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CONTENT

THE EFFECT OF ZnO NANOPARTICLES ON THE ACTIVITY OF ANTIOXIDANT ENZYMES AND CAROTENOID CONTENT AT *RHODOSPORIDIUM TORULOIDES* CNMN-Y-30 YEAST

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Keywords: yeast, ZnO nanoparticles, *Rhodosporidium toruloides,* carotenoids, catalase, superoxide dismutase.

Abstract: The present research paper provides new information on the influence of ZnO nanoparticles (ZnO NPs) of size <50 nm and <100 nm on *Rhodosporidium toruloides* CNMN-Y-30 pigmented yeast. It was established that the activity of antioxidant enzymes such as catalase, superoxide dismutase and content of carotenoid pigments in the studied strain has been modified depending on the size and concentrations of NPs. There were no significant differences between the activity of antioxidant enzymes and content of carotenoid pigments in experimental group and control at the use of significantly low concentration of ZnO NPs. The use of nanoparticles in concentration of 30 mg/l caused a decrease in activity of antioxidant enzyme catalase and contributed to the increase in the activity of superoxide dismutase. This study has revealed that the concentration of 30 mg/L of ZnO NPs initiates an significant decrease in the content of carotenoid pigments - β-carotene, torulene and torularhodin in cell biomass. The results provided opportunities for modeling cell cycle processes and highlighting of carotenoid pigments and antioxidant enzymes as parameters for determining the mode of action of nanoparticles.

INTRODUCTION

Over the last decade, the development of science and technology is characterized by intensive study of the properties of nanoparticles and the elaboration different methods for practical application of NPs. Zinc oxide nanoparticles are among the most widely used because of large total surface area, UV absorption property and chemical stability (Dastjerdi et al., 2010; Osmond et al., 2010; Bernhardt et. al, 2010; Gunawan et al., 2013). ZnO NPs are widely used in various applications including food industry. ZnO nanoparticles can be used in food packaging as antimicrobial agents to prevent contamination of foods (Sirelkhatim et al., 2015) or UV light absorbers to protect foods that are sensitive to UV light exposure (EFSA., 2016). Wide range of application of ZnO nanoparticles in the food industry highlights the problem to provide consumers a safe and contamination free food.

Numerous *in* vitro studies have demonstrated the toxic effect of ZnO nanoparticles, such as induction of oxidative stress, cell apoptosis, cytotoxic, genotoxic and inflammatory responses (De Angelis et al., 2013; Johnson et al., 2015; Song et al., 2010; Wahab et al., 2014; Cheng et al., 2017). The cytotoxicity of nanoparticles and their interaction with biological systems is still unclear. Thus, there is a need for studies that contribute to our understanding of the mechanism of influence of nanoparticles and to evaluate the risk of utilization of them. Therefore, the possible adverse effects of exposure to nanoparticles were studied on different organisms (Bhuvaneshwari et al., 2015; Hazeem et al., 2016; Prach et al., 2013). Yeasts *Rhodotorula gracilis* can serve as biological model for studying the influence of nanoparticles on molecular and cellular mechanisms. According to recent publications, scientific name *Rhodosporidium toruloides* is the synonym of *Rhodotorula gracilis*. The structural and functional organization of yeast cell, ability to biosynthesize a wide range of antioxidants position this unicellular eukaryotic organism as a model for evaluating of adaptive cell response to nanoparticle action.

The evaluation of antioxidant potential is important to determine the action of nanoparticles on yeasts cells. According to the scientific literature, reactive oxygen species (ROS) production induced by nanoparticles in high concentrations , affect cell physiology, causing DNA and cell membrane damage (Jaleel et al., 2008; Puja et al., 2015). Cells possess the antioxidant defense system that can prevent ROS mediated damage (Zannatul et al., 2009; Rahman et al., 2016). Antioxidant enzymes, including catalase and superoxide dismutase and carotenoid pigments form the first line of defence against free radicals in *R. gracilis* yeast strains.

Thus, the research aim was to evaluate modifications in the activity of intracellular antioxidant enzymes and carotenoid pigments in *R. toruloides* CNMN-Y-30 yeast strain under the influence of ZnO nanoparticles depending on size and concentration.

MATERIALS AND METHODS

Objects of study. Pigmented yeast strain *Rhodosporidium toruloides (synonim Rhodotorula gracilis)* CNMN-Y-30, producer of proteins and carotenoids, was selected for the research (Usatîi et al., 2016). The strain is preserved in the collection of Yeasts Biotechnology Laboratory and in the Collection of Nonpathogenic Microorganisms of Institute of Microbiology and Biotechnology of Moldova.

Nanoparticles: ZnO nanoparticles with particle size <50 nm in form of nanopowder, purity >97%, contains 6% Al dopant, surface area >10,8 m^2/g (ALDRICH) and with size <100 nm in form of nanopowder, purity >80%, surface area 15-25 m^2/g (ALDRICH) were used. The suspension of nanoparticles was prepared according to the method specified (Oterro-Gonzalez et al., 2013). The concentrations of nanoparticles used in experiments constituted 1.0; 5.0; 10; 15; 20; 25; 30 mg/L. The variant without application of nanoparticles was used as control sample.

Culture media: YPD media specific to yeast strains was used for the cultivation (Agiular et al., 2003). The submerged cultivation was carried out in depth capacity 1 liter Erlenmeyer flasks on shaker 200 rpm at a temperature of 25^0...28^0 C, the duration of cultivation 120 hours. Yeast cells in amount of 5%, 2x106 cells/ml were inoculated in liquid medium. The cultivation of yeasts was effectuated at the constant illumination of 2000 Lx.

Methods. Catalase activity was determined by method Aebi (1984) modified by Efremova et al. (2013). Superoxide dismutase activity was determined on PG T60 VIS Spectrophotometer, at 560 nm, according to method described by (Некрасова et al., 2008). The carotenoid content in the yeast biomass was determined by spectrophotometric techniques described by (Frengova et al., 1994; El-Banna Amr et al., 2012; Tămaş et al., 1986). Statistical analysis of results was done using computerized application statistics 7 for windows.

RESULTS AND DISCUSSIONS

An important indicator of cellular resistance under stress conditions is the activity of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) (Fridovich., 1995). Catalase is known for its ability to convert toxic hydrogen peroxide into water and oxygen. Antioxidant enzymes play a major role in reducing ROS levels. The increase in superoxide dismutase and catalase activities could be an adaptive response to the action of nanoparticles on yeasts cell. The determination of antioxidant enzymes activity of yeast *R. toruloides* CNMN-Y-30 under the influence ZnO (<50 nm) and (<100 nm) nanoparticles and demonstrated that at the use of low concentration of NPs (1-5 mg/L) the catalase activity in experimental samples was similar to the control (Figure 1). However, if the concentration of nanoparticle was increased to 30 mg/L, the activity of the catalase was reduced, so cell exhibited a diminished ability to protect themselves from consequences of oxidative stress and accumulation of hydrogen peroxide. No significant correlation between the nanoparticle concentrations and the activity of the catalase was established, $R^2 = 0.7118$ for ZnO (<50 nm) nanoparticles and $R^2 = 0.2176$ for ZnO (<100 nm) nanoparticles (Figure 1).

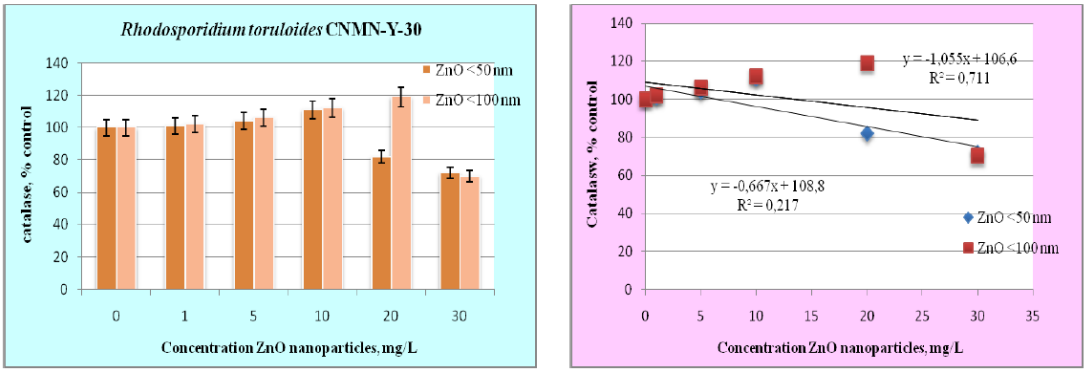

Figure 1. Catalase activity at *Rhodosporidium toruloides* CNMN-Y-30 under the action of ZnO nanoparticles depending on size and concentration

The results of the study of superoxide dismutase activity in *R. toruloides* CNMN-Y-30 under the influence of ZnO (<50 nm) and (<100 nm) nanoparticles at the concentrations from 5 mg/L to 30 mg/L revealed a trend towards an increase in the experimental samples (Figure 2). SOD increased significantly by 53% for ZnO (<50 nm) NPs and 85% for ZnO (<100 nm) NPs at the concentration of 20 mg/L. The values of superoxide dismutase activity in the control yeast biomass constituted 132...135 U/mg protein. ZnO (<50 nm) nanoparticles and ZnO (<100 nm) nanoparticles at the concentration of 20 mg/L caused an increase in the activity of SOD up to 202 U/mg protein and 250 U/mg protein. So, ZnO nanoparticles significantly affected the antioxidant enzyme system of studied yeast strain. Analysing the action of ZnO nanoparticles on SOD activity, it can be mentioned that concentrations of NPs correlate to 79% for ZnO (<100 nm) nanoparticles and practically does not depend on used concentrations for those of ZnO (<50 nm) nanoparticles, R^2 = 0 ,3667 (Figure 2).

