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Age-related changes in protease activity as cold stress response by *Penicillium* strains from different temperature classes

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Abstract: Proteases play a key role in cell defense mechanisms to cold-induced oxidative stress. Data on the relationship between cold stress, growth phase, and temperature preferences of the fungal strains isolated from different habitats are very scarce. Here, we report changes in the intra- and extracellular protease activity of three fungal *Penicillium* strains (two Antarctic and one temperate) under transient temperature downshift during exponential- and stationary growth phases. The results indicated enhanced enzyme levels in both growth phases depending on the degree of stress and strain thermal class. In order to explain the obtained data, we compared them with our previous results on the protein carbonyl content, accumulation of oxidative-stress biomarkers, and antioxidant enzyme defense in the same three fungal strains. The cell response was affected by the temperature preference of the strain, but not by the climatic distance between the locations of isolation.

Keywords: Antarctic, filamentous fungi, low-temperature stress, growth phase, secondary antioxidant defense.

Introduction

Normal cell function in aerobic conditions naturally results in reactive oxygen species (ROS) production. They have a role in the normal cellular functions maintenance. However, overproduction of ROS is associated with causing severe injury (Miteva-Staleva *et al*. 2017). Cells can tolerate a certain level of ROS thanks to a variety of defense mechanisms such as antioxidant molecules (glutathione), antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase), and repair enzymes (McCord and Fridovich 1969; Mythri *et al.* 2013). Many endogenous and exogenous sources can induce excessive radical generation leading to oxidative stress. As a result, an increased level of oxidants and reduced production of antioxidants can be observed. ROS overproduction causes damage to all intracellular biomolecules as proteins, lipids, nucleic acids, *etc.*, and thus affects many cellular functions: lipid peroxidation of the membrane, intracellular oxidative modification of proteins, and oxidative DNA damage (Su *et al*. 2019; Warraich *et al*. 2020; Juan *et al.* 2021).

Exposure to low temperatures is considered a cause of ROS accumulation and oxidative stress occurrence (Chattopadhyay *et al.* 2011). Cold environment induced oxidative stress in prokaryotic and eukaryotic organisms (Kostadinova *et al*. 2012; Miteva-Staleva *et al.* 2017; Sachdev *et al.* 2021; Seixas *et al.* 2022). There are also many studies where cellular biomolecules have been shown as a primary target of cold injury (Hasanuzzaman *et al.* 2013; Wei *et al.* 2022). Possible contributions of oxidative stress, including cold stress to aging are also discussed. According to *Free Radical Theory of Aging* of Harman (1995), ROS-induced oxidative damage can accelerate aging processes. Extensive research study the role of ROS in aging (Shields *et al.* 2021). Despite the contradictory results this concept of involving ROS in aging remains valid (Luo *et al*. 2020).

Level of protein carbonyls, lipid peroxidation, and DNA damage can be used to prove oxidative damage in cells. The cytotoxicity caused by ROS was reported to influence intracellular proteins by its oxidative modification (Mythri *et al*. 2013). The proteins' oxidative modifications obtained as a result of the oxidants' nature and the exposure level are reversible and irreversible (Yan 2014). For example, they can oxidize irreversible amino acid's chains, methionine residues and thiol-containing cysteine, resulting in structural changes to the proteins that may lead to its dysfunction (Shields *et al*. 2021). The formation of carbonyl derivatives results from the oxidation of the amino acid residues proline, lysine, arginine, and threonine. Carbonyl groups are formed in proteins after their reactions with aldehydes (4-hydroxy-2-nonenal, malondialdehyde), which are products of phospholipid peroxidation, and when interacting with ketamines, ketoaldehydes, deoxyozones (Berlett and Stadtman 1997). Reversible oxidation of protein cysteine residues leads to the S-sulfonation, S-nitrosylation, disulfides, and S-glutathionylation. A commonly used biomarker for protein oxidative damage is protein carbonylation.

