RESEARCH REGARDING THE DYNAMICS OF SOME BIOCHEMICAL MARKERS OF OXIDATIVE STRESS AT *MONILINIA LAXA* (ADRH. & RUHL.) HONEY CULTIVATED ON DIFFERENT AMINO ACIDS ENRICHED MEDIA

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Abstract: Antioxidants that make up the defense for Ascomycetes still arouses a major interest because of their hypothetical role as virulence and aggression factors and also as the enzymes that play a key role in cellular defense against ROS produced during microbial metabolic activity. A study of catalase and peroxidase activity dynamics of the species *Monilinia laxa* (Aderh & Ruhl.) Honey cultivated *in vitro* on medium supplemented with different amino acids was conducted in order to know the biology of the fungi responsible for the appearance of brown rot at various species of stone fruits. We used for this purpose the Leonian medium (in the formula changed by Bonnar), in each variant being added 0, 125 mg of the following amino acids: alanine, glutamic acid, asparagine, aspartic acid, cystine, cysteine, phenylalanine, histidine, valine, lysine, serine, methionine and leucine. We also used a control variant, without amino acids, in final resulting 14 working versions. To determine the catalase activity Sinha method was used, to monitor the peroxidase activity we used Möller method and the experimental measurements carried out at two intervals, were made both of fungus mycelium and culture fluid. We found notable differences in the activity of two enzymes, microbial culture induced both by the age of the culture medium and the type of amino acid introduced in it.

INTRODUCTION

Ubiquitous organisms, filamentous fungi have a high adaptive plasticity to different environmental changes, one of the fundamental requirements of these microorganisms for surviving is the need to adjust their activities in terms of an aerobic lifestyle, as the metabolic performance in such circumstances is followed by the emergence of reactive oxygen species and, hence, the need for it`s proper management, its absence can lead toward apoptosis and death of eukaryotic cell due to so-called oxidative stress (Avery, S.V. *et al*., 2008). Defined as being an imbalance between reactive oxygen species production and the biological system`s ability of rapid detoxification, followed by the repair and removal of damaged parts resulting from their activity, oxidative stress is characterized by disturbances in the normal redox state of the cells, capable of causing toxic effects by peroxides and free radicals production, equipped to destroy cellular components, including proteins, lipids and DNA (Sies, H., 1991, Dean R.T. *et al*., 1997, Esser, K. and Kues, U., 2006) .

The ways in which organisms are protected from the aggression of reactive oxygen species are related to cell compartmentalisation, to the ability to elaborate adaptive responses inducible in oxidative stress conditions, to repair and turnover processes that help to minimize the damage that occures from reactive oxygen species attack or by the existence of hydroxyl radical scavenger that captures a series of hidroxil radicals, superoxides and organic radicals, which chelate metal ions and prevent certain chemical reactions toxic for the organism, the so-called preventive antioxidants (Gadd, G.M., 2001) and, last but not least, the protection afforded by antioxidant compounds and enzyme systems. The protective enzyme system of eukaryotic cells include: superoxide dismutase, catalase, glutathione peroxidase, glutathione transferase, glutathione reductase and glucose-6-phosphate dehydrogenase, these enzymes having a predominantly intracellular localization, the extracellular environment being more exposed to radical attack (Bai, Z. *et al*., 2003, Li, Q. *et al*., 2009).

Present in the medium, some amino acids regulate the activity of fungal enzymes, including the oxidoreductases (Subramanian, K.N. *et al.,* 1968), data from literature indicating that interference between amino acids and the medium are responsible for the catalase inhibition, and the presence of arginine in the culture medium inhibits the catalase activity (Frederick, J.R. *et al*., 2001). Also, experimental studies show the ability of oxidative decarboxylation of some amino acids such as serine, alanine, phenylalanine, tryptophan and methionine. In the presence of dihydrofumarate, this oxidoreductase catalyzes the hydroxylation of various aromatic compounds and reduces nitrate in the presence of some specific donors (Fear, 1976, quoted by Roşu, C.M., 2007). *In vitro* experiments showed that the activity and the thermostability of peroxidase depend, in many cases, by their interaction with amino acids such as proline, tryptophan, valine, β-alanine (Bakardjeva, N. *et al.,* 1999). Shtarkman, I.N. *et al*, 2007 demonstrated that some amino acids present in the medium (methionine, cystine, tyrosine, tryptophan, phenylalalnine, lysine, leucine, arginine and proline) are responsible for protecting the intracellular DNA against damage caused by reactive oxygen species under a moderate oxidative stress, complementing the antioxidant enzyme defense system activity in the eukaryotic cells.

