

ANTIOXIDATIVE ENZYMES IN THE RESPONSE OF BUCKWHEAT (*FAGOPYRUM ESCULENTUM* MOENCH) TO COMPLETE SUBMERGENCE

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Abstract - Oxidative stress and antioxidative defense system activity were studied in buckwheat leaves after complete submergence and re-aeration. The levels of H₂O₂ and lipid peroxidation were found to be significantly higher in stressed than in untreated buckwheat leaves. Enzymes catalyzing the degradation of H₂O₂ and peroxides were shown to participate actively, whereas superoxide dismutase did not take part in the buckwheat leaf response to flooding stress. The most prominent increase in antioxidative enzyme activities was noticed upon return to air, when the strongest oxidative stress occurred and the need for antioxidative defense was the greatest.

Key words: Complete submergence, lipid peroxidation, antioxidative enzymes, buckwheat

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INTRODUCTION

Submergence refers to the situation when floodwaters rise to levels that keep shoots completely under water. Some important effects of submergence include the reduction in available light, water and nutrient uptake leading to a decrease in metabolism. The most significant consequence of flooding stress is the prevention of a direct exchange of gases between the plant and the atmosphere resulting in reduced O₂ and CO₂ levels. The low rate of gas diffusion in water compared to that in air limits the entry of CO₂ for photosynthesis and O₂ required for respiration. Low CO₂ levels hamper the rates of underwater photosynthesis (Colmer and Voesenek, 2009). As a consequence, the photosynthetic electron transport chain becomes over-reduced, forming superoxide radicals (O₂^{•-}) and singlet oxygen species (¹O₂) in the chloroplasts (Hossain et al., 2009). In addition, progressive soil flooding leads to anoxia with profound effects on the plant respira-

tory metabolism (Dat et al., 2004). At very low light intensity, mitochondria are the main source of reactive oxygen species (ROS) due to the activity of the mitochondrial electron transport chain (Skutnik and Rychter, 2008). In the absence of O₂, which is the terminal acceptor in the mitochondrial respiratory chain, the ATP level decreases and is synthesized mainly by alcoholic fermentation (Yordanova and Popova, 2002). Due to their close dependence on O₂ homeostasis for normal functioning, mitochondria may play a crucial role in sensing changes in environmental O₂ levels (Dat et al., 2004). It was shown previously that decreases of O₂ in plants, ranging from low concentrations (hypoxia) to complete absence (anoxia), have been associated with changes in the expression of genes related to ROS-mediated signal transduction (Voesnek et al., 2006). Re-aeration during the post-anoxic period, when the water recedes, may also result in serious cell damage. The depletion of ATP, increased reduction state of the cell environment and hyper-polarization of

the inner mitochondrial membrane during anoxia may create conditions of enhanced ROS production and oxidative stress after re-exposure to air called “post-anoxic injury” (Skutnik and Rychter, 2008). It is also accepted that hypoxia and anoxia lead to changes in H_2O_2 production (Voesnek et al., 2006). In particular, H_2O_2 produced by cytosolic membrane-bound NADPH oxidases has been associated with stress responses (Laloi et al., 2004). As H_2O_2 is potentially damaging, there are cellular antioxidant mechanisms that remove it very efficiently (Noctor and Foyer, 1998; Corpas et al., 2001). The amount of ROS depends on the equilibrium between the formation rate and the detoxification capacity of the antioxidative system. ROS have pleiotropic effects in plants, as they do in animals. When produced in a controlled manner within specific compartments, ROS have key roles in plant metabolism and molecular biology. On the contrary, when they are synthesized in excess, the resultant uncontrolled oxidation leads to cellular damage and eventual death. To prevent damage, and at the same time allow the beneficial functions of ROS to continue, the antioxidant defenses must keep active oxygen under control (Noctor and Foyer, 1998). In higher plants several antioxidants (ascorbate, glutathione, phenolic compounds, tocopherols) and the antioxidative enzymes (superoxide dismutase, catalase, peroxidases and glutathione reductase) are involved in the ROS detoxification system (Yordanova and Popova, 2002).

