RESPONSE OF BARLEY SEEDLINGS TO OXIDATIVE STRESS GENERATED BY TREATMENTS WITH GROWTH HORMONES

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Abstract: The effects induced by growth hormone regulators on soluble protein level and some oxidoreductases in *Hordeum vulgare* cv. *Madalin* seedlings were investigated. The study of superoxide dismutase, catalyse and peroxidase behaviour and of protein synthesis was realized in dynamics to evaluate the response of barley seedlings to oxidative stress generated by exposure to hormone factors. During experiments, peroxidase registered smaller limits of variability than superoxide dismutase and catalase. It seems that superoxide dismutase and catalase rather than peroxidase acted as components of an antioxidative protective system. Generally, the protein level not presented significant modifications in early stage, but at 6 days, the major part of variants responded by amplification of protein synthesis, excepting some kinetin and GA3 concentrations. In final stage, both control and hormone variants reduced their protein synthesis.

INTRODUCTION

Phytohormones have essentials roles in the regulation of plant physiological processes, because they serve as integrators and inductors of multicellular plant organism differentiation and in expression of hereditary information. The organ growth and development depend on endogenous hormone level. Various factors such as organism physiological state, correlation degree between characters, ontogenetic phase, gene epistasy or gene pleiotropy, and environmental conditions can induce quantitative modifications of endogenous phytohormones (DUCA et al., 2003). Plants are very often subjected to a wide variety of stressful conditions (UV-radiation, high light intensities, exposure to herbicides, extreme temperatures, toxins, air pollutants, heavy metals, wounding etc.) that generate reactive oxygen species (ROS) and create oxidative stress situations. Oxidative stress can arise from an imbalance between generation and elimination of ROS, leading to excess ROS levels which can damage all biomolecules and can lead in turn to various diseases and cell death. Increase of endogenous ROS levels and activation of antioxidant defence mechanisms are the most rapid indicators of oxidative stress. ROS (superoxide anion radical, singlet oxygen, hydrogen peroxide, hydroxyl radical) can damage many cellular components including proteins, membrane lipids, nucleic acids. A severe effect is peroxidation of membranary lipids (GONÇALVES et al., 2007). To minimize the damaging effects of ROS, aerobic organisms evolved non-enzymatic (ascorbic acid, reduced glutathione, carotenoids, tocopherols, flavonoids, alkaloids) and enzymatic (superoxide dismutase, SOD; catalase, CAT; peroxidase, POD) antioxidative protection mechanisms.

SOD - the main scavenger of superoxide radicals - is a strong antioxidant which converts the toxic superoxide in hydrogen peroxide and oxygen by so called *dismutation reaction*: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. The formed H_2O_2 in this way is also toxic and must be scavenged by catalase and diverse peroxidases which catalyze its conversion to H_2O. These enzymes that interact with superoxide and H_2O_2 are tightly regulated through a feedback system. For example, excessive superoxide inhibits POD (mainly glutathione peroxidase) and CAT to modulate the equation from H_2O_2 to H_2O; increase of H_2O_2 slowly inactivates SOD. Therefore, CAT and glutathione peroxidase, by reducing H_2O_2, conserve SOD; SOD, by reducing superoxide, conserves catalases and glutathione peroxidase. Through this feedback system, steady low levels of SOD, glutathione peroxidase, and catalase, as well as superoxide and H_2O_2 are maintained, which keeps the entire system in a fully functioning state (SCANDALIOS, 2005). Depending on H_2O_2 concentration, **CAT** exerts a dual function. At low H_2O_2 levels (<1 μM) and in presence of increased levels of other substrata (ethanol, ascorbic acid etc.), CAT acts like a POD: $RH_2 + H_2O_2 \rightarrow R + 2H_2O$. At high H_2O_2 concentrations, CAT degrades extremely rapid H_2O_2 by catalasic specific reaction: $2 H_2O_2 \rightarrow 2H_2O + O_2$. **POD** catalyzes the oxidation of many substrata (phenols, aromatic amines, ascorbic acid, glutathione, nitrites) in the presence of H_2O_2, with H_2O production: $AH_2 + H_2O_2 \rightarrow A + 2H_2O$. Peroxidase is present both in cytosol and in cell wall, in genetically different isoenzyme forms (GASPAR et al., 1982; SCANDALIOS, 2005).