Same authors complete the recent studies that aimed the research on the formation of reactive oxygen species in aqueous solutions, indicating that vary physical factors makes these mediums chemical reactive, the biological molecules,

in their turn, became the sensors of the processes taking place in aqueous mediums and thus, in agreement with the concepts of the day, the reactive oxygen species formed in the cellular and intercellular space plays an ambiguous role, causing on the one hand, damage to biological structures such as DNA, lipids, proteins, etc.. during oxidative stress, and on the other hand, ROS present in specific physiologic concentrations plays an important role as signals, showing the extent to which the liquid medium is suitable for maintaining vital activity and redox regulation of various cellular functions.

This paper is intended to be a time monitoring activity for some biochemical markers of oxidative stress such as catalase and peroxidase in the fungus *Monilinia laxa* (Aderh. & Ruhl.) Honey, parasitic on various varieties of *Prunus* sp. and *in vitro* cultivated on media supplemented with different amino acids.

MATERIALS AND METHODS

The *Monilinia laxa* strain was isolated from mummified fruit harvested from different varieties of *Prunus domestica*, incubated on PDA medium for 7 days and used as inoculum in the Leonian medium (Constantinescu, O., 1974) supplemented with 0.125 mg from the next amino acids: alanine, glutamic acid, aspartic acid, asparagine, cystine, cysteine, phenylalanine, histidine, methionine, valine, lysine, serine, leucine, and we also used a control sample, without amino acids. The cultures were maintained submerged in the dark, at a temperature of 28^0C, and the sampling for biochemical determinations was made at intervals of 7 and, respectively, 14 days after the seeding of the culture medium. The investigations were carried out on biomass and the supernatant resulted from the centrifugation of the culture medium. The methodological support for monitoring the activities of both oxidoreductase have been Artenie Vl. *et al*., 2008 for catalase and o-dianisidine method for peroxidase (Cojocaru D.C., 2009).

RESULTS AND DISCUSSIONS

The *Monilinia laxa* has the ability to biosynthesize catalase in submerged cultures, experimental determination of its activity in the fungus mycelium and in the liquid culture confirming its existence in both time intervals, in all medium variations.

The critical analysis of the graphic details about the dynamics of intracellular catalase activity during growth and development of the mycelium of *Monilinia laxa* (Aderh. & Ruhl.) Honey, as they appear in Figure 1, provides a picture of the trend curves of variation of the enzyme in all variants environment enriched with amino acids (including amino acid-free sample), except the asparagine supplemented culture, where the evolution in time had an descending allure, down from 202.9916 µmoli hydrogen peroxide/min. up to 80.8772 µmoli hydrogen peroxide/min.

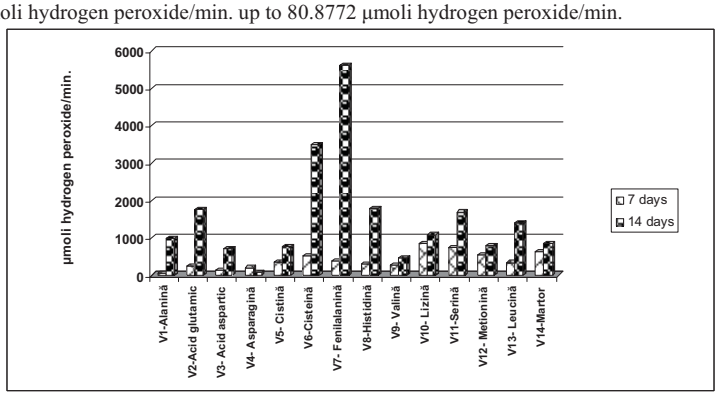

Fig. 1- The dynamics of catalase activity in mycelium of *Monilinia laxa* (Aderh. & Ruhl.) Honey species cultivated on media supplemented with different amino acids

One can appreciate that phenylalanine was responsible for the increased biosynthesis rate and the high catalase activity between the two time intervals. The enzyme present in the mycelium illustrates an ascension at a value of 389, 0312 µmoli hydrogen peroxide/min. at 5608.1527 µmoli hydrogen peroxide/min. while cysteine, present in the culture medium, had a variation curve in the two time intervals ranging from maximum 520.0003 µmoli hydrogen peroxide/min. tu minimum 3502, 9601 µmoli hydrogen peroxide/min. The addition of histidine itself undoubtedly influenced the dynamics of enzyme in a progressive direction, moving it to a minimum of 287.8543 µmoli hydrogen peroxide/min. to a maximum 1785.0845 µmoli hydrogen peroxide/min. recorded at 14 days after the inoculation of the culture medium. The same was noted in the mycelia mass developed on the liquid medium enriched with glutamic acid, the catalase having antioxidant effects with a threshold registered at the first determinations interval of 238.7318 µmoli hydrogen

