INFLUENCE OF CARBON SOURCES ON THE ACTIVITY OF BIOCHEMICAL INDICATORS OF OXIDATIVE STRESS IN SAPROPHYTIC FUNGUS *RHIZOPUS NIGRICANS*

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Abstract

Reactive oxygen species are derived from molecular oxygen used in respiration and they are capable of damaging cellular components, including proteins, lipids and DNA. Oxidative stress is caused by an imbalance between production of reactive oxygen species and the ability of biological system to detoxify reactive intermediates or to repair the inflicted damages. The purpose of this paper is the determination of the antioxidant potential in saprophytic fungus *Rhizopus nigricans*. It was determined the influence of carbon sources, represented by grinded cereal caryopses, and their concentration from culture medium on the fungus capacity to synthesize antioxidant enzymes like catalase and peroxidase. Enzymatic assays were performed at three time intervals: 5, 10 and 15 days, using both fungus mycelium and culture liquid. After analyzing the results we can point out a correlation between the nature and concentration of carbon source, the age of fungal culture and the production of oxidoreductases. In the first time period catalase and peroxidase production is maintained at low levels, but with depletion of nutrients and accumulation of toxic metabolic byproducts a significant increase takes place in the second time period. The last time period corresponds with the entering in decline phase of culture and with drastic decrease in production of both enzymes.

Key words: catalase, peroxidase, oxidative stress, *Rhizopus nigricans*, cereal caryopses.

The saprophytic fungus *Rhizopus nigricans* is an obligate aerob that is frequently found in decaying organic matter rich in complex carbohydrates. This organism has the ability to thrive in such environments because of simple growth requirements and the capacity to produce various hydrolytic enzymes (Skory C.D., *et al.,* 2009). Because it is an agent of decay, it produces significant damages during storage and transportation of perishable fruits and vegetables. It produces food rotting and may elaborate mycotoxins (Dan V., 1999, quoted by Tanase C., Șesan T.E., 2006). This fungus belongs to storage mycobiota and may affect cereal grains stored under improper temperature and humidity conditions.

The major effects of storage fungi on cereal grains consist in decrease of germinative faculty, discoloration, warming and rotting, loss of dry matter, production of mycotoxins and nutritional changes. Depending on severity, infestation with fungi may affect quality and can completely destroy their usefulness (Meronuck, R.A., 1987). Testing the sensitivity of filamentous fungi *in vitro* is becoming more important because of the frequency and diversity of infection caused by them (Meletiadis J., *et al*., 2001).

It is known that oxygen can have toxic effects on microorganism that grow aerobically, especially because of the threat arising from the formation of reactive oxygen species (ROS) (Bai Z., *et al.,* 2003). All aerobic organisms use molecular oxygen for respiration or nutrients oxidation to efficiently obtain energy, molecular oxygen being reduced to water by accepting four electrons. During the reduction of molecular oxygen several reactive oxygen species are formed, by accepting one, two or three electrons in order to form superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH). These reactive oxygen species attack almost all cellular components and occasionally causes lethal cell lesions (Inoue Y., *et al*., 1999).

Reactive oxygen species represent a variety of molecules and free radicals (chemical species with one unpaired electron) derived from molecular oxygen. Molecular oxygen in the ground state is a bi-radical, which contains two unpaired

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electrons on the outside (also known as triplet state). Because the two electrons have the same spin, oxygen can react with only one electron at a time and therefore it is not very reactive. On the other hand, if one of two unpaired electrons is excited and changes its spin (also known as singlet oxygen), it becomes a powerful oxidant because the two electrons with opposing spins can quickly react with other electrons (Turrens J.F., 2003).

Oxidative stress is caused by an imbalance between the production of reactive oxygen species and the ability of the biological system to detoxify reactive intermediates or repair the inflicted damages.

