Cold-stress response during the stationary-growth phase of Antarctic and temperate-climate *Penicillium* strains

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**Abstract**

Cold-induced oxidative stress during the aging of three *Penicillium* strains (two Antarctic and one from a temperate region) in stationary culture was documented and demonstrated a significant increase in the protein carbonyl content, the accumulation of glycogen and trehalose, and an increase in the activities of antioxidant enzymes (superoxide dismutase and catalase). The cell response to a temperature downshift depends on the degree of stress and the temperature characteristics of the strains. Our data give further support for the role of oxidative stress in the aging of fungi in stationary cultures. Comparing the present results for the stationary growth phase with our previous results for the exponential growth phase was informative concerning the relationship between the cold-stress response and age-related changes in the tested strains. Unlike the young cells, stationary-phase cultures demonstrated a more pronounced level of oxidative damage, as well as decreased antioxidant defence.

**INTRODUCTION**

Aging is considered in the context of various abiotic stresses, such as water deficit, high temperature, salinity, cold, heavy metals, mechanical wounding, etc. It is well known that the extreme level of these factors can be reflected in sharply increased production of reactive oxygen species (ROS), e.g. hydrogen peroxide (H$_2$O$_2$) and superoxide anion radicals ($O_2^-$) [1–3]. ROS interact with cellular biomolecules, such as lipids [4–6].

Activation of the antioxidant defence system is an important strategy for coping with oxidative stress. This system includes several enzymes that directly degrade ROS: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [7]. Changes in the activities of antioxidant enzymes have been found in many organisms in response to abiotic stress [8–10].

Extremely low temperatures can be very dangerous for many physiological and biochemical cell components. Cold stress induces cascades of alterations in metabolic pathways, including the activity of antioxidative enzymes and the regulation of gene expression [11, 12]. Stressful low-temperature exposure results in a loss of membrane fluidity and rigidification, generation of ROS, and production of malondialdehyde (MDA) content due to lipid peroxidation and enhanced antioxidant enzyme activities [13]. Evidence has been provided for many plants [14–16]. Similar data have been published for Antarctic and temperate bacteria [11, 17]. Although such phenomena were also found for fungal cultures, investigations concerning them are very scarce [18–20]. Our previous studies pointed out the close correspondence between long- or short-term cold exposure and oxidative stress in Antarctic fungi from different thermal classes. Growth at low temperature affected the level of oxidative stress biomarkers and the antioxidant enzyme defence [21–24].

Although basal levels of ROS are indispensable for redox signalling and cell survival, high levels of ROS have been thought to contribute to aging [25]. On the other hand, stress-induced oxidative damage to cellular macromolecules accelerated the aging processes [26]. One of the most popular theories in aging research is the free-radical hypothesis, introduced by Harman in 1956. He stated that ‘aging and the degenerative diseases associated with it are attributed basically to the deleterious side attacks of free radicals on cell constituents and on the connected tissues’ [27]. Over the past two decades it has been modified to the oxidative stress hypothesis, because oxygen species such as peroxides and aldehydes, which are not technically free radicals, also play a role in oxidative damage to cells [28]. The imbalance between prooxidants and antioxidants leads to an accumulation of oxidative damage in a variety of macromolecules with age, resulting in a progressive loss of functional cellular processes, leading to the aging phenotype [29]. Currently, strong evidence in favour of this theory has come from...
studies with different organisms [30]. However, investigations with fungi as the model system for cellular aging are not often published. Only a few fungal species have been investigated in experimental aging research. At the same time, fungi are an appropriate model system in different research areas, including aging. They are easy to cultivate under laboratory conditions, possess a complexity of organization that is much lower than that of higher organisms, and different species are well suited for genetic analysis of various phenomena and pathways [31]. It should be noted that Antarctic fungi have not been used in aging research.

