POLISH POLAR RESEARCH
vol. 43 no. 2, pp. 125–143, 2022 DOI: 10.24425/ppr.2021.138587

Cell response of Antarctic strain *Penicillium griseofulvum* against low temperature stress

Ekaterina Ts. KRUMOVA[1*], Ekaterina K. KOEVA[1], Stoyanka R. STOITSOVA[1], Tsvetelina S. PAUNOVA-KRASTEVA[1], Galina D. STOYANCHEVA[1] and Maria B. ANGELOVA[1]

[1] *The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, 26, Acad. G. Bonchev str., 1113 Sofia, Bulgaria*

**corresponding author:* <ekrumova@abv.bg>

Abstract: During the evolution organisms are subjected to the continuous impact of environmental factors. In recent years an increasing number of studies have focused on the physicochemical limits of life on Earth such as temperature, pressure, drought, salt content, pH, heavy metals, etc. Extreme environmental conditions disrupt the most important interactions that support the function and structure of biomolecules. For this reason, organisms inhabiting extreme habitats have recently become of particularly great interest. Although filamentous fungi are an important part of the polar ecosystem, information about their distribution and diversity, as well as their adaptation mechanisms, is insufficient. In the present study, the fungal strain *Penicillium griseofulvum* isolated from an Antarctic soil sample was used as a study model. The fungal cellular response against short term exposure to low temperature was observed. Our results clearly showed that short-term low temperature exposure caused oxidative stress in fungal cells and resulted in enhanced level of oxidative damaged proteins, accumulation of reserve carbohydrates and increased activity of the antioxidant enzyme defence. Ultrastructural changes in cell morphology were analysed. Different pattern of cell pathology provoked by the application of two stress temperatures was detected. Overall, this study aimed to observe the survival strategy of filamentous fungi in extremely cold habitats, and to acquire new knowledge about the relationship between low temperature and oxidative stress.

Keywords: Antarctic, filamentous fungi, oxidative stress, biomarkers, antioxidant enzymes.

Introduction

Antarctica is the continent with the coldest and driest climate on the Earth. It is the most extreme habitat on the planet and provides an interesting and unique environment for the colonization and survival of natural life. Extreme Antarctic conditions are a prerequisite for the development of stress tolerance mechanisms by the microorganisms inhabiting this inhospitable environment (Durán *et al.* 2019). In recent years, studies on microorganisms adapted to low temperatures have increased, driven by their potential value for application in biotechnology. Archaea, bacteria and fungi are the main components of microbial life in Antarctica (Teixeira *et al*. 2013; Purves *et al*. 2016). The fungal group is the most diverse in the Antarctic ecosystems (Godinho *et al.* 2015). Ecological selection as well as evolutionary adaptations have ensured fungal survival in extreme conditions (Cowan *et al*. 2014). Antarctic fungi may be cosmopolitan. Some of them can survive as air transported propagules, but are not able to grow under Antarctic conditions, while other native, well adapted fungi develop and reproduce at low temperatures (Selbmann *et al*. 2015). Fungi adapted to a cold environment are sources of new bioactive secondary metabolites and enzymes. They can survive in a wide range of stress conditions, such as drought, salinity, solar radiation and low temperatures. Fungal survival mechanisms include production of bioactive compounds, cold–active enzymes and antifreeze proteins (Robinson 2001; Krishnan *et al.* 2011; Godinho *et al*. 2013)

The link between low-temperature exposure and the manifestations of oxidative stress has attracted more scientific attention in recent years, as well as the involvement of antioxidant enzymes in survival under such drastic conditions. It should be noted that while the cellular response against cold stress has been studied in various bacteria and plants, little is known about the adaptation of fungi to survive at temperatures close to negative (Miteva-Staleva *et al*. 2011).

Despite the intensive studies on microorganisms from extreme habitats, there is still a need to address problems related to the mechanisms of survival in habitats like Antarctica. Moreover, the role of cellular response against oxidative stress as part of the survival mechanism of microorganisms, especially in fungi isolated from permanently cold habitats, has not yet been elucidated. Any new information in this context will be a contribution to the better understanding of the involvement of low temperature-induced stress in environment-related responses. Our previous results demonstrated that growth at low temperatures induced the generation of reactive oxygen species (ROS) in cells and their mitochondrial fractions in Antarctic fungi in a dose and age-dependent manner. At the same time, cold-stress response was not dependent on the cold-adaptation of the model strains (Miteva-Staleva *et al*. 2011).

The purpose of this study was to establish a relationship between oxidative stress in the cell and stress caused by low temperature exposure, and to identify stress biomarkers as well as the role of the antioxidant enzymatic defence in the cellular response of a strain of filamentous fungus isolated from Antarctica.

Materials and methods

Fungal strain and cultivation. — The fungal strain *Penicillium griseofulvum* Dierckx, R.P. 1901, isolated from a soil sample from Livingston Island, Antarctica, was used in the experiments. This strain is part of the Mycological Collection at the Bulgarian Academy of Sciences, Stephan Angeloff Institute of Microbiology in Sofia, where it is kept at 4°C on beer agar with a pH 6.3.

Experiment design. — The low temperature effect on growth, morphology and physiology of the model strain was assayed under submerged cultivation in flasks and bioreactors at different temperatures. The composition of the culture medium used was as described in Angelova *et al*. (1995). For inoculum preparation, submerged cultivation was performed for 24 hours at 25°C in 500 ml flasks. Culture medium of 74 ml and 6 ml spore suspension at a concentration of 2 x 10^8 spores ml^{-1} were used. Cultivation was performed in 3l bioreactors/ working volume 2L at optimal temperature (25°C), stirrer speed of 400 rpm, and air flow 0.5 v.v.m. During the mid-exponential phase, the temperature was reduced to 6°C or 15°C. This downshift was reached approximately in 40 min. Temperature was up-shifted to the optimum value after a 6-hour incubation period under cold stress conditions. The control variants were grown at optimal temperature throughout the whole period.

Cell-free extract preparation and enzyme activity assay. — A cell-free extract was prepared as described by Angelova *et al.* (1995). Superoxide dismutase (SOD), catalase (CAT), intracellular protein and damaged protein were determined in the cell-free extract. The specific SOD activity (EU 1.15.1.1.) was determined by reduction of NBT (nitroblue tetrazolium) by the method of Beauchamp and Fridovich (1971). For one unit of SOD activity (expressed as units/mg protein), the amount of enzyme protein required to inhibit the NBT reduction rate (A560) by 50% at pH 7.8 and 30°C was taken. Catalase (EU 1.11.1.6.) was determined by the method of Beers and Sizer (1952) and expressed as units/mg protein. The amount of enzyme protein required to break down 1 μM H_2O_2 for 1 min at 25°C and pH 7.0 was taken as one unit of activity.

Analythical methods. — Soluble reducing sugars were determined by the glucose standard method of Somogy-Nelson (Nelson 1944; Somogy 1952). The protein amount was estimated by the Lowry *et al*. (1951) method. Bovine serum albumin was used as a standard. Fungal growth was determined by measurement of biomass dry weight. For the culture filtration, a Whatman filter was used (Clifton, USA) No. 4. Biomass was washed with distilled water and dried at 105°C

to constant weight. The content of carbonyl groups in oxygen modified proteins was determined by the method of Hart *et al.* (1999) modified by Adachi and Ishii (2000). A procedure described by Becker (1978) and Vandercammen (1989) and modified by Parrou *et al.* (1997) was used to determine the glycogen and trehalose content.