Cells answer to damaged proteins with increased proteolytic activity to cope with their harmful effect (Flick and Kaiser 2012). Intracellular proteases are considered a "secondary antioxidant defense" because they effectively remove damaged proteins through intracellular degradation (Grune and Davies 1997). Proteases perform many functions in important processes such as modulation of protein-protein interactions, transduction, enhancement of molecular signals, localization, and activity of a variety of proteins. Thus, protein degradation is a biological process with great importance during stress conditions. Proteases have been also identified as hallmarks of aging (Rai *et al*. 2022). Controlled degradation of proteins holds a balance between protein biosynthesis and degradation, under both optimal and stress conditions. These processes are essential for the normal functioning of cells in all phases of development. Thus, cells obtain amino acids for *de novo* protein synthesis during recovery from stress (Kidrič *et al.* 2014). There are reports describing the role of proteases in aging organs in plants and animals (Lopez-Otin and Bond 2008; Van der Hoorn 2008). Fungi are a useful model organism for understanding cellular stress response since they are eukaryotes. The ensuing findings may be appropriate for the higher eukaryotes.

Recent studies have widely discussed the relationship between exposure to low temperatures and oxidative stress in plants and animals but fungi have not been well studied (Hasanuzzaman *et al*. 2013; Hao *et al*. 2022; Wei *et al*. 2023). Furthermore, cold stress responses in fungi isolated from extremely cold habitats are even rarer (Gocheva *et al*. 2006; Miteva-Staleva *et al*. 2011, 2015, 2017; Kostadinova *et al*. 2012). Very scarce information is available on the role of proteinase enzymes in cold stress response by both the young and senescent fungal strains isolated from different environmental conditions.

Our previous studies revealed that cold exposure to fungi isolated from Antarctica clearly induced oxidative stress events and activation of antioxidant defense in all strains tested (Gocheva *et al*. 2006, 2009; Kostadinova *et al*. 2012; Miteva-Staleva *et al*. 2011, 2015). The data obtained evidenced the disruption of the oxidant/antioxidant balance, increase in the content of carbonylated proteins, and accumulation of reserve carbohydrates. Despite the activation of antioxidant enzyme defense, oxidative damage was detected in the fungal cells. Comparison between the two exponential and stationary phase cultures showed a significantly higher level of oxidative damage in old cells. Furthermore, the exponential phase cells are more resistant to cold stress than the stationary phase cells.

This study integrates the picture of the above-mentioned results. It was focused on the protease activity changes in both exponential and stationary phases of two Antarctic (*Penicillium olsonii* p14, *Penicillium waksmanii* m12) and one temperate (*Penicillium rugulosum* t35) fungal strains cultivated under low-temperature stress conditions. As far as we know this is the first research on the participation of protease enzymes in the cellular response to cold stress in young and old cells of fungal strains, isolated from Antarctica.

Material and methods

Fungal strains. — The fungal strains included in the experiments belong to the Mycological collection of the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences. The psychrotolerant *P. olsonii* p14 and mesophilic *P. waksmanii* m12 were isolated from soil samples taken from Terra Nova Bay, Antarctica (74°41′S; 164°07′E), during an Italian expedition (2003-2004). The mesophilic *P. rugulosum* t35 was isolated from soil samples collected from the region of Kniazhevo, Bulgaria (temperate climate).

The Microbank system (Prolab Diagnostics, Richmond Hill, Canada) was used for long-term preservation of the fungal strains at −80°C. Before the experimental tests, colonies were grown on potato dextrose agar (PDA) for seven days at the corresponding optimal temperatures: 20°C for Antarctic psychrotolerant *P. olsonii* p14 and 30°C for Antarctic mesophilic *P. waksmanii* m12 and temperate mesophilic *P. rugulosum* t35 (Gocheva *et al*. 2006).