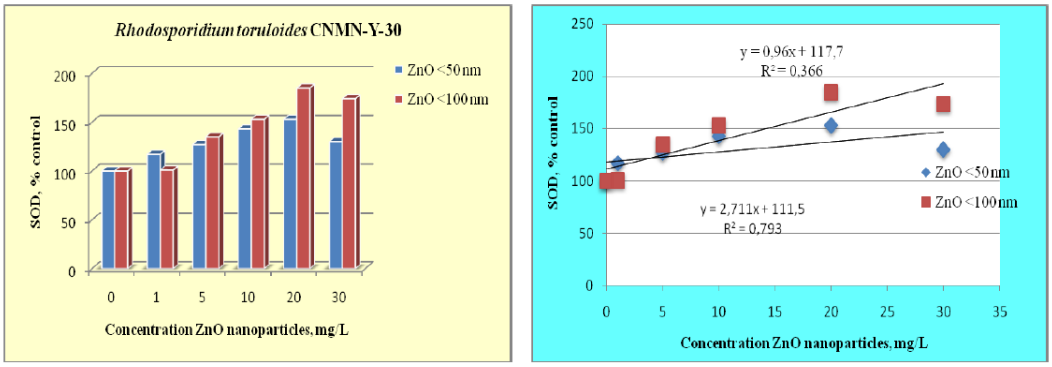

Figure 2. Superoxide dismutase activity at *Rhodosporidium toruloides* CNMN-Y-30 under the action of ZnO nanoparticles depending on size and concentration

Various studies have elucidated first line antioxidant defense mechanisms (Jamec., 2008; Yang ., 2009; Lassegue., 2010). There are a number of antioxidants that act to prevent the formation of reactive species in cells, others scavenge free radicals and inhibit pro-oxidants (Carocho et al., 2013). Carotenoid pigments also known as macromolecular antioxidants, play an important role in protecting cells from oxidation and cellular damages. Carotenoids are recognized as singlet oxygen collectors. Thus, in the conditions of oxidative stress, these pigments can be used as biological markers for assessing the degree of influence of metal oxide nanoparticles on living systems.

Therefore, to understand the effects of ZnO nanoparticles on *R. toruloides* CNMN-Y-30 yeast depending on size and concentration, it is essential to study the formation of carotenoid pigments, including β-carotene, torulene and torularhodin. So, its was established that low concentrations of ZnO (<50 nm) nanoparticles do not deviate significantly process of biosynthesis of carotenoids. A modest increase (18%) of the carotenoids content was observed in the case of utilization of 10 mg/L of nanoparticles. In other experimental variants, the content of pigments in yeast biomass do not differ from the control (Figure 3). At the same time, the statistical analysis of results of the influence of ZnO (<100 nm) NPs on yeasts highlighted that supplementation of the culture medium with 30 mg/L of nanoparticles initiates an essential (36%) decrease of the amount of carotenoid pigments in the yeast biomass. Thus, large size and high concentrations of nanoparticles can alter the biosynthesis process of carotenoid pigments. So, free radicals in excess can damage cell membranes responsible carotenoid biosynthesis. The dependence of the concentrations of ZnO (<100 nm) nanoparticles and carotenoid content is expressed by the coefficient of determination $R^2 = 0.5622$. Therefore, the concentration of nanoparticles determines the accumulation of carotenoid pigments in only 56% of cases. In the case of the use of ZnO (<50 nm) nanoparticles, the concentration determines the accumulation of carotenoid pigments in 16% of cases (Figure. 3).

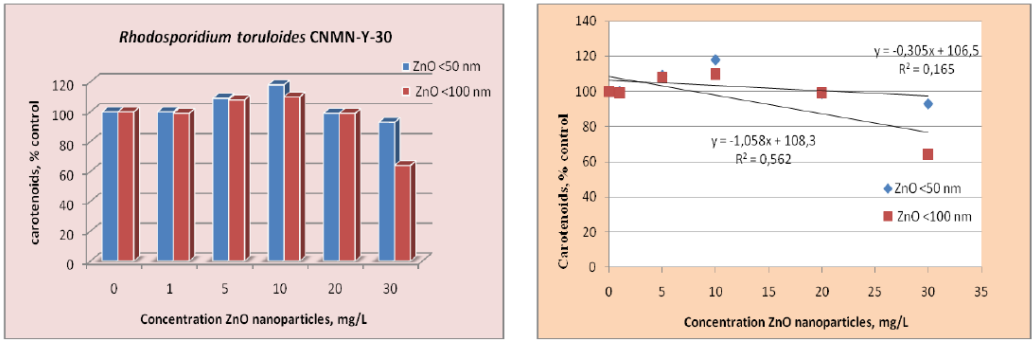

Figure 3. The content of carotenoid pigments at *Rhodosporidium toruloides* CNMN-Y-30 under the action of ZnO nanoparticles depending on size and concentration

According to recent studies on *Rhodosporidium* genus, proposed pathways for carotenoid biosynthesis including beta-carotene, torulene and torularhodin synthesis depend on the properties of used yeast strain and cultivation conditions (Singh et. al, 2016; Merhan**.,** 2017). In view of the fact that carotenoid content was influenced by the application of ZnO nanoparticles and some quantitative variations were established against the control samples, it was studied the accumulation of basic components of pigments.

The obtained results have demonstrated that, the proportions of β - carotene, torulene and torularhodin does not change significantly under the influence of ZnO (<50 nm) nanoparticles (Figure 4). This phenomenon indicates that nanoparticles in most used concentrations do not affect pathways for carotenoid biosynthesis.

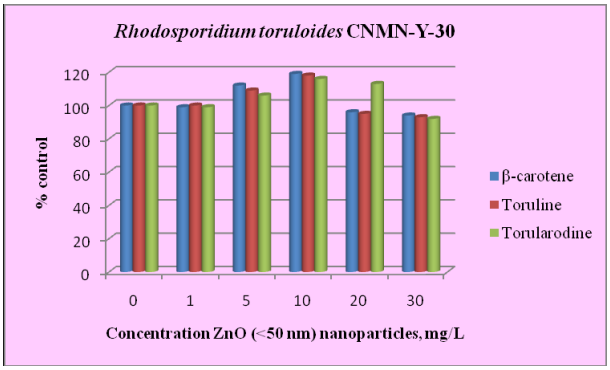

Figure 4. The level of β-carotene, torulene and torularhodin at *Rhodosporidium toruloides* CNMN-Y-30 under the action of ZnO (<50 nm)nanoparticles

Analysing the content of the basic components of carotenoid pigments under the influence of ZnO (<100 nm) nanoparticles some changes depending on the applied concentrations were established. β-Carotene, one of the indices of responses of the yeast cell, at concentration of 30 mg/L, was decreased by 33% and the content of torulene decreased further by 51% (Figure 5). Thus, the obtained results suggest that large size nanoparticles, upon contact with the surface of the yeast cells, can cause some metabolic disorder, thus modifying the specific biochemical reaction. This information provides opportunities in modeling vital cell processes and highlighting carotenoid pigments as a model for determining the degree of action of nanoparticles.

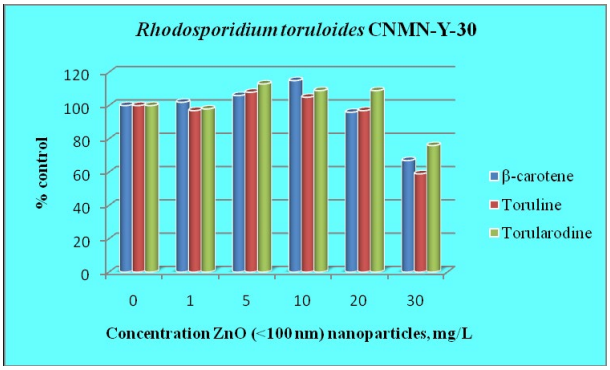

Figure 5. The level of β-carotene, torulene and torularhodin at *Rhodosporidium toruloides* CNMN-Y-30 under the action of ZnO (<100 nm) nanoparticles

CONCLUSIONS

Generalizing the results obtained in this study it can be mentioned that the activity of the antioxidant enzymes catalase, superoxide dismutase and the content of carotenoid pigments at *Rhodosporidium toruloides* strain CNMN-Y-30, in contact with the ZnO nanoparticles was changed depending on the size and applied concentration. Estimating the degree of influence of the ZnO nanoparticles (<50 nm) and ZnO (<100 nm) on the yeast *Rhodosporidium toruloides* CNMN-Y-30 was established that, in the case of low concentrations of nanoparticles (1-5 mg/L), the activity of antioxidant enzymes and content of carotenoid pigments in the experimental samples was similar to the control. Nanoparticles examined at a concentration of 30 mg/L caused the decrease in carotenoid content, the activity of the antioxidant enzyme catalase and the increase of superoxide dismutase activity. Changes in the biosynthesis of carotenoid pigments expressed by the reduction of β-carotene, torulene and torularhodine associated with the decrease in the catalase activity provide opportunities for modeling of vital processes and revealing carotenoids and antioxidant enzymes as biological test in terms of determining the degree of action of nanoparticles on the yeasts.