All experiments were performed in triplicate.

Transmission electron microscopy (TEM). — For TEM assay, samples were taken from each variant after short term exposure to low temperature. Mycelia and cultural liquid were separated by filtration. The mycelia were washed twice, e.g. with distilled water and phosphate buffer pH 7.8. The samples were fixed for 2 hours at 4°C with 4% glutaraldehyde in 0.05 M Na cacodylate buffer (CB), pH 7.2. This was followed by 1h of post-fixation in 1% OsO_4 in 0.05M CB, and dehydration in graded ethanol series, up to 100% ethanol. The samples were further incubated with propylene oxide, and impregnated and embedded in Spurr resin (Sigma) following the producer's instructions. Ultrathin sections were prepared on Reichert-Jung ultramicrotome. Counterstaining was performed with 1% uranyl acetate in 70% methanol for 30 min in the dark, followed by 20 min in 0.4 % lead citrate. The observations were made on an HRTEM JEOL JEM 2100 (JEOL, Japan) Transmission Electron microscope at accelerating voltage of 220 kV.

Results

Effect of temperature on growth and glucose consumption. — The used *P. griseofulvum* strain belongs to the psychrotrophic fungi. To investigate the relationship between low temperature and oxidative stress the model strain was subjected to short-term (6h) low temperature exposure (6°C and 15°C) under conditions of submerged cultivation. Fig. 1 show the changes in the growth curves of the selected Antarctic strain after a decrease in temperature for 6 hours and subsequent restoration of the optimal conditions.

Temperature downshift from 25°C to 6°C resulted in transient growth inhibition and even reducion in the amount of biomass over a period of 2–4 h. The strain resumed growth and after exposure was discontinued and the optimal temperature was restored, the amount of biomass increased and became comparable with the control variant. Biomass production was close to the control variant when the temperature dropped to 15°C and after the optimal temperature was restored this trend did not change.

Short-term low-temperature stress also led to changes in the consumption of the carbon source in the culture medium. Fig. 2 clearly shows that reducing the temperature to 6°C significantly slowed glucose uptake. Delayed glucose consumption in this variant was also observed after the optimal growth temperature was restored within 12 h of culture development. A similar trend

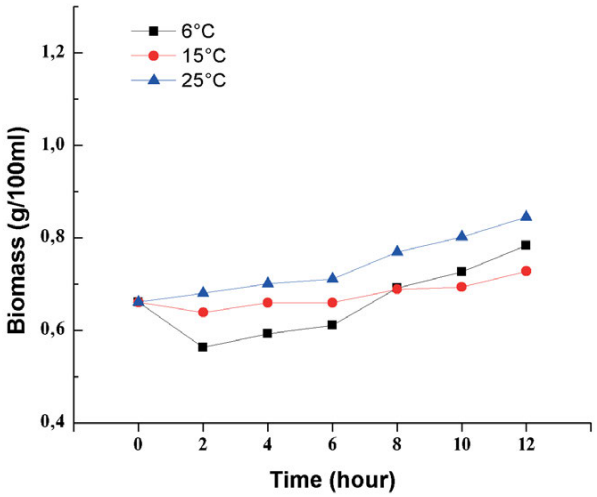

Fig. 1. Effect of temperature change on biomass production at *Penicillium griseofulvum.*

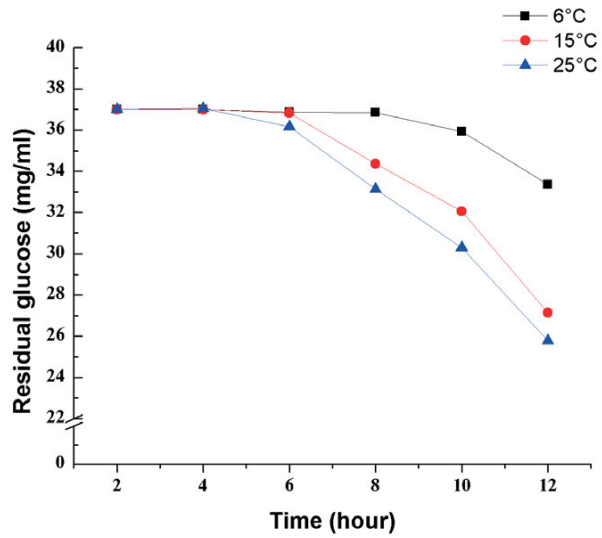

Fig. 2. Glucose consumption of *Penicillium griseofulvum* under low temperature stress, with subsequent recovery of optimal conditions.

for carbon source consumption was also observed when the temperature was lowered to 15°C but it was significantly less than in the first experimental variant.

Changes in the level of oxidative stress biomarkers: Low temperature stress and protein oxidative damage. — Proteins can be modified by a large

number of reactions, including ROS impact. The presence of carbonyl groups is used as a marker of ROS-mediated protein oxidation. The changes in the level of oxidatively damaged proteins were studied during 6 h cold stress as described above (Fig. 3).

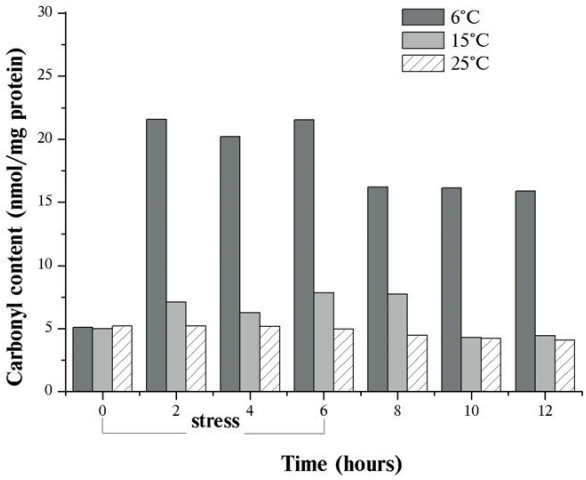

Fig. 3. Changes in carbonyl groups content in the cells of the model strain under short-term low temperature stress.

Under low-temperature stress conditions, the content of carbonyl groups of the cells cultured at 6°C significantly increased compared to the other two variants. For the stress period, the increase was about four times higher than the control. The tendency remained unchanged after the optimal temperature was restored. At the 12th hour of cultivation, the damaged proteins level was again almost four times higher than the control. The levels of carbonylated proteins in the variant with the temperature lowered to 15°C were higher than those in the control variant. For the period of short stress application, the damaged proteins content rose to 36% and to 58% after 2 and 6 hours, respectively, compared to the control. These levels were close to the control variant after the restoration of the optimal growth temperature.