Cultivation. — Cultivation was performed in 3 L bioreactors (ABR-09) equipped with control systems for temperature, pH, and dissolved oxygen monitoring. The culture method and AN-3 medium ingredients are described by Gocheva *et al.* (2009). The optimal growth temperature for Antarctic psychrotolerant *P. olsonii* p14 is 20°C and 30°C for Antarctic mesophilic *P. waksmanii* m12 and temperate mesophilic *P. rugulosum* t35 (Gocheva *et al.* 2006). In our study, a temperature downshift from the corresponding optimal temperature to 6°C or 15°C was applied to the model strains. The temperature was reduced during the mid-exponential or stationary phase from 20°C and 30°C for the psychrotolerant and mesophilic strains, respectively, to 6°C and 15°C in approximately 40 min. After cold stress for six hours, the temperature is increased to the corresponding optimal value. Control variants were grown at the respective optimal temperatures.

Cell-free extract preparation. — The preparation of cell-free extract was previously described by Gocheva *et al.* (2009). Filtration, washing in distilled H_2O and then in cold 50 mM potassium buffer (pH 7.8) were applied to obtain biomass. The fungal cell wall disruption was done by the physical disruption (quartz sand) method. Chilling in an ice-salt bath was used for the maintenance of the temperature at 4–6°C during the treatment. The homogenate was centrifuged three times at 1500 x g for 15 min and then the resulting supernatant was further centrifuged at 15000 x g for 30 min. The obtained cell-free extract (liquid fraction) was used in the experiments.

Analytical methods. — Both intracellular and extracellular protease enzyme activity assays were determined by the method of Cupp-Enyard (2008) and Anson (1938) with modification. The formation of tyrosine during the hydrolysis of casein by protease is in the basis of this method. As one unit of protease activity is taken as 1 μmol of tyrosine obtained as a result of an enzymatic reaction per ml of reaction mixture per minute. For the extracellular protease assay, cells from the

respective cultures were collected by filtration (Whatman filter no. 4, Clifton, USA) by repeatedly washing the filter with distilled water and cold 50 mM potassium buffer (pH 7.8). The supernatant was used for enzyme activity assay. The intracellular protease was detected in the cell-free extract.

Statistical evaluation of the results. — All results presented in this study were obtained and evaluated from experiments with at least three replicate using three parallel runs. Statistical evaluation of results was performed by Student's t-test (t-test) for MIE (mean interval estimate), analysis of variance (ANOVA), and Dunnet's post-hoc test, with a significance level of 0.05.

Results

Changes in the intracellular protease activity under transient temperature downshift. — The changes in the intracellular protease activity of the fungal cells from both developmental phases (exponential and stationary) of the model strains during short-term temperature downshift are investigated (Fig. 1). Cultivation of the cells at the respective optimal temperature for six hours showed an almost constant level of enzyme activity (Fig. 1A–C). A slight increase was observed at the 10^{th} hour in the mesophilic strains *P. waksmanii* m12 and *P. rugulosum* t35. Compared with the control variants, enzyme activity was higher under cold treatment conditions. The increase began simultaneously with the start of the temperature stress. After exposure to 15°C for 6 h, protease activity was 10–20% higher in comparison with the control variant. This trend continued even after the low temperature treatment had stopped. No significant difference in the degree of increase in protease activity among the used model strains was observed.

The highest levels of protease activity were determined at 6°C in comparison to the variants at 15°C. Temperature downshift caused a more pronounced increase in the two mesophilic strains than in the psychrotolerant strain *P. olsonii* p14. While mesophilic strains demonstrated about 95% and 45% higher enzyme activity for *P. waksmanii* m12 and *P. rugulosum* t35, respectively, the psychrotolerant strain showed comparably lower sensitivity to cold temperatures (18%). It should be noted that the upward tendency continued even after the return to optimal temperature.