In our previous studies we focused on assessing the cold cell response of three fungal strains (two strains isolated from Antarctica and one from a temperate region) belonging to the genus Penicillium. The decrease in growth temperature below the optimal clearly induced oxidative stress events and the activation of antioxidant defense in all of the tested strains [21]. Furthermore, transient temperature downshifts applied in the exponential phase also resulted in changes in the oxidant-antioxidant balance in comparison to growth at an optimal temperature [22]. The results also demonstrated a significant induction in the \( ^{1}O_2 \) and \( H_2O_2 \) levels in a dose- and growth-phase-dependent manner [32, 33]. Furthermore, the aging of stationary cultures of is an interesting biological process [34].

Assessment of the age-associated cell response to low temperature of cold-adapted and non-adapted strains can provide information on fungal ecology concerning the relations of fungi with the abiotic environment. The present study focused on the relationship between cold stress and aging processes in fungi from different ecological niches: the extreme cold of Antarctica and a temperate region. The aim was to evaluate the effect of oxidative stress induced by extreme cold exposure on the stationary-phase cultures of both Antarctic strains, psychrotolerant Penicillium olsonii and mesophilic Penicillium waksmanii, and the temperate mesophilic strain, Penicillium rugulosum. The changes in growth, the level of stress biomarkers, and the activities of SOD and CAT, were recorded and compared to those from the exponential-growth phase (previously reported [22]).

**Cell-free extract preparation and enzyme activity determination**

The cell-free extract was prepared as previously described [22]. All of the steps were performed at 0–4 °C.

SOD activity was measured by the nitro-blue tetrazolium (NBT) reduction method of Beauchamp and Fridovich [35]. One unit of SOD activity was defined as the amount of enzyme required for 50% inhibition of NBT reduction (A560) and expressed as units per mg protein [U (mg protein)]\(^{-1}\). Catalase activity was determined by monitoring the decomposition of 18 mM \( H_2O_2 \) at 240 nm [36]. One unit of activity is the amount that decomposes 1 \( \mu \)mol of \( H_2O_2 \) \( \text{min}^{-1} \) (mg protein)\(^{-1}\) at 25 °C and pH 7.0. Specific activity is given as U (mg protein)\(^{-1}\).

**Analytical methods**

Protein was estimated by the Lowry procedure [37] using a solution of bovine serum albumin as the standard. Glycogen and trehalose content was determined following the procedure of Becker [38] and Vandercampoen et al. [39], as modified by Parrou et al. [40]. Soluble reducing sugars were determined by the Somogy–Nelson method [41]. Protein oxidative damage was measured spectrophotometrically as the protein carbonyl content using the 2,4-dinitrophenylhydrazine (DNPH) binding assay [42], as slightly modified by Adachi and Ishii [43]. The carbonyl content was calculated using a molar extinction coefficient of 21 \( \text{mM}^{-1} \text{cm}^{-1} \), as nanomoles of DNPH incorporated (protein carbonyls) per mg of protein. The dry weight determination was performed on samples of mycelia harvested throughout the culture period. The culture fluid was filtered through a Whatman 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 mean interval estimation (MIE), and by one-way analysis of variance (ANOVA) followed by Dunnett’s post-test, with a significance level of 0.05.

**METHODS**

**Fungal strains, culture media and cultivation**

The fungal strains, *P. olsonii* p14 and *P. waksmanii* m12, isolated from Antarctic soils [21], as well as strain *P. rugulosum* t35, isolated from temperate Bulgarian soil samples, were used in the experiments. All strains belonged to the mycological collection of the Institute of Microbiology, Sofia, and they were maintained at 4 °C on beer agar (pH 6.3). The composition of the culture medium, AN-3, and the cultivation in 3 l bioreactors (ABR-09, equipped with temperature, pH and automatic dissolved oxygen monitoring equipment and a control system) were used as previously described [21].

