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Short report
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Role of Monophenols in the Recovery Process of Wild-Type Yeast Cells Exposed to Severe Environmental Stress – a Short Report

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Phenolic compounds are important plant metabolites associated with the plant's defence reactions in conditions of biotic and abiotic stress. Heterotrophic organisms are not capable of synthesizing phenolic compounds, but absorb them with foods of plant origin. Many of them exhibit antioxidant activity in chemical and biological assays. This study examined whether these compounds are able to protect cells of a heterotrophic organism from the effects of severe environmental stress. A wild-type strain of *Saccharomyces cerevisiae* yeast was used in the experiments. Yeast cells that were pre-incubated in conditions of severe thermal, osmotic or oxidative stress and then plated on a medium enriched with benzoic acid and its derivatives had a similar survival rate to the cells growing on a medium with no supplements, while in the presence of cinnamic acid derivatives and coumarin a reduction was observed in their survival. In the presence of the synthetic monophenol BHT, two different reactions were noted – a decrease in survival after 10 min of severe heat shock, and similar survival to that of the cells growing on the non-supplemented medium after 5 min of heat shock and the other types of stress. The results obtained suggest that monophenols used after severe environmental stress are not capable of eliminating the detrimental effects of these factors.

INTRODUCTION

Synthesis of plant secondary metabolites is associated with changes in the living conditions of the plant. The biological function of these substances is to protect the plant from the detrimental effects of environmental factors. The secondary metabolites include phenolic compounds, *i.e.* simple phenols (monophenols), represented by benzoic acid derivatives and phenylpropanoids; with the latter including derivatives of cinnamic acid and coumarins, which are lactones of *trans*-cinnamic acids.

Monophenols play an important role in systems that protect plants against biotrophic pathogens and UV radiation. They are also involved in stress signal transmission [Dixon & Paiva, 1995; Glazebrook, 2005].

Heterotrophic organisms are not capable of synthesizing phenolic compounds, but absorb them with foods of plant origin. These compounds exhibit health-promoting properties that result, to a large extent, from their antioxidant properties.

Saccharomyces cerevisiae yeast is a unicellular heterotrophic organisms used as a model organism in studies on various aspects of the cellular response to environmental factors. Yeast cells have various defence mechanisms that allow them to respond adequately to detrimental stimuli in their environment. However, when the action of an adverse factor is prolonged or intense, defense programmes are not activated and, in a consequence, the cells lack resistance to the stress factor. An easily monitored manifestation of this phenomenon is decreased viability of a yeast cell population under stress conditions. This type of the effect of environmental factors is known as severe environmental stress.

Under conditions of severe stress induced by various environmental factors, yeast cells exhibit symptoms of oxidative damage. These disturbances are accompanied by changes in the level of reactive oxygen species, antioxidant enzyme activity and the cellular antioxidant pool [Koziol *et al*., 2005; Swiecilo & Gardiasz, 2007; Kwolek-Mirek *et al*., 2009]. These data suggest that the oxidative stress accompanies stress reactions induced by various environmental factors.

With this in mind a study was conducted to determine whether phenolic compounds, both naturally occurring and synthetic one such as BHT (butylated hydroxytoluene), can protect cells of a wild-type yeast strain from the effects of various types of severe stress.

MATERIALS AND METHODS

The yeast used in the experiments was the wild-type strain SP4 with the genotype Mat alpha leu1 arg4 [Bilinski *et al*., 1978]. The yeast were grown in a liquid YPD medium containing 1% Difco yeast extract, 1% Difco Bacto peptone and 2% glucose. The culture was grown in aerobic conditions

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at 22°C for 24 h to obtain logarithmic-phase cells (LOG cells) at a density of 1-5×10^7 cells/mL.