Hydrogen peroxide is an inevitable byproduct of all living organisms that rely on respiration for energy production, the mithocondrion being the main place of its production (Qiang Li, *et al.*, 2008). The hydrogen peroxide cytotoxicity is given by its ability to cause damage to macromolecules, being compared with other reactive oxygen species is less toxic, but is able to diffuse into other compartments from his place of production (Branco M.R., *et al.*, 2004). H_2O_2 can oxidize Fe-S centers or cysteines in certain proteins or can react with transition metals and produce hydroxyl radical that is able to oxidize any cell molecule, causing DNA damage, protein inactivation and fragmentation and lipid peroxidation (Aguirre J., *et al.*, 2006).

Because reactive oxygen species are common in aerobic organisms, these have enzymatic and non-enzymatic systems for protection (Yu B.P., 1994). All forms of life maintain a reducing environment within their cells. This reducing environment is accomplished by enzymes that maintain the reduced state trough a constant supply of metabolic energy. Disorders of the normal redox state can cause toxic effects by producing peroxides and free radicals that damage cellular components, including proteins, lipids and DNA.

Cells contain a number of mechanisms that maintain low ROS levels, that collectively constitute the antioxidant response. For defense against reactive oxygen species, cells contain antioxidant enzymes such as superoxide dismutase, catalase, peoxidase (Izawa S., *et al*., 1996). Non-enzymatic, defensive systems include vitamins E and C and antioxidants with low molecular weight (Fridivich I., 1995, Abrashev R.I, 2008), pigments (carotenoids, melanin, etc.), phenolic compounds and proline (Belozerskaya T. A., Gessler N.N., 2007). Hydrogen peroxide is enzymatically catabolised in aerobic organisms by catalase and peroxidase, enzymes that act synergistically to protect cells from high concentration of H_2O_2. Both enzymes belongs to hemoprotein class, catalase (EC 1.11.1.6) performs hydrogen peroxide degradation with release of molecular oxygen (Kurakov A.V., *et al*., 2001), and peroxidase (EC 1.11.1.7) catalyzes the dehydrogenation of many organic compounds as: phenols, aromatic amines, hydroquinone, especially benzidine derivates. Detoxification of hydrogen peroxide is fundamental aspect of cellular antioxidant response in which catalase and peroxidase play a major role (Kuwasaki L, Aguire J., 2001).

The objective of this study is to monitor in time the evolution of biochemical indicators of oxidative stress: catalase and peroxidase in *Rhizopus nigricans* under the influence of various carbon sources represented by grinded cariopses from three cereal species: wheat, corn and barley. Studies regarding the influence of exogenous factors on the enzymatic activity were performed in the Microbiology Department of Biological Research Institute, among which we mention: Manoliu Al., *et al*., 2005, 2006, 2010.

MATERIALS AND METHODS

The species we chose to conduct our study was *Rhizopus nigricans*, fungus isolated from germinated cereal caryopses. We used three species: wheat, corn and barley. Wheat and corn grains were taken from the storage place of the Enterprise of Cereal Products from Chişinău, Republic of Moldova, and the barley grains were taken from a private household in Greblești village, from the Strășeni aria, Republic of Moldova. Pure culture was maintained on solid medium PDA (potato, dextrose,agar).

To determine the dynamic of catalase and peroxidase was used Leonian liquid medium with following composition: K_2HPO_4 1,25 g, $MgSO_4$. $7H_2O$ 0,625 g, peptone 1 g, glucose 20 g, distilled water 1000 ml (Constantinescu O., 1974). The composition of culture medium was modified by replacing the carbon source – glucose, with different amounts of grinded cereal caryopses, resulting in the final 3 medium variants: V1=10 g/l, V2=20 g/l,V3=30 g/l, plus a control version in which the composition of medium remained unmodified. Medium was distributed in amounts of 100 ml in Erlenmeyer flasks. In each flask were inoculated slices of 8 mm in diameter from a 5 days old culture of *Rhizopus nigricans*. The flasks were incubated in thermostat set at 28°C.

Enzyme assay was made at three time intervals: 5, 10 and 15 days, using both mycelium and culture liquid. The determination of catalase activity was performed using Sinha method (Artenie Vl., *et al*., 2008) and the determination of peroxidase was made on the basis of ortho-dianisidine method (Cojocaru D.C., 2009). The activity of both enzymes was reported to the

amount of soluble proteins determined by Bradford method (Artenie Vl., *et al*., 2008).

