

COLD-INDUCED RESPONSE OF BUCKWHEAT (*FAGOPYRUM ESCULENTUM* MOENCH) SEEDLINGS. B. Lučić¹, Ž. Jovanović², S. Radović¹, and V. Maksimović². ¹Faculty of Biology, University of Belgrade, 11000 Belgrade, Serbia; ²Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, 11010 Belgrade, Serbia

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Cold stress is a major environmental factor that limits the agricultural productivity of plants and has a huge impact on their survival and geographical distribution (Zhu et al., 2007). Plants differ in their tolerance to chilling (0-15°C) and freezing (<0°C) temperatures. Many changes in physiological and biochemical pathways have been observed during the exposure of plants to low temperatures, such as modified levels and activities of enzymes, appearance of new protein isoforms, and altered lipid membrane composition (Tomashov, 1999). Low temperatures induce overproduction of reactive oxygen species (ROS) that can bring about serious cellular damage by reacting with DNA, proteins, and lipids (Sattler et al., 2000). Cold exposure can alter the structure of membranes due to lipid peroxidation (Jouve et al., 1993).

The ability of plant cells to adjust their scavenging system to elevated ROS levels appears to be one of the basic elements in acquiring stress tolerance (Anderson et al., 1995). Plants employ both enzymatic and non-enzymatic systems in protecting themselves against ROS toxicity (Pastori and Foyer, 2002). The enzymatic system consists of ROS scavengers such as superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6), guaiacol peroxidase (EC 1.11.1.7), and ascorbate peroxidase (EC 1.11.1.11).

Common buckwheat (*Fagopyrum esculentum* Moench) belongs to the family Polygonaceae and generally prefers higher altitudes and temperate regions for its growth. In spite of being a potential source of cold-tolerance genes, mechanisms involved in the response of buckwheat to cold stress and the effects of low temperature on buckwheat are almost unknown.

In the present study, changes of lipid peroxidation as well as activity and composition of antioxidative enzymes were investigated in buckwheat seedlings subjected to low temperature (4°C) in order to obtain more information about the mechanisms involved in the response of buckwheat to cold.

Buckwheat seeds were germinated for 10 days on plates with cotton soaked in ½ Murashige-Skoog nutrient solution under optimal light and temperature conditions (25°C). Formed seedlings were subjected to a temperature of 4°C for 48 h in the dark (treatment), whereas control seedlings were kept at 25°C in the dark. After the treatment, crude protein extracts were prepared from treated as well as control seedlings in buffer containing 50 mM potassium phosphate (pH 7.0) and 0.1 mM EDTA. The homogenates were centrifuged at 15000 g for 20 min, and the supernatant was used for assays of enzyme activity and IEF analysis. All steps were carried out at 4°C. Protein concentration in the extracts was determined according to Bradford (1976) using a Bio Rad assay kit. Catalase (CAT) activity was assayed

according to Aebi (1984) and activity was expressed as $\Delta\text{Abs}_{240} \text{ min}^{-1}\text{mg}^{-1}$ of protein. Ascorbate peroxidase (APX) activity was assayed as described by Nakano and Asada (1981) and activity was expressed as $\Delta\text{Abs}_{290} \text{ min}^{-1}\text{mg}^{-1}$ of protein.

Soluble guaiacol peroxidase (POD) activity was determined according to Chance and Maehly (1955) and activity was expressed as $\Delta\text{Abs}_{470} \text{ min}^{-1}\text{mg}^{-1}$ of protein.

Superoxide dismutase (SOD) activity was measured according to Beuchamp and Fridovich (1971) and activity was expressed as $\Delta\text{Abs}_{560} \text{ min}^{-1}\text{mg}^{-1}$ of protein.

For determination of peroxidase isoforms, total soluble proteins (25 µg) were separated by isoelectrofocusing (IEF) on a pH gradient in the range of 3 to 9, and peroxidase isoenzymes were visualized by in-gel staining with 20 mM guaiacol or 0.6 mM 4-chloro-1-naphthol.

The level of lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) produced by the thiobarbituric acid reaction as described by Heath and Packer (1986).

All measurements were performed in three independent experiments with three replications each. All the obtained data were subjected to statistical analysis using the Sigma stat program. Comparisons with $P < 0.05$ were considered significantly different.

Possible harmful effects of low temperature treatment on buckwheat seedlings were investigated in terms of morphological changes and survival of seedlings, as well as changes in the level of lipid peroxidation.