Changes in the level of oxidative stress biomarkers: Low- temperature stress and changes in levels of reserve carbohydrates in cells. — The effect of short-term low-temperature shock on the production of glycogen and trehalose by *P. griseofulvum* cells was studied (Fig. 4, 5). The experiments in this study showed that a downshift in the temperature to 6°C led to a significant increase in the level of glycogen during the stress period, after which its levels dropped but were still higher than those of the control variant (Fig. 4). The maximum level of glycogen was reported 2 h after the stress period. It was 72% higher than the

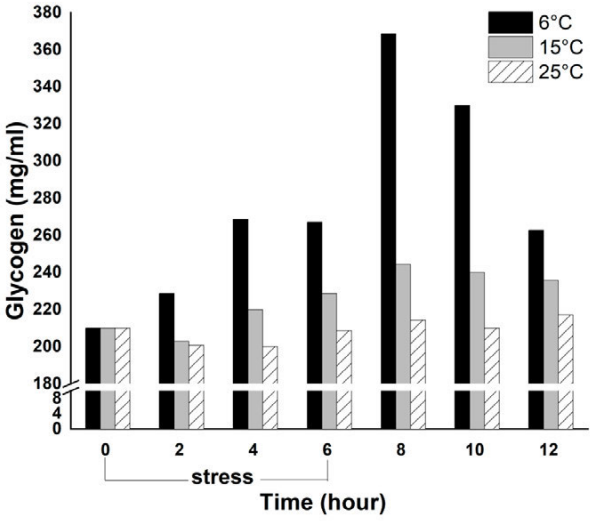

Fig. 4. Changes in glycogen levels of cells in cold stress conditions.

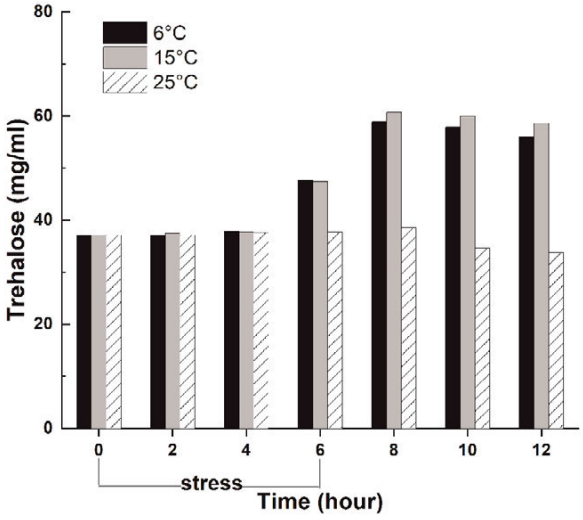

Fig. 5. Changes in the levels of trehalose of cells placed under cold stress.

control and 50% higher than the other stress variant (15°C). A similar response was observed after a temperature downshift to 15°C, but to a lesser degree compared to the control. A maximal glycogen level in this variant was also observed at the 8th hour of the experiment, and it was 14% higher than the control variant.

Changes in the levels of trehalose in the cells demonstrated a different trend. Fig. 5 clearly shows that there was a cellular response, including trehalose synthesis. An increase in the content of this carbohydrate after 4 hours of stress was recorded. This tendency continued after the optimal growth temperature was recovered. Lowering the temperature to 6°C and 15°C resulted in a 1.5-fold increase in the trehalose levels at the 8th, 10th and 12th hours compared to the control levels.

Changes in the level of oxidative stress biomarkers: Antioxidant defence in the low- temperature stress conditions. — The cellular response to oxidative stress was determined by measuring the activity of key antioxidant enzymes, such as SOD and CAT.

Exposure of the studied Antarctic strain to temperatures of 6°C and 15°C led to activation of the antioxidant enzyme defence. Changes in the activity of the first antioxidant enzyme, SOD, during the stress and post-stress period are presented in Fig. 6. A clear tendency for an increase in SOD activity was observed with

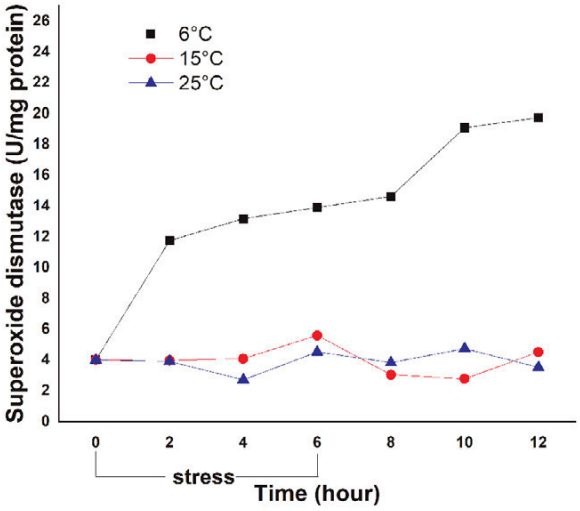

Fig. 6. SOD activity under short-term low-temperature stress.

lowering the cultivation temperature to 6°C. At the 6th hour, this increase was more than three times higher than the control variant cultivated at optimum temperature. This tendency continued in the hours after the stress application. At the 12th hour, the activity of SOD in the mycelium treated with 6°C was about six times higher than the control variant. The short-term stress caused by 15°C did not lead to significant changes, and the trend was similar to the control variant.

In the study of the second antioxidant enzyme, lowering the temperature to 15°C and 6°C also stimulated enzyme production. Antarctic strain cultures

exhibited a response with higher CAT activity at 6 °C than that at 15°C (Fig. 7). A sharp decrease in catalase was observed after short stress exposure to 6°C. After that, enzyme activity was restored.

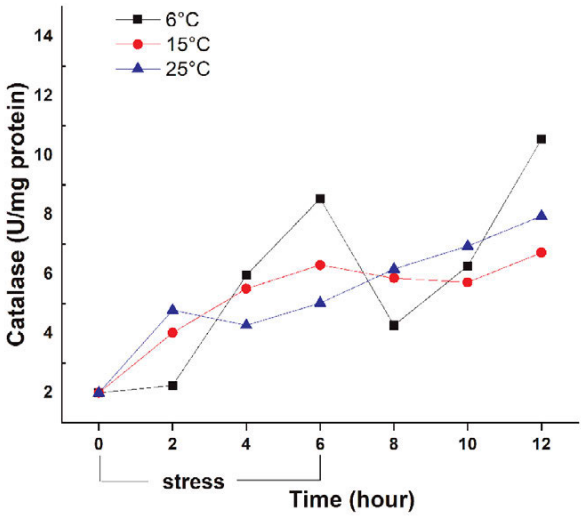

Fig. 7. Changes in the activity of catalase under conditions of low temperature stress.

Ultrastructural changes as a result of the cold stress. — The TEM of the fungus maintained at the optimal temperature of growth, 25°C, revealed a typical fungal fine structure, as well as some peculiarities of the strain (Fig. 8A–D). Among the latter, the accumulation of electron-dense material in the cytoplasm, or inside vacuoles, should be mentioned. Due to its ultrastructural similarities with the electron-dense melanin bodies described by Franzen *et al*. (2008) and Liu *et al*. (2014), the material was interpreted as "melanin-like". Inside the vacuoles, myelinic-like helical accumulations of membranes were located. Under the conditions of the present experiment, the mitochondria were with narrow cristae and electron-light matrix.

The 6 h exposure to cold stress resulted in ultrastructural changes that differed in distribution and degree. After 6 h at 15°C (Fig. 8E–H), the appearance of some hyphae did not much differ from the fungus maintained at the optimal temperature. Focal alterations were also noted. Among those, an increase in the thickness of the cell wall (Fig. 8E) could be considered as an adaptive response. Some increase in vacuolar volume was probably due to more extensive vacuolar fusion processes (Fig. 8F). Other alterations included focal destruction of the cell wall (Fig. 8G) and changes in the mitochondrial structure, mainly reduction in the number of cristae (Fig. 8H).