RESULTS AND DISCUSSIONS

The three cereal species used as carbon source: wheat, corn and barley falls in the category of the main cereals cultivated on large areas of the globe, cereal grains representing basic food for almost entire population of the world. Complex composition of cereal caryopses: starch, dextrins, simple glucides, proteins (prolamins, glutelins, albumins and globulins), lipids, cellulose, minerals - K, Ca, Fe, P, Mg, Se, Na, Cu, Mb, Mn, and B vitamin complex (B1, B2, B5, B6), vitamins PP, E, K, H, and lower amounts of vitamin A, amino acids (lysine, tryptophan, methionine, leucine, valine, histidine, leucine etc.) (Cordain, L., 1999; Starodub, V., 2008), influences the enzymatic activity of the fungus because some essential nutrients that participate together with enzymes in antioxidant processes (Halliwell B. and Gutteridge J.M.C., 2007, Sarikurkcu C. *et al*., 2010). Phytonutrients such as beta carotene and vitamins (especially E and C) are well known for their protective effects against ROS and presence of some metals, particularly iron and copper ions can cause hydroxyl radical formation by interaction of superoxide radical with hydrogen peroxide (Wu D., Cederbaum A.I, 2003), metals can also act as cofactors for many enzymatic reactions and may function as structural components in protein (Somerville G.A., and Proctor R.A., 2009).

The results on catalase activity in the mycelium of fungus *Rhizopus nigricans*, grown on media with various concentrations of caryopses from three cereal species: wheat, corn and barley, are illustrated in Figure 1. In the first time period in all experimental variants values are maintained at low levels, but in terms of carbon source nature in the medium variants with grinded wheat caryopses enzymatic activity is slightly higher. There are no sizable differences between work versions, except the variants supplemented with wheat, where catalase activity increases inversely with the concentration of grinded wheat caryopses contained in the culture medium. Maximum value was recorded in variant V1 wheat – 353.624 UC/mg protein, and the minimum value was recorded in version V2 barley – 3.174 UC/mg protein.

In the second period enzymatic activity has significantly increased, the highest values being maintained in variants with wheat caryopses (values ranging between 477.816 CU/mg protein and 636.61 UC/mg protein). As in the first studied period there are no significant differences between versions with the same type of grinded caryopses.

At 15 days after inoculation, along with the ageing of culture, values slightly decreases in variants with wheat, but still remaining the highest compared with other two cereal species (V1 – 418.209 UC/mg protein, V2 – 300.008 UC/mg protein, V3 – 313.815 UC/mg protein). In medium versions with barley and corn enzymatic activity is slightly increased compared to activity recorded in the second period.

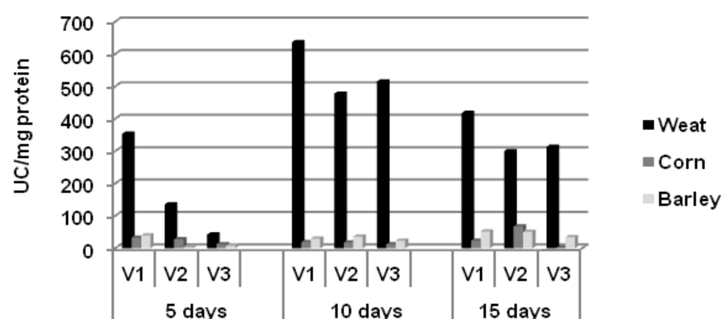

Figure 1 **The influence of carbon sources on catalase activity in mycelium of fungus *Rhizopus nigricans***

After the interpretation of data, in culture liquid of fungus, it was observed that catalase activity tended to zero or even could not be detected in most experimental variants (Fig. 2), except variants V1 (30.495 UC/mg protein), V2 (0.169 UC/mg protein) with maize in the first period, and V1 wheat (20.357 UC/mg protein), V2 corn (0,375 UC/mg protein) at 15 days after inoculation, where recorded values were relatively small, close to zero. This suggests that for the fungus *Rhizopus nigricans* grown on different carbohydrates sources, catalase biosynthesis occurs at intracellular level, its excretion in liquid culture is achieved mainly due to cell lysis caused by reactive oxygen species attack or toxic metabolic compounds for fungal cell accumulated in the extracellular space or due activation of cell apotosis followed by cell lysis and death with periplasmatic release of enzyme and other cellular constituents.

