STUDIES CONCERNING THE INFLUENCE OF SOME OLIGOELEMENTS ON THE ACTIVITY OF KREBS CYCLE DEHYDROGENASES AT *MONILINIA LAXA* (ADERH.& RUHL.) HONEY PARASITE ON PLUM TREES

CERCETĂRI PRIVIND INFLUENȚA UNOR OLIGOELEMENTE ASUPRA ACTIVITĂȚII DEHIDROGENAZELOR CICLULUI KREBS LA *MONILINIA LAXA* (ADERH.& RUHL.) HONEY PARAZITĂ PE PRUN

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Abstract. *Present in microorganisms in small quantities, trace elements have the ability to interfere with some important biological functions, including enzymatic activities on some important metabolic pathways. This study systematizes the experimental results concerning the „in vitro" activity modulation of enzymes that defines each step of the tricarboxilic acids cycle by microelements like – B, Cu, Mn, Mo, Zn, Fe, or their mixture at Monilinia laxa (Aderh.&Ruhl.) Honey parasite on different types of plum tree species. The researches were made using the fungus mycelium sampled at 7 and 14 days from the inoculation on Leonian medium. The activity of the dehydrogenases complex was determined by spectrofotometry using the Sisoev and Krasna method's (modified by Artenie). The studies showed the main differences in the enzymes activity dynamics related to the type of oligoelement added to the culture*

Key words: *Monilinia laxa*, dehidrogenases, Krebs cycle, trace elements

Rezumat: *Prezente în microorganisme în cantități foarte mici, oligoelementele posedă abilitatea de a interfera cu importante funcții biologice incluzând activități enzimatice din căi metabolice cheie. Studiul de față sistematizează rezultatele experimentale privind modularea activității „in vitro" a enzimelor ce caracterizează fiecare etapă a ciclului acizilor tricarboxilici de către microelemente de genul – B, Cu, Mn, Mo, Zn, Fe, dar si a unui amestec din acestea, la specia Monilinia laxa (Aderh.&Ruhl.) Honey parazită pe diferite soiuri de prun. Cercetările au fost efectuate în miceliul ciupercii la 7, respectiv 14 zile de la însămânțarea pe mediul Leonian, activitatea complexului dehidrogenazelor fiind determinată spectrofotometric prin metoda Sisoev şi Krasna (modificată de Artenie). Studiile au relevat diferențe semnificative în dinamica activității enzimelor în funcție de tipul oligoelementului introdus în mediul de cultură.*

Cuvinte cheie: *Monilinia laxa*, dehidrogenaze, ciclul Krebs, oligoelemente

INTRODUCTION

Present in small amounts in microorganisms, the trace elements (micronutrients) have the ability to interfere with important biological functions, including enzyme activities of some central metabolic pathways .Although most literature data includes B, Cu, Mn, Mo, Fe, Zn in the microelements category,

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according to the classification to date, Fe is only a the trace element (Şoldea C.and Mocanu M. 2011), while others consider them all invariably microbioelements (Gârban Z., 1999) found in all living organisms. Some scientific hypotheses, supported by the experimental data, offer as explanation a biochemical basis of this fact, the relevance of the metal ions is due to the fact that a large amount of enzymes requires additional components to be able to perform the catalytic roles, for the purpose of being co-opted as so-called enzymatic cofactors, whose function is accomplished by trace elements (Cojocaru D.C., 2007).

Known as the tricarboxylic acid cycle, the Krebs cycle is a major metabolic pathway of primary metabolism found in the fungal cells. The integral enyzmatic equipment of the citric cycle catalyzes among the prokaryotic organisms, a cascade of the reactions in the cytosol, although other viewpoints (Griffin D.H., 1996) situate the enzymatic set among some fungal species in the mitochondrial matrix, excepting the succinate dehydrogenase, "captive" in the internal membrane.