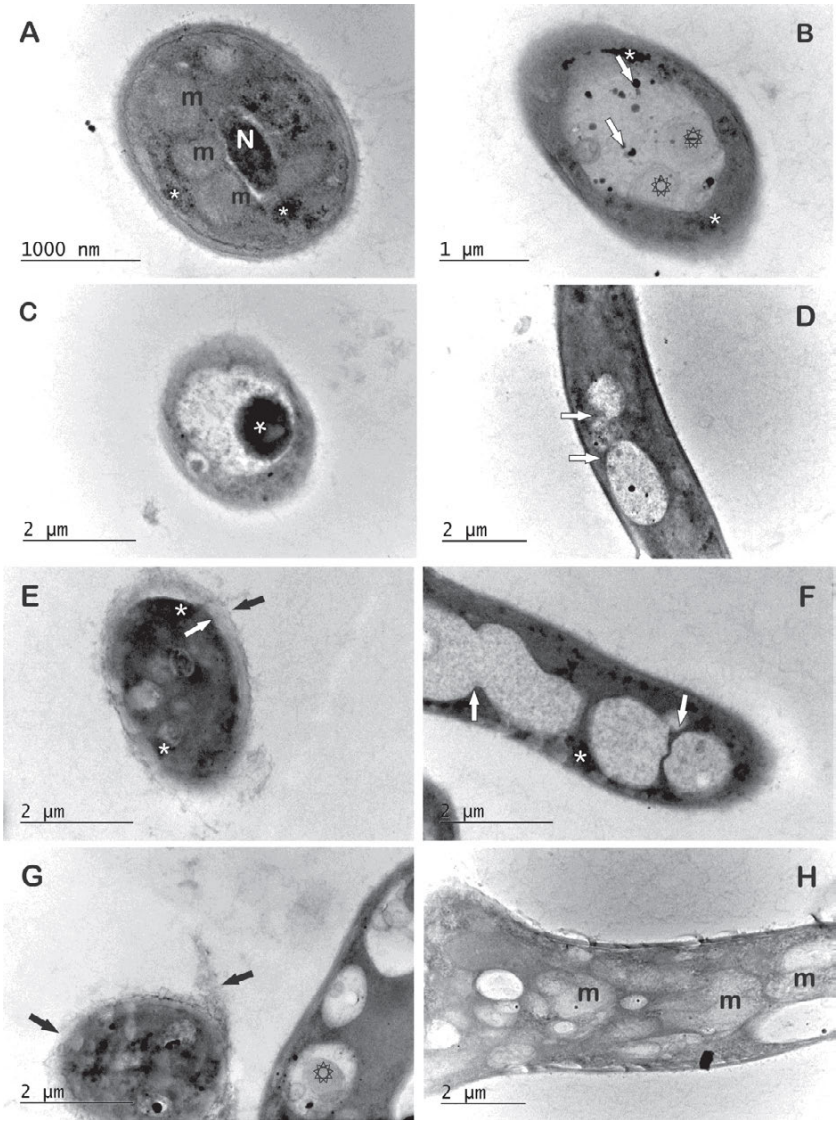

Fig. 8. A–D. Ultrastructural characteristics of the strain maintained at the optimal temperature of 25°C. **A**, Cross section through a hypha at the level of the nucleus (N), mitochondria (m); dark areas in cytoplasm or vacuoles – melanin-like accumulations (asterisks). **B**, Cross-section at the level of a vacuole. Inside the vacuole, electron-dense inclusions (arrows) and helical membrane accumulations (asters) are present. **C**, Crosssection at the level of the vacuole containing accumulated electron-dense material (asterisk). **D**, Longitudinal section showing fusion of smaller vesicles to vacuoles (arrows). **E–H**, Ultrastructural alterations after 6 h cold stress at 15°C: **E**, Cross section through a cold-stressed hypha showing thickening of the cell wall (arrows); dark areas in cytoplasm - melanin-like accumulation (asterisks). **F**, Extensive fusions between vacuoles (arrows); dark areas in cytoplasm - melanin-like accumulation (asterisks). **G**, Destruction of the cell wall (arrows). Inside a vacuole, helical membrane accummulations (asters) are observed. **H**, Longitudinal section showing disintegrated mitochondria (m).

The cold stress at 6°C resulted in a much higher incidence of cellular alterations of the strain. Even in apparently unaltered hyphae, the melanin-like substance in cytoplasm and vacuoles was apparently more than in the other two samples (Fig. 9A,B). The predominant part of the hyphae was with significantly increased electron density throughout the cytoplasm (Fig. 9C–H). This could likely be the result of overall accumulation of melanin-like material. There was deterioration of the mitochondrial structure (Fig. 9C–H), and altered organelles were seen to fuse with the vacuoles (Fig. 10C). Cell wall disintegration proceeded as initial occurrence of filaments protruding outwards (Fig. 9C,D) to detachment of some cell wall material (Fig. 9G) and complete focal lack of cell wall (Fig. 9H). There was also ultrastructural evidence of alterations of the turnover of the plasma membrane with frequent occurrence of deep plasmalemma infolds (Fig. 9 E,F), focally protruding deep into the cytoplasm up to the centre of the hyphae (Fig. 9G).

Discussion

In recent years there has been a growing interest in studying fungal cellular response to temperature stress, but the response to a downshift in growth temperature and its relation to oxidative stress needs clarification. This study was undertaken primarily to examine the effect of short-term cold exposure (from 25 to 6 or 15°C) on the level of oxidative stress in psychrotolerant fungal strain of *P. griseofulvum* isolated from Antarctica. The main findings of the present study confirmed the relationship between cold exposure and oxidative stress. The results revealed that the temperature downshift induced an oxidative cell response, indicated by significant changes in: (i) oxidative stress biomarkers and antioxidant enzyme activity; (ii) cell-growth abilities and glucose-consumption characteristics; and (iii) ultrastructural morphology.

Firstly, the temperature downshift induced oxidative stress events in the strain *P. griseofulvum*. The present results demonstrated a remarkable increase in protein carbonyl levels of cells exposed to 6 and 15°C, respectively. Protein carbonyls are thought to be an excellent marker for protein oxidation (Davies and Goldberg 1987). This is a typical response to oxidative stress caused by increased ROS generation followed by temperature stress (Li *et al.* 2008). Oxidative modification of critical residues in carbonylated proteins causes unfolding of proteins that tend to aggregate, and selective degradation in cells (Lévy *et al.* 2019). A similar response to low temperature was observed in cold-adapted fungi and yeast (Hu *et al*. 2016; Liu *et al*. 2017; Bakar *et al.* 2020). Generally, increase in protein carbonyls was reported after enhanced ROS level, for example, hyperoxygenation, heavy metal treatment, addition of oxidant species, and temperature exposure (Fedorova *et al.* 2014). Data for a positive correlation between elevated ROS levels and damaged proteins have been reported for different microbial cells