Determination of the survival of yeast cells in the presence of monophenols

Hydroxy derivatives of cinnamic acid (p-coumaric, caffeic, trans-cinnamic and trans-ferulic (trans-4-hydroxy--3-methoxycinnamic) acids), coumarin and BHT (2,6-di (tert-butyl)-p-cresol) were dissolved in ethanol. The initial concentrations of these substances were 400 mmol/L for cinnamic acid derivatives, 69 mmol/L for coumarin and 10 mmol/L for BHT. Appropriate volumes of these substances and weighed amounts of benzoic acid and its derivatives (gallic and 4-hydroxybenzoic acid) were added to molten the YPD medium containing 2% agar, cooled to about 50°C. The final concentrations of these substances are presented in Table 1. A suspension of yeast cells diluted to a density of about 1-5×10^3 cells/mL was plated on Petri dishes containing medium with or without supplements. After plating, the Petri dishes were incubated for 2 days at 28°C, and then the colonies were counted using an automatic counter (aCOLyte; Synbiosis) and the associated software (version 2.0.8). The number of colonies obtained for the control samples, *i.e.* those that were not exposed to monophenols, was given a value of 100%.

Establishing severe environmental stress conditions

The logarithmic-phase liquid yeast culture was subjected to severe environmental stress. Severe thermal stress was induced by an abrupt change in temperature from 22°C to 48°C. The culture was exposed to this factor for 1, 5 and 10 min. Severe osmotic stress was induced by adding weighed amounts of NaCL and $NaNO_3$ to the logarithmic-phase yeast culture to obtain a 1 mol/L solution. The culture was exposed to these factors for 10, 30 and 60 min. Severe oxidative stress was induced by adding hydrogen peroxide at a final concentration of 2 mmol/L. The culture was exposed to this factor for 10, 20 and 30 min. The other agent used to induce oxidative stress was menadione (2-methyl-1,4-naphthoquinone sodium bisulfite), at a concentration of 25 mmol/L. The sample was exposed to this factor for 10, 30 and 60 min.

Fluorescent microscopy analysis of live/dead cell population

The yeast cells were stained with Live/Dead stain (LIVE/DEAD® Yeast Viability Kit, Molecular Probes, OR), according to the manufacturer's directions, and then the suspension of yeast cells was observed under a BH2-TR-30 Olympus fluorescent microscope with an FITC filter. Live, actively metabolising cells, weakened cells and dead cells were counted in 20 fields of view. The cells were identified using criteria described by Millard *et al*. [1997].

Colony-forming ability following severe stress was tested by plating a suspension of these cells on solid YPD medium. First the stress agents were eliminated by 100-fold dilution of the cultures in a phosphate buffer (0.1 mol/L pH 6.8) (in the case of heat shock the temperature was additionally reduced to 22°C). A cell suspension with a density of about 1×10^3 cells/mL was plated onto solid YPD medium. The Petri dishes were incubated for two days at 28°C, and then the colonies formed were counted. The number of colonies obtained for the sample not exposed to the stress factors was given a value of 100%.

TABLE 1. Survival of wild-type yeast cells in the presence of selected monophenols.

Type of monophenols	Concentration (mmol/L) of monophenols	Survival rate (%) of yeast cells
Control	0	100.0±2
Benzoic acid	1.0	101.6±9.3
	2.5	89.3±7.6
	5.0	29.4±3.7
	10.0	0
4-Hydroxy benzoic acid	1.0	98.8±6.0
	5.0	92.1±9.8
	10.0	90.7±7.4
	20.0	52.9±8.3
Gallic acid	1.0	97.8±7.3
	5.0	99.5±6.0
	10.0	84.6±7.0
	50.0	36.2±5.6
p-Coumaric acid	1.25	101.5±7.3
	2.5	84.8±4.8
	3.75	76.8±6.2
	5.0	78.2±3.7
Caffeic acid	1.25	109.4±6.9
	2.5	69.4±7.8
	3.75	69.6±4.5
	5.0	55.9±8.5
Trans-cinnamic acid	1.25	96.8±7.0
	2.5	44.5±7.7
	3.75	27.6±5.9
	5.0	12.3±4.5
Trans-ferulic acid	1.25	98.4±6.8
	2.5	76.3±7.0
	3.75	43.7±9.4
	5.0	26.0±6.8
Coumarin	0.7	102.6±6.9
	1.25	88.4±9.4
	2.5	12.7±4.3
	5.0	0
BHT	0.05	105±6.9
	0.1	95.5±6.7
	0.2	81,5±8.5
	0.5	56.6±8.9