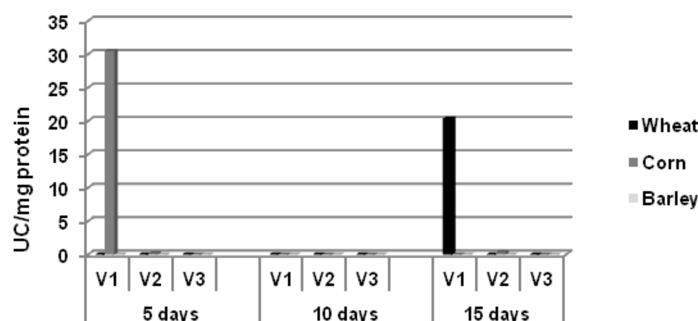

Figure 2 **The influence of carbon sources on catalase activity in culture liquid of fungus *Rhizopus nigricans***

Peroxidase activity assayed at three time intervals in mycelium of fungus *Rhizopus nigricans* is depicted in Figure 3. At 5 days after inoculation recorded values in all variants are rather low, the maximum being recorded in version V2 wheat (0.1498 UC/mg protein) and the minimum value in version V1 barley (0.0046 UC/mg protein). There is no certain correlation between the amount of cereal caryopses contained in medium and the peroxidase activity, except barley samples, where peroxidase activity increases with the increase of grinded caryopses amount, and except corn samples where enzymatic activity increases inversely with the amount of grinded caryopses from culture medium. Depending on the nutrient substrate, the highest values are recorded for work versions with wheat caryopses (V1 – 0.1404 UP/mg protein, V2 - 0.1498 UP/mg protein, V3 – 0.1187 UP/mg protein).

In the second study period, along with the culture ageing, nutritional resources depletion and accumulation of secondary toxic compounds in the culture medium, recorded values are slightly elevated, the highest values being maintained also in samples that contain wheat caryopses. There are no significant differences between work versions with the same nutrient background, the maximum being recorded in version V1 wheat (0.154 UP/mg protein), and the minimum in version V1 barley (0.0101 UP/mg protein).

At 15 days after inoculation occurs a drastic decrease in peroxidase activity in all medium variants, regardless of carbon source. Maximum value was obtained for variant V3 wheat (0.0995 UP/mg protein), and the minimum value was recorded at version V3 barley (0.0068 UP/mg protein).

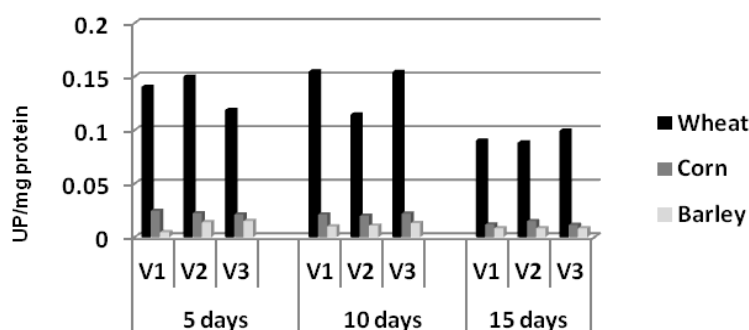

Figure 3 **The influence of carbon sources on peroxidase activity in mycelium of fungus *Rhizopus nigricans***

