Temperature Pre-Treatment Modulates Oxidative Protection of *Aspergillus niger* Cells Stressed by Paraquat and Hydrogen Peroxide

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Abstract

The protective effect of pretreatment with low oxidative stress factors is little known in relation to the growth and development of filamentous fungi. The acquisition of new knowledge can be particularly useful for industrially important fungal strains. In the present study, the adaptive response of the filamentous fungus *Aspergillus niger* 26 to oxidative stress has been examined. The findings indicate that pretreatment with a sub-lethal temperature leads to the development of resistance to lethal concentrations of paraquat (PQ), H_2O_2 and extremely high temperatures. Fungal cultures subjected to 35°C followed by exposure to high doses of stress agents showed a higher amount of biomass accumulation compared to the single treated cells. The pre-exposure strategy exerted a protective effect with respect to the amount of oxidatively damaged proteins in *A. niger* cells, which was accompanied by a corresponding increase in the intracellular protein content and induction of antioxidant enzymes compared to the non-adapted cultures. At the same time, the results demonstrated different responses in the temperature-adapted cells. Pretreatment makes cells more resistant to both PQ and temperature than to H_2O_2.

Key words: fungi, oxidative stress, adaptive response, biomarkers, antioxidant enzymes

Резюме

Ефектът на предварителното третиране с ниски дози от фактори, индуциращи оксидативен стрес върху растежа и развитието на култури от филаментозни гъби е слабо изучен феномен. Получаването на нови знания в тази област може да бъде много полезно при използването на индустриално важни щамове. В настоящото изследване проучихме адаптивния отговор на щам *Aspergillus niger* 26 към оксидативния стрес. Получените резултати показват, че пре-третирането със сублетална температура води до проявата на резистентност към летални концентрации паракват (ПК), Н2О2 и екстремно висока температура. При третиране с температура 35°C и последващо въздействие с високи дози от използваните стрес фактори се наблюдава натрупване на по-високо количество биомаса в сравнение с еднократното третирани култури. Стратегията на пре-третирането води до протективен ефект по отношение количеството на оксидативно увредените белтъци в клетките на *A. niger*, което кореспондира с повишено количество вътреклетъчен белтък и индукция на антиоксидантната ензимна защита в сравнение с не-адаптираните култури. Освен това, резултатите демонстрират разлики в отговора на клетките, адаптираните към температурата. Пре-третирането повишава в по-голяма степен резистентността към ПК и температурата, отколкото към H_2O_2.

Introduction

In filamentous fungi, as eukaryotic organisms, oxidative stress induced directly or indirectly through various abiotic factors such as heat, cold, herbicide applications (e.g., paraquat), drought, UV radiation, etc. leads to enhanced production of reactive oxygen species (ROS) (Lushchak, 2011).

These ROS include the superoxide anion radical ($^•O_2^-$), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH$^•$) and can cause peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately lead to cell death (Fridovich, 1998). They are produced by metabolic pathways localized in different cellular compartments.

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Fungi possess a complex antioxidative defence system containing non-enzymatic and enzymatic components to scavenge ROS. The enzymatic components comprise of several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPX), and glutathione reductase (GR) (Bai *et al*., 2003). These enzymes operate in different subcellular compartments and respond in concert when cells are exposed to oxidative stress. When the level of ROS exceeds the defence mechanisms, the cells are said to be in a state of "oxidative stress". SOD catalyze $\cdot O_2^-$ dismutation to H_2O_2 and molecular oxygen (Fridovich, 1998). SODs are metallo-proteins and are classified into Fe-, Mn-, CuZn- and Ni-containing SOD on the basis of the metals in their active sites. It is generally accepted that fungal cells contain Mn-SOD in the mitochondria and CuZn-SOD in the cytoplasm (Ito-kuwa *et al.*, 1999; Angelova *et al.,* 2005). CATs are ubiquitous enzymes, which protect aerobic organisms from the toxic effects of H_2O_2 by catalyzing the conversion to molecular O_2 and H_2O. CATs from filamentous fungi have several characteristics that distinguish them from their mammalian counterparts (Bussink and Oliver, 2001).