For the submerged cultivation, 74 ml of the AN-3 medium was inoculated with 6 ml spore suspension at a concentration of \( 2 \times 10^8 \) spores ml\(^{-1} \) in 500 ml Erlenmeyer flasks. The cultivation was performed at the optimal temperature (20 °C for *P. olsonii* p14 and 30 °C for *P. waksmanii* m12 and *P. rugulosum* t35, respectively [21]) for 48 h on a rotary shaker (220 r.p.m.). 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 at the optimal temperature with a stirrer speed of 400 r.p.m. and an air flow of 0.5 vol min\(^{-1} \). During the stationary phase, the temperature was reduced to 6 °C or 15 °C. This downshift was reached in approximately 40 min. After 6 h of incubation under cold-stress conditions, the temperature was upshifted to the optimal value. The control variants were grown at an optimal temperature throughout the whole period.
RESULTS

Mycelium biomass from *P. olsonii* p14, *P. waksmanii* m12 and *P. rugulosum* t35 cultures, grown until the stationary phase at the optimal temperature, was exposed to temperatures of 6 and 15 °C, respectively. The duration of the temperature downshift was chosen to be 6 h because this range was found to be wide enough to give a clear contrast between the control and the stressed cultures.

**Effect of temperature downshift on fungal growth**

Fig. 1 demonstrates the effect of a sharp decrease in the growth temperature to 15 or 6 °C on the biomass content. Maintenance of the optimal temperature (control variant) saw a typical stationary phase, with a constant level of dry weight during the whole experimental period (10 h). Exposure to cold stress resulted in a reduction of biomass production in relation to temperature. Within the first 2 h from the beginning of the stress, the growth of the Antarctic strains (*P. olsonii* p14 and *P. waksmanii* m12) ceased and the biomass decreased in comparison to the control (Fig. 1a, b). This decrease was more pronounced at 6 °C (about 24–27%) than at 15 °C (12–13%). While the biomass content of the strain *P. waksmanii* m12 continued to decline during the next 2 h (by 20 and 35% for the downshift to 15 and 6 °C, respectively), the strain *P. olsonii* p14 overcame the reduction in biomass and showed a sustained level trend. A similar trend was demonstrated for the temperate mesophilic strain *P. rugulosum* t35 after the shift from 30 to 15 °C or 6 °C (Fig. 1c), but the difference in biomass production between the control and the treated mycelia was more significant in comparison with both Antarctic strains.

**Carbonyl content of cultures after exposure to low-temperature stress conditions**

We compared the level of protein carbonylation in the treated and nontreated cultures. It can be seen in Fig. 2 that the carbonylated protein content in the control variants of the Antarctic and temperate strains also showed a notable increase during the first 6 h of the experimental period. Furthermore, a statistically significant increase over the control values was observed in all of the investigated strains under cold-stress conditions. The highest levels of carbonyl groups were observed at 6 °C in comparison to the variants at 15 °C. As is shown in Fig. 2(a), there was a sharp increase in the oxidative modification of proteins in *P. olsonii* p14 after the beginning of the stress. This trend was observed during the whole period of exposure to cold shock and subsequently decreased after the end of the treatment. The carbonyl content increased 2.0- and 1.5-fold at 6 and 15 °C, respectively, compared with the control.

Elevated production of carbonyl groups after a temperature downshift to 6 or 15 °C was also detected in the Antarctic mesophilic strain *P. waksmanii* m12 (Fig. 2b). The level of carbonyl content was significantly increased (1.7- and 1.3-fold) after exposure to 6 or 15 °C, compared with the control values. The maximum carbonyl content in this strain was detected 6 h after the beginning of the stress. It can be seen in Fig. 2(c) that the response of temperate strain *P. rugulosum* t35 to cold shock showed a similar tendency. The sharp temperature downshift induced a 1.6- and 1.3-fold increase in oxidatively damaged protein content at 15 and 6 °C, respectively.

**Effect of stress on glycogen and trehalose content**

The results for reserve carbohydrate (glycogen and trehalose) accumulation in the model strains are presented in Figs 3 and 4. There was no statistically significant difference in the glycogen content in the cells of psychrotolerant
*P. olsonii* grown in non-stress conditions (Fig. 3). In the control groups of the mesophilic strains, *P. waksmanii m12* and *P. rugulosum t35*, a slight increase in glycogen accumulation was established (23 and 29 %, respectively) after 8 h of cultivation, followed by recovery to basal level.