Transient temperature downshift to 15°C or 6°C affected protease levels in stationary-growth cells depending on the stress level and temperature preferences of the model strains (Fig. 1D–F). In the control groups of the mesophilic strains, *P. waksmanii* m12 and *P. rugulosum* t35, a lack of increase during the first 6 h of the experimental period was detected. In contrast, the psychrotolerant strain *P. olsonii* p14 showed a slightly enhanced proteinase activity immediately after the 2^{nd} hour of cultivation.

Exposure to 15°C caused a slow increase in *P. waksmanii* m12 and *P. rugulosum* t35. After exposure for 6 h, the activity increased by 50% and 54%

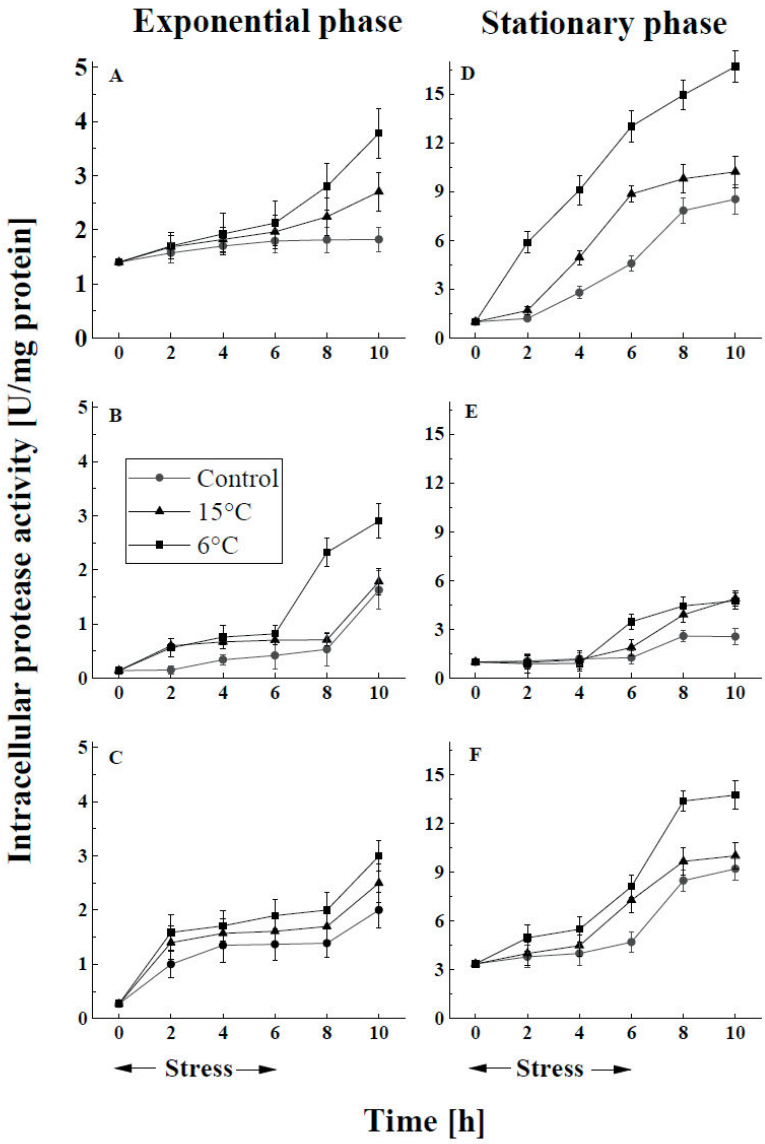

Fig. 1. Intracellular protease production in exponential (**A**, **B**, **C**) and stationary (**D**, **E**, **F**) phase cultures of *P. olsonii* p14 (**A**, **D**), *P. waksmanii* m12 (**B**, **E**) and *P. rugulosum* t35 (**C**, **F**) at optimal temperature (●) and during short-term temperature downshift from optimal to 6°C (■) or to 15°C (▲). Values are means of three replicates; bars represent the standard deviation. Temperature downshift turns out to have a statistically significant effect on the protease activity ($p<0.05$).

compared to the control, respectively. At the same time, the Antarctic strain *P. olsonii* p14 revealed a more rapid and more significant response (93%), compared with the mesophilic strains.

