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Activation of antioxidative metabolism in different growth stages of *Phycomyces blakesleeanus* mycelia exposed to vanadate

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ABSTRACT:
Understanding the effect of vanadate (V(V)) on the well-studied filamentous fungus, *Phycomyces blakesleeanus*, is of great importance for establishing the mechanisms of vanadium internalisation and metabolism. Although *P. blakesleeanus* is not a soil fungus, its short life cycle and evolutionary basal position in the fungal kingdom makes it an excellent model for studying the interactions of fungi with vanadate and other metals as well as the improvement of their use in vanadium recovery (bioaccumulation) and the remediation of contaminated soils. In this study, we investigated the effects of vanadate at three concentrations (1 mM, 5 mM, and 10 mM) on both enzymatic and non-enzymatic components of the antioxidant system of mycelia at different growth stages. The production of ROS was highest in the exponential phase, but its reducing capacity was maintained, probably due to high levels of non-enzymatic antioxidants such as phenols and glutathione. In the stationary phase, while the reducing capacity of the mycelia was somewhat impaired by vanadate (V(V)), it recovered due to the action of the glutathione system and the activation of peroxidases. In the late exponential/early stationary phase, no significant activation of the antioxidative systems was observed, and the reducing capacity was impaired. When we consider the effects of V(V) on mycelial growth, this was the only phase with reduced viability, while the exponential and stationary phases were unaffected, if not stimulated. This study provides insight into the tolerance of *P. blakesleeanus* mycelia to vanadate, even at concentrations as high as 10 mM, making this fungus a good candidate for V(V) bioaccumulation.

Keywords: mycelia, ROS, phenolics, antioxidative enzymes, glutathione

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INTRODUCTION

As an important part of the ecosystem, fungi are involved in various environmental processes directly related to metals (Xu *et al.* 2019). Many metals serve as essential micronutrients for fungi at low concentrations, however, at high concentrations, such metals can be toxic or even lethal (Das *et al.* 2009). For one such transition metal, vanadium (V), widely known as a trace contaminant in coal and oil, but not yet recognised as a problematic environmental pollutant (Schlesinger *et al.* 2017), fungi are the main pathway for its entry into the ecosystem (Anke *et al.* 2005). Fungi generally exhibit a good tolerance to it and are capable of growing in environments with vanadate concentrations in the millimolar range (Ceci *et al.* 2012; Xu *et al.* 2019). In addition to tolerance, many fungi can also accumulate or mobilise vanadium from minerals,

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indicating their potential use in the recovery and remediation of vanadium (Xu *et al*. 2019). The effects of vanadium in biological systems are diverse and depend primarily on the oxidation state, but also on the chemical form, dose, and duration of exposure (Aureliano 2016 and the references therein). In the last few decades, vanadium has attracted the attention of researchers as a potential therapeutic agent, mainly due to its insulin-mimetic effects (Treviño & Diaz 2020), linked to its ability to form complexes and exchange chelators and ligands with the environment (Scior *et al*. 2016). These complexes act as vehicles for therapeutically active "free" vanadium, but may also exert toxic effects (Scior *et al*. 2016). Metabolic transformations of vanadium in those organisms which adopt it, such as fungi, might provide a promising way to obtain V complexes with desirable pharmaco-kinetic properties.

Vanadate can induce the formation of free radicals either via a Fenton-like or a Haber–Weiss reaction (Valko *et al*. 2005; Soriano-Agueda *et al*. 2016). In addition, it can also influence the phosphorylation and dephosphorylation of proteins, regulate the activity of numerous enzymes, and trigger various signalling pathways (Scior *et al*. 2005). Normally, reactive oxygen species – (ROS) are part of the physiological processes in fungi and are involved in germination, development, intercellular communication, differentiation, etc. (Aguirre *et al*. 2005; Gessler *et al*. 2007). However, increased radical concentrations and the induction of oxidative stress by various stimuli, such as high metal concentrations, affect antioxidant defence systems and may lead to various changes in growth, metabolism, or the production of bioactive compounds (Bai *et al*. 2003; Belozerskaya & Gessler 2007). The extent of metal toxicity depends on the fungal species, the metal concentration in the soil, the duration of exposure, and the properties of the metal such as solubility, absorbability, transport, and chemical reactivity (Priyadarshini *et al*. 2021; Robinson *et al*. 2021). Oxidative stress is often associated with transition metals because they can easily change their oxidation state. After entering the cell, vanadium ions undergo oxidation-reduction processes involving various reducing agents and enzymes, such as glutathione reductase, ascorbate, nicotinamide adenine dinucleotide phosphate (NADPH), thiols, and others (Soriano-Agueda *et al*. 2016).

The objective of this study was to investigate the effects of increasing concentrations of vanadium in its oxidation state 5, vanadate (V(V)), on *Phycomyces blakesleeanus* mycelia at different growth stages. *Phycomyces blakesleeanus* is a well-studied filamentous fungus commonly used for physiological studies (Galland *et al*. 2007; Živanović *et al*. 2018, 2023). Vanadium can enter the mycelia in both V(V) and V(IV) form, which are its most common oxidative states (Soares *et al*. 2008). The study of the effects of vanadate on the enzymatic and non-enzymatic defence systems in this fungus is a step towards gaining a better understanding of the response of fungi to exposure to a complex transient metal such as vanadium.