MATERIAL AND METHODS

Biological material consisted in *Hordeum vulgare* L. cv. *Mădălin* caryopses, from Podu Iloaie Agricultural Research Center. They were subjected to 4 hours treatments, by immersion in growth regulators solutions: 2,4-D (A), kinetin (B), gibberellic acid (C), 2,4-D + Kin mixture (D), as follows:

Control		distilled water
A	A1	1 mg l^{-1} 2,4-D
	A2	10 mg l^{-1} 2,4 D
	A3	25 mg l^{-1} 2,4-D
	A4	50 mg l^{-1} 2,4-D
B	B1	1 mg l^{-1} kinetin

	B2	10 mg l^{-1} kinetin
	B3	25 mg l^{-1} kinetin
	B4	50 mg l^{-1} kinetin
C	C1	1 mg l^{-1} GA3
	C2	10 mg l^{-1} GA3
	C3	25 mg l^{-1} GA3
	C4	50 mg l^{-1} GA3
D	D1	1 mg l^{-1} 2,4-D + 1 mg l^{-1} Kin
	D2	10 mg l^{-1} 2,4-D + 10 mg l^{-1} kin
	D3	25 mg l^{-1} 2,4-D + 25 mg l^{-1} Kin
	D4	50 mg l^{-1} 2,4-D + 50 mg l^{-1} Kin

In 3, 6 and 10 days old seedlings, SOD, CAT and POD activities have been determined, as well the soluble protein level. Distilled water, without any hormone addition, was used for control.

Determination of superoxide dismutase (SOD) was carried out by method described by MINAMI and YOSHIKAWA (1979). Method principle is based on the ability of this enzyme to inhibite the tetrazolium salt (nitroblue tetrazolium – NBT) reduction by superoxid radicals generated by riboflavin photoreduction. Extinction is measured at λ=560 nm. One SOD unit represents the quantity of enzyme which produces 50% inhibition in the standard conditions.

Catalase activity was determined by iodometric titration (ARTENIE et al., 1981). Method principle is based on potassium iodide oxidation by H_2O_2 remained undecomposed after one minute incubation with catalase, and on titration with $Na_2S_2O_3$ of iodine released in presence of starch as indicator. A catalase unit represents that enzyme amount decomposing 1 μM H_2O_2 (0.034 mg)/min. The results are expressed in mg H_2O_2/g fresh matter.

Peroxidase activity was established by MÖLLER and OTTOLENGHI (1966) method. Method principle is based on the measurement of colour intensity of o-dianisidine oxidation product with hydrogen peroxide, in the presence of peroxidase. Colour intensity is measured with green filter, at a photocolorimeter (λ=540 nm). The calculus of results uses the coefficient of micromolecular extinction (0.0128). A peroxidase unit corresponds to the enzyme amount catalyzing the decomposition of 1 μM H_2O_2/min, in optimal conditions.

The protein determination was carried out according to the Bradford method (1976). Method principle is based on binding of Coomassie Brilliant Blue G-250 at aromatic amino acid radicals and measuring of extinction at λ = 595 nm. Finally, the results are expressed in mg protein/g fresh weight.