The highest protease activity was measured in the cultures exposed to 6°C compared to the control. Downshift from optimal temperature to 6°C caused an

immediate remarkable increase in activity of all tested strains by 73%, 175%, and 189% for *P. rugulosum* t35, *P. waksmanii* m12, and *P. olsonii* p14, respectively. This rising trend was also observed after the cessation of stress.

Changes in the extracellular protease activity under transient temperature downshift. — The effect of low-temperature stress on the extracellular protease activity of the three model strains was investigated (Fig. 2). Data from

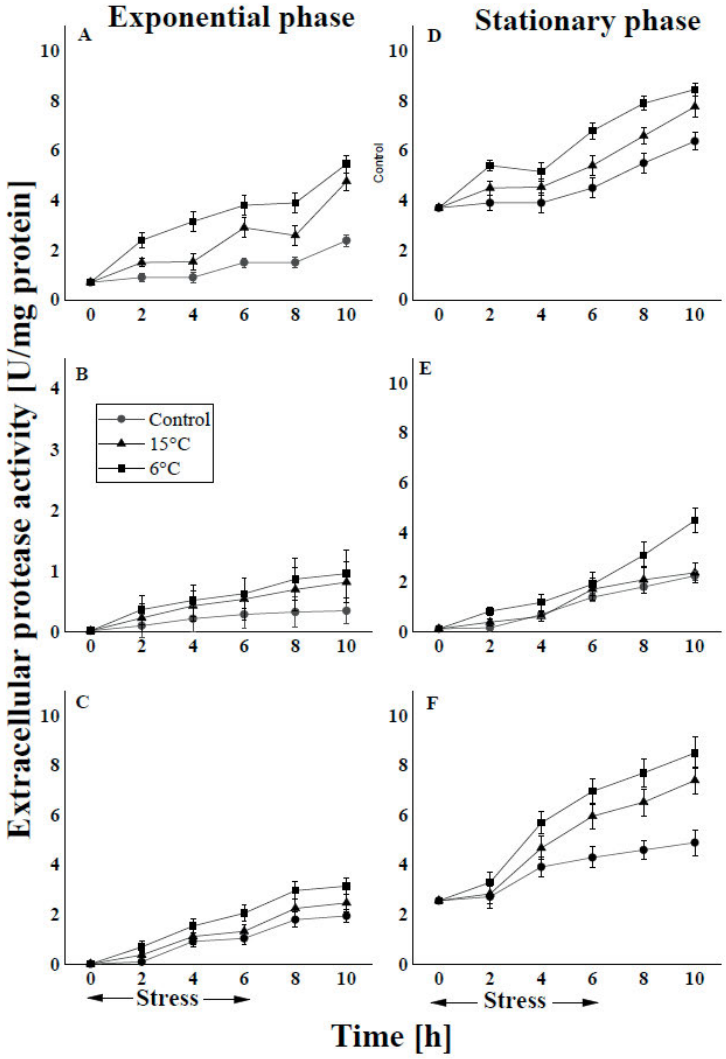

Fig. 2. Extracellular protease production in exponential (**A**, **B**, **C**) and stationary (**D**, **E**, **F**) phase cultures of *P. olsonii* p14 (**A**, **D**), *P. waksmanii* m12 (**B**, **E**) and *P. rugulosum* t35 (**C**, **F**) at corresponding optimal temperature (●) and during short-term temperature downshift from optimal to 6°C (■) or to 15°C (▲). Values are means of three replicates; bars represent the standard deviation. Temperature downshift turns out to have a statistically significant effect on the protease activity ($p<0.05$).