There are only a few reports presenting data on changes in the antioxidative enzyme activities in leaves in response to complete submergence and subsequent re-aeration. In this study we report that both complete submergence and re-aeration cause oxidative damage in buckwheat leaves and induce their antioxidative defense system.

MATERIALS AND METHODS

Plant materials and stress treatments

Buckwheat plants were grown in the greenhouse of the Institute for Molecular Genetics and Genetic

Engineering in Belgrade for two weeks. The environmental conditions were: 12h photoperiod, temperature 24°C. Prior to submergence, control leaf samples were taken. The plants were then submerged completely in water and flooded leaf samples were taken after 48 h. Following re-aeration for 1 h and 2 h, new leaf samples were taken from the same plants. The leaves were frozen immediately in liquid nitrogen and stored at -70°C.

Histochemical detection of H_2O_2 in buckwheat leaves

Both control and treated leaves were submerged in a solution containing 1 mg/mL DAB (3,3-diaminobenzidine 4-hydrochloride), pH 3.8 and held in the dark for 4 h. Afterwards the chlorophyll was removed by sinking the leaves in boiling ethanol for 10 min. Brown precipitation products of the reaction indicated the accumulation of H_2O_2 in leaf tissue.

Determination of lipid peroxidation level

The extent of lipid peroxidation was determined by spectrophotometric measurement of malondialdehyde (MDA), synthesized in a reaction with thiobarbituric acid (Heath and Packer, 1968).

Protein extraction

The whole cell protein extract used for determination of enzyme activity was obtained by grinding 0.5g of leaf tissue in liquid nitrogen, followed by homogenization in 1mL of extraction buffer containing 10 mM Tris pH 6.8, 0.5 M NaCl and 1% triton. Homogenates were centrifuged for 30 min at 13000×g at 4°C. The protein concentration in the extracts was measured spectrophotometrically according to Bradford (1976).

Enzyme assays

CAT (EC 1.11.1.6) activity was determined spectrophotometrically by monitoring the consumption of H_2O_2 at 240 nm for 30s (Aebi, 1984) in a reaction mixture containing 50 mM potassium phosphate

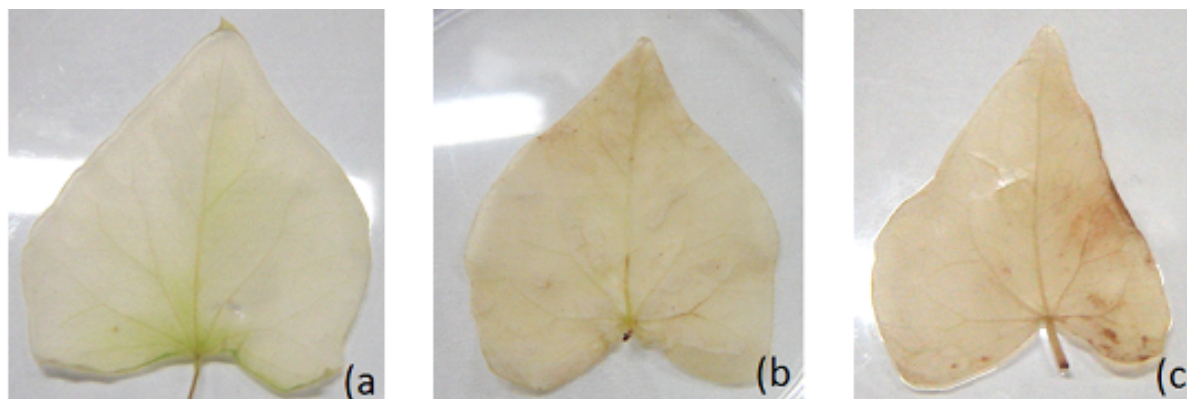


Fig. 1. Histochemical detection of H_2O_2 in buckwheat leaves subjected to complete submergence following re-aeration in buckwheat leaves a) control, b) submerged for 2 days, c) upon 2h re-aeration