peroxide/min. ., the peak line moving from 1756, 7359 µmoli hydrogen peroxide/min. in the second period of enzymatic determinations. Serine caused a progressive dynamic in time for the endocellulare catalase, the value of 731.1005 µmoli hydrogen peroxide/min. acquiring an additional activity, amounting to 1690.7072 µmoli hydrogen peroxide/min. Serine is essential for catalase activity because, along with a water molecule, formes a hydrogen bonding system, an hydrophobic substrate channel with a narrow base that allows small peroxides to quickly spread at the enzyme active site, guiding histidine located at the C terminus end of catalase N-terminus point. Thus, the histidine imidazole ring, unlike the other hemoprotein in which it is parallel to the heme plane, in the catalase the orientation is parallel to it, which favors it energetically, because intensive interactions of π-π type between the imidazole ring of histidine and porphyrin lower the reactivity of the compound with the reduced substrate (Fita, I. and Rossman, M.G., 1985, Zamocky, M. and Koller, F., 1999).

Leucine induced a similar behavior, the dynamic variation curve going from 341,949 µmoli hydrogen peroxide/min. at 1412.0509 µmoli hydrogen peroxide / min., and alanine caused the emergence of a pattern of behaviour similar to the enzyme, its values moving from one minimum noted at 7 days of 47.9643 µmoli hydrogen peroxide/min. to an upper limit of 998.9287 µmoli hydrogen peroxide/min. at registered 14 days. Alanine, as glutamine, is essential for increasing air hyphae, glutamine and alanin transaminase have an active feature for aerial hyphae (Cárdenas, M.E. and Hansberg, W., 1984), and catalase activity, as a consequence of hiperoxidant status is high during their growth and during the formation of conidia, given that activity in the conidia is 60 times higher than in the mycelium developed in liquid culture medium (Avery, S.V. *et al.,* 2008). Given the fact that the endoenzime activity is related to increased synthesis during the maturation and germination of conidia (Gessler, N.N. *et al.,* 2007), the biosynthesis of the enzyme usually increases at the end of exponential growth phase and early hyphae aggregation (Michán, S.H.. *et al.,* 2002), when the spore biosynthesis begins and, also the cell differentiation and the fungus enters the stationary phase (Hansberg, W. *et al.,* 1993). The oxidoreductase dynamics as an expression of physiological response of the fungal cell to oxidative stress imposed by the presence of amino acids in submerged culture is closely related to the establishment, stabilization, magnitude and metabolism consequences of an aging mycelia culture, the enzyme being a complex mechanism of control for reactive oxygen species, acting at different levels, just to prevent retrograde adaptive responses in the fungal cell and genomic instability.

The fluctuations in the catalase dynamics in the culture fluid indicate several different behaviors of extracellular enzyme activity in the two experimental determinations. So, we note such a situation in which the enzyme had an infinitesimal variation while under the influence of culture medium supplementation with phenylalanine - while remaining practical almost constant at values of as 136.2484 µmoli hydrogen peroxide/min. respectively, 136 099 µmoli hydrogen peroxide / min.

In other cases, the enzyme had increased in value over time, smaller in the environments with added cysteine (from 187.1649 µmoli hydrogen peroxide / min. at 207.6749 µmoli hydrogen peroxide / min.), histidine (from 110.1101 µmoli hydrogen peroxide / min. to 122 647 µmoli hydrogen peroxide / min.), cystine (from 113.9688 µmoli hydrogen peroxide/min. at 158.5504 µmoli hydrogen peroxide/min. , average ups (of about three times) from glutamic acid (from 30.4856 µmoli hydrogen peroxide/min. at 93.1739 µmoli hydrogen peroxide/min. nearly four times more in the case of aspartic acid - from 44.5003 µmoli hydrogen peroxide/min. at 168.4431 µmoli hydrogen peroxide/min. The largest variation curve of catalase activity in liquid culture of *Monilinia laxa* species was recorded in the medium variant enriched with alanine where, although as value, the enzyme activity was low, while the width has increased by about 13 times - from 6.9808 µmoli hydrogen peroxide/min. at 91.7467 µmoli hydrogen peroxide/min. We also found that there were various degrees decreases of the enzyme activity in the extracellular space, the largest variation curve in this direction is noted in the variant without amino acids, where catalase activity in liquid culture dropped six times, going 248.1864 µmoli hydrogen peroxide/min. at a level to 39.5022 µmoli hydrogen peroxide/min. The same significant decrease, similar to the ones registered in the supernatant version control sample were induced by the presence of leucine at the start of the submerged culture, with serine, methionine, asparagine and valine.