Starting from premise that fungi are able to excrete various synthesized substances, with different roles, in the external environment, including enzymes, we have resort to enzyme assay in liquid culture, obtained values being plotted in Figure 4. Similarly as in mycelium, recorded values from culture liquid of the fungus are maintained at low levels in all three periods of study, leading to conclusion of mostly endocellular biosynthesis of the enzyme, likewise catalase. Highest values are recorded in variants supplemented with wheat, and lowest in those with corn. In first time interval values obtained from working options underscores the influence of grinded caryopses concentration on peroxidase activity, metabolizing of this substrate is correlated with the release of reactive oxygen species. In medium versions with wheat caryopses (V1 – 0.1367 UP/mg protein, V2 – 0.1113 UP/mg protein, V3 – 0.0868 UP/mg protein) and corn caryopses (V1 – 0.01 UP/mg protein, V2 – 0.0105 UP/mg protein, V3 – 00132 UP/mg protein)

enzyme activity increases inversely with the concentration of substrate used by fungus for growth and development, and for work versions with barley caryopses it increases with amount of barley caryopses which supplemented culture medium (V1 – 0.0166 UP/mg protein, V2 – 0.0093 UP/mg protein, V3 – 0.0079 UP/mg protein).

In the second period under study this correlation between enzyme activity and concentration of carbon source is maintained in variants supplemented with wheat and barley caryopses, while after 15 days of incubation takes place a decrease in peroxidase activity in all variants regardless of carbon source used, except variant V1 with wheat, where peroxidase activity still has a high amplitude (0.10729 UC/mg protein).

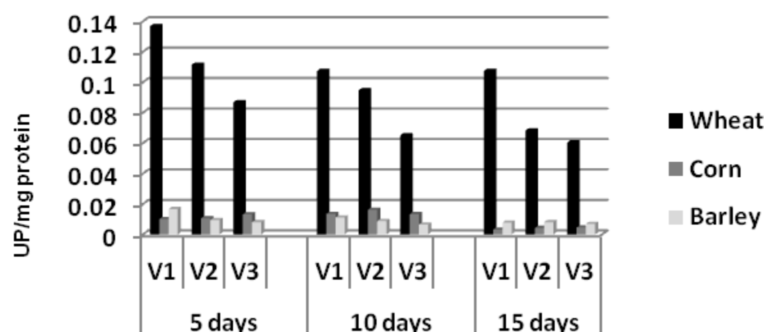

Figure 4 **The influence of carbon sources on peroxidase activity in mycelium of fungus *Rhizopus nigricans***

The high catalase and peroxidase activity in wheat samples might explain due the diversity of chemical composition of carbon sources used. For example, wheat grains contain copper and iron, and free ions of this metals could interact with H_2O_2 (Fenton reaction) to form a highly reactive radical OH (Sigler K., *et al.*, 1999), in addition wheat caryopses are poor in vitamin A, and the lack of vitamin A can lead to increased production of reactive oxygen species (Haw-Jyn Chiu, *et al.*, 2008). Instead corn grains are rich in phenolic compounds, contain carotenoid pigments (zeaxanthin, cryptoxanthin, carotene, lutein) and vitamin E that are part of non-enzymatic defense equipment, and may act together with catalase and peroxidase to counteract oxidative stress (Kähkönen M.P., 1999; Huda-Faujan N., *et al.*, 2009; Wong J.C., *et al.*, 2004; Rocheford T.R., 2002). Barley grains also contain considerable amounts of phenolic compounds.

CONCLUSIONS

● The nature of nutrient substrate strongly influenced enzyme activity, thus the highest values of catalase and peroxidase activity were recorded in medium variants supplemented with wheat grains, and the lowest values were observed in medium variants containing grinded barley grains.

● Nutrient substrate concentration which supplemented medium variants did not significantly influenced the catalase and peroxidase activity, not being recorded significant differences between variants.

● Catalase biosynthesis is endocellular and not extracellular at *Rhizopus nigricans*, because in culture liquid of the fungus catalase activity tended to zero or could not be detected in most experimental variants.

● Oxidative stress enzyme (catalase and peroxidase), regardless of carbon source used, had the highest activity in the second study period - at 10 days after inoculation, along with culture ageing and accumulation of metabolic secondary compounds, in the first time period values were maintained at low levels, and in last time period a decrease in both enzymes activity has been noted.

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