Numerous reported in the literature recorded the influence of micronutrients on the enyzmes involved in the Krebs cycle in the filamentous fungi. So, the role of $Mn^{2+}$and Mg^{2+} is mentioned to actuates directly on the izocitrat-dehydrogenase due to the enzyme NADP + dependence's (Punckar N.S. *et al.*, 1984; Yasutake Y. *et al.*, 2003, Bertini I., 2007), of the Fe^{2+} which form a chelated with the citrate, essential for the aconitase's activity (Beinert H. and Kennedy M.C., 1993), influencing implicit throughtout the Krebs cycle progress and that play both structural and functional role in the succinate dehydrogenase (Frey P.A. and Hegeman A.D., 2007), the inhibitory activity that have Fe^{2+}, Zn^{2+} and Cu^{2+} on the malate-dehydrogenase, the Cu^{2+} inhibiting completely at 0.1 mM concentration the activity of this flavoprotein, specifying that the three metal ions competes for the catalytic site of the protein (Jernejc K. , Legis M., 2002), the antagonism and the synergism between micronutrients being reported in other papers (Fraústo da Silva J.J.R., Williams R.J.P., 2001). Given that the Krebs cycle is amphibolic, some intermediaries being as the precursors of secondary compounds of the fungi (Olteanu Z. *et al*, 1988) and also known as the influence of micronutrients on the efficiency of these products (Mn^{2+}, Fe^{2+}, Zn^{2+} reduces for example, the efficacy of the citric acid to *Aspergillus niger* (Soccol C.R. *et al*, 2006; Hang Y.D. and Woodams E.E., 1998), implicit being affected and the Krebs cycle enzyme's production.

Going on the line to other studies (Manoliu Al. *et al.,* 2004, 2005) this paper aims to study the influence of various micronutrients that have on the Krebs cycle dehydrogenase in *Monilinia laxa*.

MATERIAL AND METHOD

The inoculum of the *Monilinia laxa* has been isolated from mummified fruit that were harvested from the Research and Development Station for Fruit Tree Growing Iasi, Miroslava farm, from varieties of the *Prunus domestica*. The "*in vitro*" cultivation of the fungus was the Leonian medium (in the changed Bonnar formula), distributed in the Erlenmeyer flasks, which was supplemented with the following trace elements (in the quantities described by Constantinescu, O., 1974). So, we added as H_3BO_3, $CuSO_4$ x H_2O, $MnCl_2$ x $4H_2O$, Na_2MoO_4 x $2H_2O$, $FeCl_3$ x $6H_2O$, $ZnSO_4$ x $7H_2O$, B -10

mg, Cu - 100 mg, Mn - 20mg, Mo - 20mg, Fe - 20 mg, Zn - 200 mg separately, one nutrient in each flask, as well as all nutrients in a single variant. The control sample consisted of media without the addition of micronutrients. The culture media were seeded with disks 8 mm in diameter, cut-out from a culture of *Monilinia laxa* aged 7 days and incubated under stationary conditions at 28^0C, at the thermostat. The experiments, conducted in fungus mycelium were performed at 7 days and 14 days after inoculation of the culture.

The Krebs cycle dehydrogenases activity was determined by the Sîsoev and Krasna method, in modifying Artenie, Vl. (Cojocaru, 2009). On the basis of this method of estimation of the total microbial dehydrogenase activity is the ability of these enzymes to transfer hydrogen from various substrates to 2,3,5 – trifeniltetrazoliu chloride, which is reduced, passing in triphenylformazan, colored in red, the intensity of the it's colour is proportional to the dehydrogenases activity.

RESULTS AND DISCUSSIONS

The trace elements influence on the Krebs cycle dehydrogenases activity in *M. laxa* is shown graphically in the figures below. The isocitrate-dehydrogenase activity in fungus mycelium, quantified and statistically analyzed, as shown in figure 1, where the control sample value achieved 1.2375 μg formazan/g. Stimulative effect on the isocitrate-dehydrogenase activity had boron - the most intensively - 2.9226 μg formazan/g, followed by a mixture of trace elements - 2.5744 μg formazan/g, manganese - 2, 1808 μg formazan/g and copper - 1.7201 μg formazan/g. Inhibitory effect in the 7 days old mycelium culture is observed in the molybdenum - 0.3681 μg formazan/g which was found the lowest isocitrate-dehydrogenase activity, followed in ascendingly by zinc - 0.5663 μg formazan/g, respectively, iron - 0.9312 μg formazan/g.