Determination of the survival of yeast cells following severe environmental stress in the presence of monophenols

The yeast cells were subjected to severe environmental stress according to the procedure described above. The exposure time was 5 and 10 min for thermal shock, 1 h for osmotic shock, 1 h for oxidative shock induced by menadione and half an hour for oxidative shock induced by hydrogen peroxide. Next the stress factors were removed as described above and a 1-5×10^3 cell suspension was plated on Petri dishes with a medium enriched with monophenols. The final concentrations of these substances are presented in Table 3. The substances were applied at the highest concentrations which do

not reduce the survival of this strain of yeast cells. Following incubation for two days at 28°C, the colonies formed were counted. The number of colonies obtained for a sample not exposed to stress factors or to monophenols was given a value of 100%.

The menadione, coumarin, and p-coumaric, caffeic and gallic acids were purchased from Sigma Chemical Co.; the *trans*-cinnamic, *trans*-ferulic, and 4-hydroxybenzoic acids from Aldrich; BHT at the Organic Industry Institute in Poland; and the remaining reagents from POCH S.A. in Poland.

The results obtained constitute means of three or more independently conducted experiments. The mean values and standard deviation were calculated using an Excel 7.0 spreadsheet.

RESULTS AND DISCUSSION

The percentage of wild-type yeast cells which survived in the presence of selected monophenols was presented in Table 1. Monophenols were added to the growth medium, so they had long-term effects on the yeast cells. Of the compounds analysed, BHT exhibited the strongest cytotoxic properties, manifested as a reduction in the survival of the wild-type yeast cells. This compound decreased the survival of the yeast cells when applied at concentrations higher than 0.05 mmol/L. Similar effects were observed in the case of the other monophenols when applied at about 25 times higher concentration. In this group, the strongest cytotoxic properties were noted in coumarin, benzoic acid, *trans*-ferulic acid and *trans*-cinnamic acid. Coumarin at a concentration of 5 mmol/L completely inhibited the ability of the yeast cells to form colonies, while *trans*-cinnamic acid at the same concentration led to a more than 8-fold reduction in the survival of the cells. At this concentration, benzoic acid and *trans*-ferulic acid reduced the survival of the yeast cells more than 3-fold.

Other authors have also observed the detrimental effect of phenolic acids and BHT on various physiological processes in yeast [Klinke *et al*., 2003; Endo *et al*., 2008; Passone *et al*., 2005]. The cytotoxic and cytostatic effects induced by monophenols, presented in this study and observed by other authors, may result from their pro-oxidant activity. It has been suggested that when cells growing in standard growth conditions are exposed to high concentrations of phenolic compounds that scavenge free radicals under oxidative stress conditions, these compounds can induce a shift in the oxidative/antioxidative balance towards oxidation [Kim *et al*., 2006].

According to data from the literature, derivatives of both hydroxybenzoic acid and hydroxycinnamic acid (gallic, gentisic, p-hydroxybenzoic and p-coumaric acids) exhibit strong antioxidant properties in tests of total antioxidant capacity based on the inhibition of R-phycoerythrin oxidation (ORAC test) and reduction of the ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonate) cation radical [Yeh & Yen, 2003]. BHT has also been shown to have antioxidant capabilities in chemical assays determining its capacity to sequester ABTS, DPPH (2,2-diphenyl-1-picrylhydrazyl) and hydroxyl free radicals [Soares *et al*., 2003].

The antioxidant properties of substances determined based on their ability to scavenge free radicals in *in vitro* tests are not always equivalent to their functions *in vivo*. For this reason, the present study investigated the ability of monophenols to repair damage that has occurred in conditions of severe thermal, osmotic or oxidative stress.

