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ACTIVITIES OF ANTIOXIDATIVE ENZYMES DURING *CHENOPODIUM RUBRUM* L. ONTOGENESIS *IN VITRO*

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Abstract — For the short-day plant *Chenopodium rubrum*, a 14 h/10 h photoperiod is inductive for flowering, while continuous light (CL) is noninductive. Plants of one group were grown continuously under an inductive photoperiod, while in the other group flowering induction was delayed by 17 days of CL in order to separate on the time scale different developmental phases in plants of the same age. Regardless of the photoperiodic conditions the plants were exposed to, seed maturation occurred in 10 weeks. Activities of catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) were determined in different phases of development (vegetative growth, flowering, seed development, and maturation). The activities of antioxidative enzymes depended on both the phase of development and the photoperiod. In plants grown continuously under an inductive photoperiod, high CAT and POD activities were detected at the time of flowering and decreased during seed development and maturation. In plants in which flowering induction was delayed by 17 days of CL, the activities of POD and SOD were lowest in the vegetative phase of development and attained maximum values in the phase of seed maturation. In both groups of plants, the highest CAT activity was measured at the time of flowering.

Key words: *Chenopodium rubrum,* growth, flowering, seed maturation, catalase, peroxidase, superoxide dismutase

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INTRODUCTION

Continuous production and removal of reactive oxygen species is, besides being a phenomenon with negative consequences (damage to cell membranes and organelles), linked with a signal role in plant developmental processes (Elstner, 1982; Hendry and Crawford, 1994). The antioxidative enzymes catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) are engaged in the scavenging of free radicals and activated oxygen species (Van Loon, 1986; Bowler et al., 1992; Khan and Panda, 2002) and thereby participate in regulation of plant growth and development processes or protection against pathogens or abyotic stress.

CAT and POD metabolize H_2O_2 in different ways, different metabolic patways of H_2O_2 degradation probably coresponding to differences of plant metabolism correlated with different phases of development or protection against biotic or abiotic stress (Marinescu et al., 2000). Catalase protein synthesis is linked with the photosynthetic and photorespiratory pathways (Schmidt et al., 2002). Since it was shown that H_2O_2 is involved in hormone-dependent developmental signaling processes, cell wall growth, and control of stomatal closure (Schroeder et al., 2001), it was supposed that CAT regulation serves to limit H_2O_2 accumulation while allowing essential signaling functions to occur (Luna et al., 2004). Changes in CAT activity are linked with desiccation during seed maturation (Bailly et al., 2004), seed germination (Bailly et al., 2002; Prodanović et al., 2007), and plant growth and development (Bailey and Mc Hargue, 1943; Matters and Scandalios, 1986). PODs are the most investigated enzymes since they have a role in very important physiological processes such as seed germination and seedling growth (Belani et al., 2002; Dučić et al., 2003/4; Prodanović et al., 2007); root growth (Gaspar et al., 1992, Kukavica et al., 2007); plant growth

and development (Baiey and Mc Hargue, 1943; Fielding and Hall, 1978); and lignin biosynthesis in cell walls (Bruce and West, 1989).

SODs are a group of metalloenzymes that catalyse the disproportionation of superoxide molecules (Mc Cord and Fridovich, 1968), constituting the first line of defense against reactive oxygen species within the cell (Alcher et al., 2002). Changes of SOD activities are associated with seed germination (Gidrol et al., 1994), as well as with plant growth and development (Matters and Scandalios, 1986; Lall and Nikolova, 2003).

Chenopodium rubrum L. sel. 184 is a qualitatively short-day weedy annual with a defined critical night length of 8 h (Tsuchiya and Ishiguri, 1981). It is sensitive to photoperiodic stimulus for flowering as early as at the cotyledon stage (Seidlová and Opatrná, 1978), when six appropriate photoperiodic cycles are sufficient for photoperiodic flower induction. As an early flowering species (Cumming, 1967), the plant flowers after 15 days under suitable photoperiodic conditions *in vitro* (Živanović et al., 1995) and produces seeds after 10 weeks (Mitrović et al., 2007). *C. rubrum* plants modify their growth and development in accordance with the photoperiod they are exposed to (Cook, 1975; Mitrović et al., 2007).

By exposing one group of plants to a photoperiod inductive for flowering at the cotyledon stage and another 17 days later, we separated (on the time scale) vegetative growth, flowering, and seed development and maturation in plants of the same age in order to register changes in activities of antioxidative enzymes linked with different developmental phases.