Although the oxidative stress responses in fungi is insufficiently studied, it has been shown that fungal cells respond to different oxidative stressors in distinct ways (Bai *et al*., 2003). For example, herbicides as paraquat (PQ), menadion (MD) and their derivatives are strong inductors of ROS generation, mainly $\cdot O_2^-$. On the other hand, H_2O_2 is a reactive oxygen species and universally cytotoxic at high concentrations, mainly due to strong oxidant, hydroxyl radical. To our knowledge, there are few reports regarding the effects of antioxidant enzymes over expression on fungal sensitivity to PQ, MD and H_2O_2. Li *et al*. (2008) showed that *A. niger* B1-D adapts to exposure to H_2O_2 by reducing growth and inducing a number of antioxidant enzyme activities, of which the induction of catalase is the most pronounced. Similar results have been published for *Penicillium chrysogenum* cells treated by H_2O_2. Conversely, after addition of MD the same strain demonstrated enhanced activity of Mn- and Cu/Zn-SOD, but CAT did not included in the cell response (Emri *et al*., 1997). Our previous study confirmed the distinct antioxidant response to PQ and H_2O_2 in 18 fungal species (Angelova *et al*., 2005).

At the same time, the pre-treatment with low concentrations of PQ, MD and H_2O_2 significantly increased survival of the lethal doses of each oxidant, indicating the existence of an adaptive response to oxidative stress (Lee *et al*., 1995; Izawa *et al*., 1995; Li *et al.,* 2008). *Candida albicans* is able to acquire adaptive oxidative tolerance by pretreatment with non-stressing concentration of H_2O_2 before exposure to a drastic oxidative challenge (Gonzàlez-Pàrraga *et al*., 2003). While MD caused protection against cell killing of *Saccharomyces cerevisiae* by subsequent higher concentrations of MD or H_2O_2, the pretreatment with H_2O_2 did not protect cells against the enhanced dose of MD (Jamieson, 1992).

Temperature pre-treatment also affected the survival of fungal cultures against induced oxidative stress. Yeast cells treated with a temperature of 40°C showed less accumulation of ROS than nontreated cells in response to heat shock and H_2O_2 (Liu *et al*., 2011). The authors suggest that the overall improvement in stress tolerance is associated with the induction of TPS1 gene expression and trehalose accumulation. Heat-shock enhanced the trehalose content in *S. cerevisiae* and reduced the damage caused by ROS (Benaroudj *et al*., 2001). It is important to clarify the role of the major enzymes of antioxidant defence, SOD and CAT, in the induced adaptive response. Whereas this problem is widely studied in plants, little is known about the effect of temperature pre-treatment on the modulation of antioxidant enzyme activity of fungi. The knowledge can be useful for industrially important fungal strains.

Our previous investigation demonstrated the effect of enhanced temperatures on the morphology, the level of oxidative stress biomarkers, and activity of antioxidant enzyme defence in the fungal strain *Aspergillus niger* 26 (Abrashev *et. al.*, 2014). The aim of the present study was to investigate if pretreatment with sub-lethal temperature causes an adaptive response to oxidative stress induced by heat shock, PQ and H_2O_2 as exogenous sources For this purpose, growth, intracellular protein content, carbonylated protein level, and the antioxidant enzyme activity (SOD, CAT) variations were evaluated.

Materials and methods
Materials

Nitro blue tetrazolium (NBT), paraquat (PQ), 2,4-dinitrophenylhydrazine (DNPH), were obtained from Sigma-Aldrich (Deisenhofen, Germany). All other chemicals used in this study were of the highest analytical degree.

Fungal strain, culture conditions and temperature pre-treatment

The fungal strain, *A. niger* 26 from the Mycological Collection at the Stephan Angeloff Institute of Microbiology, Sofia, was used throughout and maintained at 4°C on beer agar, pH 6.3. All experiments under submerged conditions were carried out in the medium AN-3 (Abrashev *et al*., 2005).