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SALINITY AND SELENIUM NANOPARTICLES EFFECT ON ANTIOXIDANT SYSTEM AND MALONDIALDEHYDE CONTENT IN *OCIMUM BASILICUM* L. SEEDLINGS

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Abstract: Selenium (Se) is an essential nutritional element and its presence has a crucial role in fortification of crops. The aim of the work was to investigate the possible use of selenium nanoparticles (SeNPs) in the mitigation of salinity stress on basil (*Ocimum basilicum* L.) seedlings. Non-enzymatic compounds, enzymatic activity and malondialdehyde (MDA) as indicator of lipid peroxidation products were quantified under the influence of SeNPs and two concentrations of NaCl (50 mM and 100 mM) applied singular or combined. The activity of antioxidant enzymes in *O. basilicum* seedlings was generally lower in the control regardless of the treatment applied singular or combined. The same decreasing trend was noted in the case of total polyphenols and MDA content. On the other hand, both combined treatments stimulated the soluble protein content in relation to the single variant, the increase being more pronounced at 50mM + SeNPs in respect with the singular treatment.

INTRODUCTION

Sal stress conditions are known to affect plants physiological and biochemical potential, which in turn affect crops primary and secondary metabolism (Hebbara et al., 2003; Hendawy and Khalid, 2005). Salinity is one of the world environmental and agricultural constraints to crop production that not only delays, but also decreases germination of most crops. Lower levels of salinity delay germination, whereas higher levels reduce the final percentage of seed germination (Ghoulam and Fares, 2001). High saline sodic condition affects field and horticultural crops performance particularly in arid and semi-arid regions of the world (Grigore et al., 2014; Grigore and Toma, 2017). On the other hand salinity hinders plant development and diminishes plant growth.

The sweet or common basil, *O. basilicum* L. is the most important species of the genus *Ocimum* which belongs to *Lamiaceae* family. Basils ("king of the herbs"), especially the sweet basil, are commonly used in gastronomy and oral health care (Baritaux et al. 1992; Khatri et al., 1995; Sajjadi, 2006). Although traditionally used as a medicinal herb to treat various conditions, basil has a long history as a culinary herb due to its foliage, which adds a distinctive flavour to many foods (Labra et al., 2004). Since these plants are quite rich in essential oils, they are commonly produced for economic purposes such as diuretic in pharmaceutics or as fragrance in cosmetics (Caliskan et al., 2017). Basil is popular among consumers year-round and controlled environmental conditions enabling stable production worldwide (Park et al., 2016, Oprica, 2016).

Selenium (Se) is an essential nutritional element, but excessive Se can be toxic to animals and humans. Selenium can exist in the (+6), (+4), (0), and (-2) oxidation states, the major feature of Se chemistry that affects the Se solubility and movement in nature. In waters, dissolved inorganic Se is normally present as (+6) selenate (SeO_4^{2-}) and as (+4) selenite (SeO_4^{2-}) (Long et al., 1990). However it is first removed from soils by plants and soil microorganisms, which can take it into their proteins and produce volatile forms such as dimethyl selenide (El-Ramady Hassan et al., 2014). The obtained SeNP are reported as novel compounds with excellent antioxidant properties and lower cellular toxicity compared with other selenospecies (Estevez et al., 2014). In addition, results of Heba and Ibrahim, (2014, indicate that Se pretreatment enhanced tolerance of wheat plants against drought oxidative stress through modulation of the plant's antioxidant system.

Due to the distinctive properties of Se nanoparticles (SeNP), there is inquisitiveness in its synthesis for nanotechnology applications. SeNP have been exploited for medical purposes such as antimicrobial, antifungal and anticancer. The nanoform of Se can be used for various applications due to its advantages over the bulk form such as low toxicity, better reactivity, and low dosage (Chhabria and Desai, 2016).

Studies have highlighted that Se can offset the damaging effects of abiotic stress, such as drought (Hasanuzzaman and Fujita, 2011), High-Temperature (Djanaguiraman et al., 2010) and heavy metals (Tedeschini et al., 2015, Kumar et al., 2012), UV-B (Yao et al., 2013, Mostafa and Hassan, 2015), salinity (Ardebili et al., 2014, Diao et al., 2014). Using antioxidants up-regulation and its enzymes, Se exposure has been linked at optimal levels to the reduction of

various reactive oxygen species (ROS) (Feng et al., 2013), inhibition of uptake and translocation of heavy metals, changing in heavy metals speciation and rebuilding of cell membrane, chloroplast structures as well as recovery of the photosynthetic system.

On the other hand, Hussein and Abou-Baker, (2018), showed that the foliar application of nano-Zn led to mitigate the adverse effect of salinity of the cotton plants which were irrigated with diluted seawater. In addition, the results of Shalaby et al., (2017), showed that Se addition mitigated the detrimental effect of salinity on lettuce growth and its development.

Therefore in this context, the aim of this study was to report a novel approach in using Se-NPs under abiotic stress. The study also provides an estimation of the response of enzymatic and non-enzymatic antioxidant defense system in *O. basilicum* to Se-NPs application under salinity condition.

MATERIAL AND METHODS

2.1. Experimental design of plant materials and growth conditions

Study of *Ocimum basilicum* seedlings tolerance to salinity and responses to SeNPswas conducted in laboratory conditions, based on completely randomized design with three replications. Sweet basil seeds used in the present experiments were supplied from Agricultural Research and Development Station, Secuieni Neamt. Intact seeds, which were homogeneous and identical in size and colour, and free from wrinkles, were chosen. These seeds were sterilized with 10% sodium hypochlorite for 30 seconds and then were washed with sterile distilled water.

2.2. Treatment of biological material

Twenty-five seeds of *Ocimum basilicum* were placed for 4 hours in distilled water solutions and 100µM Selenium nanoparticles (SeNPs) solution (which was prepared at our partner V.F.Kuprevich Institute of Experimental Botany of the National Academy of Sciences of Belarus).
After application of this pre-treatment, the seeds were placed in Petri dishes and watered with water for 7 days. Treatments with selenium nanoparticles and NaCl solution (50 mM, 100 mM) were applied singular or combined, on the sweet basil seedlings, resulting the following variants from Table 1. Biochemical analyses were performed on 14-day-old *Ocimum basilicum* seedlings.

Table 1. Description of SeNPs and saline solution treatments applied singular or combined

Variants	Treatment
Control	Water only
SeNPs	Watered only with SeNPs solution
50 mM NaCl	Watered only with saline solution
50mM NaCl + SeNPs	Watered with saline solution and nanoparticle solution (1: 1)
100 mM NaCl	Watered only with saline solution
100mM NaCl + SeNPs	Watered with saline solution and nanoparticle solution (1: 1)

2.3. Oxidative stress index

Malondialdehyde (MDA) as indicator of lipid peroxidation products was quantified in enzymatic extracts according to the method described by Hodges et al. (1999). One ml of the enzymatic supernatant was mixed with 2 ml of 0.5% thiobarbituric acid (TBA) solution (in 10% TCA). The mixture was kept at 95 °C for 60 min, and cooled at room temperature, then centrifuged at 12.000 rpm for 10 min to remove the interfering substances. Absorbance was read at 532 nm using UV–Vis spectrophotometer.

2.4. Antioxidant system

2.4.1. Non enzymatic constituent

Total polyphenol content assay

The total polyphenols content was determined by using a modified Folin-Ciocalteau method Singleton et al., (1999). The absorbance of resulting bleu-colored solution was read at 765 nm after two hours, against the blank (distilled water). The amount of the total polyphenolic content was expressed as mg gallic acid equivalent (mg GAE/g DW) (R^2 = 0.99). Three readings were taken for each sample and the result averaged.

2.4.2. Antioxidant enzymes

Preparation of enzyme extracts

16-days old seedlings sample was homogenized with 0.1 M phosphate buffer (pH=7.5). After that the homogenates were centrifuged at 15,000 × g for 15 min at 4°C, and the supernatants were used for enzyme assays.