MATERIALS AND METHODS

Growth conditions and preparation of the mycelia for analysis. All the experiments were performed using the mycelia of the wild-type strain *Phycomyces blakesleeanus* (NRRL 1555(-)), originally from the Northern Regional Research Laboratory (Peoria, Il., USA; Bergman *et al*. 1973). The vegetative spores were kept at -20°C at a concentration of 10^7, and heat-shock activated (48–50°C, 15 min) immediately prior to inoculation. The activated spores were grown in modified minimal medium SIV (Sutter 1975) containing (in mM): 110 glucose, 13.1 L-asparagine, 36.7 KH_2PO_4, 2 $MgSO_4 \times 7H_2O$, 0.376 $CaCl_2$, 36.7 KH2PO4, 2 MgSO4 × 7H2O, 0.376 CaCl2, and (in μM): 3 thiamine × HCl, 1 citric acid × H_2O, 3.7 $Fe(NO_3)_3 \times 9H_2O$, 3.5 $ZnSO_4 \cdot \times 7H_2O$, 1.8

$MnSO_4 \cdot \times H_2O$, 0.2 $CuSO_4 \cdot \times 5H_2O$, 0.2 $NaMoO_4 \cdot \times 2H_2O$, pH 6.7, in Erlenmeyer flasks shaken on a digital orbital shaker at 120 rpm (Sea Star, Heathrow Scientific, USA) at 22°C, under continuous white fluorescent light at an intensity of 10 W/m^2 (Osram, Germany). The standard SIV medium has a pH of 4.8, and the vanadate stock solution has a pH of 10. To avoid the pH influence of V(V), the growth medium was adjusted to pH 6.7 with KOH, which is the pH of the medium containing 10 mM V(V). The stock solution of 200 mM sodium orthovanadate (Na_3VO_4) (Sigma-Aldrich, Taufkirchen, Germany), was prepared according to the method described by Gordon (1991). The growth curve was determined by vacuum filtration of 20 mL of mycelial suspension through pre-weighed filter paper every 4 h from 12 h to 68 h of growth, and the biomass was oven-dried for 24 h at 50°C. Glucose was determined from the supernatant using the 3,5-dinitrosalicylic acid method (Jain *et al*. 2020). Three time points were chosen for treatment with vanadate: 20 h (the early to mid-exponential phase), 36 h (the late exponential phase), and 56 h (the stationary phase). At the selected time points, the mycelia were collected by vacuum filtration, washed with sterilised distilled water and resuspended in fresh SIV medium containing 1 mM, 5 mM, or 10 mM V(V). After 1 h or 5 h treatment, the mycelia were washed twice with 50 mM Na_2EDTA pH 6.7 at 4°C, and centrifuged for 7 min at 4300 × g. The control samples were exposed to the same set of steps as the treated ones, but without the addition of V(V). A portion of the mycelia was freeze-dried and used for the ABTS assay and total phenolic content, while fresh mycelia were used for the glutathione determination and enzymatic assays. All the samples were stored at -70°C until use.

Cell survival – "culturability". At selected time points, 500 mg of fresh weight (FW) mycelia was transferred to 50 mL of fresh minimal medium containing 1 mM, 5 mM, or 10 mM V(V) for 5 h. After treatment, the mycelia were washed with 50 mM Na_2EDTA pH 6.7 and transferred to 50 mL of fresh minimal medium for another 12 h and then filtered through pre-weighed filter paper. The filtrate was oven-dried for 24 h. Cell survival or "culturability" was monitored by the growth rate as a function of dry biomass weight (DW). The growth rate is expressed in $mg_{DW}mL^{-1}\ h^{-1}$.

Detection of intracellular ROS. ROS were determined using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) method (Kalyanaraman *et al*. 2012). DCFH-DA was dissolved in 96% ethanol and used at a final concentration of 20 μM. The mycelia were collected after treatment for 0.5 h, 1 h, or 5 h and diluted with sterilised distilled water to OD_{750} ~ 0.5, washed twice and incubated with DCFH-DA in the dark for 1 h at room temperature. DCF fluorescence at ex/em 485/530 nm was measured using a Tecan Infinite M Nano microplate reader (Tecan Group Ltd, Männedorf, Switzerland) in clear bottom black microplates. The results are presented as the ratio of fluorescence intensity of the treatment and control.

Radical scavenging activity, total phenolic content and total glutathione content. Freeze-dried mycelia (50 mg per sample) were used to determine the radical scavenging activity (ABTS assay) (Re *et al*. 1999) and total phenolic content (Singleton & Rossi 1965) of the samples. The extraction solvents were prepared using 50% ethanol at a ratio of 1/8 w/v for the ABTS assay, and 80% methanol at a ratio of 1/10 w/v for the total phenolic content. The samples were homogenised in half the solvent volume with 5 mm stainless steel beads (Qiagen, Germany) twice for 30 s at a frequency of 30 Hz on the Tissue Lyser (Qiagen, Germany), and the remaining buffer was then added. After a 4-min extraction the samples were centrifuged at 16100 × g for 10 minutes at 4°C, and the supernatants were immediately used for the tests. Ascorbic acid

was used as the standard (0.1–1 mM) for the ABTS assay, and the results were expressed as mmol ascorbic acid equivalents (AscE) per gramme dry weight (mmolAscE/g_{DW}). The total phenolic content was determined spectrophotometrically using the method proposed by Singleton & Rossi (1965). Gallic acid was used as the standard (0.1–2 mM) and the results were expressed as mmol gallic acid equivalents (GAE) per gramme dry weight (mmolGAE/g_{DW}). For the total glutathione content (G_{tot}), 100 mg of FW mycelia was frozen in liquid N_2 and homogenised on Tissue Lyzer as described above. Extraction was performed according to Matsumoto *et al*. (1996) and absorbance was measured every 2 minutes for 6 minutes at 405 nm. The concentration of total glutathione was determined from the standard curve and expressed in µg per gramme fresh weight (µg/g_{FW}).

Enzyme assays. For the enzyme extraction, 200 mg of FW mycelia and 1 ml of 50 mM potassium phosphate buffer composed of KH_2PO_4 and K_2HPO_4, pH 7.2 with/without 2 mM D-iso ascorbic acid sodium salt and 2 mM PMSF were used. Buffers with D-iso ascorbic acid and PMSF were used for the extraction of glutathione peroxidase (GPx) and glutathione reductase (GR), while buffers without these components were used for the determination of pyrogallol/guaiacol peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD). Frozen samples were homogenised as previously described, and the extraction was performed on ice for 15 min with continuous shaking. The extracts were stored at -70°C until use. The activities of GPx, GR, and POD were determined spectrophotometrically as U/mg protein, while CAT was determined polarographically. The total protein content was measured according to Bradford (1976) using bovine serum albumin as the standard. The activity of glutathione peroxidase (EC 1.11.1.9.) was measured according to Drotar *et al*. (1985) by observing the decrease in NADPH concentration at 340 nm. The glutathione reductase (EC 1.8.1.7) activity was determined as described by Smith *et al*. (1988) by monitoring the reduction of DTNB at 412 nm. The peroxidase (EC 1.11.1.7) activity was determined by monitoring the increase in absorbance at 420 nm, as a result of pyrogallol oxidation by H_2O_2 in the presence of peroxidase (Maehly & Chance 1954). The catalase (EC 1.11.1.6) activity was determined polarographically using the Clark-type oxygen electrode (Hansatech Instruments Ltd, UK) according to the method proposed by del Río *et al*. (1977). SOD (EC 1.15.1.1) forms were separated by native PAGE according to Laemmli (1970), and the activity visualised in gel following Beaucham & Fridowich (1971).