With the aging of the culture mycelia, the activity of this biochemical parameter decreased in the control sample to 0.6054 μg formazan/g, higher levels than it's being observed in the case of copper - 0.7059 μg formazan/g, for molybdenum - 0.6790 μg formazan/g. In the other medium variant, the isocitrate- dehydrogenase it registered decreased levels, the strongest being due to the cumulative effect of the trace elements in the mixture - 0.1630 μg formazan/g, followed by manganese - 0.2474 μg formazan/g, iron - 0.3201 μg formazan/g, zinc - 0.5218 μg formazan/g and boron - 0.5296 μg formazan/g. Rigorously analyzing the dynamics of the isocitrate-dehydrogenase activity in time, it can be concluded that the senescence culture has induced a decrease in the activity of this enzyme, the only exception being a variant V4 with molybdenum, which was increased the level of the enzyme activity in time.

Another studied enzyme was α-ketoglutarate-dehydrogenase, the impact of the trace elements on the activity of this enzyme in *Monilinia laxa* (Aderh.&Ruhl.) Honey specie's being graphically illustrated in figure 2.

The best potentiation of the α-ketoglutarate-dehydrogenase enzyme activity at 7 days was achieved by boron, registering 2.4780 μg formazan/g, compared with control sample value which were noted 0.4414 μg formazan/g. A strong stimulative effect on the α-ketoglutarate-dehydrogenase in 7-day-old mycelium was observed at the zinc - 1.6684 μg formazan/g, iron - 0.9256 μg formazan/g, respectively, molybdenum - 0.8969 μg formazan/g. The inhibition of the α-ketoglutarate-dehydrogenase activity was recovered in the case of medium variants containing manganese - 0.3945 μg

formazan/g, a mixture of micronutrients - 0.0247 µg formazan/g and that containing the copper - 0.1633 µg formazan/g, suggesting that, the α-ketoglutarate -dehydrogenase in *Monilinia laxa* species is inhibited by copper but that its combination with other trace elements enhances this inhibitory effect.

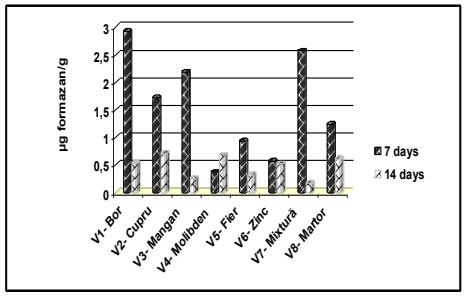

Fig. 1 - The influence of the trace elements on the isocitrate-dehydrogenase in *Monilinia laxa*

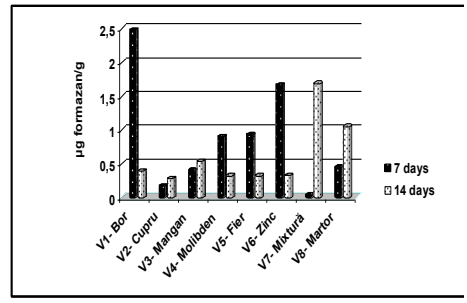

Fig.2 - The influence of the trace elements on the α-ketoglutarate-dehydrogenase in *Monilinia laxa*

After 14 days of incubation, the α-ketoglutarate-dehydrogenase activity value in the control sample was 1.0481 µg formazan/g. The simultaneity of action of the trace elements mixture - induced as α-ketoglutarate dehydrogenase a strong increase in contrast with other medium supplemented with trace elements - 1.6872 µg formazan/g. All other trace elements have caused a significant inhibition of this biochemical parameters compared with the control sample, copper, iron, zinc, boron molybdenum giving relatively uniform values, while manganese, compared with them, was comparatively more tolerant - 0.5292 µg formazan/g.

The results of the time-course monitoring of the succinate-dehydrogenase activity in *Monilinia laxa* mycelium cultivated on medium enriched with the various trace elements are reproduced graphically in figure 3. As a first observation, it appears that, at 7 days after inoculation of the culture, all medium variants have as this enzyme activity the values were lower than the control sample, who had a value of 1.2590 µg formazan/g.