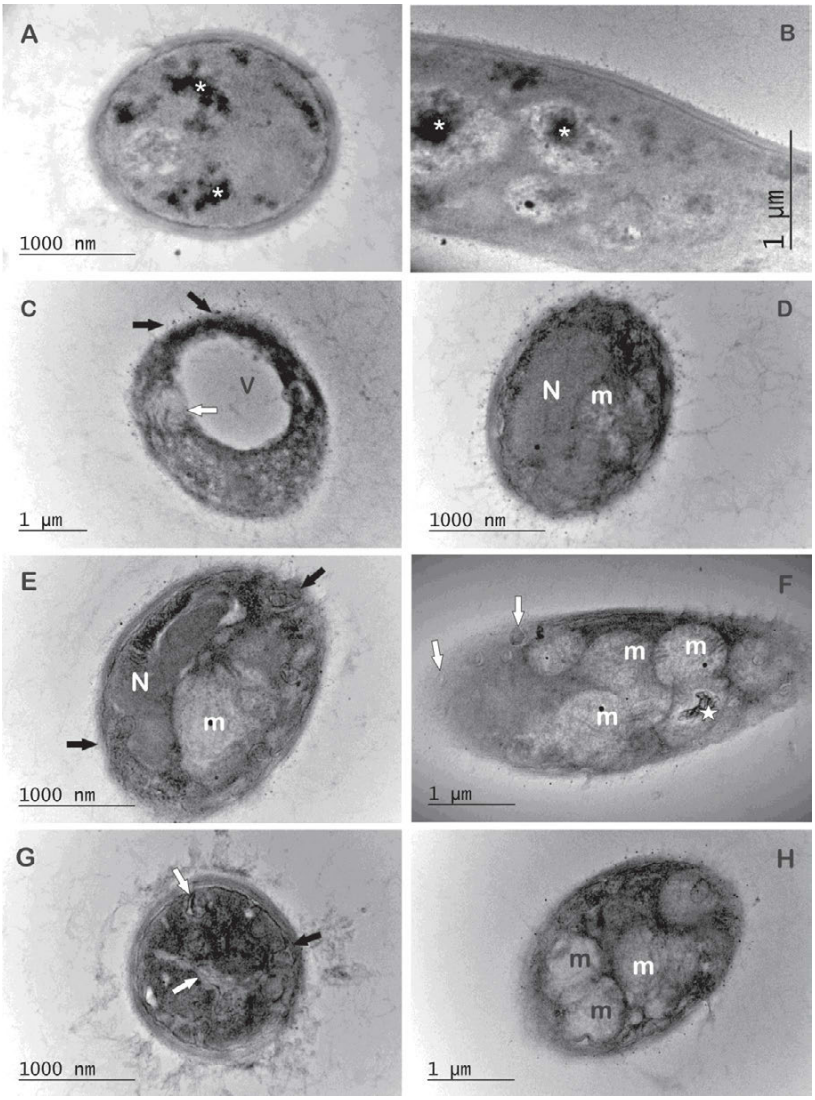

Fig. 9. Ultrastructural alterations of hyphae subjected to 6 h stress at 6°C. **A, B**, Crosssection and longitudinal section through comparatively unaltered hyphae, respectively. Cytoplasmic (**A**) and vacuolar (**B**) accumulation of electron-dense, melanin-like material (asterisks). **C**, Spot-like electron dense material located over filamentous protrusions of the cell wall (black arrows). White arrow points to a fusion between a destructed mitochondrion (m) and a vacuole (V). **D**, Cross section at the level of the nucleus (N). Destruction of the cell wall with formation of protruding filaments containing electrondense dot-like dense bodies. Mitochondrion (m) with altered ultrastructure. **E**, Crosssection showing nucleus (N), disintegrated mitochondrion (m) and foci of formation of deep plasmalemma infolds (arrows). **F**, Deep infolds of the plasma membrane (arrows), altered mitochondria (m). Aster indicates a completely disintegrated mitochondrion. **G**, Cross section through an area whith cell wall disintegration and filamentation. Arrows point to plasmalemma infolds going inwards as deep as the cell centrum. **H, A**, locus with completely lacking cell wall, altered mitochondria.

(Nyström 2005; Li *et al*. 2009). Our previous research also demonstrated enhanced ROS levels in Antarctic fungal cells under low-temperature treatment (Miteva-Staleva *et al*. 2011; Kostadinova *et al*. 2012).

Glycogen and trehalose contents in aerobic cells after exposure to unfavourable growth conditions could also be used as stress indicators (Shi *et al*. 2010; Cherkas *et al*. 2020). They play a different physiological role in the cell: trehalose is a major stress protector and helps chaperones to control the denaturation and renaturation of proteins while glycogen is a reserve carbohydrate. Here, our data showed a significant change in the levels of both the reserve carbohydrates as a function of cold stress duration. The trehalose content kept unchanged at the first 4 h compared to the control (0 h). However, its content increased rapidly and continuously with the incubation time. A similar trehalose behaviour was reported for basidiomycete fungi *Volvariella volvacea* (Bull.) Singer (Zhao *et al*. 2018) and *Hebeloma* spp. (Tibbett *et al*. 2002), and bakers' yeasts (Hirasawa *et al.* 2001) subjected to low temperature treatment. Fungal cells probably respond to cold stress by activating enzymes of the trehalose metabolism with an overall effect of trehalose consumption (Sahara *et al.* 2002; Voit 2003). This may be due to the fact that trehalose is re-metabolized into amino sugars or other polyols requiring its enhanced enzymatic breakdown (Tibbett *et al*. 2002). The subsequent accumulation of trehalose could be a compensatory mechanism for maintaining energetic homeostasis for better survival.

In contrast, there was glycogen accumulation in response to cold after 6 h of treatment. Moreover, glycogen accumulation seems to be of primary importance for viability at 6°C. Such cold stress response was demonstrated by other fungi and yeasts (Aguilera *et al*. 2007; Gocheva *et al*. 2009; Kostadinova *et al*. 2017). Some studies reported data linking glycogen to microbial viability, suggesting that it is involved in the stress response (Perez-Torrado *et al.* 2002). One possible explanation could be related to the induction of genes involved in glycogen synthesis, which continues over time, even after restoration of the optimal temperature (Aguilera *et al*. 2007).

Taken together, the above results showed clear signs of increased oxidative stress in *P. griseofulvum* cells treated with low temperatures. As a consequence, activation of the antioxidant enzyme defence was observed with the fungal cells responding with strong antioxidant protection to scavenge ROS.

A similar increase in the levels of antioxidant enzymes under low temperature conditions has been reported by other authors for various pro- and eukaryotic organisms (Park *et al.* 1998; Smirnova *et al*. 2001; Suzuki and Mittler 2006). Our previous results proved that Antarctic fungi also incorporate antioxidant protection into their cellular response at low growth temperatures (Gocheva *et al*. 2006, 2009; Tosi *et al.* 2010; Krumova *et al.* 2012a).

Secondly, rapid inhibition of mycelium growth and glucose consumption were found. The *P. griseofulvum* cells responded to temperature downshifts by

a lag period (2 h) before growth resumed at a rate characteristic of the new temperature. This response was temperature-dependent; the damage induced by the lower temperature stress was more pronounced than the higher one. Similar biomass reduction was reported for bacterial and fungal cells treated with cold or heat shock (Nevarez *et al*. 2010; Feng *et al*. 2018). Yamanaka *et al*. (1997) found a drastic reduction in OD_{650} of *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 cells (50 to 70%) after temperature downshift from 37 to 0°C. The authors suggested a so-called cold shock-induced autolysis associated with the activation of genes encoding peptidoglycan hydrolase synthesis. According to Piñas *et al*. (2008), stress-induced autolysis is one of the most evident mechanisms of programmed cell death. It should be noted that the temperature downshift resulted in a temporary growth retardation of the psychrotolerant *P. griseofulvum*. The growth resumed due to the stress resistance mechanisms of cold adapted microorganisms, including modification of their energy metabolic pathways, increase in the production of chaperones to minimize misfolded protein production, and regulation of the aminoacid synthesis (Bakar *et al.* 2020).