Cultivation was carried out in 3 L bioreactors, ABR-09, developed and constructed by the former Central Laboratory for Bioinstrumentation and Automatisation (CLBA) of the Bulgarian Academy of Sciences. The bioreactor was equipped with automatic temperature, pH and dissolved oxygen (DO) monitoring equipment and a control system. For the inoculum, 80 ml of medium AN-3 was inoculated with 10^9 spores in 500 ml Erlenmeyer flasks. The cultivation was performed on a shaker (220 rpm) at 30°C for 24 h. For bioreactor cultures, 200 ml of the seed culture was brought into the 3 l bioreactor, containing 1800 ml of the medium AN-3. The cultures were grown for 18 h at a temperature of 30°C with a stirrer speed of 600 rpm air flow, 1.0 v.v. m. At that time, a single administration of stress factors, PQ (1, 3 or 5 mM), H_2O_2 (5, 10 or 30 mM) or temperature (35. 40 or 50°C), was performed and cultivation was carried out for 12 h. Experiments with PQ and H_2O_2 were continued at the initial temperature. Experiments without the stress agents were also performed under the same conditions, as controls.

For adaptive stress response assays, 12-hour bioreactor cultures of *A. niger* 26 were transferred from 30 to 35°C for 6 h. The pre-treated fungal cells were then immediately challenged with 5 mM PQ, 30 mM H_2O_2 (incubation at 30°C) or 50°C for 12 h. The control variants were grown at an optimal temperature without stress agents during the whole period. Results were evaluated from repeated experiments using three parallel runs.

Cell-free extract preparation and enzyme activity determination

The cell-free extract was prepared as described earlier (Abrashev *et al*., 2005). Briefly, mycelium biomass was harvested by filtration, washed in distilled H_2O and then in cold 50 mM potassium buffer (pH 7.8), and resuspended in the same buffer. The cell suspension was disrupted by homogenizer model ULTRA Turrax T25 IKA WERK. The temperature during treatment was maintained at 4-6°C by chilling in an ice-salt bath and by filtration through a Whatman filter, No 4 (Clifton, USA). Cell-free extracts were centrifuged at 13 000 x g for 20 min at 4°C.

SOD activity was measured by the nitro-blue tetrazolium (NBT) reduction method of Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the amount of enzyme protein required for inhibition of the reduction of NBT by 50% (A_{560}) and was expressed as units per mg protein. Catalase activity was determined by monitoring the decomposition of 18 mM H_2O_2 at 240 nm (Beers and Sizer, 1952). One unit of activity is that which decomposes 1 µmol of H_2O_2 min^{-1} mg $protein^{-1}$at 25°C and pH 7.0. Specific activity is given as U (mg protein)$^{-1}$. Protein was estimated by the Lowry procedure (Lowry, 1951) using crystalline bovine albumin as standard.

Measurement of protein carbonyl content

Protein oxidative damage was measured spectrophotometrically as protein carbonyl content using DNPH binding assay (Levine *et al*. 1990), slightly modified by Adachi and Ishii (2000). The cell-free extracts were incubated with DNPH for 1 h at 37°C, proteins were precipitated in 10% cold TCA, washed with ethanol: ethylacetate (1:1), to remove excess of DNPH, and finally dissolved in 6 M guanidine chloride, pH 2. Optical density was measured at 380 nm, and the carbonyl content was calculated using a molar extinction coefficient of 21 mM^{-1} cm^{-1}, as nanomoles of DNPH incorporated (protein carbonyls) per mg of protein.

Determination of dry weight

The dry weight determination was performed on samples of mycelia harvested throughout the culture period. The culture fluid was filtered through a Whatman (Clifton, USA) No 4 filter. The separated mycelia were washed twice with distilled water and dried to a constant weight at 105°C.

Statistical evaluation of the results

The results obtained in this investigation were evaluated from at least three repeated experiments using three or five parallel runs. The statistical comparison between controls and treated cultures was determined by Student's *t*-test for MIE (mean interval estimation) and by one-way analysis of variance (ANOVA) followed by Dunnett's post-test, with a significance level of 0.05.

Results

Cell response to single administration of stress factors
Fungal growth

The ability of *A. niger* cultures to produce biomass under conditions of oxidative stress indu-

ced by PQ, H_2O_2 or temperature is presented in Fig. 1. The value of stress agents was chosen based on preliminary experiments where the range was found to be wide enough to give clear contrast between control and stressed cultures.