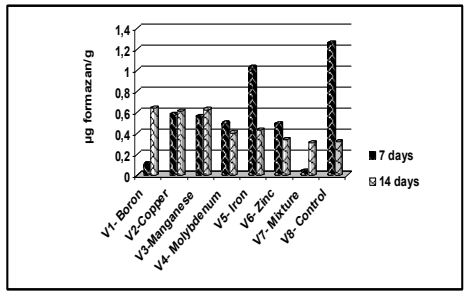

Fig. 3 - The influence of the trace elements on the succinate- dehydrogenase in *Monilinia laxa*

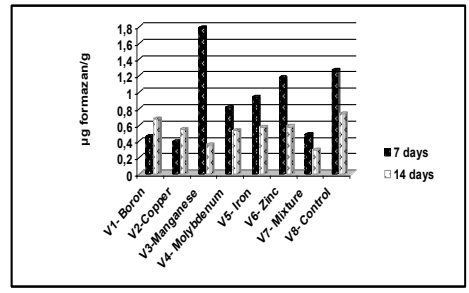

Fig. 4 - The influence of the trace elements on the malate- dehydrogenase in *Monilinia laxa*

The aging of the mycelial culture has induced a decrease of the succinate-dehydrogenase activity in the control sample- 0.3186 µg formazan/g. Has been

noted, however, that in the medium variants containing boron - 0.6384 μg formazan /g, manganese - 0.6266 μg formazan/g, copper - 0.6090 μg formazan /g, iron - 0.4297 μg formazan/g, molybdenum - 0.4064 μg formazan/g, zinc - 0.3387 μg formazan/g, the trace elements have had an stimulating effects on the succinate-dehydrogenase activity. It is noteworthy that the simultaneous effect of the micronutrients in the V7 medium variant-produces on the succinate dehydrogenase an inhibitory effect at 14 days - 0.3093 μg formazan/g.

The chart of the malate dehydrogenase activity, the modulated enzyme "in vitro" of the different trace elements in *M. laxa* fungus mycelium, show that in fungal culture for 7 days, the enzyme activity in the control sample reached a value of 1.2631 μg formazan/g and that the medium variant containing manganese was recorded, a value of 1.7825 μg formazan/g. Other trace elements have determined the inhibition of the malate-dehydrogenase activity, the copper strong hindering most strong the activity of this enzyme - 0.3899 μg formazan/g. The enzymatic activity value in the medium variant supplemented with zinc was approximately equal to the control sample calue activity - 1.1719 μg formazan/g which supports the hypothesis that zinc has a very peached inhibitory effect on the malate dehydrogenase activity in a young culture.

In a secondary series of tests, it has been observed that all variants had lower values of the malate dehydrogenase activity below the control sample, which leads towards the conclusion that in an aging culture, trace elements, either alone or together, inhibit the activity this enzyme in the *M. laxa* mycelium. Thus, it has been observed that the manganese slowed the enzyme activity the most - 0.3586 μg formazan/g compared with the control sample - 0.7358 μg formazan/g and that, in combination with other micronutrients, has increased its inhibitory effect - 0.2932 μg formazan/g.

A careful analysis of the malate-dehydrogenase activity dynamic's shows that, over time, the enzyme activity decreases including the control sample, except for medium variants containing boron and copper.

CONCLUSIONS

1. The studies on the Krebs cycle dehydrogenases activity in *Monilinia laxa* fungus cultivated "in vitro" on the medium supplemented with various micronutrients found that it was influenced in different ways depending on the type of enzyme, on the micronutrient type introduced in the culture medium and the mycelial culture age.

2. In a young culture, at 7 days the isocitrat-dehydrogenase activity was stimulated by boron, the mixture of trace elements, manganese and copper and inhibited by molybdenum, zinc and iron. In 14 days culture the isocitrat-dehydrogenase activity was stimulated by copper and molybdenum.

3. After 7 days of incubation, the α-ketoglutarate-dehydrogenase activity was stimulated by boron, zinc, iron, molybdenum and inhibited by manganese, copper and trace element mixture. After 14 days of incubation, the α-ketoglutarate-dehydrogenase activity was stimulated by the mixture of trace elements.

