W303-1A. Since yeast strains with different genetic background may differ in stress resistance (Bayliak *et al.* 2006; Semchyshyn 2009), we also examined survival under stress exposure in parental W303-1A strain. The survival of W303-1A yeast cells, pre-treated with 1 and 5  $\mu$ l/ml of *R. rosea*, did not differ from control upon heat shock (Table 2). In the case of exposure to hydrogen peroxide, W303-1A behaved similarly to YPH250 strain.

M. M. Bayliak and others

**TABLE 2.** Effect of hydrogen peroxide and heat shock on survival (%) of exponentially growing *S. cerevisiae* W303-1A (wt) and Wmsn2msn4 ( $\Delta$ msn2 $\Delta$  msn4) cells after pre-incubation with *R. rosea* aqueous extracts.

| Stressor                            | Strain                                   | Concentration of <i>R. rosea</i><br>aqueous extract, $\mu$ l/ml medium |             |             |
|-------------------------------------|--|--|-------------|-------------|
|                                     |  | 0  | 1           | 5           |
| 10 mM H <sub>2</sub> O <sub>2</sub> | W303-1A (wt)                             | 83 $\pm$ 1   | 95 $\pm$ 5* | 49 $\pm$ 10 |
|                                     | Wmsn2msn4 ( $\Delta$ msn2 $\Delta$ msn4) | 5 $\pm$ 2  | 3 $\pm$ 1   | 3 $\pm$ 1   |
| Heat shock (40°C)                   | W303-1A (wt)                             | 87 $\pm$ 6   | 88 $\pm$ 8  | 72 $\pm$ 4  |
|                                     | Wmsn2msn4 ( $\Delta$ msn2 $\Delta$ msn4) | 57 $\pm$ 12  | 85 $\pm$ 8* | 76 $\pm$ 12 |

Cells growing exponentially in YPD medium were pre-incubated with *R. rosea* aqueous extracts during 2 h, and then they were collected by centrifugation and resuspended in 50 mM K-phosphate buffer (pH 7.0) and exposed to stresses for 1 h, n=5-6. \*Significantly different from respective control values (without *R. rosea* extract) with  $P < 0.01$  using Student's *t*-test. Results are shown as means  $\pm$  SEM (n=5-6).

At concentration of 1  $\mu$ l/ml, *R. rosea* extract enhanced the resistance of yeast cells to 10 mM H<sub>2</sub>O<sub>2</sub>, but at higher concentration (5  $\mu$ l/ml), *R. rosea* extract enhanced sensitivity of W303-1A cells to hydrogen peroxide. The  $\Delta$ msn2 $\Delta$ msn4 mutant, similarly to  $\Delta$ yap1 mutant, was more susceptible to hydrogen peroxide, than to heat shock (Table 2). Pre-treatment with *R. rosea* extract did not influence the sensitivity of  $\Delta$ msn2 $\Delta$ msn4 strain to 10 mM H<sub>2</sub>O<sub>2</sub>, but enhanced the resistance to heat shock.

In general, the obtained results suggest that regulatory proteins, Yap1 and Msn2/Msn4, are required for realization of complete protective action of *R. rosea* on yeast resistance at least to selected stressors. It may be supposed, that in our experiments *R. rosea* extract might act due to connection with Msn2/Msn4 and Yap1 transcription factors inducing synthesis of protective proteins, including primary antioxidant enzymes. Most likely, it is not direct interaction of components of *R. rosea* extract with Yap1, or with Msn2/4. This assumption is supported by reports which showed indirect activation of Yap1p by low concentrations of some plant compounds, such as resveratrol and green tea extract (Maeta *et al.* 2007; Escoté *et al.* 2012). In some studies, it was shown that *R. rosea* may exert its positive effects preventing depletion of glutathione (Calcabrini *et al.* 2010; Kim *et al.* 2006b). This situation may take place in our experiments, because both yeast transcriptional regulators activate expression of genes of glutathione biosynthesis. *R. rosea* components may also affect other pathways, in particular, biosynthesis of heat shock proteins, as it was observed in *C. elegans* (Panossian *et al.* 2009). This mechanism may explain the enhanced resistance of  $\Delta$ msn2 $\Delta$ msn4 cells, pre-incubated with *R. rosea* extract, to heat shock. Particularly, induction of heat shock proteins in *S. cerevisiae* is controlled predominantly by Hsf1p (heat shock factor) and can be additionally regulated by Yap1 and Msn2/4 (Costa and

*Effect of Rhodiola rosea on yeast stress resistance*

Moradas-Ferreira 2001). One may suggest also that the effects observed in this study might be associated somewhat with antioxidant properties of *R. rosea* compounds. Earlier it was shown, that the direct and indirect antioxidant action of salidroside, a marker compound of *R. rosea* rhizome, can partly protect fibroblasts from H<sub>2</sub>O<sub>2</sub>-induced premature senescence (Mao *et al.* 2010) and attenuated H<sub>2</sub>O<sub>2</sub>-induced cell damage by down-regulation of intracellular levels of Ca<sup>2+</sup> and ROS via a cAMP-dependent pathway (Guan *et al.* 2011).

Taken together, our results suggest, that action of *R. rosea* on *S. cerevisiae* lifespan and stress resistance could involve hormetic mechanisms (Wiegant *et al.* 2008). That is because *R. rosea* extract only at low concentrations (1 and 5 µl/ml) boosted the resistance of yeast cells against different stresses and provided life-prolonging effect, whereas at high concentrations (20 µl/ml) it had adverse effects on yeast stress resistance. It seems that the stress-protective action of *R. rosea* is connected with involvement of transcription factors Msn2/Msn4 and Yap1. These findings suggest that a shift towards stress resistance may be due to changes in gene expression. At high concentrations, *R. rosea* extract sensitizes *S. cerevisiae* cells to stressors and shortens yeast lifespan. Nevertheless, the “beneficial” doses for “young” exponential-phase cells and “old” stationary-phase cells are different, clearly demonstrating importance of physiological state in *R. rosea* extract effects. These results support the utilization of yeast as a useful model to study *in vivo* molecular mechanisms responsible for putative health beneficial effects of natural phytochemicals.

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## M. M. Bayliak and others

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*Effect of Rhodiola rosea on yeast stress resistance*

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