As expected, the dry weight content decreased with an increase temperature. The results showed that the moderate temperature stress (35°C) decrease the biomass of the fungal culture by 11% compared to the control variant. However the acute stress at 50°C reduced biomass accumulation by 42% compared to the control. Similarly to the trend of tolerance to high temperatures, the growth of *A. niger* 26 decreased with exposure to increased concentrations of PQ and H_2O_2. About 20, 41 and 68% lower levels of biomass were achieved after exposure to 1, 3 or 5 mM PQ, respectively. The treatment with 5, 10 and 30 mM H_2O_2 led to approximately the same results. Thus, both agents were the more powerful stress factors that affected *A. niger* development.
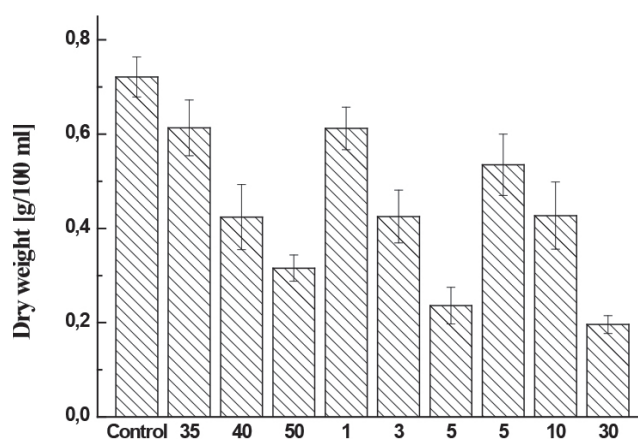
Fig. 1. Biomass production by *A. niger* 26 in response to a single administration of the stress factors, PQ, H_2O_2 or temperature. The effect of treatment was significant ($p \leq 0.05$)

Table 1. Effect of single administration of stress factors on the intracelluual protein, carbonylated protein content, and specific activity of SOD and CAT

Variants	Intracellular protein [mg/g d.w.]	Carbonyled protein [nM/mg protein]	SOD [U/mg protein]	CAT [U/mg protein]
Control	40.82	1.82	32,8	11.8
Temperature [°C]				
35	33.56	3.56	58.5	13.5
40	23.12	6.02	82.2	16.2
50	16.42	8.12	109.3	18.7
PQ [mM]				
1	29.92	3.98	64.6	14.1
3	21.51	7.11	95,1	15.1
5	11.87	13.87	121,7	18.5
H_2O_2 [mM]				
5	26.11	5.12	33.9	18.7
10	12.82	8.82	35.8	22.3
30	8.06	15.06	37.6	29.6

Changes in protein content and antioxidant enzyme activities

The agents used (temperature, PQ and H_2O_2) are known as inducers of oxidative stress. Their effect on the stress biomarkers, such as intracellular protein content, oxidatively damaged proteins and antioxidant enzyme activities is demonstrated in Table 1.

When cells of the tested fungal strain were exposed to the above-mentioned factors, the intracellular protein level decreased significantly as compared with the control cultivation. After 12 h, there was a trend for a dose-dependent decline in protein content. The most dramatic reduction was related to the H_2O_2 exposure (from 40 to 80% compared to the control). PQ affected protein level to a similar extent, while increasing the temperature from 35 to 50°C resulted in a lower reduction (from 18 to 60% compared to the control).

The above-mentioned reduction in the intracellular protein content coincided with a remarkable enhancement in the oxidatively damaged proteins, measured by the protein carbonyl content. At the end of the stress treatment with 35 and 40°C, carbonyls increased about 2- and 3,3-fold in comparison with the control. Exposure at 50°C caused about 4.5-fold higher increase in carbonyls than in the control. Carbonyls in total protein of the PQ and H_2O_2-exposed fungal cells showed the same trend of increase but to a greater extent.

As expected, under oxidative stress conditions induced by $^{\bullet}O_2^{-}$ and H_2O_2 generating agents, the antioxidant enzyme response of *A. niger* cultures varied greatly. The activity of SOD and CAT increased by PQ and temperature treatment, but the level of induction varied for both enzymes: about 2 - 3.7-fold for SOD compared to 35 -58% for CAT. In contrast, peroxide stress caused a 2-3-fold increase in catalase activity compared to the control, while SOD only showed a modest increase with H_2O_2.