It can be seen in Fig. 3 that the temperature downshifts to 15 and 6 °C affected glycogen content in the tested strains, depending on their thermal classes. Glycogen accumulation in the psychrotolerant *P. olsonii* was initiated at an earlier stage but remained at low levels (Fig. 3a). By contrast, both mesophilic strains exhibited a two-step response. During the first 4 h of cultivation, the glycogen level remained almost unchanged. In contrast, a considerable increase occurred at the sixth hour of cultivation: 1.3- and 1.6-fold for *P. waksmanii m12*, and 1.1- and 1.4-fold for *P. rugulosum t35*, compared with the control at 15 and 6 °C, respectively (Fig. 3b, c). In the statistical analysis, a significant difference (0.05 level) was observed in the glycogen content between the mesophilic strains cultivated at optimal temperature and those cultivated at temperature downshift to 6 and 15 °C.

Furthermore, we monitored the changes in the trehalose content of the model strains belonging to different thermal...
1.7-fold increase compared to the control, and the differences proved to be statistically significant. The maximum trehalose content was achieved 6 h after the temperature downshift.

**Activation of antioxidant enzyme defence**

To evaluate the changes in antioxidant defence we measured the level of SOD and CAT activity in fungal cells taken from the stationary growth phase and subjected to 15 and 6 °C for 6 h (Fig. 5). The time-courses for the SOD activity for all three strains during the cold-shock treatment, as well as under optimal conditions, are shown in Fig. 5a–c. Compared with the control cultures, the SOD activity was statistically higher under the cold treatment conditions. Exposure to 15 °C caused a slow and insignificant increase in SOD in the strain *P. rugulosum* t35. At the same time, both Antarctic strains showed a more rapid and significant response compared with the temperate strain. This trend was seen most clearly in the psychrotolerant strain *P. olsonii* p14. The highest SOD activity was measured at 6 °C. Statistical analysis indicated that the downshift from optimal temperature to 6 °C caused an immediate and remarkable increase in SOD activity (0.05 level) for all of the tested strains.

Transient exposure to 6 and 15 °C also resulted in statistically elevated CAT activity compared with the control variant, and the increase occurred in a temperature- and time-dependent manner until the end of the treatment (Fig. 5d–f). The highest percentage of increased CAT activity was found in both Antarctic strains. Whilst the temperate strain *P. rugulosum* t35 showed an approximately 1.5-fold increase in CAT activity, the psychrotolerant strain *P. olsonii* p14 showed 1.4- and 2.0-fold increases after the downshift from the optimal temperature to 15 or 6 °C, respectively, compared with the control. The antioxidant cell response of the mesophilic Antarctic strain *P. waksmanii* m12 included a similar increase in CAT activity (approximately 2- and 1.5-fold) compared with the control cultures. During the recovery phase, the model strains showed a significant reduction in SOD activity compared to the control. In contrast, CAT activity was maintained 2 or 4 h after the return to the optimal temperature.

**DISCUSSION**

The oxidative stress theory of aging states that aging is due to the accumulation of unrepaired ROS-inflicted oxidative damage to DNA, proteins and membrane lipids [44, 45]. Although various evidence supporting this theory has been published, it is still an open question whether such increases in oxidative damage are the main cause of aging. Here we compare the effect of oxidative stress induced by temperature downshift on the exponential-growth (previous study [22]) and stationary-phase cultures (present data) of three *Penicillium* strains belonging to different thermal classes. Furthermore, our previous reports demonstrated that cold stress caused a drastic increase in ROS generation in cells of the same *Penicillium* strains in the exponential and stationary phases of growth [32, 33]. The ROS levels showed a more drastic increase during the...
stationary phase in both intact cells and mitochondria compared to the exponential phase. Downshift from the optimal temperature to 15 and 6 °C led to an enhanced level of oxidatively damaged proteins, increased accumulation of reserve carbohydrates and increased activity of antioxidant enzyme defence in exponentially growing cells [22]. In the present study we evaluated changes in the stress biomarkers caused by the same temperature downshift in stationary-phase cultures.