extracellular proteolytic activity in exponential phase (Fig. 2A–C) showed a similar trend to that of intracellular proteolytic activity in exponential phase (Fig. 1A–C). The protease activity of untreated cultures is relatively low. At the same time the temperature downshift to 15°C or 6°C caused a pronounced increase in protease activity compared with the control values. The changes in activity were in a temperature- and time-dependent manner until the end of the treatment. In addition, temperature downshift affected the proteolytic activity of the strain tested depending on their thermal classes. The most significant response was observed after exposure to 6°C in the psychrotolerant Antarctic strain *P. olsonii* p14, followed by mesophilic Antarctic strain *P. waksmanii* m12, and mesophilic temperate strain *P. rugulosum* t35, by 153, 117, and 96%, respectively, compared to the untreated variant.

The stationary growth-phase cultures demonstrated significantly higher initial proteolytic activity compared to the exponential cultures, which increases continuously throughout the experiment. Transient exposure to 6°C and 15°C resulted in elevated enzyme activity compared with the control variant, and the increase was in a temperature-dependent manner until the end of the treatment. A considerable increase occurred at the sixth hour of cultivation: 20 and 51% for *P. olsonii* p14, 22 and 37% for *P. waksmanii* m12, and 38 and 61% for *P. rugulosum* t35, compared with the control at 15°C and 6°C, respectively.

Discussion

In this study, we describe the changes in extra- and intracellular protease activity in three *Penicillium* strains belonging to the different thermal classes (one psychrotolerant and two mesophilic) and their role in the cell response against cold stress. The results revealed a dependence of the protease activity on the stress level, time of treatment, growth phase, and temperature preference of the strain.

The main finding is that the increase in the protease activity participates in the cell response to the (transient) temperature downshift. The extra- and intracellular protease activity steadily increased with increasing stress levels, and time of treatment. This trend was observed already in the first two hours of the onset of stress, followed by a linear increase in the following four hours, and continued even after the cessation of exposure. The induced protease synthesis demonstrated a pronounced dependence on the degree of cold stress. Temperature downshift to 6°C led to an enhanced protease level compared to the variants treated with 15°C. These results suggested that thermal stress promotes increased protein degradation. A drastic temperature downshift is known to cause accelerated ROS generation in many pro- and eukaryotic organisms, including fungi (Chu *et al*. 2016; Hao *et al*. 2022; Jiang *et al*. 2022; Joudmand *et al*. 2021). Our previous studies also evidenced the occurrence of oxidative stress in fungal cells after low-temperature treatment (Gocheva *et al*. 2009; Miteva-Staleva

et al. 2017). It should be noted that the strains used in the present experiments have demonstrated enhancement in the ROS level, stress biomarkers, and antioxidant enzyme defense during temperature downshift from the optimal temperature to 15°C and 6°C (Gocheva *et al.* 2009; Miteva-Staleva *et al.* 2011, 2015, 2017). Furthermore, a significant increase in carbonyl content was found. Protein carbonyls are irreversible products and are thus susceptible to proteolytic degradation. It is noteworthy that the accumulation of carbonylated proteins could be a consequence of many basic situations, such as carbohydrate metabolism, protein maintenance, and homeostasis as well as cellular motility (Ciacka *et al.* 2020). However, oxidative stress significantly accelerated this process. Thus, it is no surprise the upward trend of proteolytic activity. In recent study we added information on changes in protease levels in the cultures of the same three *Penicillium* strains (Gocheva *et al*. 2009; Miteva-Staleva *et al*. 2017).

Many studies showed that the degradation of oxidatively modified proteins is essential for cell survival under stressful conditions (*e.g.* Jung *et al*. 2014; Chu *et al*. 2016; Castaño *et al*. 2021). Maintaining the balance between protein synthesis and degradation allows the cell to rid itself of damaged protein molecules and thus adapt to changing conditions. Activation of the proteolytic system has been observed under various types of stress.For example, the treatment of plant cells with heavy metals results in a 20% increase in proteolytic activity (Romero-Puertas *et al*. 2002). In the stress response system, chloroplastic proteases play a significant role (Mamaeva *et al*. 2020).