4. At 7 days after inoculation of the culture, the succinate-dehydrogenase activity was inhibited by all micronutrients, while at 14 days after seeding, the enzyme activity was stimulated in all medium variants.

5. After 7 days, the malate-dehidrogennase activity was stimulated only by manganese and inhibited to 14 days for all trace elements, either separate or combined.

REFERENCES

1. **Beinert H., Kennedy M.C., 1993** - *Aconitase, a two-faced protein: enzyme and iron regulatory factor. FASEB J.* **7** (15), pg. 1442–9.
2. **Brown D.A., Cook R.A., 1981**- *Role of metal cofactors in enzyme regulation. Differences in the regulatory properties of the Escherichia coli nicotinamide adenine dinucleotide phosphate specific malic enzyme, depending on whether magnesium ion or manganese ion serves as divalent cation.* Biochemstry, Volume 20(9), 2503-12
3. **Cojocaru D.C., Olteanu Z., Ciornea E., Oprică L., Cojocaru S.I., 2007** - *Enzimologie general.* Editura Tehnopress Iaşi, pg. 273-278
4. **Fraústo da Silva J.J.R., Williams R.J.P., 2001** - *The biological chemistry of the elements: the inorganic chemistry of life*. Oxford UNiversity Press, 575 pages
5. **Frey P.A., Hegeman A.D., 2007** – *Enzymatic reaction mechanisms*. Oxford University Press, pg. 831
6. **Gârban Z., 1999** – *Biochimia, tratat comprehensiv*. Ed. Did. si Pedagogică Bucuresti, pag 29
7. **Gupta S.K., Maggon K.K., Venkitasubramanian T.A., 1977** - *Effects of zinc on tricarboxilic acid cycle and enzymes in relation to aflatoxin biosyinthesis.* J Gen Microbiol., 99(1):43-8.
8. **Griffin D.H., 1996** – *Fungal physiology*, John Willey and Sons Ltd, pages 132-133
9. **Hang Y.D., Woodams E.E., 1998** – *Production of citric acid from corncobs by Aspergillus niger.* Bioresource Technolog., 65(3), pg. 251-253
10. **Jernejc K., Legisa M., 2002** - *The influence of metal ions on malic enzyme activity and lipid synthesis in Aspergillus niger*. FEMS Microbiol.Lett.Volume 217 (2), pg. 185-190
11. **Manoliu Al., Oprică. L., Olteanu Z., Humă A., Artenie V., Creangă D., E., Bodale I., 2004 -** *Magnetic field effect on some cellulolytic fungi* Source: 3rd International Workshop on „Biological effects of Electromagnetic fields" October 4-8, Kos Greece.
12. **Manoliu Al., Oprică L, Creangă D.E., 2005 –** *Ferrofluid and cellulolytic fungi*. Journal of magnetism and magnetic materials, volume 289, pg. 473-475, Proceedings of the 10th International Conference on Magnetic Fluids.
13. **Punckar N.S., Vandyanathan C.S., Appaji R.A.O., 1984** – *Mechanism of citric fermentation by Aspergillus niger*. Journal of Scientific and Industrial Reasearches, vol. 43, pg. 398-404.
14. **Olteanu Z., Surdu S., Tănase A., Tănase D., 1988** - *Activitatea unor dehidrogenaze ale ciclului Krebs la tulpini de Claviceps purpurea (Fr.) Tul. în culturi submerse*. Microbiologie industrială şi biotehnologie, Iaşi, pg. 459-464.
15. **Soccol C. R, Vandenberghe L. P. S, Rodrigues C., Pandey A., 2006** - *New Perspectives for Citric Acid Production and Application*. Food Technol Biotechnol, 44, 141–9.
16. **Şoldea C., Mocanu A.M., 2011** – *Biomolecule. Biotransformări*. Edit. Matrixromn.
17. **Yasutake Y., Watanabe S., Yao M., Takada Y., Fukunaga N., Tanaka I., 2003** - *Crystal Structure of the Monomeric Isocitrate Dehydrogenase in the Presence of $NADP^+$*. Journal of Biological Chemistry 278, pg. 36897–36904.