First the metabolic condition of yeast cells was tested after different times of exposure to the factors that induced severe thermal, osmotic and oxidative stress. Live/Dead staining was used to determine the percentages of actively metabolising, weakened (with low metabolism) and dead cells. The effect of conditions of severe stress on the ability of cells to form colonies on a solid medium was also evaluated.

The percentages of cells with reduced metabolism and of dead cells increased with the length of exposure to the stress factors (Table 2). The number of actively metabolising cells in the Live/Dead assay corresponds to the num-

TABLE 2. The metabolic activity and survival of yeast cells in severe stress conditions.

Type of stress factors	Duration of stress factors (min)	Number of cells (%)			
		actively metabolising	weakened	dead	capable of colony forming
Control	0	97.3±5.2	2.1±1.1	0.6±0.4	100
Thermal stress	1	91.5±8.4	6.8±1.8	1.7±0.2	89.4±7.8
	5	61.4±7.4	31.0±5.4	8.6±1.6	59.0±2.5
	10	18.5±2.4	62.1±4.1	19.5±2.3	23.1±3.6
Osmotic stress 1 mol/L NaCl	10	94.0±9.5	4.4±1.3	1.6±0.6	91.8±9.4
	30	75.6±5.8	10.3±2.3	14.1±2.7	89.7±5.7
	60	50.3±6.4	37.4±5.2	12.8±0.9	59.4±5.1
Osmotic stress 1 mol/L $NaNO_3$	10	91±7.5	6.8±2.4	2.2±0.5	95.5±7.3
	30	86.0±7.3	10.1±6.3	3.9±0.3	73.5±5.2
	60	45.9±5.2	27.6±3.1	26.5±3.2	51.5±8.3
Oxidative stress 25 mmol/L Menadion	10	95.4±9.2	3.7±0.2	0.9±0.2	105.3±5.3
	30	79.9±5.5	9.5±1.9	13.6±2.1	89.2±6.4
	60	50.4±2.2	36.8±5.3	12.8±3.1	57.6±5.3
Oxidative stress 2 mmol/L H_2O_2	10	74.3±8.4	13.2±2.6	12.5±2.1	86.3±5.9
	20	67.6±5.3	20.3±4.2	12.1±2.1	56.3±6.4
	30	47.3±6.3	39.1±3.2	13.6±0.9	55.7±4.3

ber of cells capable of division, determined by plating on Petri dishes. These data suggest that cells with reduced metabolism, which are observed directly following stress, have damage which hinders the completion of the division cycle and the formation of a daughter cell, so that they do not form colonies when plated on a solid medium.

To test the protective capacity of monophenols, stress conditions were selected which induce a high percentage of cells with a low level of metabolism. The fact that this condition is not accompanied by structural damage to the cell membranes (which could be seen when the Live/Dead-stained cells were observed) suggests that at this stage it may be reversible. Hence, in further testing heat shock was applied for 5 and 10 min, osmotic shock for one hour, menadione-induced oxidative shock for one hour and oxidative shock induced by hydrogen peroxide for half an hour.

The ability to form colonies was most strongly inhibited in conditions of severe thermal shock induced by a temperature of 48°C for 10 min. In the case of the other factors, the viability of the yeast cells decreased by about one half (Table 3).

The percentage of yeast cells surviving under conditions of severe thermal, osmotic and oxidative shock, and then grown in the presence of benzoic acid and its derivatives, was similar to that of cells grown in the non-supplemented medium (Table 3). In the case of cells plated on a medium containing cinnamic acid derivatives or coumarin, however, the percentage of surviving cells decreased compared to the level characteristic for a population subjected to environmental stress. Caffeic, *trans*-ferulic and *trans*-cinnamic acids had a particularly detrimental effect on this parameter. Two types of reaction were noted in the presence of BHT. Following 10 min of severe heat shock, the survival of yeast cells decreased by about half compared to stressed cells growing on a medium without BHT. Following 5 mins of heat shock and the other types of stress, the percentage of cells surviving in the presence of BHT was similar to that of the cells growing on the non-supplemented medium.