Statistical data analysis. The results are presented as box and whisker plots including all points from at least two independent experiments (n = 4–6). Filamentous fungi are significantly inhomogeneous in terms of organelle distribution and cytoplasm density within the hyphae which, in addition to the presence of a cell wall, poses a challenge for successful cytoplasm isolation. This is particularly pronounced in coenocytic (non-septate) fungi such as *P. blakesleeanus*. Another problem posed by the pooled analysis of separate experiments is the remarkable phenotypic plasticity and ability of *P. blakesleeanus* to adapt to almost imperceptible changes in the environmental conditions with alterations in physiology and morphology (Schinagl *et al*. 2016). For this reason, the calculation of the enzymatic and non-enzymatic antioxidant activities of the *P. blakesleeanus* mycelia was adjusted. For each individual experimental series, the activities were expressed relative to the control mean for that series. The individual controls were also normalised to the control mean for the statistical calculations. This protocol was deemed valid as the response trend to vanadate treatment was similar across different experimental series despite large differences in absolute values.

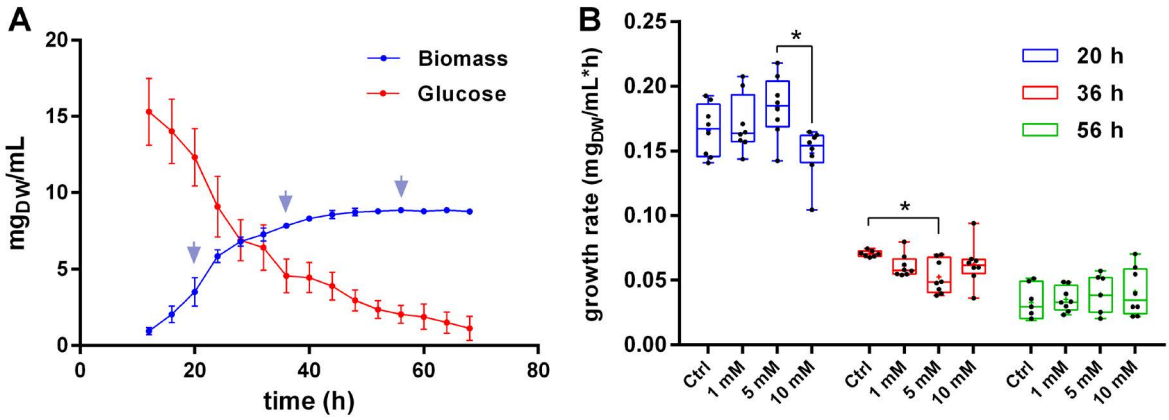

Fig. 1. A. The growth curve of *Phycomyces blakesleeanus* mycelia (n ≥ 6). Arrows denote the different growth phases selected for the experiments: mid-exponential (20 h), late exponential (36 h), and stationary (56 h) phase. **B.** The growth rate of *Phycomyces blakesleeanus* mycelia during 12 h cultivation in V(V) free medium after exposure to increasing concentrations of vanadate for 5h in three selected growth phases, expressed as mg$_{DW}$/mL*h (n = 7–8).

For comparisons between the control and the treated samples (growth rate, radical scavenging activity, total phenolic content, total glutathione content and enzyme activity), a two-tailed non-parametric Mann-Whitney test was used (n = 4–6). Due to the short life cycle of *P. blakesleeanus*, treatments of different durations were compared to corresponding controls. For comparisons between the control samples at different growth stages (ROS, radical scavenging activity, and total phenolic content), the controls from both 1 h and 5 h were pooled. Given the higher number of samples (8–24), one-way ANOVA at a significance level of 0.05 was used. The statistical data analysis and graphs were generated in GraphPad Prism 6.01.

RESULTS AND DISCUSSION

Growth rate dependence on vanadate dosage. The growth curve of *P. blakesleeanus* mycelia was obtained by determining DW every 4 hours (Fig. 1A). The exponential phase lasted from 12 h to about 36/40 h of growth, followed by a stationary phase. Three characteristic time points were selected for the vanadate exposure experiments: the mid-exponential phase (20 h), the late exponential/early stationary phase (36 h) and the stationary phase (56 h). To determine the toxic effects of V(V) on *P. blakesleeanus*, the mycelia were exposed to 1 mM, 5 mM, and 10 mM V(V), separately, for 5 h at each of the aforementioned time points, then washed and grown for a further 12 h in fresh vanadium-free medium. In the exponential and stationary phases V(V) exhibited no detrimental effects on mycelial survival, with only a slight inhibitory effect at the late exponential phase (Fig. 1B). Although there were no statistically significant differences between the control and the treatments at 20h, the 10 mM treatment resulted in a significantly lower growth rate than that with 5 mM V(V), indicating possible hormetic effects of 5 mM V(V). *Phycomyces blakesleeanus* shows a high tolerance toward V(V) stress, and these results agree with those reported in Žižić *et al.* (2013), where V(V) up to 5 mM had a stimulatory effect on the mycelia in the exponential phase. The mycelia of different fungal species exhibit very different responses to V(V) treatment. Thus, treatments with up to 10 mM V(V) had little to no effect on the growth of basidiomycetes *Bjerkandera adusta* and *Xerocomus badius*, but inhibited the growth of *Armillaria cepistipes* and *Amanita muscaria* in particular (Xu *et al.* 2019). Notably, vanadium content in *A. cepistipes* and *A. muscaria* was significantly higher than in *B. adusta* and *X. badius* (Xu *et al.* 2019), which could