2.4.2.1. Superoxide dismutase (SOD) activity was estimated by recording the decrease in absorbance of superoxide-nitroblue tetrazolium complex by the enzyme (Winterbourn et al., 1975). About 3 ml of reaction mixture, containing 0.1 ml of 1.5 mM nitroblue tetrazolium (NBT), 0.2 ml of 0.1 M EDTA, 2.55 ml of 0.067 M potassium phosphate buffer, and 0.01 ml of enzyme extraction, were taken in test tubes in duplicate from each enzyme sample. One tube without enzyme extract was taken as control. The reaction was started by adding 0.05 ml of 0.12 mM riboflavin and placing the tubes below a light source of 215 W florescent lamps for 5 min. The reaction was stopped by switching off the light and covering the tubes with black cloth. Tubes without enzyme developed maximal color. A non-irradiated complete reaction mixture, which did not develop color, served as blank. Absorbance was recorded at 560 nm and 1 unit of enzyme activity was taken as the quantity of enzyme that reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes.

2.4.2.*2. Peroxidase (POD) activity* was determined spectrophotometrically by measuring the oxidation of o-dianisidine (3, 30-dimethoxybenzidine) at 540 nm (Möller and Ottolenghi, 1966) with slight modification. The reaction was started by adding 0.1 H_2O_2 0.05% on mixture reaction containing 0.2 ml of enzyme extraction, 0.8 ml distilled water and 1.5 ml 1% o-dianisidine. After 5 min. the reaction was stopped with 2.5 ml H_2SO_4 50%. One unit of POD activity was expressed as the amount of enzyme that produced a change of 1.0 absorbance per min.

2.4.2.3. Catalase (CAT) activity was measured according to the method described by Sinha, (1942). Briefly, the assay mixture consisted of 0.4 ml phosphate buffer (0.01 M, pH 7.0), 0.5 ml hydrogen peroxide (0.16 M) and 0.1 ml enzymatic extract in a final volume of 3.0 ml. About 2 ml dichromate acetic acid reagent was added in 1 mL of reaction mixture, boiled for 10 min, cooled. Changes in absorbance were recorded at 570 nm. CAT activity was expressed as the amount of enzyme needed to reduce 1 µmol of H_2O_2 per min. The activity of these enzymes (SOD, POD and CAT) was expressed as unit per mg proteins (U/mg protein).

The determination of soluble protein content was determined according to Bradford method (1976) with bovine serum albumin as standard. Thus, this assay is refers to the binding of Coomassie Brilliant Blue G-250 at aromatic amino acid radicals and measuring the colour at 595nm.

Statistical analysis. All experiments were carried out with three independent repetitions and the results were expressed as the mean values ± standard deviation (STDEV).

RESULTS AND DISCUSSIONS

In the conducted study, heterogeneous responses regarding the growth (Fig.1) and activity of antioxidant defense system determined on *O. basilicum* 14 days old seedlings were obtained as a result of saline treatments with different concentrations of NaCl and a solution of 100 µM SeNPs (Figure 2, 3 , 4).

● All treatments, singular and combined applied to *O. basilicum* seedlings determined a decrease in SOD activity in comparison with plants control. There is about a half (45%) reduction in SOD activity in SeNPs treatment applied to seedlings (Figure 2). Singular treatments with 50mM NaCl indicated a comparable SOD activity like in the control. Interestingly, this enzyme activity was reduced by half (46%) in 100mM NaCl treatments. Enrichment of medium of basil seedlings with SeNPs reduced the SOD activity compared with control with 46% (50mM+SeNPs) and 56% (100mM+SeNPs).

● Catalase, enzyme involved in the removal of reactive oxygen species (ROS), converts H_2O_2, which results from reaction catalyzed by SOD, in H_2O and O_2 in peroxisomes. Treatments applied to the species *O. basilicum* lead to an extremely low activity in singular treatment with

SeNPs, the decrease being of 83% compared to control (Figure 3). Combined treatments determined an increase comparatively with simple treatment (both with SeNPs and 50 mM NaCl) in the case of 50mM + SeNPs. Interestingly, in the 100mM saline treatment and combined with SeNPs, CAT activity showed very close values.

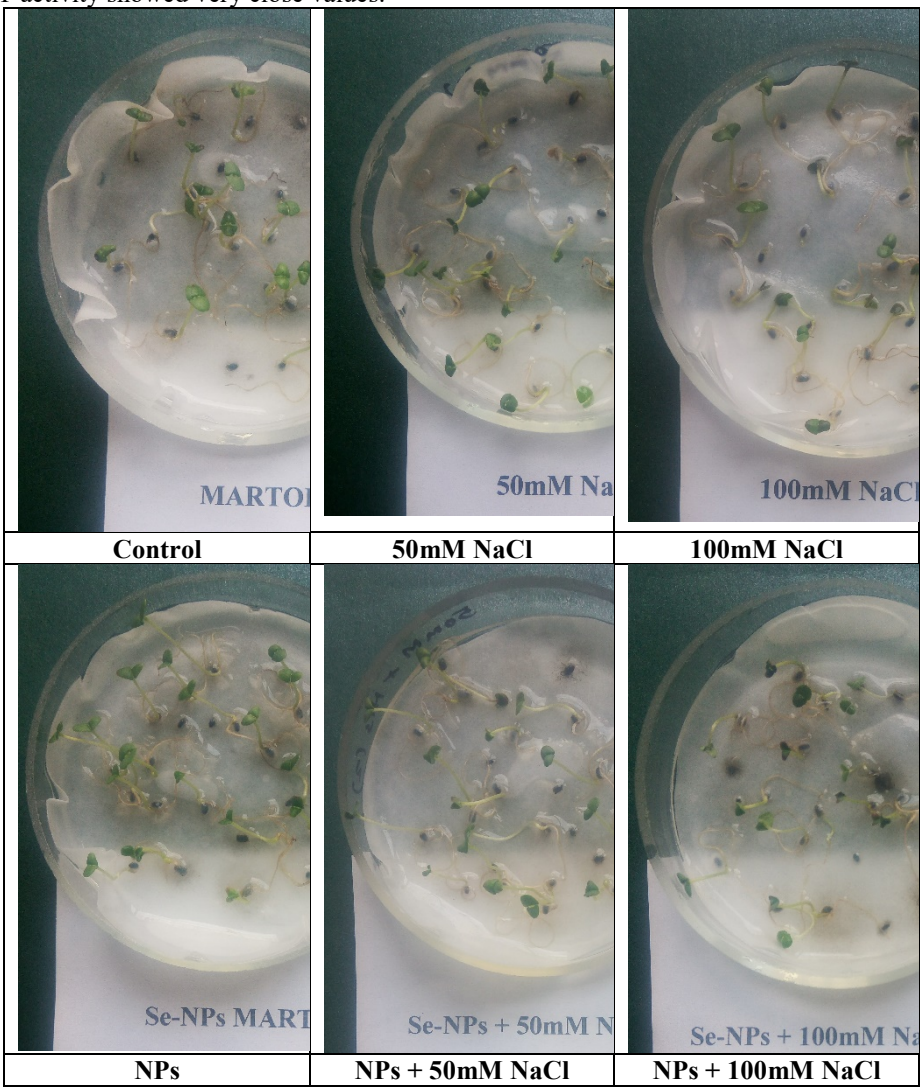

Fig. 1. Effect of treatment of Se NPS and NaCl solution (singular or combined) in 14-day-old *Ocimum basilicum* seedling

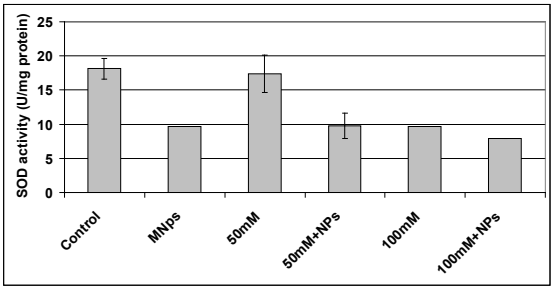

Fig. 2. SOD activity in 14-days-old *Ocimum basilicum* seedlings

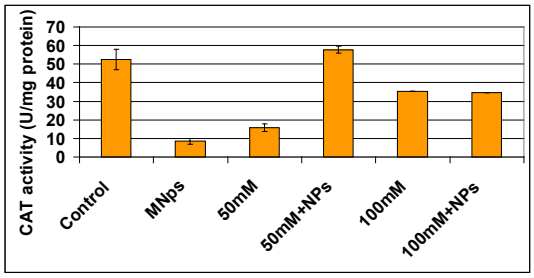

Fig. 3. CAT activity in 14-days-old *Ocimum basilicum* seedlings

● Peroxidase is involved in the oxidation of many substrates in the presence of H_2O_2 with the production of H_2O: $AH_2 + H_2O_2 \rightarrow A + 2H_2O$. This enzyme is stimulated by the accumulation of H_2O_2, being able to remove this toxic compound. Compared to CAT, POD has an increased affinity for H_2O_2 but has a low processing rate. The obtained results indicate a decrease with 28% in POD activity in *O. basilicum* seedlings treated with SeNPs compared to the control. The combined treatment 50mM NaCl + SeNPs indicates stimulation of POD activity compared to 50mM NaCl singular treatment and with the plants control (with 51%). In contrast, treatment combined with 100mM NaCl + SeNPs reveals a relatively similar activity to singular treatment and an increased compared to control (48%) (Figure 4).