Adaptive response of A. niger 26 to temperature pretreatment

To test the adaptive response induced by pretreatment with moderate heat stress (HS) biomass production, protein content and antioxidant enzyme activities of pretreated cultures subsequently exposed to 5 mM PQ, 30 mM H_2O_2 and temperature 50°C were compared with non-pretreated cells challenged with the same agents.
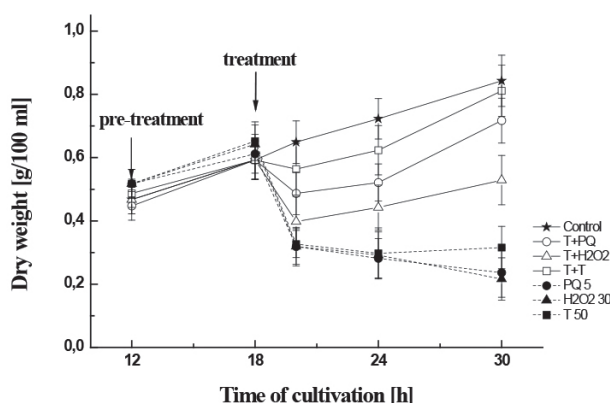
Fig. 2. Time course of biomass production for two different experiments: cultures without pretreatment (dash lines; closed symbols) and cultures pretreated with 35°C (solid lines; open symbols), when 5mM PQ (○, ●), 30 mM H_2O_2 (△, ▲) and temperature 50°C (□, ■) was added. The control (✱) without treatment.
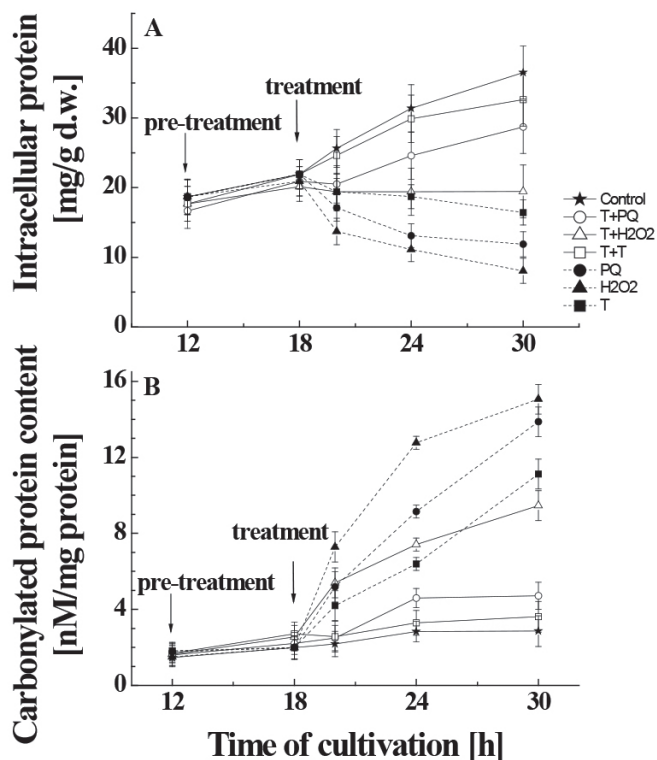
Fig. 3. Adaptive response on intracellular protein (A) and carbonylated protein content (B) cultures without pretreatment (dash lines; closed symbols) and cultures pretreated with 35°C (solid lines; open symbols), when 5mM PQ (○, ●), 30 mM H_2O_2 (△, ▲) and temperature 50°C (□, ■) was added. The control (✱) without treatment.

Fungal growth

Experiments to evaluate changes between pretreated and non-pretreated cells of *A. niger* 26 in relation to the growth versus time were performed and the results are shown in Fig. 2.

Direct exposure to high concentrations of PQ, H_2O_2 and temperature lead to reduced biomass content, these doses are lethal to this culture. Sharp changes occurred during the first 2 hours of the treatment and continued thereafter until the end of cultivation. However, pretretment with 35°C for 6 h prior to exposure to lethal doses removed the deleterious effects to a great extent. This effect was more prominent in the temperature and PQ-treated cells than in the variants with H_2O_2. When pretreated cells were subjected to oxidative stress agents, the dry weight increased 3- and 2.6-fold respectively, compared to the non-pretreated cells.