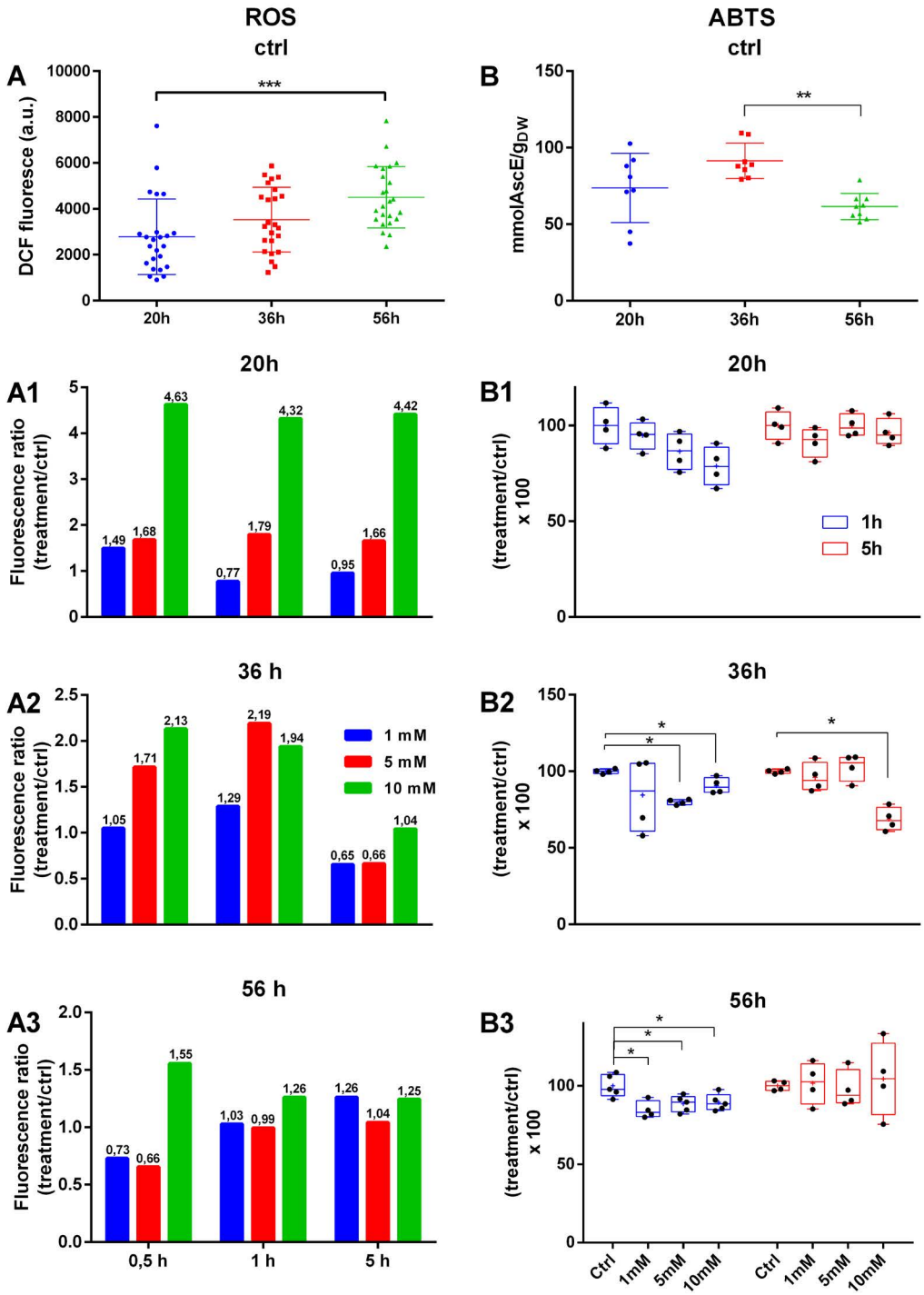

Fig. 2. The production of ROS and changes in radical scavenging potential after V(V) treatment. The left column (**A**) shows the production of ROS, while the right column (**B**) depicts the results of the ABTS test. First row (**A** and **B**) – the control values at selected time points were compared by one-way ANOVA followed by Tukey's multiple comparison test. **A1**, **A2**, and **A3** represent the intracellular ROS content at selected time points after treatment with 1 mM, 5 mM, and 10 mM V(V) (n = 6). The changes in ROS content are depicted as the fluorescence ratio of the treatment and control. **B1**, **B2**, and **B3** – the radical scavenging potential (ABTS test) at selected time points after 1 h or 5 h of treatment with 1 mM, 5 mM, and 10 mM V(V), depicted as the treatment and control ratio x 100 (n = 4). The control of each group was compared to treatment using the Mann-Witney rank sum test. The x - axes of **A1** and **A2**, and **B1** and **B2**, correspond to the x - axes of **A3** and **B3**, respectively.