● Saline and SeNPs treatments applied singular and combined determined a stimulation of soluble protein content in basil seedlings compared to the plant control. Thus, singular SeNPs treatment improved the stimulation of soluble protein content with 51%. Both combined treatments stimulated the soluble protein content in relation to the single ones, the increase being more pronounced at 50mM + SeNPs compatively with the singular treatment (Figure 5). On the other hand, the highest amount of protein was obtained when applying 100mM + SeNPs treatments. It can be seen the SeNPs role in alleviate the adverse effect of salinity in terms of increasing the soluble proteins content.

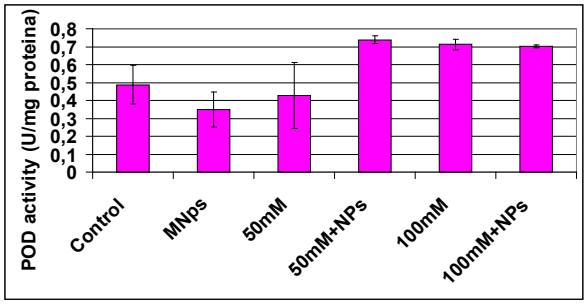

Fig. 4. POD activity in 14-days-old *Ocimum basilicum* seedlings

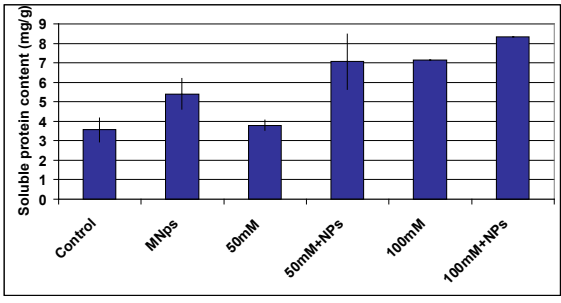

Fig. 5. Soluble protein content in 14-days-old *Ocimum basilicum* seedlings

● Lipid peroxidation induced by free radicals is also important in cell membrane damage. The level of lipid peroxidation, measured by the MDA content, was considered an indicator of salinity induced oxidation and useful for determining the salt tolerance of plants (Hernandez and

Almansa, 2002). Data of MDA content in 14-days-old *O. basilicum* seedlings in the presence of SeNPs and salinity are depicted in Figure 5. Therefore, singular SeNPs treatment applied to *O. basilicum* caused a slight decrease (10%) in MDA content as opposed to control. The lowest NaCl concentration applied singular leaded to the MDA content very similar to the control. In addition, both concentrations of NaCl in combination with SeNPs determined a decrease in MDA content compared to singular treatments (Figure 6).

● High antioxidant activity is reported from different medicinal plants, and phenolic compounds and flavonoids are the usual antioxidants present in them. Figure 7 showed a decrease of total polyphenol content in *O. basilicum* seedlings after the applied of treatments.

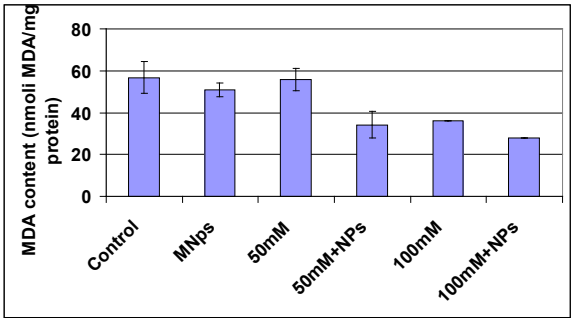

Fig. 6. MDA content in 14-days-old *Ocimum basilicum* seedlings

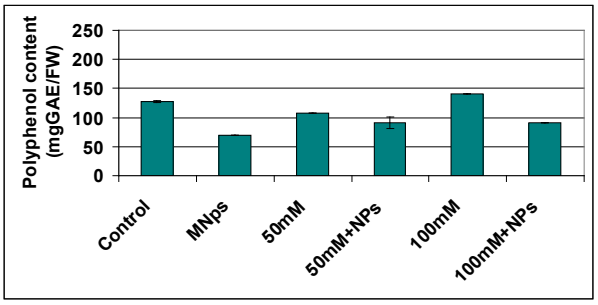

Fig. 7. Polyphenol content in 14-days-old *Ocimum basilicum* seedlings

Basil seedlings treated with singular SeNPs indicated a half-reduced (45%) polyphenol content compared to the plant control. Combined treatments with SeNPs and two NaCl concentrations applied determined a decrease in polyphenol content in relation to the corresponding saline treatments; the decrease being more pronounced at 100mM + SeNPs versus 100mM NaCl (140,71 mgGAE/FW and 90,7464, respectively).

CONCLUSIONS

Singular treatments with SeNPs determined in 14-days-old *O. basilicum* seedlings, a decrease in the activity of antioxidant enzymes (SOD, CAT, POD), in total MDA and total polyphenols compared to the plant control. Furthermore, the same treatment stimulated the soluble protein content.

Combined treatments with SeNPS and different concentrations of NaCl have resulted in a decrease in SOD activity in basil seedling as compared to the corresponding single treatments. The same downward trend was noted for MDA and total polyphenols contents when saline stress was alleviate by applying SeNPs. On the other hand, the protein content was stimulated by the application of combined treatments, the mitigating salinity being more pronounced at low NaCl concentration.

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PINUS NIGRA ARN. AND THE INFRATAXON PINUS NIGRA SSP. BANATICA A SHORT REVIEW

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Keywords: *Pinus nigra* ssp. Banatica, subspecies, present situation.

Abstract: Banat black pine is an infrataxon sometimes considered as subspecies, sometimes as variety of black pine, with natural spreading area in southwestern Romania, on Banat Mountains, from where it slightly extends into south of the Danube, in Serbia. The controversy surrounding the position of Banat black pine in southwestern Romania has been known since the last century. The Banat black pine population from this part of Romania has never been thoroughly studied at the molecular level. A future direction of study could be that variability analysis and genetic structure of individuals from natural population of *Pinus nigra* ssp. Banatica in southwestern Romania, using genetic markers.

INTRODUCTION

Pinus genus belongs to the Pinaceae family; it has its origin in the northern hemisphere and grows in many types of forests in Europe, Asia, North Africa, North America and Central America. It is a diverse genus that includes over 100 species and dates back to the Mesozoic period (Keeley, 2012). Most species, about 70, are from North America and Central America. Another area with a wide variety of species, about 25, is located in Asia, especially in China (Price et al, 1998). According to European Flora taxonomy (Tutin et al, 1993), the Pinus genus is divided in two sub-genes: Diploxylon and Haploxylon. Research on chloroplast DNA analyses (Gernandt et al., 2005) also led to delimitation of two sub-genes: Pinus sub-genus (Diploxylon or Hard pines) respectively Strobus (Haploxylon or Soft pines), with several sections each. Black pine is included in Pinus section, Pinus subsection, which includes predominantly species with Eurasian, Mediterranean spreading area and others with North America and Cuba spreading area.

Black pine is one of the most important species in Europe, both economically and adaptively. The largest natural spreading area of black pine in Europe includes Mediterranean regions, on both side of the 40° parallel. It has its origin in the eastern half of the Iberian Peninsula, continuing eastwards across France, Italy, Balkan Peninsula, to the Asian part of Turkey. The southern limit of the spreading area is in north Africa, close to Gibraltar and Algeria. The northern limit at about 46° apart from the black pine in Banat and another island presence is reported in Crimea (Șofletea et Curtu, 2008). Black pine presents very wide variability at a species and exotype level, including many subspecies and varieties, with obvious structural genetic individualization, depending on the origin area and according to biochemical determination of isoenzymes (Bonnet et Bikay, 1978). Thus, we can distinguish: Pinus nigra ssp. clusiana Clem. et Arias (Pinus salzmanni Dunal), including black pine populations in North Africa, Spain and southern France; Pinus nigra ssp. laricio Poiret (Pinus nigra poiretiana Asch. et Graebn), spread across Corsica (var. corsicana) and Calabria (var. calabrica); Pinus nigra ssp. nigricans Host, including populations in the central and eastern European distribution areas; Pinus nigra ssp. pallasiana Lamn, includes populations of black pine from Asia Minor; Pinus nigra ssp. caramanica Loud, from Crimea; Pinus nigra ssp. banatica (Born.) Novak (Pinus nigra var. banatica Endl. George. et Ion) – Banat black pine (Șofletea et Curtu, 2008) – an infrataxon sometimes considered as subspecies, sometimes as variety of black pine, with natural spreading area in southwestern Romania, on Banat Mountains, from where it extends moderately towards southern Danube, in Serbia. Therefore, in the Romanian specialty literature it is known as Banat black pine.