As expected, substantially enhanced ROS levels in the stationary-phase cells exposed to low temperatures gave rise to oxidative stress. The results demonstrated a pronounced reduction in biomass content, depending on the location of isolation, the degree of stress and the thermal classification of the strains. The best resistance was shown by the Antarctic psychrotolerant strain, followed by the Antarctic mesophilic strain. The temperate mesophilic strain *P. rugulosum* t35 proved to be the most sensitive to temperature downshift.

Very little information is available on the oxidative cell response of from cold habitats [11, 23, 46, 47]. Furthermore, there are no data about the effect of cold stress on stationary-phase cells of Antarctic fungi. A similar biomass reduction has been reported for the exponential-phase
cultures of two fungal strains (psychrotolerant and mesophilic) isolated from Livingston Island, Antarctica [24], as well as bacterial and fungal cells treated by cold or heat shock [48–50]. A possible explanation or cause for this discrepancy is that most of the Antarctic mycoflora are metabolically active whenever a combination of favourable abiotic conditions occurs during the short growing summer season [51–53]. Furthermore, although hyphal autolysis is a natural part of the fungal life cycle, its onset can be accelerated by oxidative stress factors [50, 54, 55]. According to Yamanaka et al. [48], the amount of N-acetylmuramoyl-l-alanine amidase is the main factor for the cold-shock-induced autolysis in *Bacillus subtilis*. This phenomenon may be due to an ordered degradation of cellular reserves [56] or differential expression of genes in response to stress [50]. Under cold-stress conditions, gene down-regulation may explain growth reduction. To combat such harsh conditions, fungi (including yeasts) have adapted to have special features that are still not fully understood. The published data suggest that these organisms use a combination of strategies involving the production of cold-adapted enzymes, cryoprotectant wall carbohydrates and pigments, as well as higher amounts of polyunsaturated fatty acids in cytoplasmic membranes and a high amount of intracellular lipids [46, 57, 58].

If we compare the effect of temperature downshift on the growth of stationary-phase cells with our previous results [22], we have to note that the exponential-phase cultures of the same strains exhibited clearer resistance to cold stress. The young fungal cells also demonstrated reduction in biomass content, but to a lesser extent.

The present finding that the carbonylated protein content increased markedly in comparison to the control variants was not surprising. An increase in the rate of ROS production or a decrease in their rate of scavenging accelerated the oxidative modification of cellular molecules, including proteins [59, 60]. Low-temperature stress induces ROS accumulation in cells, which leads to the production of oxidized proteins [61]. Temperature-dependent enhancement of carbonylated proteins has been reported for exponential-phase cultures of Antarctic fungi [24]. A similar response has been observed in fungal cells against the oxidative stress induced by different abiotic factors [8, 22, 62–64].

The above-mentioned remarkable increase in oxidatively damaged protein content in stationary-phase cells was also established in the young cells of the same three *Penicillium* strains. The collation of data for both growth phases revealed that the exponential-phase cells exhibit higher resistance to cold stress. It is noteworthy that: (i) the protein carbonyl content in the exponential-phase cells is significantly lower than in the stationary-phase cells in the controls as well as in the stressed cultures; (ii) the increase in the accumulation of carbonylated proteins was more sharply outlined in the stationary-phase cells; (iii) while the control in the experiments with exponential-phase cells demonstrated insignificant changes to protein carbonyl content during the whole fermentative period, the stationary-phase cells showed a trend of continuous and significant increase in comparison with the starting value.

As known, aging is associated with the accumulation of oxidized proteins (protein carbonyls) [65]. An enhanced level of oxidized proteins in stationary-phase cells has been determined for yeasts and filamentous fungi [66, 67]. De Castro et al. [68] reported that the transition to stationary phase of the fungus *Phycomyces blakesleeanus* was accompanied with a higher physiological oxidative damage illustrated by the higher protein carbonylation.