RESULTS AND DISCUSSIONS

1. Enzyme response of barley seedlings to oxidative stress generated by treatments with growth hormones. Numerous studies performed on animal and plant organisms analysed the role of xenobiotic factors (heavy metals, pesticides, mutagen agents etc.) in generation of oxidative stress and induction of reactivity of respective organisms by activation or stimulation of non-enzymatic or, especially, enzymatic antioxidative mechanisms. Activation or increased synthesis of antioxidative enzymes (POD, SOD, CAT, glutathion reductase, polyphenol oxidase) takes often place as response to the biotic and abiotic stress at which the plant is exposed. Higher levels of these enzymes are attributed, in these situations, to their role in xenobiotic oxidation (SPRECHER and STEWART, 1995). Many studies are dedicated to the effects induced by growth regulators. A number of *in vivo* and *in vitro* experiments evidenced an increase of leaf peroxidase activity in the treatments with phytohormones (2,4-D, IAA, ethylene) (AOSHIMA and TAKEMOTO, 2006). For example, all 2,4-D containing media determined higher levels of enzyme activity, the maximum value being registered for 10 mg/l 2,4-D. In *in vitro* wheat cultures, it seems that SOD and CAT activities are stimulated in regenerative callus, while POD responses are less specific. Generally, SOD responded in a positive manner in all growth hormone additions, but the reaction was more marked in auxin treatment (SZECHYNSKA-HEBDA et al. 2007). Several experiments regarding 2,4-D effects on mice CAT, POD, and SOD evidenced the inhibitory effect of this auxin on all enzyme activities, comparatively to control. The most severe effect was exerted on catalase activity (YILMAZ et al., 2004).)

In our studies, performed on barley seedlings obtained from seed treatments with growth regulators, the commentaries relative to antioxidative enzymes behaviour will be made on the basis of the results expressed in specific *enzymatic units/mg protein.*

Superoxide dismutase is the main enzymatic detoxifiant of superoxide anion that is converted to H_2O_2. Catalyzing the conversion of superoxide anion radical to H_2O_2 and molecular oxygen, SOD helps prevent tissue damage by $O_2^{\cdot -}$ and its metabolites. The principal scavenger of so formed H_2O_2 is the CAT and, in certain situations, the PODs. The increase of CAT activity constitutes an indicator of a toxic accumulation of H_2O_2 which becomes inhibitory for SOD activity. Thus, the SOD - CAT tandem serves as front-line antioxidant defence.

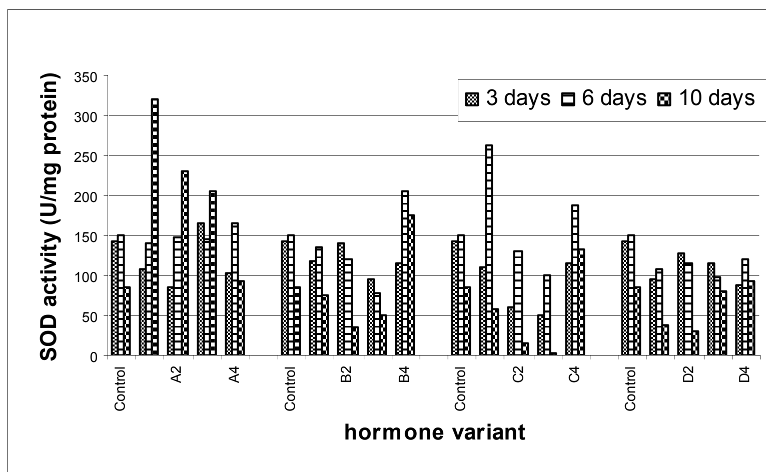

Fig. 1. Dynamics of SOD activity in barley seedlings, after treatments with growth hormones