Thirdly, the ultrastructural data, against the background of the biochemical results, showed a different pattern of cell pathology provoked by the two stress temperatures. At 15°C cold stress, there was ultrastructural evidence of presence of both comparatively unaltered and focally altered hyphae. The organelles most sensitive to the temperature shift were the mitochondria. This is not an unusual phenomenon but likely a more general stress response, and it has earlier been registered in fungi subjected to heat stress (Abrashev *et al.* 2014) or to the action of toxic or antifungal substances (Krumova *et al.* 2012b; Wang *et al.* 2018). The increase in cell wall thickness observed in some hyphae was similar to that reported in experiments with the application of heat stress on fungi (Abrashev *et al.* 2014; Ikezaki *et al.* 2019). This can also be considered a more general stress response, most likely of an adaptive nature, aiming to reinforce and protect the cells subjected to stress. At that temperature, an apparent increase in the volume of vacuoles in comparison with the 25°C control was registered, accompanied by some intensification of the fusion processes between individual vacuoles. One of the important functions of the fungal vacuole is the sequestration and processing of damaged cellular components (Klionsky *et al.* 1990; Klionsky and Eskelinen 2014; Ren *et al.* 2017; Aufschnaitera and Büttnera 2019), by analogy with the lysosomes of higher eukaryotes. Vacuoles may use both altered cell organelles like damaged mitochondria, and altered molecules as substrata for lysosomal-like digestion. Thus, the increase in carbonylated protein content after 6 h at 15°C observed in this study well agrees with the increased vacuolar volume and, probably, activity. The suggestion about the adaptive role of these processes was also supported by the biochemical data showing a comparatively rapid normalization of the amount of carbonylated proteins in the absence of stress, four to six hours after restoration of the optimal growth temperature for the samples subjected to 15°C stress (see Fig. 3). All in all, the present experimental

data showed the 15°C low-temperature stress as responsible of alterations that were reversible and predominantly adaptive in their character.

In contrast, the 6 h stress at 6°C was the cause of serious damage to the hyphal cells. A deterioration of the mitochondria was recorded overall in the materials examined by TEM. While damaged mitochondria were observed to fuse with the vacuoles, the vacuolar system did not seem enlarged as in the 15°C-stressed hyphae. Moreover, the biochemical data for the amount of carbonylated proteins (Fig. 3) showed no diminution in the amount of the damaged molecules in the absence of the stress factor within the followed 12 h interval. These data imply that this stress temperature caused pathomorphological processes that were unlikely to be reversible, at least within short-term intervals. On the other hand, the significantly increased accumulation of melanin-like material by the fungus at this temperature should be taken into account. Melanin is considered to be involved in the protection of fungi against environmental stress (Eisenman *et al.* 2020; Smith and Casadevall 2019). Therefore, its increase in the 6°C-stressed hyphae might be evidence of the compensatory efforts of *P. griseofulvum.*

In conclusion, short-term temperature downshift is clearly able to induce oxidative stress in fungal cells, which is a signal for an enhanced level of oxidative damaged proteins, accumulation of reserve carbohydrates and increased activity of the antioxidant enzyme defence. The significant changes in growth, glucose consumption, and ultrastructural morphology found in this study suggest that different sets of adaptive responses may be involved in an attempt by the fungus to survive the cold environmental stress caused by the two different sub-optimal temperatures.

Acknowledgements. — This work was supported by the Bulgarian National Science Fund (DN-01/1-2016) to which we owe our sincere thanks. We thank the anonymous reviewers whose comments helped improve and clarify this manuscript.

References

Abrashev R., Stoitsova S., Krumova E., Pashova S., Paunova-Krasteva T., Vassilev S., Dolashka-Angelova P. and Angelova M. 2014. Temperature-stress tolerance of the fungal strain *Aspergillus niger* 26: physiological and ultrastructural changes. *World Journal of Microbiology and Biotechnology* 30: 1661–1668. doi: 10.1007/s11274-013-1586-8

Adachi H. and Ishii N. 2000. Effects of tocotrienols on life span and protein carbonylation in *Caenorhabditis elegans*. *Journals of Gerontology Series A, Biological Sciences and Medical Sciences* 55: B280–B285. doi: 10.1093/gerona/55.6.b280

Aguilera J., Randez-Gil F. and Prieto J. 2007. Cold response in *Saccharomyces cerevisiae*: new functions for old mechanisms. *FEMS Microbiology Reviews* 31: 327–341. doi: 10.1111/j.1574-6976.2007.00066.x

Angelova M., Genova L., Slokoska L. and Pashova S. 1995. Effect of glucose on the superoxide dismutase production in fungal strain *Humicola lutea*. *Canadian Journal of Microbiology* 41: 978–983. doi: 10.1139/m95-136

Aufschnaitera A. and Büttnera S. 2019. The vacuolar shapes of ageing: From function to morphology. *BBA - Molecular Cell Research* 1866: 957–970. doi: 10.1016/j.bbamcr.2019.02.011

Bakar N.A., Karsani S.A. and Alias S.A. 2020. Fungal survival under temperature stress: a proteomic perspective. *PeerJ* 8: e10423. doi: 10.7717/peerj.10423

Beauchamp C. and Fridovich I. 1971. Superoxide dismutase: Improved assay and an assay applicable to acrylamide gels. *Analytical Biochemistry* 44: 276–287. doi: 10.1016/0003-2697(71)90370-8

Becker J. 1978. A method of glycogen determination in whole yeast cells. *Analytical Biochemistry* 86: 56–64. doi: 10.1016/0003-2697(78)90318-4

Beers R.F. and Sizer I.W. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry* 195: 133–140. doi: 10.1016/S0021-9258(19)50881-X

Cherkas A., Holota S., Mdzinarashvili T., Gabbianelli R. and Zarkovic N. 2020. Glucose as a major antioxidant: when, what for and why it fails? *Antioxidants (Basel)* 9: 140. doi: 10.3390/antiox9020140. doi: 10.3390/antiox9020140

Cowan D.A., Makhalanyane T.P., Dennis P.G. and Hopkins D.W. 2014. Microbial ecology and biogeochemistry of continental Antarctic soils. *Frontiers in Microbiology* 5: 154. doi: 10.3389/fmicb.2014.00154

Davies, K.J.A. and Goldberg, A.L. 1987. Proteins damaged by oxygen radicals are rapidly degraded in extracts of red blood cells. *Journal of Biological Chemistry* 262: 8227–8234. doi: 10.1016/S0021-9258(18)47553-9

Durán P., Barra P. J., Jorquera M. A., Viscardi S., Fernandez C., Paz C., Mora M. de la Luz. and Bol R. 2019. Occurrence of soil fungi in Antarctic pristine environments. *Frontiers in Bioengineering and Biotechnology* 7: 28. doi: 10.3389/fbioe.2019.00028