buffer pH 7.0, 0.1 mM EDTA, 20 mM H_2O_2 and 33 μ L extract in 1mL total volume. The reaction was started by the addition of H_2O_2 . CAT activity was calculated and expressed as $\Delta A_{240} \text{ min}^{-1}(\text{mg of protein})^{-1}$. POD (EC 1.11.1.7) activity was measured by monitoring the change of absorption at 470 nm due to the formation of tetraguaiacol (Pole et al., 1994). The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 0.1 mM EDTA, 10 mM guaiacol, 10 mM H_2O_2 and 33 μ L extract in 1mL volume. The reaction was started by the addition of H_2O_2 and monitored for 50 s. POD activity was calculated and expressed as $\Delta A_{470} \text{ min}^{-1}(\text{mg of protein})^{-1}$. The activity of APX (EC 1.11.1.11) was determined in 1 mL reaction mixture containing 50 mM potassium phosphate buffer pH 7.0, 0.1 mM EDTA, 0.5 mM ascorbate and 33 μ L extract. The H_2O_2 -dependent oxidation of ascorbate was determined by measurement of the decrease in absorbance at 290 nm (Nakano and Asada, 1981). The activity of APX was expressed as $\Delta A_{290} \text{ min}^{-1}(\text{mg of protein})^{-1}$. SOD (EC 1.15.1.1) activity was determined by measurement of NBT photochemical reduction (Beauchamp and Fridovich, 1971). The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 0.1 mM EDTA, 13 mM methionine, 2 μ M riboflavin, 75 μ M NBT and 33 μ L extract in 1 mL volume. Fifteen min after exposure to white fluorescent light the reaction tube contents were mixed and absorbance was measured at 560 nm. SOD activity was calculated according to the equation $V/v-1$,

where v represents the activity of the sample and V the activity of the control which contained no enzyme. SOD activity was expressed as $\Delta A_{560} \text{ min}^{-1}(\text{mg of protein})^{-1}$

Statistical analysis

The results are presented as mean values \pm standard errors. Lipid peroxidation level and enzyme activities were determined in three independent experiments repeated in triplicate. The results were analyzed by Student's t test using SPSS statistical software (SPSS Inc., Chicago, IL, USA) and p values <0.05 were considered significant.

RESULTS

Histochemical detection of H_2O_2 and lipid peroxidation

H_2O_2 was detected histochemically in the leaves of plants subjected to complete submergence lasting 2 days, followed by re-aeration for 2 h. Brown precipitation products indicating the accumulation of H_2O_2 in the leaf tissue were more pronounced in the re-aerated leaves than in those subjected to submergence only (Fig. 1). The concentration of MDA was used as a general marker of oxidative stress as well as an indicator of the level of lipid peroxidation, since it is possible that carbohydrates and some amino

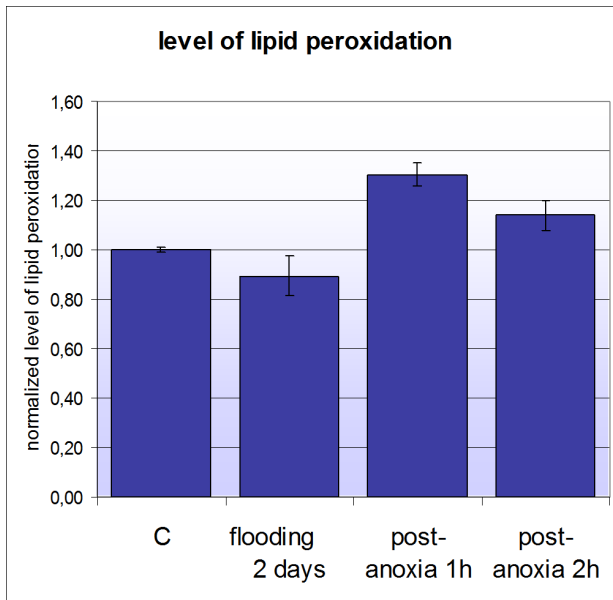


Fig. 2. Level of lipid peroxidation estimated by measuring MDA concentration in buckwheat leaves subjected to anoxia and re-aeration.