TAXONOMIC CLASSIFICATION OF THE BANAT BLACK PINE

The controversial position of the Banat black pine in south-eastern Romania has been known since the last century. Boșcaiu and Boșcaiu (1999) mention that Rochel (1828) and Schwarzott (1831) announced for the first time the presence of the black pine in south-eastern Romania, on Domogled Mountain and on Danube valley, near Sviniţa, under Pinus pinaster Auct. non Aiton. Thus the association of the Banat black pine with Pinus pinaster is inappropriate, considering that its spreading area is in the Mediterranean, where it is found especially in France, Spain, Portugal and North Africa. According to the morphological description, the maritime pine has longer needles than the Banat black pine, 10-22 cm,

slightly stinging, cones are longer than 8-18 cm, with bright reddish apophysis. Banat black pine has needles 8-12 cm long, dark green and cones are 6-10 cm long, with bright yellowish apophysis (Debazac, 1964).

Later, Heuffel (1858) described the same populations as Pinus nigra laricio auct. non Poiret (Boşcaiu et al, 1999). In relation to this classification, morphological characters of Pinus nigra ssp. laricio and Pinus nigra ssp. banatica are different; needles are 12-15 cm long, cones (4-7cm long) with brown-yellowish or bright reddish apophysis. Bud scales are light brown, whereas in Banat black pine, bud scales are whitish-gray (Debazac,1964).

Subsequently, Romanian black pine populations, because of their peculiarities, are separately mentioned by some authors as the endemic taxon Pinus nigra subs. banatica (Endel ex Borb.) Novak. According to Georgescu (1936), these black pine populations should be treated as independent species, as Pinus banatica (Georgescu et Ionescu) (Boşcaiu et al, 1999). Morphologically, it differs from the typical Pinus nigra species through the pyramid-shaped crown of young trees, irregular, tabular shaped at middle and old age trees. Tendrils, yellowish-greenish, to purplish blue – green, cones yellow to dirty yellow – greenish (Şofletea et Curtu, 2008). Needles are very rigid and stinging (Negulescu et Săvulescu, 1965).

The same source (Boşcaiu and Boscaiu, 1999) debate Borza's (1947) mentions, who classify populations of black pine in Banat and Oltenia as Pinus nigra subsp. pallasiana (Lamb.) Holmboe. This classification was taken over in 1946 by Gaussen et al, in the first edition of European Flora 1 and therefore maintained in the second edition of the same volume in 1993. Jalas et Suominem (1973) in the atlas of European Flora 2 classified almost all black pine species in Balkans as Pinus nigra subsp. pallasiana. Related to the association with Pinus nigra ssp. pallasiana the information about the spreading area of this species do not include populations in southeastern Romania, it is found in Greece, Turkey, Crimea and Bulgaria the latter being considered the furthest spreading area to the West (Isajev, 2004).

SPREADING AREA AND PARTICULARITIES OF HABITATS OCCUPIED BY BANAT BLACK PINE

Sporopolinic spectra performed in Peştera lui Veterani (Veterans Cave) showed that Banat black pine from Tricule formed compact stands of pine since the last glaciation, 12,000-14,000 years ago. Endemic to Romanian Carpathians, with low demands and high resistance for arid and sunny hills, Banat black pine covered these resort areas a long time ago, before replanting other broad-leafed species. The existence of relic specimens of Banat black pine shows that the connection between Crimea and the southern Carpathian population might have been possible through Balkan Peninsula, not directly through Crimea (Matacă, 2005).

The natural spreading area of the species includes areas in Banat and Western Oltenia. It is commonly found on limestone cliffs from low altitudes (about 150 m on Danube Valley) to full mountain areas (1500 m in Mehedinţi Mountains) (Şofletea et Curtu, 2008). In Banat: along the Cerna Valley, it starts at Pecinişca and Herculane to Corcoaia, with interruption in Cheile Cernei (Cerna Gorge), on steep cliffs in the Domogledului peak (Domogled, Şuşcu, Hurcu), on Culmea Desiminului (Desiminului Summit) and Arjanei on the right of Cerna (Clepeneac, Stone of Banitei); on Danube Valley at Tricule and on Trescovăţ cliffs (Svinița). In Olt: between Baloteşti and Păuneşti on Topolnița valley; Izverna (Baia de Aramă), Runcu on Sohodol Valley of Runc, on Cleanţul Cucului and Dosul Macrişului (Târgu Jiu) (Săvulescu et al, 1952).

In 1958, Paskovski and Leandru described four types of forest where we can find Banat black pine as well (Pinetum nigri banatica-orni; Quercetum-Pinetum nigri banatica-myrtilli, Pinetum nigri banatica-sessilis and glades of black pine with shrubs).

Black pine with flowering ash on limestone (Pinetum nigri banatica-orni), includes altitudes of about 700-900 m, southern and south-eastern exposure, rocky slopes and groves, with superficial soil, and tithonic limestone sublayers. The stand is composed of black pine in variable consistency with an active rhythm of growth. The sub-stand is made of flowering ash and eglantine in smaller quantity. Black pine with oak species (Quercetum-Pinetum nigri banatica-myrtilli) on siliceous rocks: this type of forest is rare, found only in a spot on Cerna Valley and at Svinița. It can be found at altitudes of 250 – 300 m, on skeletal soil, very superficial with sublayers of siliceous rocks (granite in Cerna Valley, siliceous breccia at Svinița). Some specimens are 20 m tall, but most of them are shorter (12-14 m). A rare sub-stand is made of shrub specimens of sessile oak, Italian oak, Turkey oak, mountain ash, flowering ash, dribs and drabs of beech. Plants such as Chrysosogon gryllus, Festuca duriuscula, Vulpia myurus, Genista pilosa, Asperula cynanchica, Hieracium pavichii, Achillea sp. are also present; Cerna Valley resort is characterized by a conspicuous frequency of Vaccinium myrtillus, Cytisus nigricans and Cytisanthus radiatus, which cannot be found at Svinița where the plant cover is generally weak. In this area, the glade with wood of black pine can also be found at Trescovăţ Stone, not far from Svinița, the approximate altitude being 500-670 m. Here the pine is joined by sessile oak, flowering ash, and fewer beech, white beech, trembling poplar, silver linden, like shrubby or at most as short trees. Among shrubs, the most abundant is lilac. Mix of black pine with broad-leaved trees on limestone (Pinetum nigri banatica-sessilis), furrows steep slopes of Domogled and Şuscu, being the lithological layer limestone. In the first altitudinal plant belt are species as sessile oak, European haze, linden, fewer birch are present, the second altitudinal plant belt come across of flowering ash, mountain ash, and rarely oriental hornbeam. The sub-stand is

composed of smoke tree, Turkey hunzel, blackthorn, cornel tree, red dogwood, rock buckthorn etc. Glades of black pine with shrubs (Pinetum nigri banatici-radiati, Pinetum nigri banatici-junipereum, Pinetum banaticum-Syringeum nanae) were found on Cerna Valley and Oltenia, at altitudes of about 600-1000 m always on sunny slopes, with limestone sublayer. The sub-stand is represented by thickets of different shrubs (common juniper and black cytisus, dwarfish juniper and lilac, flowering ashes and lilac).

Doniță et al. (2005) identified and characterized in the Cernei Mountains' habitat R4218 – Southeast Carpathian glade – forests of black pine (Pinus nigra ssp. banatica) with Genista radiate, on limestone, rendzina soils, superficial, skeletal, saturated in bases, water balanced, with possible deficits in summer, eutrophic. Phytocoenosis within it are performed by European forest and sub-Mediterranean species. The altitudinal plant belt is made, in the upper level of black pine trees (Pinus nigra ssp. banatica), which do not form a finished layer, and in the lower altitudinal plant belt of flowering ash (Fraxinus ornus), Turkish hazel (Corylus colurna), silver linden (Tilia platyphyllos), sessile oak (Quercus petraea), Oriental Hornbeam (Carpinus orientalis); the shrub level is missing or it is poorly developed, made of Cotoneaster integerrima, Cotinus coggygria, Cornus mas, Sorbus cretica, Rhamnus saxatilis, local Syringa vulgaris, Juniperus communis. The plant cover and sub shrubs is dominated by Carex humilis and Sesleria rigida.

Located in the southwestern part of Romania, the natural spreading area of the Banat black pine is predominantly subject to the influence of western and southwestern atmospheric circulation. Western circulation, in the cold period, brings polar air masses or, rarely, tropical, maritime, favourable to mild winters with mostly heavy rainfall at low altitudes. During the summer, it causes a higher degree of thermal instability, evidenced by the frequency of the downpours, accompanied by electric discharge. (Buza et al., 1981).

The Banat black pine as genetic resource and afforestation-reforestation material in Romania

A stand of Banat black pine was selected for the production of forestry materials in the "qualified" category (fig. 1), with a total area of 83.20 ha, of which 60.40 ha represents the buffer zone, with altitudes ranging from 730 m to 970 m (Băile Herculane, Caraș-Severin County) (Pârnuță et al., 2011).