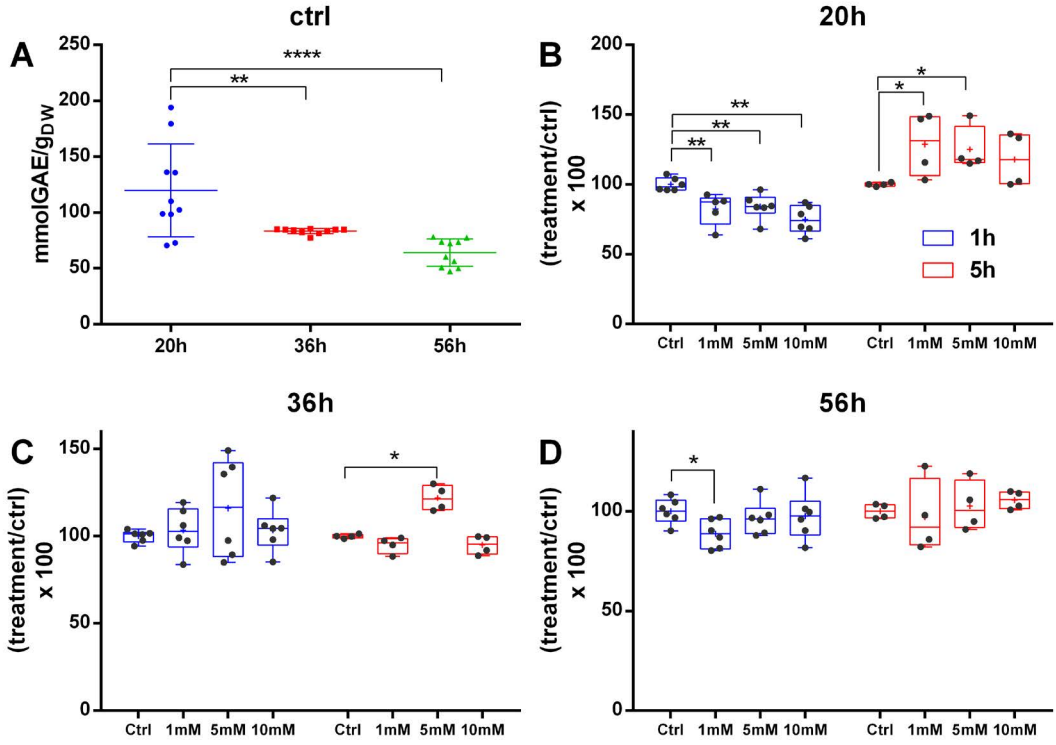

Fig. 3. Phenolic content in the mycelia of *Phycomyces blakesleeanus* in the controls and after 1 h and 5 h treatments with 1 mM, 5 mM, and 10 mM V(V), determined as mmolGAE/g_{DW}. **A**. The phenolic content of the control in selected growth phases compared by one-way ANOVA followed by Tukey's multiple comparison test (n = 10). **B**, **C**, and **D** – the phenolic content of the mycelia after 1 h and 5 h treatments with 1 mM, 5 mM, and 10 mM V(V) at selected growth phases, depicted as the treatment and control ratio x 100. The control of each group was compared to treatment using the Mann-Witney rank sum test (n = 4–6).

explain the higher toxic effect. Currently, although we do not know the extent of vanadate uptake by *P. blakesleeanus*, it is unlikely to reach the exceptionally 5% of dry weight as in *A. muscaria*. In *Saccharomyces cerevisiae*, 5 mM V(V) caused growth arrest, but 1 mM had no effect (Willsky *et al.* 1984). As far as the authors are aware, no research has been conducted on the mechanisms of the stimulatory effects of vanadate on the growth of fungi, but studies have been carried out on plants with the ability to accumulate this metal. The stimulatory effect of vanadium on the growth of Chinese cabbage *Brassica rapa* was reported by Tian *et al.* (2014), and the authors suggested enhanced growth as the mechanism for the dilution of metals within cells. Similar effects were observed in pepper plants, where V(V) up to a certain concentration stimulated an increase in plant height, stem diameter, the number of leaves and floral buds, the root volume, and the fresh and dry biomass, through an increase in free amino acids and sugars in the cytoplasm (García-Jiménez *et al.* 2018). Some fungi, especially those of the genus *Amanita* are known to be vanadium bioaccumulators (Rehder 2015).

The accumulation of ROS and polyphenols is related to the developmental stage. The effects of vanadium are related to the vanadate-phosphate analogy, and to its involvement (IV and V) in both the generation and annihilation of ROS (Rehder 2013). The level of intracellular ROS after treatment with V(V) shows an increase in intracellular H_2O_2 content. In untreated *P. blakesleeanus*

mycelia, the ROS content rose in the stationary phase (Fig. 2A), which is expected for aging cultures. Laun *et al*. (2001) showed that there is an accumulation of ROS in aging mother cells of *S. cerevisiae* before apoptosis, while in some filamentous fungi, such as *Penicillium chrysogenum*, there was no accumulation of ROS during the stationary growth phase (Sámi *et al*. 2003).

De Castro *et al*. (2013) indicated that oxidative stress may play a role in the ageing of *P. blakesleeanus*, which is consistent with the decrease in the radical scavenging potential of the mycelia during the stationary phase (Fig. 2B). The highest ROS levels were caused by 10 mM V(V) in the mid-exponential mycelia (Fig. 2A1), while in the 36 h old mycelia they doubled in the first hour of the treatment, but returned to normal with prolonged incubation (Fig. 2A2). In the stationary phase mycelia, the increase in ROS levels is barely noticeable (Fig. 2A3). This could be due to the reduced permeability of the cell wall and membrane in ageing mycelia (Sousa-Lopes *et al*. 2004) and reduced metabolic activity. In addition, the basal levels of ROS were elevated in this growth stage (Fig. 2A). V(V) induced a decrease in the radical scavenging potential in 36 h and 56 h old mycelia after 1 h of treatment (Fig. 2B2, B3), while recovery was observed after 5 h of treatment, indicating some kind of adaptation. Such a rapid adaptation may explain the lack of any significant effect on the mycelial growth (Fig. 1B). Since the changes in the antioxidative system in this study (both enzymatic and non-enzymatic) were not extensive enough to explain the unaffected growth, the adaptation mechanism could be related to V(V) reduction and internalisation, similar to *Ganoderma lucidum*, where the accumulation of Se and simultaneous avoidance of selenite toxicity relies primarily on the expression of transporters with different kinetic properties (Xu *et al*. 2024).