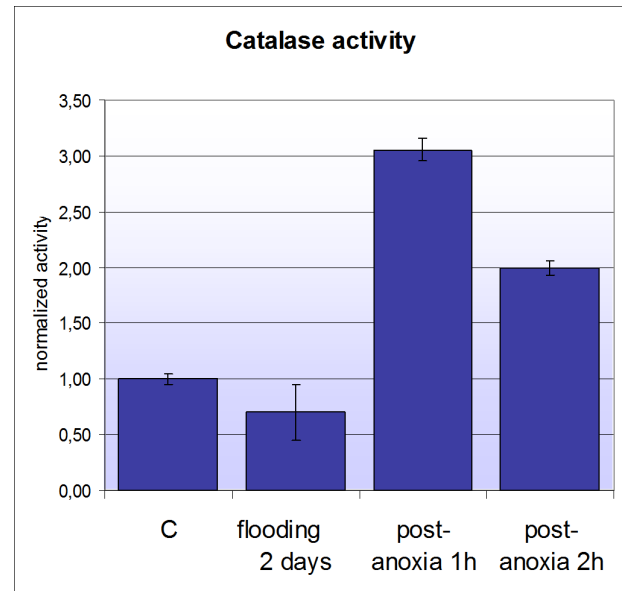


Fig. 4. CAT activity of buckwheat leaves during exposure to anoxia, and after re-aeration.

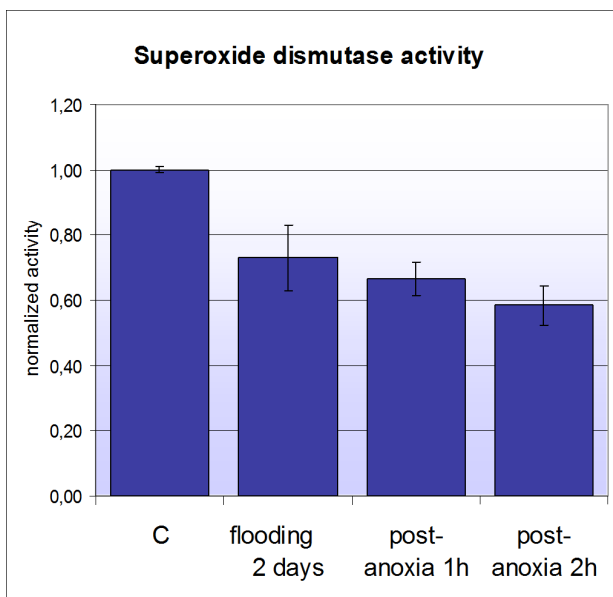


Fig. 3. SOD activity of buckwheat leaves during exposure to anoxia, and after re-aeration.

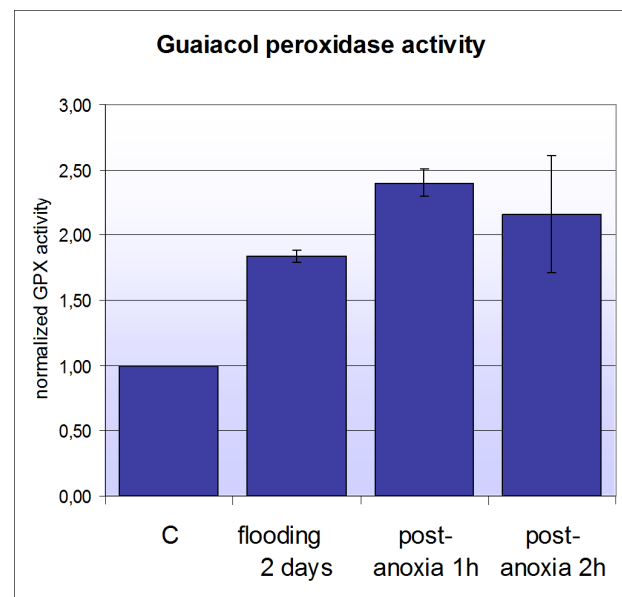


Fig. 5. APX activity of buckwheat leaves during exposure to anoxia, and after re-aeration.