Alterations in SOD activity (Fig. 1) showed different trends and registered enough important variations, its behaviour being similar from this point of view to the other two antioxidative enzymatic systems. Relative to control plants, in 3 days old seedlings, all hormone variants – excepting 25 mg l^{-1} 2,4-D (A3) – have inferior average values. This auxin concentration determined also the increase of peroxidase, comparatively to control, and slightly inhibited the plant height growth (TRUTA et al., 2008). The higher value of SOD activity in A3 variant is an indicative of generation of an oxidative stress by superoxide radical, probably in the presence of respective 2,4-D concentration. The strongest decline of SOD activity (60-65% lower than control) encountered in C2 and C3 variants was associated with different levels of catalase, namely lower than control in C2, respectively higher than control in C3, where the seedlings growth was inhibited. The depression both of SOD and CAT in B3 (25 mg l^{-1} Kin), C2 (10 mg l^{-1} GA_3) and D4 (50 mg l^{-1} 2,4-D + 50 mg l^{-1} kin) is equivalent to a lower protection against superoxides and hydrogen peroxide, fact that is favourable to their accumulation. Concerning 6 days old seedlings, in 2,4-D treated variants, SOD values are enough closed to control, slightly higher in A4 variant which also has a 25 % lower catalase activity.

In the case of kinetin treated variants, if in B1 (1 mg l^{-1} kin) all enzymes showed depressed activities (especially CAT), B2 variant has a more marked decrease of SOD (119.39 U/mg protein, comparatively to 150.59 U/mg protein for control), but the CAT and POD levels significantly surpass the control value probably as result of accumulation of hydrogen peroxide that becomes inhibitive for SOD and activate the CAT and POD-based antioxidative defence mechanisms. Increase of kinetin to 50 mg l^{-1} determined the augmentation of SOD activity of 1.4

times relative to control as response to the superoxide accumulation in the presence of chemical stressor. In this case, the catalase is low, but peroxidase is two times higher than control, fact confirming the existence of alternative mechanisms which can remove hydrogen peroxide so compensating for catalase reduced activity. One of these mechanisms is peroxidase that in certain conditions can metabolize H_2O_2 formed in reaction catalyzed by SOD in a first stage. The increase in the production of SOD without a subsequent elevation of catalase (as in B4 case) or peroxidase leads to the accumulation of hydrogen peroxide, which gets converted into the toxic hydroxyl radical. The toxicity of maximum tested kinetin concentration is also visible in strong inhibition of seedling plants during experiment.

At 240 hours, SOD showed a large variability between hormone treated variants, the bigger values being noted for 2,4-D treated variants and the smallest ones for hormone mixture treated variants. The most marked inhibition of SOD activities was present in C3 variant (2.44 U/mg protein) and in C2 variant (15.07 U/mg protein). In GA3 treatments, 1 mg l^{-1} concentration stimulated in 6 days old seedlings the activity of all tested oxidoreducing enzymes, whereas C2 and C3 variants have low SOD and POD levels. C3 variant (25 mg l^{-1} GA3) that in first germination stage had a significant decrease of SOD activity maintains and amplifies the descendant trend towards the third determination. The SOD depression accompanied by a 2.4 times smaller CAT activity shows a lower protection against ROS generating mechanisms.

2,4-D + Kin mixture was associated with SOD activity decrease at all tested concentrations. In variant D3, SOD was significantly inhibited (97.58 U/mg protein). Also appeared the possibility that superoxide anions to accumulate in cells, but the increase of CAT (220.80 U/mg protein, comparatively to 149.92 for control) and POD (11.51 U/mg protein, comparatively to 8.95 U/mg protein for control) activities prevents the risk of ROS accumulations. Oxidative stress induced in D4 seedlings is indicated not only by the SOD and CAT significant decline and slight POD decrease but also by strong growth inhibition of seedlings. Decrease of these enzymes suggests that plants have lost their system of defence.

Concerning _the dynamics of catalase activity_ (Fig. 2), 2,4-D treatments induced – excepting 6 days old seedlings of A1 variant – an augmentation of enzyme activity from one stage to another as barley plants grew and metabolic processes intensified. Relative to control, it can not be established any direct, linear relationship between 2,4-concentrations and catalase phenotype expression for none of the three stages of plants growth. A2 variant (10 mg l^{-1} 2,4-D) has a response superior to control at all the three moments of biochemical analyses, whereas A4 variant (50 mg l^{-1} 2,4-D) have in all three situations levels smaller than control, especially in 6 old days seedlings (the decrease was the most severe – with 25%).