All treated seedlings survived the low temperature treatment and recovered rapidly after cessation of the treatment. The level of lipid peroxidation was increased by the low temperature treatment, being 54% above the control (not shown). This increase suggests enhanced production of ROS in buckwheat seedlings during low temperature exposure. There is much evidence indicating that at least some effects of chilling are mediated by ROS, which may lead to irreversible damage or adjustment to stress conditions (Dat et al., 2000). The results presented here showed that in buckwheat seedlings during cold treatment, only reversible changes leading to adjustment to this condition occurred because all seedlings survived the treatment and could after it grow to adult plants. This observation suggests that buckwheat seedlings possess efficient systems for ROS detoxification. Analysis of antioxidative enzyme activity revealed that activities of SOD and CAT were not changed

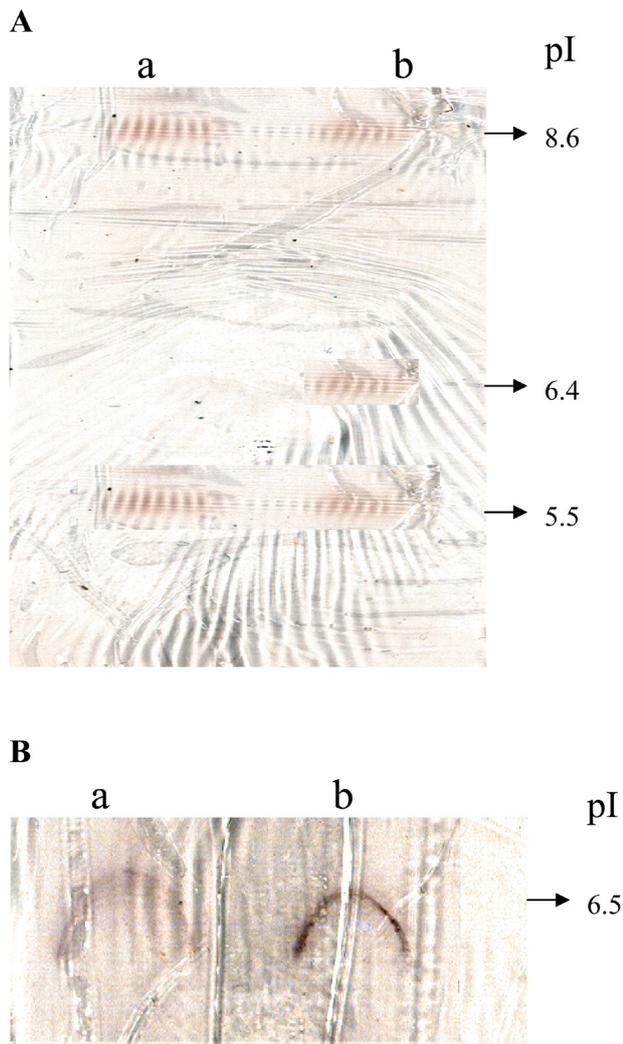


Fig. 1. Isoforms of peroxidases (PODs) in buckwheat seedlings. Total soluble proteins (25 μ g) from untreated (a) and cold-treated buckwheat seedlings (b) were separated by IEF and visualized by activity staining using guaiacol (A) and 4-chloro-1-naphthol (B) as substrates. pI - isoelectric point.

significantly by the cold treatment, whereas those of PODs and APX were increased by 33 and 22%, respectively, in comparison with the control (not shown). These results indicate that the activity of SOD was enough to transform superoxide anions into

hydrogen peroxide, which was subsequently degraded by ascorbate peroxidase and guaiacol peroxidase. Coordinated activities of antioxidative enzymes could be responsible, at least in part, for the observed cold tolerance of buckwheat seedlings.

Changes in peroxidase isoforms were revealed by guaiacol-specific staining of IEF-separated total soluble proteins (Fig. 1A). The new isoform with pI 6.4 that appeared upon cold treatment can be considered as a specific cold-induced form. Regarding the multiple functions performed by PODs in plant cells (Passardi et al., 2005) and the reinforcement of seedling cell walls observed in our experiments, the isoform with pI 6.4 could be responsible for lignification of cell walls. In addition, this isoform could perform other functions important for cold tolerance. When 4-chloro-naphthol was used as a substrate, only one form of peroxidases appeared in treated as well as in control seedlings (Fig. 1B), indicating different substrate specificity and affinity of various forms of peroxidases. Further investigation of activity, inducibility, and stress- and substrate-specificity of POD isoforms, as well as isolation and characterization of particular genes, could help to reveal the precise functions of these enzymes in the response of plant to stress, with possible application in improving plant stress tolerance.

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