Protein changes

The effect of the adaptive response on the level of intracellular protein and carbonylated pro-

tein content was examined. As seen in Fig. 3A, the intracellular protein concentration in the pretreated bioreactor cultures of *A. niger* was different from that of non-pretreated cells.

Although the evaluated values were lower than those of the control, a significant increase in protein level was measured in the cultures adapted to HS compared to the non-pretreated. Heat-pre-treated PQ-, H_2O_2- or temperature-stressed fungal cells showed 2-, 2.5-fold higher protein level compared with those with direct exposure. As expected, the level of carbonyl groups in both pretreated and non-pretreated variants increased remarkably. But the previous adaptation resulted in 3- or 1.6-fold lower carbonyl protein level after PQ and temperature treatment and H_2O_2, respectively. when compared with the cells subjected to stress agents without heat pretreatment. The marked effect was observed immediately after treatment and this trend continued until the end of cultivation.

Antioxidant enzyme activities

To compare the effect of heat-pretreatment on the induction of antioxidant defence of *A. niger* 26, the SOD and CAT activities were analysed. The results are illustrated in Fig. 4.

Oxidative stress caused by direct treatment with PQ and temperature immediately induced SOD and CAT to a large extent (Fig. 4A). The pre-treated cultures showed a further remarkable increase in both enzyme activities. For example, 12 h after exposure to the lethal dose of PQ and temperature, 25 and 40% higher SOD activity, respectively, was measured compared to the non-pretreated cultures. At the same time, the estimated values were about 4.5-fold higher when compared with the control cells. In contrast, the effect of peroxide stress turned out to be insignificant in the variants with non-pretreated or pretreated cultures.

On the other hand, H_2O_2 addition to *A. niger* cells resulted in a 2- and 4-fold increase in CAT activity for non-pretreated and pretreated cultures, respectively, compared to the control. Enhanced activity was also found in the heat-pretreated and non-pretreated variants exposed to lethal dose of PQ and temperature.

Discussion

Adaptive stress response plays a major role in microbial cells, in particular those used in different industrial applications. Filamentous fungi involved in real biotechnological processes are subjected to multiple stresses often occurring simultaneously

(Bai *et al.*, 2003). They have evolved the ability to survive and produce valuable compounds using a system of stress response mechanisms. In spite of the great interest to clarify these mechanisms, the number of studies on adaptive stress response in biotechnologically important fungi is limited. The strain *A. niger* 26 has been selected as a promising candidate for industrial production of pectinolytic enzymes, mainly polymethylgalacturonase (PMG) (Angelova *et al.* 1998, 2000; Pashova *et al.* 1999). Moreover, our previous investigations showed that this strain is a good producer of the first antioxidant enzyme SOD. Short-term HS treatment of the spores (Abrashev *et al.*, 2005) and mycelia in mild-exponential growth phase (Abrashev *et al.*, 2008) markedly enhanced SOD activity.