10 mg l^{-1} kinetin (B2) induced the increase of catalase activity with 40% comparatively to control in 6 days old barley seedlings. Important diminutions were identified in B1 variant (6 days) but also in 6 days and 10 days old seedlings obtained by 50 mg l^{-1} kinetin treated seeds. This concentration generally determined important alterations of all analyzed enzymes in all stages and also produced the most severe inhibitions of plantlets growth during experiment.

The effects of gibberellic acid on catalase behaviour became pronounced in the second stage of analysis – 6 days. In dynamics, the enzyme generally registered increases of activity regarding substrate decomposition from one stage to another, in all concentration variants. At 1 mg l^{-1} GA3, the catalase activity was significantly stimulated (238.31 U/mg protein, comparatively to 149.92 U/mg protein, for control).

The pronounced catalase decrease in C3 variant was accompanied by important modifications especially of SOD levels.

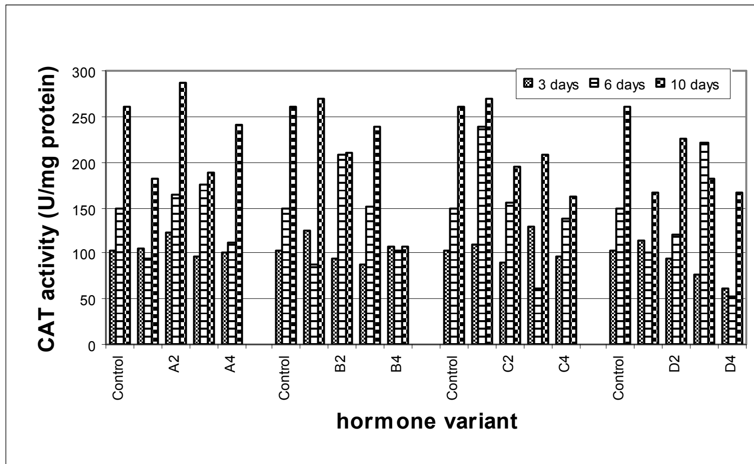

Fig. 2. Dynamics of catalase activity in barley seedlings, after treatments with growth hormones

In barley plantlets obtained from hormone mixture treated seeds, low levels of catalase activity have been registered for all tested concentrations and all three stages, with exception of 6 days old seedlings – D3 variant.

SOD augmentation observed in many studies performed on plant stress exposition can be the result of two factors: increase of superoxide radicals amount and *de novo* enzyme synthesis that in turn can be associated with SOD gene expression induction by superoxide mediated signalling transduction (FATIMA and AHMAD, 2005). Either as result of SOD activity or of direct formation in diverse metabolic pathways it is expected that H_2O_2 intracellular level to increase. In these conditions, catalase can intervene by converting H_2O_2 to H_2O. So, SOD and CAT are complementary in their action to diminish the effects of oxidative stress. Increase of some antioxidative enzymes such SOD and CAT can modify the endogenous phytohormone level, with repercussions on plantlets growth and development. Unlike most other organisms that have only one of each type of SOD in the various cellular compartments, plants have multiple forms of each type encoded by more than one gene because they evolved more complex antioxidant defence strategies. As in the case of SOD, multiple catalase isozymes encoded by specific genes are found in plants, whereas animals exhibit one form of CAT. Both *cat* and *sod* genes respond in a differential manner to various stresses known to generate ROS. This makes more difficult the explanation of some contradictory results relative to variable trends of the studied enzymes.

The variability amplitude of peroxidase (Fig. 3) was more restrained than that of catalase and superoxide dismutase. Some differences appeared in relation to seedlings growth and increase of metabolic pathways complexity, from one stage to another. A and D experimental variants presented insignificant fluctuations of peroxidase activity, comparatively to control in all three determinations.