We also found a temperature-shift-dependent increase in glycogen and trehalose content in the stressed *Penicillium* cells (Figs 3 and 4). Both reserve carbohydrates could help to protect fungal cultures from ROS generated in cold-stress conditions. According to Schade et al. [69], the accumulation of the carbohydrate reserves trehalose and glycogen is induced during the cold response in *Saccharomyces cerevisiae* cells due to the induction of the genes involved in their synthesis. *Propionibacterium freudenreichii* strains exhibited similar responses when placed at 4 °C [70]. The authors suggested that this rerouting of the carbon metabolism toward trehalose and glycogen synthesis is part of the molecular basis of long-term survival in the cold. Both trehalose and glycogen accumulate in cells subjected to heat shock, oxidative stress, or osmotic stress [71, 72]. A positive correlation between carbohydrate storage and cell survival has been reported in yeast and fungal cells [64, 73].

By comparing the exponential and stationary cultures it can be seen that both phases were characterized by a significant increase in the amount of reserve carbohydrates, depending on the degree of stress and the temperature characteristics of the strains. However, it should be noted that the old cells showed a low basic level of glycogen in comparison with the young cells. In contrast, the non-treated cells showed a significantly higher level of trehalose when they reached the stationary phase. The published data are contradictory. Trehalose is found in various eukaryotic and prokaryotic organisms, in which it accumulates during the stationary phase due to its nonutilization, and when the glycogen reserves are being utilized [74]. A possible reason for trehalose accumulation could be the reduced activity of trehalose hydrolysing enzymes (acid-trehalase and iso-aspartyl methyl transferase) [75]. At the same time, Hu et al. [76] suggested that the levels of both trehalose and glycogen decreased in *S. cerevisiae* with age. Samokhvalov et al. [77] also reported that aging led to a drastic drop in the cellular content of trehalose and glycogen.

The *Penicillium* strains tested in this study responded to cold stress with an increase in SOD and CAT activity in the stationary-growth phase, which was more pronounced for the psychrotolerant strain *P.olsonii* p14. These results suggest that the antioxidant enzymes might also be crucial for old-cell survival. Longo et al. [78] reported that SOD is a major antioxidant in stationary-phase yeast cells. This
enzyme plays a very important role in their survival under stress conditions through the inhibition of ROS levels. Stationary-phase cells of the fungal pathogen *Candida glabrata*, when treated with H$_2$O$_2$, showed extreme resistance to oxidative stress, and this resistance was mediated by the CAT activity [79]. An increase in antioxidant enzyme defence after stress exposure has been detected in various fungi [80, 81]. Temperature downshift caused significant enhancement in SOD and CAT activity in exponentially growing fungi [24]. However, the effect of cold stress on antioxidant activity during the stationary phase has not been studied sufficiently.

On the other hand, the levels of both enzymes, under normal or stress conditions, were lower when the cultures reached the stationary phase. Oxidative stress during the aging of *S. cerevisiae* in stationary culture also caused a decrease in the activities of antioxidant enzymes [34]. This decline could be due to a decrease in the rate of enzyme synthesis or to the appearance of inhibitory factors.

**Conclusion**

This study combined different biochemical analyses to investigate the relationship between cold-induced oxidative stress and the aging of three *Penicillium* strain isolated from permanently cold and temperate climates. Surprisingly, we did not observe an impact from the climatic distance between the locations of isolation on the fungal response. Taken together, our data provide further support for the role of oxidative stress in the aging of fungi in stationary cultures. Cold stress during the aging of Antarctic and temperate fungal strains belonging to the genus *Penicillium* caused oxidative stress that was evidenced by the disruption of the oxidant/antioxidant balance, an increase in the carbonylated protein content and the accumulation of reserve carbohydrates. Despite the activation of the antioxidant enzyme defence, oxidative damage was detected in the fungal cells. If we compare both exponential- [22] and stationary-phase cultures (present study), we can conclude that the old cells demonstrated a more pronounced level of oxidative damage in comparison with the young cells. Thus, the exponential-phase cells are more resistant to cold stress than the stationary-phase cells.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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