Abbreviations: ROS - reactive oxygen species, MDA - malondialdehyde, DAB - (3,3-diaminobenzidine 4-hydrochloride), SOD - superoxide dismutase, CAT - catalase, POD - peroxidases, APX - ascorbate peroxidase, NBT - nitroblue tetrazolium, TBARS - thiobarbituric acid reactive substances, PUFA - polyunsaturated fatty acid.

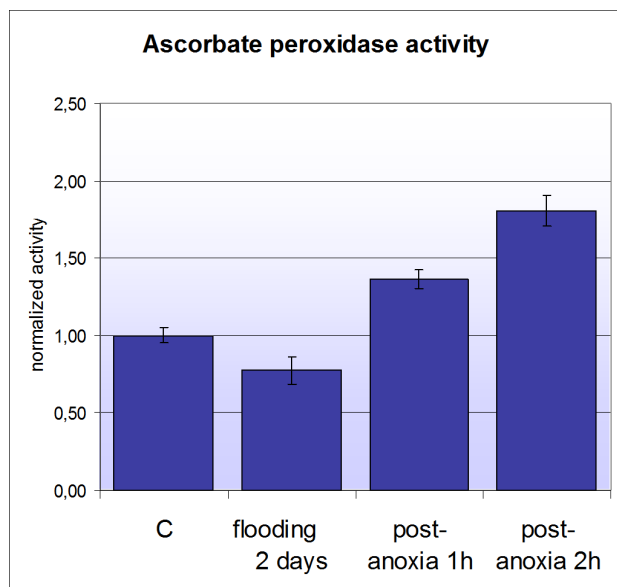


Fig. 6. POD activity of buckwheat leaves during exposure to anoxia, and after re-aeration.

acids were catabolized to MDA as an end-product (Hossain et al., 2009). An 11% decrease of the MDA content was detected in the buckwheat leaves during submergence, while re-aeration for 1 h resulted in a further increase of 30%. After 2 h in air, the lipid peroxidation level was still 14% higher than in the untreated controls (Fig. 2). Expression of the effect of flooding and post-anoxia was normalized to the control.

Antioxidative enzyme activities

Superoxide dismutase activity decreased in both the submerged and post-hypoxic leaves by 27% and 33% to 42%, respectively (Fig. 3) Catalase activity was found to be 30% lower in the submerged leaves, while 3-fold and 2-fold increases were detected after re-aeration for 1 and 2 h, respectively (Fig. 4). Ascorbate peroxidase activity declined in the submerged leaves by 23%. On the other hand, re-aeration for 1 h provoked a 36% increase in activity. APX activity continued to increase during the second hour of post-hypoxia, reaching 1.81 times the activity measured in the untreated control (Fig. 5). Guaiacol peroxidase activity was found to increase by 84% in

leaves submerged for 2 days. The activity increased further after the first hour back in air, reaching 2.4 times the activity measured in the untreated control. It remained high (2.16 fold) during the second post-hypoxic hour in air (Fig. 6).

DISCUSSION

It is evident that ROS produced during various abiotic stresses, act as signal molecules which activate plant defense mechanisms. Since the reactive species produced in plant organs during submergence induced stress, and especially after readmission to air, are also extremely toxic, their production and elimination must be under strong control of the cellular enzyme and non-enzyme protective mechanisms. Concerning anoxia tolerance, the formation of ROS (H_2O_2) is less pronounced and postponed in time in the anoxia-tolerant than in the anoxia-intolerant species. In the rhizomes of *Iris pseudacorus*, an extremely anoxia-tolerant plant, H_2O_2 was detected only after 45 days of anoxia (Chirkova, 1998). Submerged buckwheat plants showed an increasing trend in foliar H_2O_2 formation during both hypoxia and re-aeration. These results confirm the fact that buckwheat is considered as a hypoxia-intolerant species (Nishimaki, 1983; Takemae, 1986).