Eisenman H.C., Greer E.M. and McGrail C.W. 2020. The role of melanins in melanotic fungi for pathogenesis and environmental survival. *Applied Microbiology and Biotechnology* 104: 4247–4257. doi: 10.1007/s00253-020-10532-z

Fedorova M., Bollineni R. and Hoffmann R. 2014. Protein carbonylation as a major hallmark of oxidative damage: Update of analytical strategies. *Mass Spectrometry Reviews* 33: 79–97. doi: 10.1002/mas.21381

Feng J., Ma L., Nie J., Konkel M. and Lu X. 2018. Environmental stress-induced bacterial lysis and extracellular DNA release contribute to *Campylobacter jejuni* biofilm formation. *Applied and Environmental Microbiology* 84: e02068-17. doi: 10.1128/AEM.02068-17

Franzen A.J., Cunha M.M.L, Miranda K., Hentschel J., Plattner H., da Silva M.B., Salgado C.G., de Souza W. and Rozental S. 2008. Ultrastructural characterization of melanosomes of the human pathogenic fungus *Fonsecaea pedrosoi*. *Journal of Structural Biology* 162: 75–84. doi: 10.1016/j.jsb.2007.11.004

Gocheva Y., Krumova E., Slokoska L., Miteva J. and Angelova M. 2006. Cell response of Antarctic and temperate strains of *Penicillium* spp. to different growth temperature. *Mycological Research* 110: 1347–1354. doi: 10.1016/j.mycres.2006.08.007

Gocheva Y., Tosi S., Krumova E., Slokoska L., Miteva J., Vassilev S. and Angelova M. 2009. Temperature downshift induces antioxidant response in fungi isolated from Antarctica. *Extremophiles* 13: 273–281. doi: 10.1007/s00792-008-0215-1

Godinho V., Goncalves V., Santiago I., Figueredo H., Vitoreli G., Schaefer C., Barbosa E., Oliveira J., Alves T., Zani C., Junior P., Murta S., Romanha A., Kroon E., Cantrell C., Wedge D., Duke S., Ali A., Rosa C. and Rosa L. 2015. Diversity and bioprospecting of fungal communities present in oligotrophic soil of continental Antarctica. *Extremophiles* 19: 585–596. doi: 10.1007/s00792-015-0741-6

Godinho V., Furbino L., Santiago I., Pellizzari F., Yokoya N., Pupo D., Alves T., Junior P., Romanha A., Zani C., Cantrell C., Rosa C. and Rosa L.H. 2013. Diversity and bioprospecting

of fungal communities associated with endemic and cold-adapted macroalgae in Antarctica. *ISME Journal* 7: 1434–1451. doi: 10.1038/ismej.2013.77

Hart R.G., Benavente O., McBride R. and Pearce L. 1999. Antithrombotic therapy to prevent stroke in patients with atrial fibrillation: a meta-analysis. *Annals of Internal Medicine* 131: 492–501. doi: 10.7326/0003-4819-131-7-199910050-00003

Hirasawa R., Yokoigawa K., Isobe Y. and Kawai H. 2001. Improving the freeze tolerance of bakers' yeast by loading with trehalose. *Bioscience, Biotechnology & Biochemistry* 65: 522–526. doi: 10.1271/bbb.65.522

Hu B., Luo M., Ji X., Lin L., Wei Y. and Zhang Q. 2016. Proteomic analysis of *Mortierella isabellina* M6-22 during cold stress. *Archives of Microbiology* 198: 869–876. doi: 10.1007/s00203-016-1238-0

Ikezaki S., Cho T., Nagao J.I., Tasaki S., Yamaguchi M., Arita-Morioka K.I., Yasumatsu K., Chibana H., Ikebe T. and Tanaka Y. 2019. Mild heat stress affects on the cell wall structure in *Candida albicans* biofilm. *Medical Mycology* 60: 29–37. doi: 10.3314/mmj.19-00001

Klionsky D. and Eskelinen E. 2014. The vacuole vs. the lysosome: When size matters. *Autophagy* 10: 185–187. doi: 10.4161/auto.27367

Klionsky D., Herman P. and Emr S. 1990. The fungal vacuole: composition, function, and biogenesis. *Microbiological Reviews* 54: 266–292. doi: 10.1128/mr.54.3.266-292.1990

Kostadinova N., Krumova E., Stefanova T., Dishliyska V. and Angelova M. 2012. Transient cold shock induces oxidative stress events in Antarctic fungi. *In*: Lushchak V. (ed.) *Oxidative Stress*. Book 1. InTech, Rijeca: 75–99.

Kostadinova N., Tosi S., Spassova B. and Angelova M. 2017. Comparison of the oxidative stress response of two Antarctic fungi to different growth temperatures. *Polish Polar Research* 38: 393–408. doi: 10.1515/popore-2017-0015

Krishnan A., Alias S., Wong C.M. V.L., Pang K.L. and Convey P. 2011. Extracellular hydrolase enzyme production by soil fungi from King George Island, Antarctica. *Polar Biology* 34: 1535–1542. doi: 10.1007/s00300-011-1012-3

Krumova E., Abrashev R., Kostadinova N., Dishlijska V., Miteva-Staleva J., Pashova S., Vasilev S., Spasova B., Stephanova L. and Angelova M. 2012a. Novel cold-active antioxidant enzymes from Antarctic fungi. *In:* Najdenski H., Angelova M. and Stoitsova S. (eds.) *New trends in microbiology* (*65-th Anniversary of the Stephan Angeloff Institute of Microbiology*). The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences: 291–306.

Krumova E., Stoitsova S., Paunova-Krasteva T., Pashova S. and Angelova M. 2012b. Copper stress and filamentous fungus *Humicola lutea* 103 – ultrastructural changes and activities of key metabolic enzymes. *Canadian Journal of Microbiology* 58: 1335–1343. doi: 10.1139/w2012-112

Lévy E., El Banna N., Baïlle D., Heneman-Masurel A., Truchet S., Rezaei H., Huang M-E., Béringue V., Martin D. and Vernis L. 2019. Causative links between protein aggregation and oxidative stress: A review *International Journal of Molecular Sciences* 20: 3896. doi: 10.3390/ijms20163896

Li Q., Harvey L. M. and McNeil B. 2008. The effects of elevated process temperature on the protein carbonyls in the filamentous fungus, *Aspergillus niger* B1–D. *Process Biochemistry* 43: 877–881. doi: 10.1016/j.procbio.2008.04.006

Li L., Ye Y.R., Pan L., Zhu Y., Zheng S.P. and Lin Y. 2009. The induction of trehalose and glycerol in *Saccharomyces cerevisiae* in response to various stresses. *Biochemical and Biophysicals Research Communication* 387: 778–783. doi: 10.1016/j.bbrc.2009.07.113

Liu D., Wei L., Guo T. and Tan W. 2014. Detection of DOPA-melanin in the dimorphic fungal pathogen *Penicillium marneffei* and its effect on macrophage phagocytosis in vitro. *PLoS ONE* 9: e92610. doi: 10.1371/journal.pone.0092610

Liu J-Y., Men J-L., Chang M-C., Feng C-P. and Yuan L-G. 2017. iTRAQ-based quantitative proteome revealed metabolic changes of *Flammulina velutipes* mycelia in response to cold stress. *Journal of Proteomics* 156: 75–84. doi: 10.1016/j.jprot.2017.01.009

Lowry O.H., Rosenbrough H.J., Faar A.L. and Randall R.J. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193: 265–275. doi: 10.1016/S0021-9258(19)52451-6

Miteva-Staleva J., Stefanova Ts., Krumova E. and Angelova M. 2011. Growth-phase-related changes in reactive oxygen species generation as a cold stress response in Antarctic *Penicillium* strains. *Biotechnology and Biotechnological Equipment (sup1)*: 58–63.