Polyphenols are known to be strong antioxidants, therefore the total phenolic content was determined. The highest content of gallic acid equivalents was found in the mid-exponential phase, with a marked decrease at 36 h and 56 h (Fig. 3A). *Phycomyces blakesleeanus* produces exceptionally high amounts of gallic and protocatechuic acids, especially in the sporangiophores, but the mycelia are also rich in these compounds (Weinkove *et al*. 1998). This result differs from that of de Castro *et al*. (2013), but it can be attributed to different growth conditions, foremost the presence or absence of light, which has a marked effect on the accumulation of gallic and protocatechuic acids (Barrero *et al*. 1996). Significant changes in the phenolic content as a result of V(V) treatment were noted in 20 h old mycelia, where the phenolic content decreased after 1 h of treatment for all the applied concentrations (Fig. 3B). Phenolic substances can be directly involved in both redox and complexation reactions with V(V) and V(IV), thus buffering the toxicity of vanadate (Garau *et al*. 2015). Redox reactions are more pronounced at lower pH values such as those in the vacuoles, where the hydroxybenzoic acids of *P. blakesleeanus* are preferentially located (Weinkove *et al*. 1998). Micro-XRF chemical imaging showed that the predominant intracellular localisation of vanadium in *P. blakesleeanus* is probably the vacuole (Žižić *et al*. 2015). In the exponential phase mycelia treated with 1 mM and 5 mM V(V) for 5 h showed an increase in phenolic content compared to the control (Fig. 3B). This may be due to stress stimulating carbon fluxes into the secondary metabolic pathways, inducing a shift of available resources to the synthesis of secondary products, such as phenolic compounds (Lattanzio 2013). Stressful conditions such as temperature stress (Fink-Boots *et al*.1999), and treatments with paraquat and H_2O_2 increase the concentration of phenolics in cultures of basidiomycetes *Trametes versicolor* and *Abortiporus biennis* (Jaszek *et al*. 2006).

The response of antioxidant enzymes to vanadate. The accumulation of ROS leads to changes in the activities of antioxidative enzymes such as CAT, SOD, POD, and GPx. In this study, no changes were noted in CAT and SOD activi-

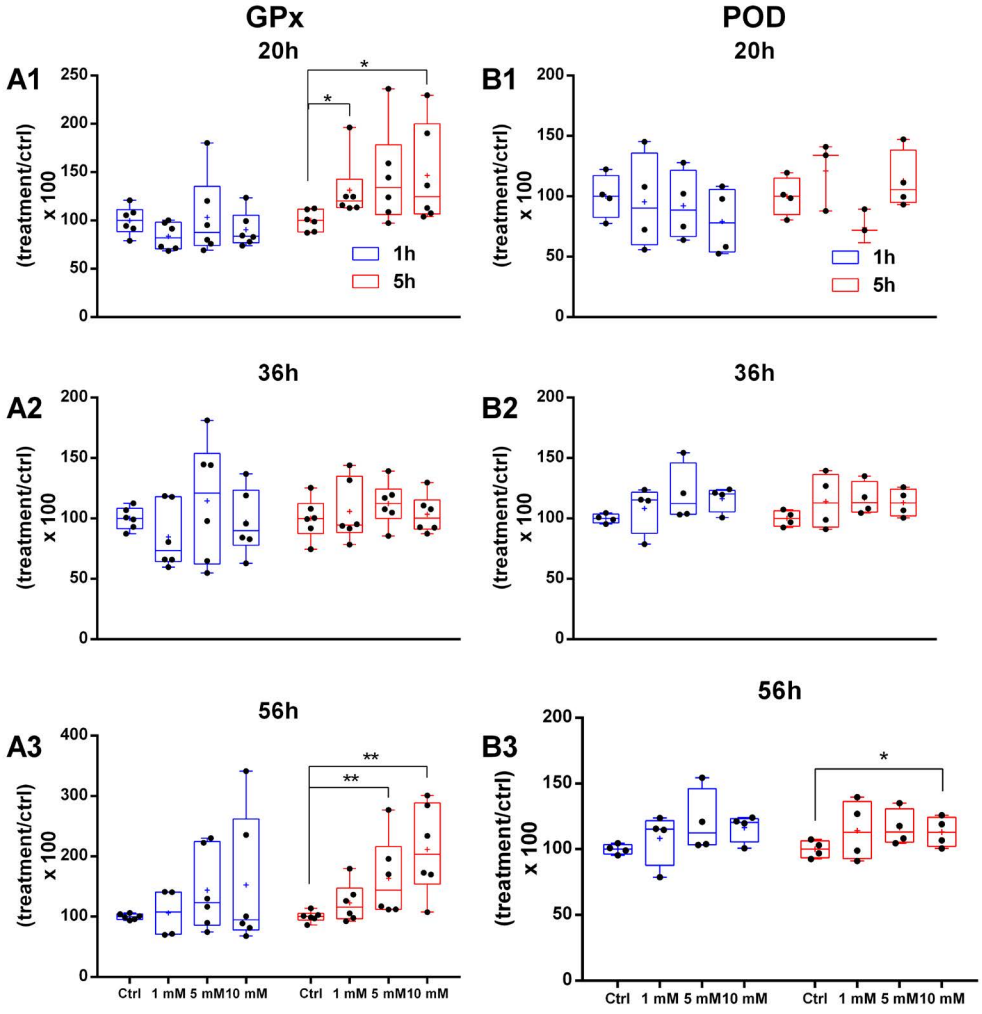

Fig. 4. The activities of GPx (**A**) and POD (**B**) in the mycelia of *Phycomyces blakesleeanus* after 1 h and 5 h treatments with 1 mM, 5 mM, and 10 mM V(V) at selected growth phases, determined as U/mg protein and depicted as the treatment and control ratio × 100 (n = 3–6). The control of each group was compared to treatment using the Mann-Witney rank sum test. The x - axes of **A1** and **A2**, and **B1** and **B2**, correspond to the x - axes of **A3** and **B3**, respectively.

ties as a result of V(V) incubation, but interestingly, the stationary phase mycelia expressed an additional form of Mn SOD, which was also unaffected by V(V) (Fig. S1). De Castro *et al.* (2013) showed that CAT exists in two forms in *P. blakesleeanus*, a constitutive form and a form inducible by H_2O_2. Their results indicate that the constitutive form is inhibited by H_2O_2 after short treatments, while prolonged exposure leads to the activation of the inducible form, but only after treatment with a lower concentration of H_2O_2. Loss of CAT activity in *S. cerevisiae* had no effect on H_2O_2 sensitivity (Izawa *et al.* 1996; Grant *et al.* 1998), suggesting that other enzymes such as peroxidases can remove H_2O_2 (Zadrąg-Tęcza *et al.* 2018). In line with this, GPx activity increased with prolonged V(V) incubation in 20 h and 56 h old mycelia (Fig. 4A1, A3). In addition, POD activity increased in 56 h old mycelia treated with 10 mM V(V) for 5 h (Fig. 4B3).