There were three preservation nucleus established for black pine of native origin, with a total area of 8.1 ha. The nucleus have been chosen from three district of Romania (Brașov, Caraș-Severin și Mehedinți) (Pârnuță et al., 2012).

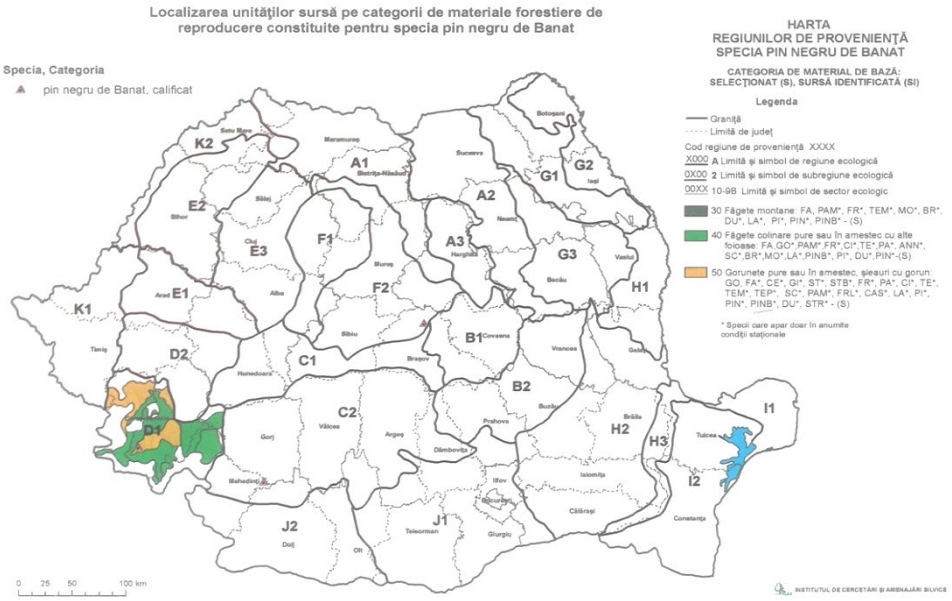

Fig.1. *Location of the establishments of origin by categories of forest reproductive materials constituted for the Banat black pine (Pârnuță et al., 2011).*

GENETIC DATA ABOUT THE BLACK PINE

The black pine is a species that has been genetically analyzed over time through isoenzyme, terpene, cpDNA and core DNA studies. Genetic differentiation between subspecies of Pinus nigra species has been achieved with these analyzes.

Variation of isoenzymes was studied in 28 populations of Pinus nigra, 9 known subspecies and two unknown in Cyprus. With the help of the dendrogram showing relations between populations, studies were divided into three main categories: Corsican and Moroccan, Laricio and Cyprus and the rest (Nikolic et al., 1983). The geographic variation of the terpenoid composition of Pinus nigra was studied using 109 samples, representing 72 natural and 11 planted populations. Five geographical groups were distinguished: Spanish, continental French, Corsican, Calabrian and eastern (including Austria, the Balkans, Turkey and the Crimea). Results from terpenoid composition and from data on the anatomic, morphological and growth characteristics and on frequencies of terpenoids, flavonoids and allozymes support the use of Wheeler et al. classification system (Gerber, 1995). Genetic variation in five natural black pine populations in Austria, Bulgaria, Greece, Corsica (France) and Calabria (Italy) was analyzed by starch gel electrophoresis with 10 enzymatic systems. There was a clear differentiation between ssp. laricio (Calabria, Corsica) and austrian ssp. (Austria, Bulgaria), and the Greek population was more similar to the austrian ssp (Scaltsoyiannes et al., 1994). Three subspecies of Pinus nigra Arn. (ssps. nigra, salzmannii, laricio) were analyzed using 23 izoenzymatic loci for four morphologic characters (length, width, wing scar and weight/10 seeds). The dendrogram obtained using genetic distances among populations indicated the existence of 3 groups, corresponding to the 3 subspecies. The data clearly showed that the Corsican population is the furthest, with relative lack of genetic variation, perhaps due to its geographical isolation (Aguinagalde et al., 1997). The geographic variation of Pinus nigra terpenes in southwestern Europe was studied in 16 Corsica Herault (France) and Eastern Pyrenees (France and Spain) populations. Differences in quantitative content of the selected compounds which divided the populations in two basic geographic groups: on the one hand populations of Herault and the eastern Pyrenees and, on the other hand, populations of Corsica. Some trees, as well as populations, share similarities, although they are not part of the same geographical region. These analyzes confirm the hypothesis that afforestation of Herault and Eastern Pyrenees was also achieved with Corsica black pine (Bojovic et al., 2005). A study on canonical discrimination analysis (CDA) was conducted in Serbia to verify the hypothesis of intraspecific chemical separation of Pinus nigra (ssp. nigra, var. gocense, ssp. pallasiana, and var. banatica) taxons, based on terpenes. The division of seven natural populations of Pinus nigra resulted in 3 groups (ssp. nigra, ssp. pallasiana and var. banatica). Individuals in pallasiana group represented the smallest proportion in (E) - caryophyllene and terpinolenes and richer in α-humulene, and those in ssp. nigra var. banatica had the highest content of α-pinene and myrcene (Sarac et al, 2014).

Genetic structure and genetic diversity were analyzed in 9 black pine populations in Bulgaria, using chloroplast microsatellite and terpene analyzes as markers. The cpSSP analyzes divided the black pine into four groups, and the most representative terpenes were α-pinene, followed by β-pinene. The results suggest that the structural model of the genetic diversity of chloroplastic DNA in black pine populations is the consequence of historical biogeographical processes (Naydenov et al, 2006). According to the hypothesis which states Western European populations survived during the last ice age rather than being recolonised in the post-glacial period was tested. Genetic variation was evaluated using a set of 10 chloroplastic DNA microsatellites. 311 specimens were analyzed, with 235 haplotypes revealing high level of diversity in most populations. With the help of bayesian analysis, 10 groups corresponding to 6 studied samples were differentiated. Temporal estimation places separation between the Alps and other regions about 150,000 years ago, and the most recent separation was found in southern France about 30,000 years ago. Analyzing these data, it was deduced that chloroplastic DNA of Western European populations is likely to have been present during the last ice age (Zara et Richard, 2006).

Eight black pine populations in Southern Spain and Northern Morocco were analyzed using ISSR markers. Analysis of main components shows the presence of two groups, while baysian analysis revealed the presence of three groups. Low genetic diversity noticed in two of the five populations is probably a direct consequence of inadequate management because no genetic variability estimation was performed before forestry treatments. Testing genetic variability of populations before any management was recommended (Rubio-Moraga et al, 2012).

CONCLUSIONS

In the past 200 years the Banat black pine in south-western Romania has been classified under various subspecies such as pallansiana, laricio and banatica. A future direction of study could be analysis of variability and genetic structure of individuals from natural populations of Pinus nigra ssp. banatica in southwest Romania, with the help of genetic markers.

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SITOSTEROLEMIA
RARE CAUSE OF HYPERCHOLESTEROLEMIA IN CHILD

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Abstract: Sitosterolemia is a rare disorder of lipid metabolism, caused by pathogenic variants in either of two genes, ABCG5 and ABCG8. We report a sitosterolemia case of a little girl with severe hypercholesterolemia at the age of 11 months. The occurence of the some linear xanthoms at the level of the Achilles tendon, bilaterally and the extremely high cholesterol (total cholesterol: 949.6 mg%, LDL-cholesterol: 837 mg%) have constituted the starting point for subsequent investigations. Cardiac and abdominal ultrasound does not have pointed out the changes. No family history of hypercholesterolemia has been reported. Completing genetic tests confirmed the diagnosis of sitosterolemia. By sequencing the entire genome two mutations were detected in the ABCG5 gene (Q16X and R446X). We present this clinical case due to the rarity and particularity of the disease. Genetic tests play the most important role in diagnosing the disease and in an appropriate therapeutic approach for the child patient. Proper diet and family responsibility is the key to dispensing the case and to avoiding complications.

INTRODUCTION

Sitosterolemia is a rare autosomal-recessive disorder characterized by increased plant sterol levels, xanthomas and increased risk of premature atherosclerosis. The disease is caused by mutations in ABCG5 or ABCG8 (ABCG5 and ABCG8 sterol-transporter are defectives), leading to increased intestinal absorption and to a diminution of the biliary excretion of plant sterols (sitosterol, campesterol, stigmasterol) and cholesterol. The diagnosis of this disease is based on up to 50-fold increased plasma levels of sterols from plants, which are normally very low (<1 mg/dl). Vegetal sterols are very structurally similar to cholesterol, but differ in the presence of an ethyl or methyl group (sitosterol or campesterol) or a double bond (stigmasterol) (Eun, 2016). Sitosterol is the most commonly found vegetable sterol in the diet and the predominant form found in patients with sitosterol. In addition to plant sterols, their saturated derivatives - stanols are also present at high levels (Ajagbe et al., 2015). The cholesterol level is variable compared to other genetic hyperlipidemias. Patients with sitosterolemia exhibit extreme phenotypic heterogeneity, ranging from near asymptomatic individuals to those with severe hypercholesterolemia, which leads to accelerated atherosclerosis and premature cardiac death. High levels of cholesterol (up to 1000 mg/dl) have been reported, especially in children, because the immature child's intestine can absorb higher amounts of cholesterol than adults. The aim of the article is to highlight the diagnostic value of genetic tests and sitosterols dosing in a case of sitosterolemia with severe hypercholesterolemia in a child, without which a final diagnosis of homozygous familial hypercholesterolemia would have been wrong.