MATERIAL AND METHODS

Plants *in vitro*. The experiments were carried out with intact *C. rubrum* plants derived from seeds sown *in vitro*. Seeds (1-year-old) were collected from plants grown *in vitro* under a 16 h/8 h photoperiod at 25°C. They were surface sterilized with 4% Na-hypochlorite for 2 min, washed with sterile distilled water, and aseptically sown on moistened filter paper in Petri dishes. Uniform germination was attained with suitable temperature and dark/light cycles (24 h of darkness at 32°C, 24 h of darkness at 10°C, and 48 h white light at 32°C). Four-day-old seedlings were transferred to glass jars containing 100 ml of MS (Murashige and Skoog, 1962) mineral solution supplemented with sucrose (5%) and gelled with agar (0.7%) and exposed to two different photoperiodic treatments: 65 days of a 14 h/10 h photoperiod, or 17 days of continuous light followed by 43 days of 14 h/10 h. Irradiance was about 70 μmol m^{-2} s^{-1}. Temperature in the growth chambers was 25 ± 2°C.

Plants were checked (plant height, number of leaves, percentage of flowering, number of matured seeds) after 17, 37, and 65 days of culturing *in vitro* before freezing in liquid nitrogen prior to extraction.

Extraction of plant material. Samples (four replicates of 0.2 g for each experimental point) of plant material were powdered in liquid nitrogen. The extraction buffer contained 0.05 M Tris (pH 7.4), 0.25 M sucrose, and 1 mM EDTA. Frozen powder was added to the extraction buffer in a 1:5 ratio. The mixture were centrifuged for 10 min at 10000 g, and the obtained supernatant was used for determination of CAT, SOD, and POD activity and protein concentration.

Enzyme assays. CAT activity was determined spectrophotometrically at 240 nm by measuring decrease in absorbance of H_2O_2 in 3 ml 100 mM sodium phosphate buffer (pH 7.5) at 25°C.

SOD acvtivity was determined spectrophotometrically at 550 nm in 50 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA and 0.02 mM sodium azide by measuring the percent of SOD-induced inhibition of cytochrome c reduction using a xanthine/xanthine oxidase system as the source of $O_2^{\cdot-}$.as described by McCord and Fridovich (1968).

POD activity was determined spectrophometrically with guaiacol as the substrate in a total volume of 3 ml. The assay mixture contained 50 mM sodium acetate buffer (pH 5.5), 92 mM guaiacol, 18 mM H_2O_2, and variable amounts of enzyme at 25°C. The

reaction was monitored at 470 nm. The reaction rate was calculated from the coefficient of absorbance of guaiacol: 25.5 $cm^2 \mu mol^{-1}$.

One unit of CAT and POD activity was defined as the amount of enzyme that converts one micromole of substrate to product in one minute.

Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Isoelectric focusing was performed horizontally in the LKB 2117 Multiphor II system using 1-mm-thick polyacrylamide gels (5% T, 3% C) containing 4% 3.5 -10.0 ampholites.

Isoenzymes of SOD were detected on the gels by the method of Beauchamp and Fridovich (1971).

Peroxidase was stained on the gel with 9.2 mM guaiacol and 5 mM H_2O_2 in sodium acetate buffer (pH 5.5) for 10 min at 24°C.

RESULTS AND DISCUSSION

Effect of photoperiodic treatment on growth, flowering, and seed maturation. Seventeen days after germination, there were no differences of plant height between plants grown under two different photoperiodic regimens (Fig. 1A). But on the 37th and 65th day, plants grown for the first 17 days under noninductive CL were about twice as high as plants grown continuously under an inductive 14 h/10 h photoperiod (Fig. 1A). This could be attributed to timing of the transition to flowering, which took place 17 days later in plants grown for the first 17 days under CL, since it is well known that the transition to flowering is accompanied by transient inhibition of growth (Opatrnà et al., 1980; Ullmann et al., 1980; Mitrović 1998).

Leaf development was stimulated by 17 days of CL compared to a 14 h/10 h photoperiod, and this effect was maintained after plants of that group were transferred to the same 14 h/10 h photoperiod (Fig. 2B). We showed earlier (Mitrović et al., 2007) that vegetative and reproductive development of *C. rubrum* is determined by the photoperiod the seedlings experience during a precise short period early in their life cycle, and that increase of day length is accompanied by increases in plant height and the number of leaves.