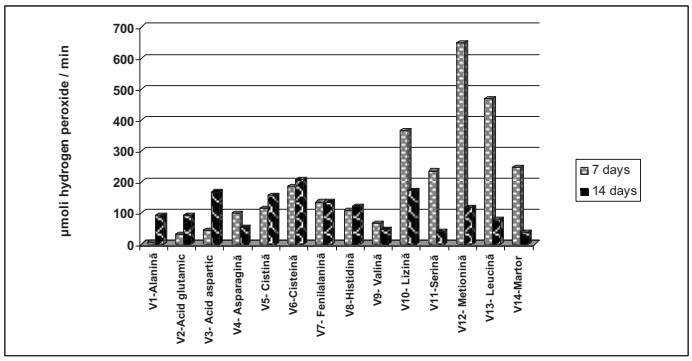

Fig. 2- The dynamics of catalase activity in culture liquid of *Monilinia laxa* (Aderh. & Ruhl.) Honey species cultivated on media supplemented with different amino acids

The peroxidase easily operates on low concentrations of hydrogen peroxide, unlike catalase, which has a lower efficacy in such conditions. The experimental results on the modulator effect of amino acids at *Monilinia laxa* (Aderh & Ruhl.) Honey species on peroxidase activity, both at 7 and 14 days after inoculation revealed the activity of this oxidoreductase, its varies depending on the type of amino acid source and on the age and culture mycelia.

In order to achieve a clear image of the in vitro activity of this oxidoreductase in the mycelium of the fungus, we quantified the data from the experiments carried out and the graphical representation of their average values as seen in Figure 3.

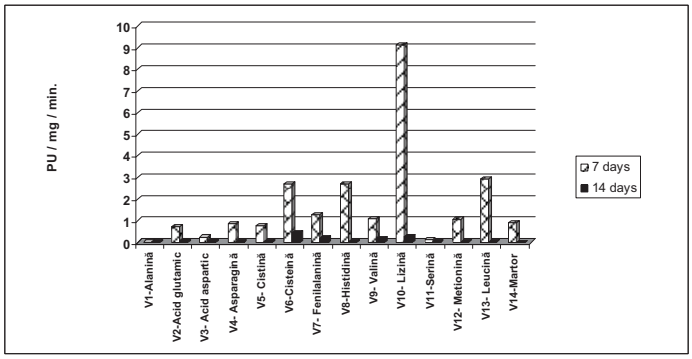

Fig. 3- The dynamics of peroxidase activity in mycelium of *Monilinia laxa* (Aderh. & Ruhl.) Honey species cultivated on media supplemented with different amino acids

Lysine plays a constitutive role in the peroxidase molecule, but the metabolic role of this amino acid apears is the oxidative damage mediated by L-lysine-α-oxidase in aqueous medium that leads to the formation of hydrogen peroxide (Lukasheva, E.V. and Berezov, T.T., 2002 , Gomez, D.P. *et al*., 2006). Perhaps this explains the very high intensity of peroxidase activity at 7 days after the sowing of the species mycelium - 9.1424 PU/ mg / min. (ten times higher than the enzyme activity in the control version). Leucine was responsible for the biosynthesis of the enzyme to three times higher than in the version without amino acids - PU 0.9109 / mg / min. We found the intervention of peroxidase in the fungus mycelium developed on medium with histidine and cysteine (2.7193 PU / mg/min respectively, 2.7085 PU / mg / min.) and that the endoenzyme activity was tripled compared to that of control sample, possibly because the culture physiological needs to balance the energy consumption requires a major involving for the redox processes. During the phenylalanine oxidation in eukaryotic organisms, the production rate of hydrogen peroxide is increased (Sauret-Ignaz, G.*et.al.,* 2007).

Peroxidase displays high activity for the substrate represented by phenylalanine, this amino acid being oxidative deaminated via phenylpropanoids pathway with trans-cinnamic acid formation, an intermediate in the phenols biosynthesis (Dixon, R.A. and Paiva, NL.., 1995). Peroxidase is, with polyphenoloxidase responsible for their oxidation. In mycelium of *Monilinia laxa* grown in submerged culture with phenylalanine, the peroxidase rate of production after 7 days of incubation showed a level of value -1.2873 PU / mg / min. By comparison, close oxidoreductase levels were