MATERIALS AND METHODS

A 11 month-old girl presented with severe hypercholesterolaemia. The presentation to the doctor was determined by the appearance of some linear orange lesions, arranged at Achilles tendon level, bilaterally (possibly xanthomas), difficult to observe because of very well represented subcutaneous cellular tissue. At the clinical exam, I did not detect anything pathologically. We specify that the diet was exclusively natural up to 6 months, after which it was diversified according to the recommendations, with the maintenance of breast milk. The weight index and nutritional index were in the normal range. Initial detection of extremely high cholesterol (total cholesterol: 949.6 mg%, LDL-cholesterol: 837 mg%) was the starting point for further investigations. The girl was dermatological, cardiological, ophthalmological, pediatric (St. Maria Iasi Hospital, IOMC Bucharest) tested, the results being normal. Also, the lipid profile in the dynamics was studied, keeping high values. During 6 weeks, the values were as it follows: total cholesterol 949, 1023, 756 (mg/dl), LDL-cholesterol 837, 945, 667 (mg/dl), HDL cholesterol 48, 54, 40.7 (mg/dl) and triglycerides 215, 120, 241 (mg/dl). Laboratory investigations performed by parents revealed normal levels of cholesterol and triglycerides. Although no family history of

hypercholesterolaemia has been reported so, the young age, the presence of xanthomes and the extremely high LDL-cholesterol level (> 600 mg/dl) were considered arguments to be homozygous familial hypercholesterolaemia. The next step was a transfer to Fundeni hospital, the recommended therapeutic solution being hepatic transplant.
After two months, the parents decided to go to a specialist clinic in Belgium, where the paraclinical investigation protocol started again and, very important, blood samples were sent to the USA for genetic testing. For the first time, the sitosterols were dosed, the calculated value being 2.37 mg%, above the normal limit (0.2-1 mg%). The lipid profile was comparable to the previous ones, the values being high (cholesterol-total: 752 mg%, LDL-cholesterol: 678 mg%, HDL-cholesterol: 42 mg%, triglycerides: 159 mg%). Treatment with ezetimibe 20 mg/day and atorvastatin 20 mg/day was initiated, so that, acute toxic drug hepatitis with severe hepatic cytolysis (TGP = 3610 IU/l, TGO = 2760 IU/l) was reported in 1 week. Hepatic function normalized in 4 weeks after discontinuation of treatment. The first results of genetic testing have excluded the known causes of severe hypercholesterolemia by sequencing the responsible genes (LDLRAP, LDLR, PCSK9, APOE and APOB) so that, liver transplantation was no longer considered an emergency.
Therefore, at the age of 1 year and 5 months, treatment with ezetimibe at the initial dose of 2.5 mg/day is gradually resumed, with a gradual increase to 7.5 mg/day. After 2 months, atorvastatin is combined at a dose of 2.5 mg/day. After 6 months of ezetimibe and atorvastatin, the lipid profile looks encouraging (total cholesterol: 203 mg%, LDL-cholesterol: 147 mg%). After 18 months of combined therapy, at 3 years and 1 month age, high cholesterol values (total cholesterol: 309 mg%, LDL-cholesterol: 249 mg%) and high sitosterol values were found (sitosterol: 7 mg%). That is because of giving up medication for specific periods and calling for alternative medicine, as well as addressing a diversified diet, restricted only to animal fats. The treatment with ezetimibe at the dose of 3.3 mg/day was resumed.
After 2 years and 6 months of expectations and uncertainties, sequencing of the entire genome confirms the diagnosis of sitosterolemia, by detecting two mutations in the ABCG5 gene (Q16X and R446X). She continued the treatment with ezetimibe alone, with a gradual increase in the dose up to 10 mg/day, the actual recommended dose. In recent years, total cholesterol ranged between 179-216 mg%, LDL-cholesterol between 121-169.6 mg% and beta-cholestanol between 6.9-10.9 μg/mL vs. normal (1.6- 6.2 μg/mL).
In terms of diet, the little girl also benefits from sustained and individualized counseling, based on observing basic principles, the diet being poor in plant sterols (nuts, seeds, olives, avocados, vegetable oils, margarine and chocolate) and in animal fats.
As the family chose to leave the country, access to the girl's personal data is limited.

RESULTS AND DISCUSSIONS

Although no family history of hypercholesterolaemia has been reported, the extremely high LDL-cholesterol level and the occurrence, at a very young age, of xanthomies could have been criteria for supporting the diagnosis of homozygous familial hypercholesterolaemia (Turgeon et al., 2016). Once the genetic tests have been completed, any diagnostic hypothesis has been removed, sitosterolemia being the diagnosis of certainty. It should be underlined that genetic diagnosis, although being the most relevant, are not at all accessible, both because of high costs and other factors. In our case, the family has overcome any barrier. A proper diagnosis is followed by adequate therapeutic behavior, benefits for the patient being huge.
Linear xanthomas were quite relevant to the case assessment and possible, in their absence, the disease would remain undiagnosed for a certain period of time. The true prevalence of sitosterolism cannot be explained precisely because of underdiagnosis and is probably more common than previously thought (Kidambi et al., 2008).
The first dosing of sitosterols revealed slightly high values (2.37 mg%), but much below the values observed in sitosterolemia (14-65 mg%) due to the fact that the girl at 1 year and 1 month was still breast-fed. Since vegetal sterols come entirely from the diet, the baby was exposed to much lower sitosterolytes during breastfeeding, as the heterozygous mother's plasma sitosterol should only be slightly increased. With the interruption of breast milk and the introduction of fruit and vegetables in the diet, the level of vegetable sterol increased (Park et al., 2014). Also, in the case of our baby, before finding out the genetic diagnosis and diet change, the sitosterolemia was 7 mg%. We need to keep in mind that a normal individual absorbs less than 5% of vegetal sterols, while the patient with sitosterolemia absorbs 15-60% of the ingested sitosterol.

The initial extremly high cholesterol levels are in agreement with the underlying disease. However, we note that cholesterol intake can be increased at a breast fed infant due to high cholesterol content in human milk (90-150 mg/liter), but also that the infant's intestinal mucosa can absorb higher amounts of cholesterol compared to that of an adult (Kamelska et al., 2012).
Diet is essential and must be strictly followed, although in pediatric age it is very difficult to achieve this goal. Dietary restriction of both cholesterol and vegetable sterols (vegetable oils, margarine, nuts, seeds, avocado and chocolate) is required. Also some crustaceans (shells, oysters) should be avoided (Gregg et al., 1986). A diet without sterols is almost impossible, because plant sterols are found in almost all herbal foods and the low diet of plant sterols led to just a 30% reduction in sterol levels (Izar et al., 2011). There are authors who claim that a diet low in cholesterol and plant sterols in infants and children would be safe and effective (Simell et al., 2000).
Ezetimibe remains the first choice as a drug therapy, being an inhibitor of intestinal sterol absorption (Othman et al., 2017). Specialty literature shyly supports, with observational studies, treatment with ezetrol under the age of 10 years (Niu et al., 2010; Tsubakio-Yamamoto et al., 2010). In our patient, initiation of treatment with ezetimibe has come with extremely severe cholesterol levels. Despite the hepatic impairment at the initial dose of a very high dose for a child patient (2 mg/kg body weight) subsequently, at lower doses, 1 mg/kg body weight, it has been shown to be safe and well tolerated. If we analyze the levels in the dynamics of LDL-cholesterol (minimum 121 mg% - maximum 169.6 mg% and vegetal sterols - beta-cholestanol (minimum 6.9 µg/ml - maximum 10.9 µg/ml), we notice that cholesterol levels are significantly reduced, only plant sterols remain at slightly higher levels than the reference ones. No beta-cholestanol has been dosed before initiation treatment with ezetimibe in order to make a more accurate comparison. So the efficacy of ezetimibe therapy is clear, especially if we take into consideration that in sitosterolemia, the levels of sitosterol can be increased up to 50 times.
Starting from the fact that in sitosterolemia there is an increased risk of premature atherosclerosis, by the accumulation of plant sterols and/or plasma stanols, we underline once again the important role of an early and accurate diagnosis of the disease (Othman et al., 2013). Our patient has no cardiac complications so far. Appropriate case management significantly improves prognosis and contributes to reducing the morbidity and mortality associated with this disease.

CONCLUSIONS

Genetic tests play the most important role in diagnosing the disease and in an appropriate therapeutic approach for the child patient. Proper diet and family responsibility is the key to dispensing the case and to avoiding complications.

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