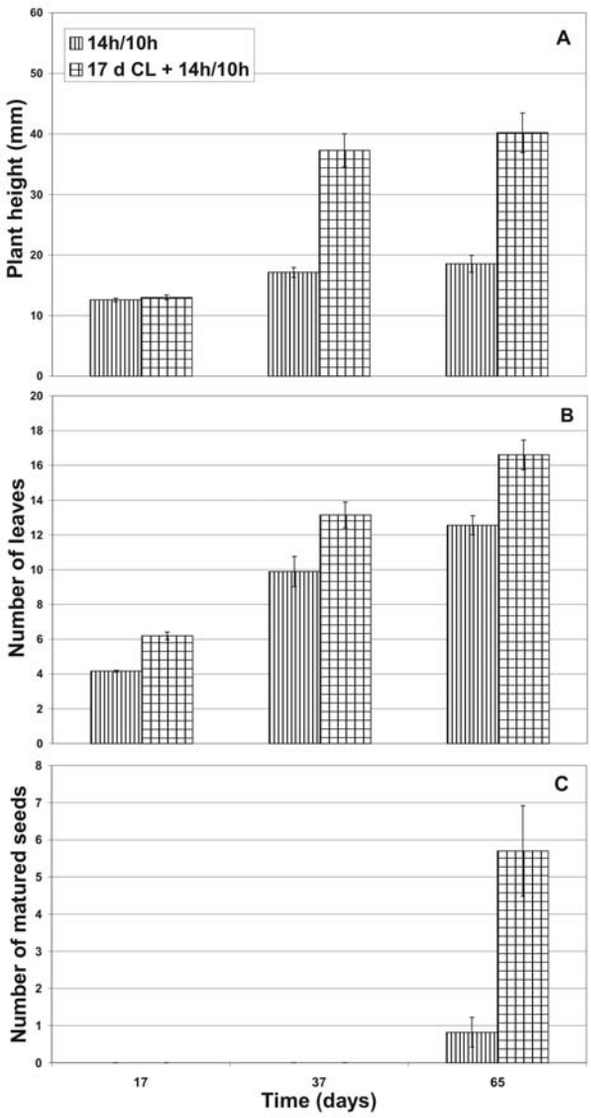

Fig. 1. Effect of different photoperiodic regimens (65 d of 14 h/10 h photoperiod, or 17 d of CL followed by 48 d of 14 h/10 h photoperiod) on *C. rubrum* vegetative and reproductive development in vitro: A) plant height, B) number of leaves, C) number of matured seeds per plant; means ± SE, n = 48; CL – continuous light, d – days.

On the 17th day, 100% of plants grown under an inductive 14 h/10 h photoperiod flowered, while

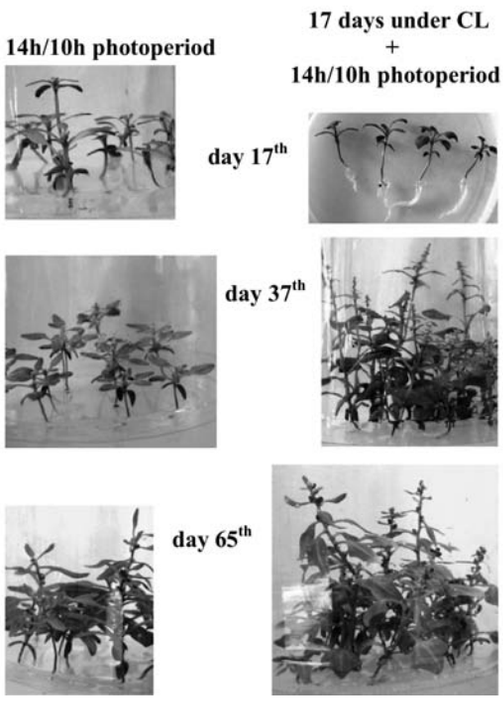

Fig. 2. Effect of photoperiod (65 d of 14 h/10 h photoperiod, or 17 d of CL followed by 48 d of 14 h/10 h photoperiod) on *C. rubrum* growth after 17, 37, and 65 d of culturing in vitro; CL – continuous light, d – days.