In the present study, HS pretreatment (35°C for 6 h) of *A. niger* 26 cells was shown to be effective in improving fungal cell tolerance to stress agents such as PQ, H_2O_2 and high temperature. Of the agents used, H_2O_2 generated extracellular oxidative stress, whereas PQ, a redox-cycling agent, served as the source of intracellular oxidative stress
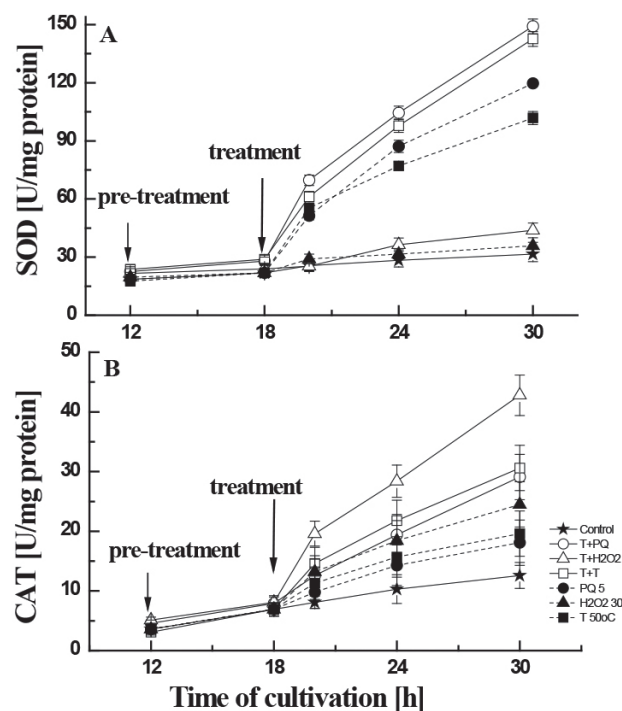
Fig. 4. Effect of heat pretreatment on activity of SOD (A) and CAT (B) in cultures without pretreatment (dash lines; closed symbols) and cultures pretreated with 35°C (solid lines; open symbols), when 5mM PQ (○, ●), 30 mM H_2O_2 (△, ▲) and temperature 50°C (□, ■) was added. The control (∗) without treatment.

generating a flux of $\cdot O_2^-$ in fungal cells (Angelova et al., 2005; Li et al., 2008; Ponts et al., 2009; da Silva Dantas et al., 2015). A similar increase in $\cdot O_2^-$ and H_2O_2 levels after heat shock treatment has been demonstrated in different aerobic cells including fungal cells (Bai et al., 2003, Abrashev et al., 2008). The major findings in this study are that: (1) the pretreatment with mild HS induces an adaptive response, which protects cells from the lethal effects of the subsequent challenge with higher concentrations of these oxidants; (2) the mechanism of enhanced fungal resistance includes suppression of the oxidative stress; (3) the adaptive responses to superoxide and peroxide stress agents are distinct, although there is a significant overlap between many of the responses.

It was shown here that a combination of mild heat stress and subsequent exposure to lethal dose of PQ, H_2O_2 or temperature cause a significant improvement of fungal growth compared to the single treatment. Despite the evaluated decline in biomass as a result of an increase in cell autolysis in the first 2 hours of exposure, the pre-treated cultures quickly overcome the harmful effect. Probably, a preliminary exposure to 35°C might induce in cells the necessary adaptation to keep their growth. Several examples of this have been reported previously. Transient exposure to a sub-lethal HS induce tolerance to a more extreme stress in *Metschnikowia fructicola* (Liu et al., 2011). Combinatorial H_2O_2 plus nitrosative stresses or cationic (NaCl) plus nitrosative stresses appear to exert adaptive effects upon the growth of *Candida albicans* cells (Kaloriti et al., 2012). Li et al. (2008) found that the pretreatment of *A. niger* B1-D with H_2O_2 at a non-lethal concentration confers greatly enhanced resistance to killing by H_2O_2 at lethal concentrations in the early exponential phase of growth. In addition to improved biomass production of *A. niger* 26, HS pretreatment also provided increased tolerance to oxidative stress. Such inducible adaptive responses have been observed at every level of DNA damage repair to the induction of antioxidant enzymes such as SOD, CAT, peroxidase as well as small molecules, such as glutathione, atocopherol and ascorbate that scavenge reactive oxygen species before they cause damage (see Patra et al., 1997).

The results of this study indicated reduced level of carbonyl groups, which can be used as a marker of oxidatively damaged proteins. As has been reported previously, when cells are exposed to severe stresses, the accelerated generation of intracellular ROS correlated well with enhanced content of oxidatively damaged protein (Davies and Goldberg, 1987). In contrast, the pretreatment with a sub-lethal dose of oxidative stress factors significantly declined ROS level in different fungal cells such as *Candida oleophila* (Reverter-Branchat et al., 2004), *A. niger* B1-D (Li et al., 2008), etc. This situation led to a decrease in the content of carbonylated proteins in stress-adapted cells compared with non-adapted cells. Protein carbonylation is an irreversible oxidative process leading to a loss of function of the modified proteins. These oxidized proteins are selectively recognized and degraded by proteolytic enzymes (Nystrom, 2005), followed by *de novo* protein synthesis or reparation (Crawford and Davies, 1994).