Glutathione is one of the major antioxidants in fungi which reacts non-enzymatically with various reactive oxygen species (Pócsi *et al.* 2004).

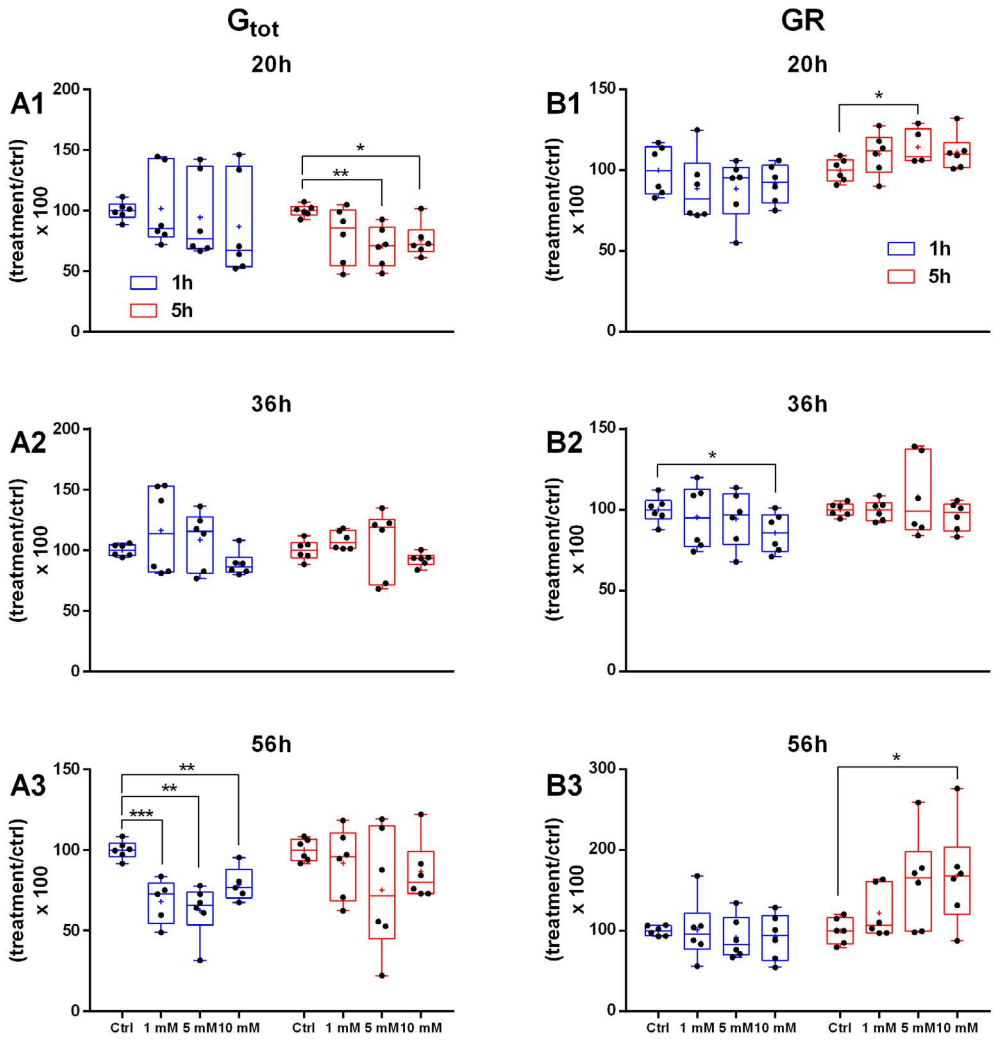

Fig. 5. G_{tot} content determined as mg/g_{FW} (**A**), and GR activity determined as U/mg protein (**B**) in the mycelia of *Phycomyces blakesleeanus* after 1 h and 5 h treatments with 1 mM, 5 mM, and 10 mM V(V) at selected growth phases, depicted as the treatment and control ratio × 100 (n = 5–6). The control of each group was compared to treatment using the Mann-Witney rank sum test. The x - axes of **A1** and **A2**, and **B1** and **B2**, correspond to the x - axes of **A3** and **B3**, respectively.

In addition, V(V) compounds undergo rapid reduction under physiological conditions in the presence of thiols such as glutathione or cysteine (Crans *et al.* 2010). Thiol-containing ligands also form stable complexes with V(V), and GSH-derived phytochelatins prevent the progression of cell damage caused by heavy metals by chelating and sequestering the metal ions (Pócsi *et al.* 2004; Crans *et al.* 2010). The content of total glutathione (G_{tot}) decreases with the depletion of both reduced (GSH) and oxidised (GSSG) glutathione. Its concentration in *S. cerevisiae* varies between 1 and 10 mM or even higher (Herrero *et al.* 2008), and under physiological conditions the GSH concentration can be 10 to 100 times higher than that of GSSG (Le Moan *et al.* 2006), so it is more likely that the decrease in total glutathione concentration is due to a lack of GSH (Kobayashi *et al.* 2002; Pócsi *et al.* 2004). In the 20 h old mycelia of *P. blakesleeanus*, G_{tot} decreased after prolonged exposure to V(V) (Fig. 5A1). At the same time, the activity of the peroxide-eliminating GPx, which catalyses

the redox reaction between GSH and H_2O_2 and produces GSSG and H_2O, increased (Fig. 4A1). This led to an increase in GSSG, which in turn increased the activity of GR (Fig. 5B1), a GSH-regenerating enzyme whose substrate is GSSG. In the stationary phase mycelia (56 h old) G_{tot} levels already decreased after 1 h of V(V) exposure (Fig. 5A3), which is reflected in the reduced radical scavenging potential (Fig. 2B3). As in the 20 h old mycelia, an increase in GPx and GR activity was detectable (Figs. 4A3 and 5B3), which resulted in the partial recovery of G_{tot} levels (Fig. 5A3) after 5h of incubation.