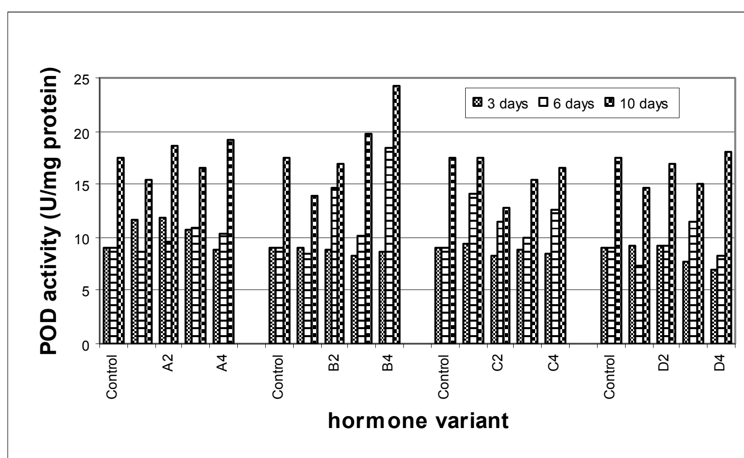

Fig. 3. Dynamics of POD activity in barley seedlings after treatments with growth hormones

Particular aspects presented B2 – 6 days, B3 – 10 days and B4 – 6 and 10 days variants where not only peroxidase suffered modifications but also the other two studied antioxidative enzymes. A slight reduced peroxidase activity had the C3 variant (GA3, 25 mg l^{-1}) that also presented alterations of SOD and CAT level. POD increase can be also the consequence of liberation of those enzyme forms located in cell wall in conditions of stress to which the plant is subjected (DEY et al., 2007).

In our studies carried out on barley seedlings obtained from seed treatments with growth regulators, the results generally evidenced the modification of antioxidative enzyme activity. The enzyme behaviour was fluctuant, sometimes contrastive and was influenced by quality and quantity of hormone addition, physiological moment when the biochemical analysis was performed and, doubtlessly, by endogenous hormone level of barley seedlings. Generally, our results concerning oxidoreductase responses to xenobiotic stress are closed to those published in literature. SOD, CAT and POD activities depend on quality, concentration and combination of hormones and on ontogenetic moment when the analysis of antioxidative enzymes was carried out. Certainly, the endogenous level of plant hormones is very important. Therefore, augmentation of activity of some oxidoreductases as response to exposure to growth regulators is a proof of detoxifying ability of seedlings by removal of ROS and their derivatives generated in stressed organisms. PPOD registered a smaller variability than the two other enzymes. It seems that SOD and CAT rather than POD acted as components of system of antioxidative defence.

2. Influence of growth hormones on protein content in barley seedlings. The treatments with various xenobiotic factors induce a large scale of morphological, physiological, biochemical. At molecular level, effects are reflected in change of expression pattern of some genes coding, for example, proteins or other compounds. There are studies evidencing the stimulation of nucleic acids in pea and artichoke by kinetin addition (SHININGER, 1980). In tobacco, soybean and radish, others researchers detected non nucleic acids increases but ribosome aggregations in so called polysomes, associated with augmentation of protein synthesis and cell division (FOSKET et al., 1977). SPRECHER and STEWART (1995) sustain that it is expected an increase of RNA and protein levels and of oxygen consumption in hormone addition. In stress conditions, the protective system can be surpassed by rapid production and

accumulation of ROS, fact determining protein structural modifications. Oxidative damage of ROS on proteins refers to site-specific amino acid modifications, fragmentation of the peptide chain; aggregation of cross-linked reaction products, altered electrical charge. Oxidation of specific amino acids because of increased reaction rate of superoxide anions with side chains of amino acids “marks” proteins for degradation by specific proteases and can lead to cross-linking and to an increased susceptibility to proteolysis (BABIOR, 1997; DAVIES, 2003; SCANDALIOS, 2005; CARGNELUTTI et al., 2006; RELLÁN-ÁLVAREZ et al., 2006). Protein oxidation in this way is often accompanied by increase of soluble protein amount as it was demonstrated in species of *Cucumber* (GONÇALVES et al., 2007).