recorded in the mycelium of the fungus developed on media supplemented with valine and methionine (1.0792 PU / mg / min., respectively, 1.0742 PU / mg / min.). Peroxidase was slightly inhibited in cultures enriched with cystine, asparagine and glutamic acid (0.7861 PU / mg / min, in version V5, PU 0.8519 / mg / min., in the variant V4, 0.7443 PU / mg / min respectively. in version V2. This effect is not necessarily due to the existence of a low rate of cystine oxidation by hydrogen peroxide, for example, in the reaction catalyzed by peroxidase (Stelmaszynska, T. and Zgliczynsky, JM, 1963), but given that this enzyme operates at low concentrations of hydrogen peroxide, reducing its activity or its inactivation occurs when you exceed a certain concentration of the substrate. The same explanation can be given to values recorded by the oxidoreductase in the mycelium grown on media with aspartic acid (0.2470 PU / mg / min.), serine (0.1465 PU / mg / min.) and alanine (0, 0164 PU / mg / min.).

Amplitude variation levels found in the values of peroxidase activity reports made from the mushroom mycelium after 14 days from inoculation of culture media enriched with various amino acids, was different depending on the type of amino acid used, the intensity of oxidoreductase activity in this time interval that is much different from that recorded after 7 days of incubation, in the sense of an enzyme activity reduction in the mycelium. This effect is not due to the existence of a necessarily low oxidation rate, for example, for cystine by hydrogen peroxide in the reaction catalyzed by peroxidase (Stelmaszynska, T. and Zgliczynsky, JM, 1963), but given that this enzyme operates on low concentrations of hydrogen peroxide, reducing its activity or inactivation occurs when you exceed a certain concentration of this substrate or when excessive acidification of the culture medium destabilizes the enzyme structure, inactivating it.

The last stage of the study was the research on the influence that amino acids have on the peroxidase activity in liquid cultures for *Monilinia laxa* (Aderh. & Ruhl.) Honey species, when it was found that the enzyme has different fluctuations over time, its activity being dependent of the nature of the amino acid introduced in the culture medium, as can be seen in Fig. 4, the strongest incentive effect after 7 days of incubation being induced by aspartic acid, asparagine and cystine, and after 14 days by phenylalanine and aspartic acid and that the exoperoxidase dynamics complements the exocatalase one, these two oxidoreductase being significantly marked by oxidative stress generated by the presence in the environment of amino acids as a source for carbon and nitrogen.

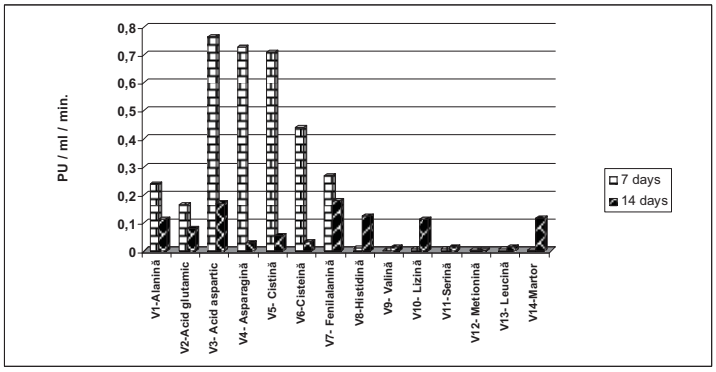

Fig. 4- The dynamics of peroxidase activity in culture liquid of *Monilinia laxa* (Aderh. & Ruhl.) Honey species cultivated on media supplemented with different amino acids

CONCLUSIONS

The studies on the influence of amino acids on catalase and peroxidase activity in the mycelium and the supernatant of *Monilinia laxa* species showed that the activity of the two oxidoreductase was significantly influenced by the amino acid introduced in the medium and by the age of culture.

After 7 days of incubation, the catalase activity in the mycelium was stimulated by lysine and serine and in the 14 days old mycelium the enzyme biosynthesis was stimulated by phenylalanine, cysteine, histidine, glutamic acid, alanine, serine, leucine and lysine. The catalase biosynthesis efficiency in fluid culture medium of *Monilinia laxa* species after 7 days after inoculation was increased in the samples with methionine, leucine and lysine, while in the supernatant of the aged culture, all the studied amino acids had a stimulating effect.

In the young mycelium, aged for 7 days, lysine, leucine, histidine, cysteine phenylanine, methionine and valine induced a very high peroxidase activity and in the aged culture all amino acids increased, to varying degrees, the oxidoreductase activity. After 7 days of incubation in the culture supernatant peroxidase activity was highly stimulated by aspartic acid, asparagine, cystine, cysteine, phenylalanine, alanine and glutamic acid, while in liquid culture at the age of 14 days, the biosynthesis of the enzyme was positively influenced by phenylalanine, aspartic acid and histidine.

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