The comparative analysis indicated remarkable similarities in the level of the extra- and intracellular protease. According to Vardi *et al*. (2007), extracellular protease activity may be involved in the cleavage of a domain from transmembrane proteins. The role of extracellular proteolytic enzymes in the cell response was reported for *Bacillus anthracis* (Wang *et al*. 2020), *Aspergillus flavus* and *Aspergillus niger* (4% salt stress) (Okpara *et al.* 2019), and *A. niger* B1-D (heat stress) (Li *et al*. 2008). Bacterial *Pseudomonas* and *Flavobacterium* strains isolated from Arctic and Antarctic regions produce a higher level of extracellular protease at 4°C growth temperature than at 30°C (Martínez-Rosales and Castro-Sowinski 2011).

Second, present results provide information on changes in protease activity under stress conditions depending on culture age. Both the phases investigated here were characterized by a significant increase in protease activity (extra- and intracellular) depending on the degree of stress and temperature preferences of the strains. However, the young cells showed low basic enzyme levels in comparison with the old cells (Figs. 1 and 2). High values of proteolytic activity during the stationary phase have also been reported in other fungi, for example, *Trichoderma* spp. (Matátá *et al.* 2019). Activation of genes encoding proteolytic enzymes during the stationary phase has been found in *Candida albicans* (Uppuluri and Chaffin 2007). Four mutations involved in the lifespan of *Schizosaccharomyces pombe* during stationary phase and stress have been

described (Vega *et al.* 2022). The exposure to 6°C caused a more significant induction of protease activity than that at 15°C in both exponential- and stationary growth cultures in comparison with the start value. The increase of the intracellular enzyme was more noticeable in the old cells than in the young cells. Although the growth ceases in the stationary phase, the cells remain *metabolically active* (Jaishankar and Srivastava 2017). These results coincided with our previous data demonstrating significantly higher oxidative damaged protein content in the stationary-phase cells compared to the exponentially-phase cells (Miteva-Staleva *et al*. 2017). Numerous reports note that protein carbonylation correlates well with the biological age of many organisms, including filamentous fungi (Mazheika *et al*. 2021). This is one of the most used markers of aging and oxidative stress among researchers evidenced by the increased carbonylated protein content in the old fungal cells (Fu *et al.* 2008; Landolfo *et al*. 2008; Li *et al*. 2008; De Castro *et al*. 2013). The elimination of oxidatively modified proteins during stress conditions is a crucial mechanism for the cellular homeostasis maintenance.

Third, we also found strain-depending changes in protease levels. The increased enzyme activity is more a function of the temperature preference of the strain than of the location from which it was isolated. The effect of low-temperature stress was associated with a substantial protease induction in the psychrotolerant *Penicillium* strain, isolated from Antarctica, and more smoothly in the other mesophilic strains, isolated from Antarctica and temperate region, respectively. Previous data also indicated that the psychrotolerant strain *P. olsonii* p14 is the most adapted to survive temperature downshift conditions (Gocheva *et al*. 2009; Miteva-Staleva *et al*. 2015; 2017). This strain overcame the cold stress to the greatest extent compared to both mesophilic strains during exponential- and stationary-growth phases.

Conclusions

Taken together the present results complete the information on the age-dependent cellular response to cold stress of fungal cells. The transient temperature downshift led to the activation of proteases to cleave oxidatively damaged proteins. Stress-induced protease activity was most pronounced in the old cells, which corresponds to increased values of stress biomarkers in these cells compared to young ones. The cell response was dependent on the growth phase and temperature preferences of the strain, but not on the climatic distance between the locations of isolation. Our results add new information to support Harman's *Free Radical Theory of Aging*.

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