Nelson N. 1944. Photometric adaptation of the Somogyi method for determination of glucose. *Journal of Biological Chemistry* 153: 375–380. doi: 10.1016/S0021-9258(18)71980-7

Nevarez L., Vasseur V., Debaets S. and Barbier G. 2010. Use of response surface methodology to optimise environmental stress conditions on *Penicillium glabrum*, a food spoilage mould. *Fungal Biology* 114: 490–497. doi: 10.1016/j.funbio.2010.03.011

Nyström T. 2005. Role of oxidative carbonylation in protein quality control and senescence. *The EMBO Journal* 24: 1311–1317. doi: 10.1038/sj.emboj.7600599

Park J., Grant C.M., Davies M.J. and Dawes I.W. 1998. The cytoplasmic Cu, Zn superoxide dismutase of *Saccharomyces cerevisiae* is required for resistance to freeze-thaw stress: generation of free radicals during freezing and thawing. *Journal of Biological Chemistry* 273: 22921–22928. doi: 10.1074/jbc.273.36.22921

Parrou J.L., Teste M.A. and Francois J. 1997. Effects of various types of stress on the metabolism of reserve carbohydrates in *Saccharomyces cerevisiae*: genetic evidence for a stress-induced recycling of glycogen and trehalose. *Microbiology* 143: 1891–1900. doi: 10.1099/00221287-143-6-1891

Pérez-Torrado R., Gimeno-Alcan˜iz J.V. and Matallana E. 2002. Wine yeast strains engineered for glycogen overproduction display enhanced viability under glucose deprivation conditions. *Applied and Environmental Microbiology* 68: 3339–3344. doi: 10.1128/AEM.68.7.3339-3344.2002

Piñas G.E., Cortes P.R., Orio A.G. and Echenique J. 2008. Acidic stress induces autolysis by a CSP-independent ComE pathway in *Streptococcus pneumoniae*. *Microbiology* 154: 1300–1308. doi: 10.1099/mic.0.2007/015925-0

Purves K., Macintyre L., Brennan D., Hreggviðsson G., Kuttner E., Ásgeirsdóttir M., Young L.C., Green D.H., Edrada-Ebel R. and Duncan K. 2016. Using molecular networking for microbial secondary metabolite bioprospecting. *Metabolites* 6: 2. doi: 10.3390/metabo6010002

Ren W., Zhang Z., Shao Z., Yang Y., Zhou M. and Chen C. 2017. The autophagy-related gene BcATG1 is involved in fungal development and pathogenesis in *Botrytis cinerea*. *Molecular Plant Pathology* 18: 238–248. doi: 10.1111/mpp.12396

Robinson C.H. 2001. Cold adaptation in Arctic and Antarctic fungi. *New Phytologist* 151: 341–353. doi: 10.1046/j.1469-8137.2001.00177.x

Sahara T., Goda T. and Ohgiya S. 2002. Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. *Journal of Biological Chemistry 277*: 50015–50021. doi: 10.1074/jbc.M209258200

Selbmann L., Zucconi L., Isola D. and Onofri S. 2015. Rock black fungi: excellence in the extremes, from the Antarctic to space. *Current Genetics* 61: 335–345. doi: 10.1007/s00294-014-0457-7

Shi L., Sutter B., Ye X. and Tu B. 2010. Trehalose is a key determinant of the quiescent metabolic state that fuels cell cycle progression upon return to growth. *Molecular Biology of the Cell* 21: 1982–1990. doi: doi: 10.1091/mbc.e10-01-0056

Smirnova G., Zakirova O. and Oktiabr'skiĭ O. 2001. Role of the antioxidant system in response of *Escherichia coli* bacteria to cold stress. *Mikrobiologiia* 70: 55–60 [in Russian].

Smith D.F.Q. and Casadevall A. 2019. The role of melanin in fungal pathogenesis for animal hosts. *In:* Rodrigues M. (ed.) *Fungal Physiology and Immunopathogenesis*. Current Topics in Microbiology and Immunology 422, Springer: 1–30.

Somogy M. 1952. Notes on sugar determination. *Journal of Biological Chemistry* 195: 19–23. doi: 10.1016/S0021-9258(19)50870-5

Suzuki N. and Mittler R. 2006. Reactive oxygen species and temperature stresses: A delicate balance between signaling and destruction. *Physiologia Plantarum* 126: 45–51. doi: 10.1111/j.0031-9317.2005.00582.x

Teixeira L.C., Yeargeau E., Balieiro F.C., Piccolo M.C., Peixoto R.S., Greer C.W. and Rosado A.S. 2013. Plant and bird presence strongly influences the microbial communities in soils of Admiralty Bay, Maritime Antarctica. *PLoS ONE* 8: e66109. doi: 10.1371/journal.pone.0066109

Tibbett M., Sanders F.E. and Cairney J.W.G. 2002. Low-temperature-induced changes in trehalose, mannitol and arabitolassociated with enhanced tolerance to freezing in ectomycorrhizal basidiomycetes (*Hebeloma* spp.). *Mycorrhiza* 12: 249–255. doi: 10.1007/s00572-002-0183-8

Tosi S., Kostadinova N., Krumova E., Pashova S., Dishliiska V., Spassova B., Vassilev S. and Angelova M. 2010. Antioxidant enzyme activity of filamentous fungi isolated from Livingston Island, Maritime Antarctica. *Polar Biology* 33: 1227–1237. doi: 10.1007/s00300-010-0812-1

Vandercammen A., François J. and Hers H. 1989. Characterization of trehalose-6-phosphate-synthase and trehalose-6-phosphate phosphatase of *Saccharomyces cerevisiae*. *European Journal of Biochemistry* 182: 613–620. doi: 10.1111/j.1432-1033.1989.tb14870.x

Voit E.O. 2003. Biochemical and genomic regulation of the trehalose cycle in yeast: review of observations and canonical model analysis. *Journal of Theoretical Biology* 223: 55–78. doi: 10.1016/s0022-5193(03)00072-9

Wang L., Jin J., Liu X., Wang Y., Liu Y., Zhao Y. and Xing F. 2018. Effect of cinnamaldehyde on morphological alterations of *Aspergillus ochraceus* and expression of key genes involved in ochratoxin A biosynthesis. *Toxins* 10: 340. doi: 10.3390/toxins10090340

Yamanaka K., Araki J., Takano M. and Sekiguchi J. 1997. Characterization of *Bacillus subtilis* mutants resistant to cold shock-induced autolysis. *FEMS Microbiology Letters* 150: 269–275. doi: 10.1111/j.1574-6968.1997.tb10380.x

Zhao X., Song X., Li Y., Yu C., Zhao Y., Gong M., Shen X. and Chen M. 2018. Gene expression related to trehalose metabolism and its effect on *Volvariella volvacea* under low temperature stress. *Scientific Reports* 8: 1–14. doi: 10.1038/s41598-018-29116-z

Received 18 June 2021
Accepted 31 August 2021