The monophenols applied in the study did not improve the growth of the wild-type yeast cells damaged by severe stress, and some of them further reduced their survival. The lack of positive effects of the monophenols in the case of cells previously incubated under conditions of thermal or osmotic shock may result from the specific mechanisms of action of these stress factors.

What was unexpected was the lack of a positive effect of these substances on yeast cells exposed to severe oxidative stress. Reports in the literature confirm that the adverse effects of pro-oxidant stress factors can be eliminated by applying exogenous antioxidants, both natural and synthetic [Soares *et al*., 2003; Krzepilko *et al*., 2004; Zyracka *et al*., 2005; Kwolek-Mirek *et al*., 2008; Wu *et al*., 2011].

In these studies, however, antioxidants were applied together with pro-oxidants. Applied in this manner they could have reacted directly with oxidizing agents. This type of mechanism of action is characteristic of preventive antioxidants, which participate in the first stage of defence against an oxidizing agent. Phenolic antioxidants (monophenols or BHT), on the other hand, act as chain-breaking antioxidants. They can react with intermediate oxidation products, which free radicals usually are. Thus they take part in the later stages of defence against stress factors by directing free radical reactions towards termination, which prevents further damage to cellular macromolecules. It was expected that this type of effect would be observed in the conditions of the present study. However, the phenolic acids and BHT used in the experiment did not protect the yeast cells that had been damaged in conditions of severe environmental stress.

When other authors used an identical approach to test antioxidants which had been shown to protect the hydrophobic or hydrophilic environment of cells, they observed no positive effect of these substances against toxicity induced by pyrethroids or high temperatures. The data presented in this study and obtained by these authors [Eubanks & Beuchat, 1982; Krzepilko & Swiecilo, 2009] suggest that the antioxidant activity of the compounds studied is not very effective in the later stages of the cellular response to severe stress.

CONCLUSIONS

1. The monophenols tested exhibit concentration-dependent cytotoxic activity against cells of the wild-type strain of *Saccharomyces cerevisiae* yeast.

TABLE 3. Survival of yeast cells in the presence of selected monophenols following previous incubation in severe stress conditions.

Type of monophenols	Concentration (mmol/L) of monophenols	Type and condition of stress					
		Thermal stress		Osmotic stress		Oxidative stress	
		5 min	10 min	NaCl	$NaNO_3$	H_2O_2	Menadione
Control	0	59.0±2.5	23.1±3.6	59.4±5.1	51.5±8.3	55.7±4.3	57.6±5.3
Benzoic acid	1.0	50.0±3.7	20.9±5.4	51.7±4.0	68.2±8.3	56.1±7.3	54.1±4.7
Gallic acid	5.0	41.6±3.6	18.8±5.3	55.7±7.3	66.6±7.0	53.4±4.9	50,3±8.8
4-Hydroxybezoic acid	1.0	65.1±5.4	15.3±2.1	59.0±6.5	50,0±8.4	51,5±5.7	56,4±7.8
p-Coumaric acid	1.25	10.6±3.2	11.6±5.4	49.5±6.9	57.5±3.8	43.9±7.9	41.5±6.1
Caffeic acid	1.25	7.6±4.6	6.4±6.2	37.4±5.4	30.3±8.9	36.3±5.9	37.7±2.9
Trans-cinnamic acid	1.0	5.3±3.6	6.2±2.5	34.3±6.0	36,3±9.4	30,3±4.7	26,0±6.5
Trans-ferulic acid	1.0	15.0±7.3	8.9±6.6	35,6±4.0	30,3±7.6	37.1±8.6	33.6±8.7
Coumarin	0.7	13.6±5.3	13.3±5.9	36.8±5.8	30.3±8.3	21.2±6.5	35.9±5.4
BHT	0.05	56.4±7.6	13.8±5.8	45.7±7.5	52,4±4.0	49,4±9,5	48,6±8.7

2. The natural and synthetic monophenols used at sublethal concentrations do not protect cells of the wild-type strain of yeast against the harmful effects of severe environmental stress.

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