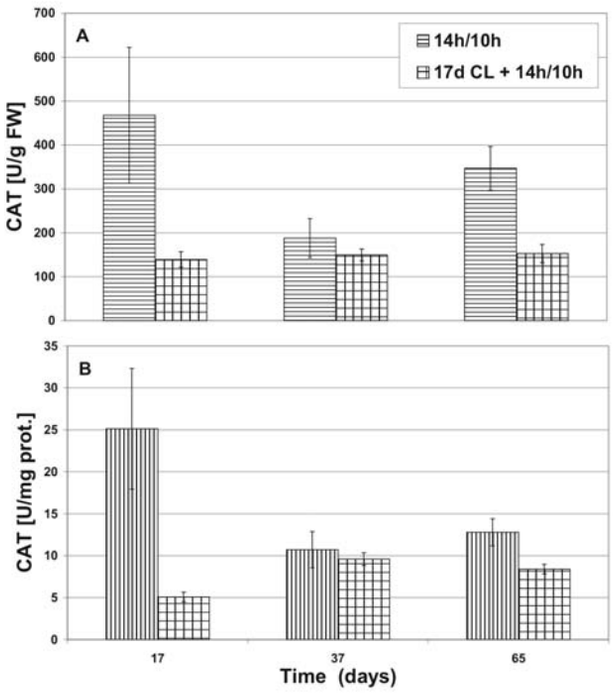

Fig. 3. Specific catalase activity per fresh weight (A) and per mg of proteins (B) in C. rubrum plants after 17, 37, and 65 d of culturing in vitro under two different photoperiodic regimens (65 d of 14 h/10 h photoperiod, or 17 d of CL followed by 48 d of 14 h/10 h photoperiod); CL – continuous light, d – days.

plants grown for the first 17 days under noninductive CL stayed vegetative. In those plants, flowering was delayed by 17 noninductive cycles of CL, 100% flowering was registered on the 37th day, and even on the lower nodes immature seeds were barely visible to the naked eye (Fig. 2). At the same experimental point, the 37th day, plants grown continuously under an inductive photoperiod were in the phase of seed development, while on the 65th day both groups of plants were in the phase of seed maturation and black matured seeds were visible (Fig. 2). Regardless of the 17-day difference in the start of reproductive development, ontogenesis in both groups of plants lasted about the same time. We showed earlier (Mitrović et al., 2007) that regardless of the day length *C. rubrum* plants are exposed to at the cotyledon stage of development, seed maturation occurs in 10 weeks *in vitro*. In other words, the duration of ontogenesis is not determined neither by day length nor by the age of plants at which reproductive development begins, suggesting the possible existence of "autonomous control of the duration of ontogenesis" in *C. rubrum* plants, which could be connected with the existence of autonomous control of flowering in plants with obligatory photoperiodic requirements, as confirmed by Chailakhyan (1988).

Day length during the first 17 days after germination also affected seed maturation and the number of matured seeds per plant (Fig. 1C). About five times more seeds were collected from plants in which flowering induction was delayed by 17 days of CL compared to those grown continuously under inductive 14 h/10 h. This leads to the conclusion that besides day length during induction and evocation of flowering (Mitrović et al., 2007; Cook, 1975), day length before flowering induction is also significant for seed development and maturation.

Effect of photoperiodic treatment on antioxidative enzyme activities during ontogenesis ***in vitro*****.** In plants grown continuously under an inductive photoperiod, the same trend is evident in changes of CAT (Fig. 3) and POD (Fig. 4) activities.

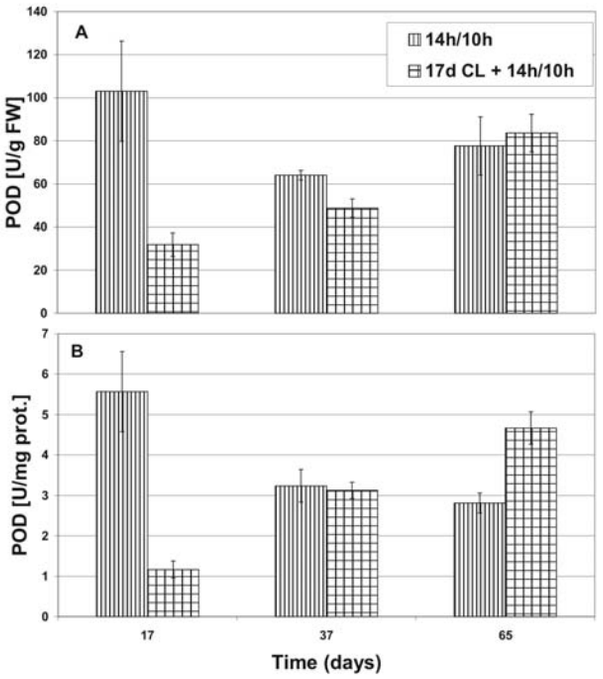

Fig. 4. Specific peroxidase activity per fresh weight (A) and per mg of proteins (B) in *C. rubrum* plants after 17, 37, and 65 d of culturing in vitro under two different photoperiodic regimens (65 d of 14 h/10 h photoperiod, or 17 d of CL followed by 48 d of 14 h/10 h photoperiod); CL – continuous light, d – days.