Furthermore, the lower level of oxidative damage may be due to the stimulation of antioxidant systems in stress-adapted cells. High activity levels of SOD and CAT were induced immediately after exposure to the stress agents in both non-adapted and adapted cells. The enhanced activity levels found for SOD and CAT in the experiments without pretreatment suggested that the cells were strongly stressed. It is noteworthy that moderate exposure to temperature promoted additional activation of the antioxidant enzymes compared to the non-adapted cultures. Thus, the increase in high-temperature tolerance caused by low-temperature pretreatment was a result of increasing ROS scavenging enzyme activities. Moreover, the enhanced antioxidant enzyme activity was implicated in the cross-tolerance of *A. niger* cells to PQ and H_2O_2 stress induced by HS treatment at 35°C. A strong correlation between HS pretreatment and antioxidant defence activity has been reported for plants (Mei and Song, 2011; Mansoor and Naqvi, 2013; Zhao et al., 2014), but results about fungi could be very seldom found. Increased antioxidant enzyme activity in fungi has been demonstrated after PQ, MD, H_2O_2, air pressure etc. (Lee et al., 1995; Pinheiro et al., 2002; Bai et al., 2003; Li et al, 2008). Similar results have also been reported for *Candida oleophila*, *Methanosarcina barkeri*, *Fusarium decemcellulare* (see Liu et al., 2012). It was reported that the enzymatic detoxification of ROS in adapted cells is dependent on the upregulation of several antioxidant genes at transcriptional level including peroxisomal catalase, cytochrome c peroxidase, peroxiredoxin TSA1, thioredoxin reductase, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase (Liu et al., 2012). Spiró et al. (2012) reported evidence that a post-transcriptional element participates in the regulation of heat stress

adaptation under oxidative conditions.

It is noteworthy that the stress agents used in the present study elicited a different response in HS-adapted cells of *A. niger* 26. Our results provided clear-cut evidence that HS pretreatment makes cells more resistant to both PQ and temperature than to H_2O_2. Although the adaptive response to H_2O_2 challenge also included improved growth, reduced oxidatively damaged proteins content and enhanced SOD activity compared to non-pretreated cells, the evaluated levels were significantly lower than those in adapted cells treated with PQ and temperature. In contrast, the variants with H_2O_2 demonstrated extremely high specific CAT activity. The levels of resistance observed in HS-adapted cells exposed to PQ were similar to those found in variants with temperature treated cultures, suggesting that the same mechanism of resistance may be operative. The response to H_2O_2 appeared to be distinct from that induced by PQ, on the basis of cross-protection experiments. Similarly to our results, the adaptive HS response in yeasts was able to confer protection against stress caused by H_2O_2, superoxide anion and lineolic acid hydroperoxide (Jamieson, 1992; Evans *et al.*, 1998). Adaptation to heat increased the resistance of *Listeria monocytogenes* to H_2O_2 (Lou and Yousef, 1997). Furthermore, *Saccharomices cerevisiae* possesses at least two distinct adaptive stress responses to oxidants: one induced by H_2O_2 and the other by exposure to compounds such as menadione, which produce a flux of superoxide anions in the cells (Jamieson, 1992; 1998). According to Hasanuzzaman *et al.* (2013), high temperature induced expression of inducible genes responsible for synthesis of heat-shock proteins (HSPs), which protect intracellular proteins against denaturation and preserve their stability and function through protein folding; thus it acts as a chaperone. The results reported by Troschinski *et al.* (2014) reveal that besides the well-documented HSPs stress response, antioxidant defence plays a crucial role in snails' (*Xeropicta derbentin)* competence to survive extreme temperatures.

Conclusion

HS-pretreatment improved growth and intracellular protein synthesis in the fungal cultures of *A. niger* 26 exposed to PQ, H_2O_2 and an extremely high temperature. The pretreatment strategy reduced the harmful effect of oxidative stress on carbonylated proteins and increased the activities of the antioxidant enzymes SOD and CAT. Taken together, the results demonstrated that the HS-pre-liminary exposure induced a coordinated response that declined the oxidative stress degree in *A. niger* cells when this pre-exposure was followed by severe stress.

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