In early ontogenetic phases (Fig. 4), the passage from undifferentiated meristematic state to differentiation state is characterized by alteration of protein and enzyme pattern.

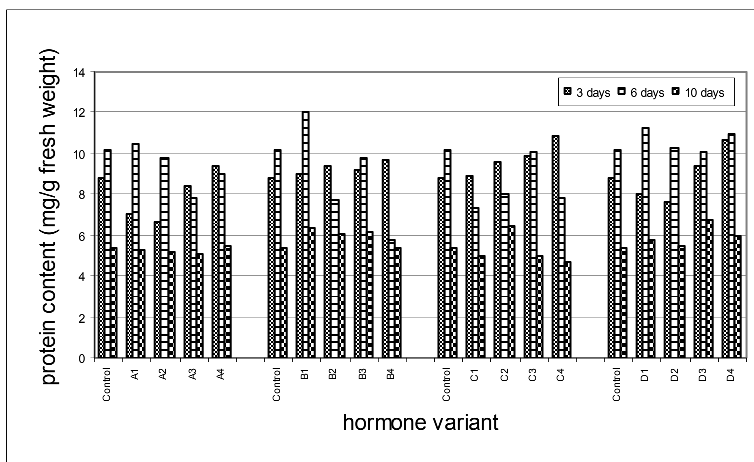

Fig. 4. Dynamics of soluble protein content in barley seedlings after hormone treatments

The 3 days old seedlings responded to 2,4-D low concentrations by a diminished protein synthesis, comparatively to control. The highest auxin concentration (50mg l^{-1}) had a slight stimulant effect on protein synthesis. All kinetin concentrations (B variants) induced increases with 2 – 10 % of protein amount. Increases of soluble protein can be the consequence of *de novo* synthesis of some stress proteins as result of exposure to exogenous hormone factor. In GA3 treatments a direct relation was evidenced between concentration increase and protein level, at first biochemical determination. Hormone mixture at lower concentrations slightly inhibited protein synthesis, comparatively to control, but the high concentrations determined increases of protein levels, with 6.5%, for 25 mg l^{-1}, respectively 21%, for 50 mg l^{-1}.

At 144 hours, A1 variant showed a small stimulation of protein synthesis so that protein level exceeded the control, but the other three concentrations have lower values than control, especially A3 variant. Concerning kinetin effects, B1 variant intensified the protein synthesis. In B2, B3 and B4, after initial protein stimulation, the repression mechanism of genes coding protein synthesis became functional fact evidenced by low level of proteins – with 25%, respectively 43 % - in barley seedlings, comparatively to control. The reduced protein content is also correlated with inhibition of seedling elongation, especially in B4 variant. It seems that the 10 mg l^{-1} and 50 mg l^{-1} concentrations strongly exert their cytotoxicity in this moment of barley

seedling ontogeny, because important modifications of antioxidative enzymes were registered in these variants at 6 days. GA3 generally decreased the protein level, whereas the variants treated with hormone mixtures synthesized higher protein amounts than control in 6 days old seedlings.

In 10 days old seedlings both control and all treated variants had significant decreases of protein levels. The fluctuations are not important comparatively to specific control. For each tested hormone the constancy of this quantitative parameter is visible, indifferently of concentration.

CONCLUSIONS

In seedlings of *Hordeum vulgare* L. cv. *Madalin*, SOD, CAT and POD activities registered enough important variations depending on quality, quantity and combination of hormones, ontogenetic moment and physiological state of seedlings.

POD showed smaller limits of variability than SOD and CAT. It seems that SOD and CAT rather than POD acted as components of antioxidative protective system.

Generally, the protein level not presented significant modifications in 3 days old seedlings. At 6 days, the major part of variants responded by amplification of protein synthesis. In 10 days old seedlings, both control and hormone variant reduced their protein synthesis.

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