High enzyme activities are registered on the 17th day, which corresponds to full flowering in this group of plants, followed by decline of activities on the 37th and 65th days, during seed development and maturation. SOD activity (Fig. 6A) increased on the 65th day (seed maturation).

In plants in which flowering induction was delayed by 17 days of CL, the lowest activities of POD and SOD were registered on the 17th day (vegetative plants) (Figs. 4 and 6). Later in the course of ontogenesis, SOD and POD activities rose during flowering and seed development, reaching a maximum in the phase of seed maturation, that is with the beginning of senescence (65th day). Our results agree with those of A b a r c a et al. (2001), who showed that ROS such as $^{\bullet}O_2^-$ are involved in induction and development of the senescence stage and that total POD activity rises in senescent *A. thaliana* tissues. Higher POD activity may be associated with reduction of H_2O_2, while higher SOD activity could be linked with high $^{\bullet}O_2^-$ concentration. CAT activity in this group of plants

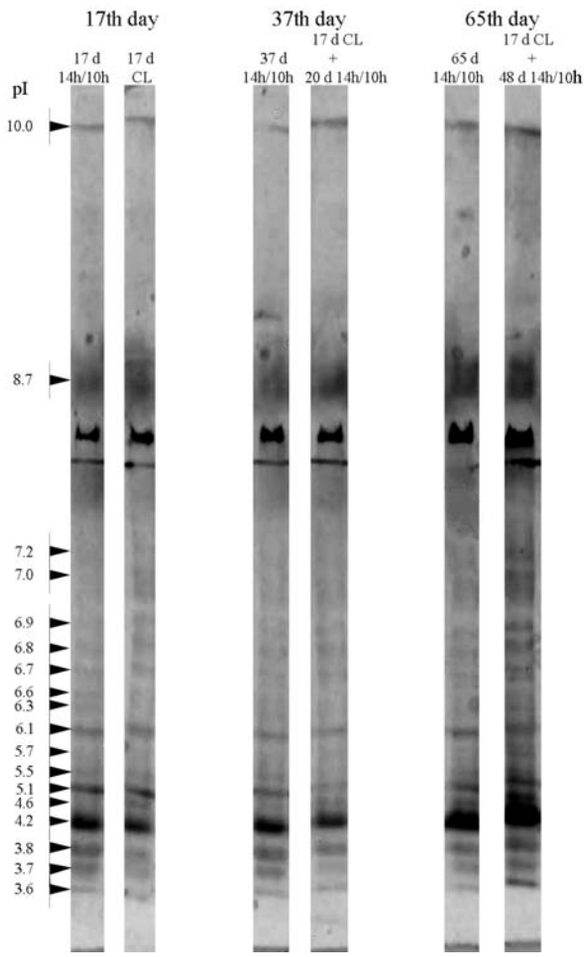

Fig. 5. Isoelectrofocusing of POD izoenzymes from *C. rubrum* plants after 17, 37, and 65 d of culturing in vitro under two different photoperiodic regimens (65 d of 14 h/10 h photoperiod, or 17 d of CL followed by 48 d of 14 h/10 h photoperiod); CL – continuous light, d – days.

slightly increased at the time of flowering, on the 37th day after the germination (Fig. 3B).

The greatest difference between these two groups of plants was registered in the activities of CAT and POD on the 17th day after germination (Figs. 3 and 4). This could be attributed both to different phases of development and to the effect of CL. Plants grown under an inductive photoperiod were in the flowering phase, while plants grown under noninductive CL were in the vegetative phase of development. On the other hand, exposure to CL brings about an increase of ROS production (A s a d a, 2006), and