Overall, the production of ROS was highest in the exponential phase with 10 mM V(V), but its reducing capacity was preserved, probably due to high levels of non-enzymatic antioxidants such as phenols and G_{tot}, which also act as chelating agents. The application of 1 mM V(V) led to an initial decrease in the antioxidative capacity of 20 h old mycelia and elevated ROS production. Although the ABTS test showed a slight decrease in the antioxidative capacity after 5h, ROS accumulation was notably lower after 1 and 5 h of V treatment, indicating the activation of antioxidative mechanisms. This may be linked to the slight increase in phenolics after 5 h of treatment, as well as the increase in GPx activity, and the decrease in G_{tot} which triggered GR activation. The elimination of ROS is probably partly mitigated by D-erythroascorbate as a significant antioxidant in *P. blakesleeanus* (Baroja-Mazo *et al*. 2005). After 5 h in 20 h old mycelia the total antioxidative capacity together with the phenolic content seemed to stabilise even at higher V(V) doses.

In the stationary phase, the reducing capacity of the mycelia was impaired by V(V), but recovered through the action of the GSH system and the activation of peroxidases. In the late stationary phase, 10 mM V(V) led to a fast but short-lived ROS increase, while treatments with 1 mM and 5 mM V(V) resulted in a decrease in ROS. However, after 1 h and 5 h, the ROS levels returned to the control level, with only a slight increase observed with the 10 mM treatment. Both antioxidative capacity and phenolic content remained at control levels. The role in ROS scavenging here lies with GPx and POD, which significantly increased in 5 mM and 10 mM treatments of mycelia in the stationary phase. The leading role of GPx is also reflected in the decreased G_{tot} content in the first hour of treatment, and the increased GR activity leading to G_{tot} restoration after 5 h of treatment. A contribution in this apparent ROS elimination could be made by D-erythroascorbate glycoside, which was not measured in our work, but could complement the enzymatic antioxidative activity, as its concentration has been shown to be significantly increased in the stationary phase *P. blakesleeanus* mycelia (Baroja-Mazo *et al*. 2005).

The most sensitive developmental phase proved to be the late exponential/early stationary phase. Only here did V(V) have a mild inhibitory effect on growth rates. The reducing capacity of the mycelia was impaired and remained so even after prolonged exposure to 10 mM V(V). No activation of peroxidases was observed, and there was even a slight inhibition of GR when 10 mM V(V) was applied. During this phase, ROS production is lower than in the exponential phase mycelia, but notable after both 1 h and 5 h of treatment, possibly due to lower metabolic activity upon transition to the stationary phase. However, the slight (albeit not statistically significant) decrease in G_{tot} content upon treatment with 10 mM V(V) treatment is probably due to the significant decrease in GR activity, while POD may contribute to H_2O_2 elimination rather than GPx.

CONCLUSIONS

Vanadium enters the cells of *P. blakesleeanus* in both V and IV oxidation forms, and as such can be the subject of intracellular redox chemistry, which leads to the production of ROS. Different components of both enzymat-

ic and non-enzymatic antioxidative response were shown to be involved in the defence against vanadium-induced ROS at different growth stages of *P. blakesleeanus*. Phenolics and glutathione peroxidase seem to be responsible for stress attenuation in the exponential growth phase. The activation of peroxidase in concert with GPx mitigates oxidative stress in the stationary phase. The mycelia are at their most sensitive during the transition from the exponential to the stationary phase, with vanadate treatment leading to growth inhibition and the impaired reducing capacity of the mycelia.

This study has confirmed that *P. blakesleeanus* is a fungus with a high tolerance to V(V) and has revealed several mechanisms responsible for its resilience. Thus, *P. blakesleeanus* is a plausible candidate for V(V) bioaccumulation and further investigation of the properties of vanadium forms and complexes obtained during its metabolism in this fungus is warranted.

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COMPREHENSIVE PLANT BIOLOGY

REZIME

Aktivacija antioksidativnog metabolizma micelije *Phycomyces blakesleeanus* u različitim fazama rasta

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Razumevanje efekta vanadata (V(V)) na dobro istraženoj filamentoznoj gljivi, *Phycomyces blakesleeanus*, značajno je za utvrđivanje mehanizama internalizacije i metabolizma vanadijuma. Iako *P. blakesleeanus* nije zemljišna gljiva, njen kratak životni ciklus i evoluciona pozicija u bazi carstva gljiva čine je odličnim modelom za proučavanje interakcija gljiva sa vanadatom i drugim metalima, što može dovesti do poboljšanja primene gljiva u bioakumulaciji vanadijuma i remedijaciji kontaminiranih zemljišta. U ovom istraživanju smo proučavali efekte tri koncentracije vanadata (1 mM, 5 mM i 10 mM) na enzimske i neenzimske komponente antioksidativnog sistema micelije u različitim fazama rasta. Proizvodnja ROS (reaktivnih kiseoničnih vrsta) bila je najviša u eksponencijalnoj fazi, ali je redukujući kapacitet održan zahvaljujući visokom nivou neenzimskih antioksidanata kao što su fenoli i glutation. U stacionarnoj fazi, redukujući kapacitet micelije je donekle bio narušen prisustvom vanadata (V(V)), ali se oporavio zahvaljujući delovanju glutationskog sistema i aktivaciji peroksidaza. U kasnoj eksponencijalnoj/ranoj stacionarnoj fazi nije primećena značajna aktivacija antioksidativnih sistema, i redukujući kapacitet je bio narušen. Kada uzmemo u obzir efekte V(V) na rast micelije, ovo je bila jedina faza sa smanjenom vitalnošću, dok je stopa rasta u eksponencijalnoj i stacionarnoj fazi bila nepromenjena, ako ne i povećana. Ovo istraživanje pruža uvid u toleranciju micelije *P. blakesleeanus* na vanadat, čak i pri visokim koncentracijama, do 10 mM, što čini ovu gljivu dobrim kandidatom za bioakumulaciju V(V).

Ključne reči: micelija, reaktivne kiseonične vrste, fenolna jedinjenja, antioksidativni enzimi, glutation