On the other hand, quantification of the lipid peroxidation level indicated oxidative damage only in re-aerated leaves. A burst of ROS occurred when aerobic conditions were restored. The results are very interesting because they are similar to those obtained with the anoxia-tolerant species, *I. pseudacorus*, where anoxia induced no TBARS production until after 45 days of anoxic treatment (Blokhina et al., 2003). In terms of membrane damage, the post-anoxic burst of oxidative reactions had a more pronounced effect than anoxia itself (Blokhina, 2001). Since *de novo* synthesis of lipids needs molecular oxygen, desaturases and large amounts of ATP, it stops during anoxia. Therefore, the most efficient way to maintain functional membranes under anoxia is to preserve preformed PUFA and lipids instead of their synthesis (Blokhina, 2000).

The involvement of the antioxidative system in the regulation of free-radical metabolism was followed by measuring changes in enzyme activities. Submergence produced a continuous decrease in SOD activity, and the same trend continued during re-aeration. Similar results were obtained with rice (*Oryza sativa* L.) roots (Chirkova et al., 1998), rice seedlings (Ushumaru et al., 1999), *Iris germanica* L. rhizomes (Monk et al., 1987) and maize (*Zea mays* L.) roots (Yan et al., 1996), although there are a number of opposing results showing an increase of SOD activity in different plant species under anoxia: soybean (*Glycine max* L.) seedlings (Van Toai and Boles, 1991), barley (*Hordeum vulgare* L.) roots (Kalashnikov et al., 1994), *I. pseudacorus* L. rhizomes (Monk et al., 1987). These contradictions could be explained by the different tolerance to anoxia between the species, or the existence of several mechanisms involved in protection against oxidative stress under these conditions. The activation of oxygen can proceed through different mechanisms which may lead to the formation of highly reactive singlet oxygen ($^1\text{O}_2$), for example. It is also possible that the formation of ROS under hypoxic conditions and the oxidative burst after re-aeration could cause rapid substrate overload of constitutive SOD, while induction may be hindered by other factors [e.g. time, activity of downstream enzymes in the ROS detoxification cascade, inhibition by the end product (H_2O_2) and the consequences of anoxic metabolism] (Blokhina et al., 2003). The gradual increase in H_2O_2 concentration in continuously flooded plants may be partially responsible for the slow decline in SOD activity (Hossain et al., 2009). Catalase activity was found to decrease in submerged plants and increase after re-aeration. An interesting similarity exists with the findings obtained for rice seedlings, where the SOD activity did not change significantly in response to submergence, while the catalase activity increased upon re-aeration (Ushumaru et al., 1999). Increased activity of CAT was also shown in citrus leaves subjected to waterlogging and subsequent drainage (Hossain et al., 2009). As shown previously for some other stress conditions, CAT could be one of the most important antioxidative enzymes

in buckwheat leaves (Jovanovic et al., 2006). It appears that in our case CAT does not participate in the early response to submergence. Buckwheat leaf APX did not contribute to the oxidation of ascorbate during the hypoxia, since a decline in activity was observed. Similar results were shown in barley plants subjected to anoxia and post-anoxia (Skutnik and Rychter, 2008) as well as in experiments carried out on wheat roots (Biemelt et al., 1998). Proteomic analyses of soybean seedlings responding to flooding were conducted to identify the key proteins involved in the response. One of the altered proteins was dominantly down-regulated under flooded conditions and was identified as cytosolic ascorbate peroxidase 2 (cAPX2). Northern-hybridization showed that the abundance of cAPX2 transcripts decreased significantly after flooding, as did its enzymatic activity (Shi et al., 2008). However, upon return to air, the APX activity in buckwheat leaves increased. These results suggest that APX is involved in the response to the re-aeration after submergence in buckwheat leaves. Guaiacol peroxidase was increased during submergence and the first hour of air readmission. Similar data have been reported for rice seedlings (Ushumaru et al., 1999), and also for barley leaves (Yordanova and Popova, 2002) subjected to submergence. These results indicate that this enzyme reacts very rapidly at the early stage of stress, thus playing an important role in defense against flooding stress in buckwheat leaves.

From the results obtained in this study we can conclude that the enzymes catalyzing H_2O_2 and peroxide degradation (CAT, APX and GPX) actively participate in the buckwheat leaf response to flooding stress. The most prominent increase of antioxidative enzyme activities was noticed upon return to air, when the strongest oxidative stress occurred and the need for antioxidative defense was the highest.

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