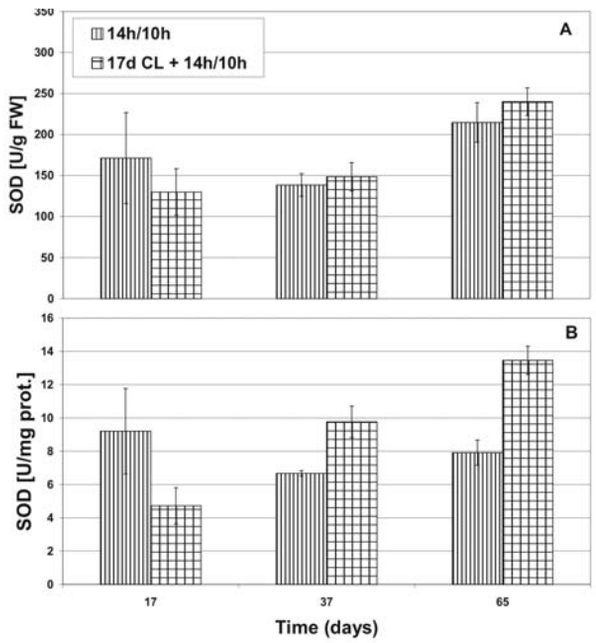

Fig. 6. Specific superoxide dismutase activity per fresh weight (A) and per mg of proteins (B) in *C. rubrum* plants after 17, 37, and 65 d of culturing in vitro under two different photoperiodic regimens (65 d of 14 h/10 h photoperiod, or 17 d of CL followed by 48 d of 14 h/10 h photoperiod); CL – continuous light, d – days.

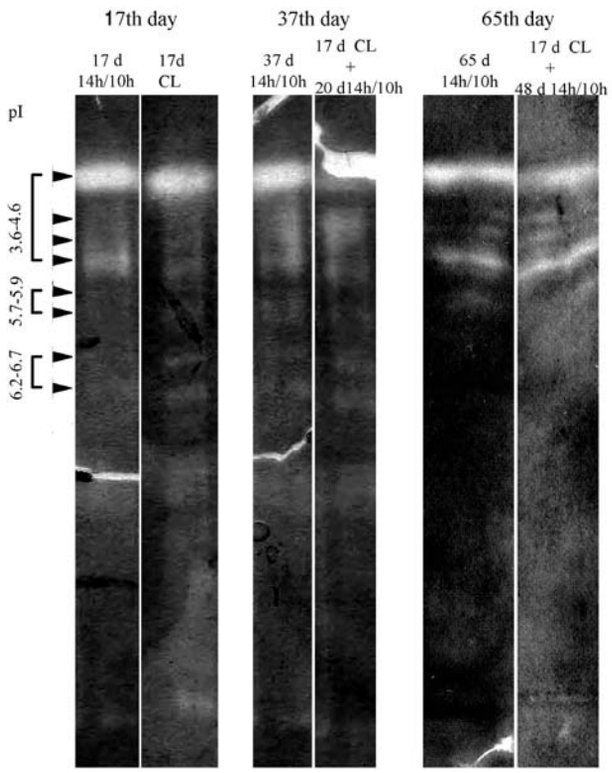

Fig. 7. Activity of SOD on polyacrylamide gel after isoelectrofocusing in *C. rubrum* plants after 17, 37, and 65 d of culturing in vitro under two different photoperiodic regimens (65 d of 14 h/10 h photoperiod, or 17 d of CL followed by 48 d of 14 h/10 h photoperiod); CL – continuous light, d – days.

in *Phaseolus vulgaris* cotyledons causes increase in content of all nonenzymatic antioxidants and decrease in production of antioxidative enzymes (Procházková and Wilhelmová, 2004).

On the 37th and 65th days POD (Fig. 4A) and SOD (Fig. 6A) activities in the two groups of plants showed no significant differences. This could be explained by the fact that plants of both groups were exposed to the same photoperiod after the 17th day and were in about the same phases of development.

On the gel, eight SOD isoforms are visible on the 17th and 37th days in both groups of plants, regardless of the photoperiod plants were exposed to (Fig. 7). On the 65th day, isoforms with pI values of 5.7 – 6.7 are missing, which could be linked with seed maturation, i.e., plant senescence. The increase in SOD activity on the 65th day in both groups of plants (Fig. 7) must be due to increase in the relative amounts of isoforms with pI 3.6 – 5.7.

Eighteen POD isoforms are visible on the gel (Fig. 5). Seventeen of them are visible in all samples, but their intensities differ depending on photoperiod plants were exposed to. The POD isoform with a pI value 4.6 is not on the visible 17th day in samples of plants grown continuously under an inductive photoperiod; it appears on the 37th day and is most intensive on the 65th day (Fig. 5). In plants in which flowering induction was delayed by 17 days of CL, this isoform is visible in all samples, being most intensive on the 65th day. It could be supposed that this isoform is associated with stress induced both by exposure to CL and by senescence.

The low intensities of three isoforms (pI 3.6, 3.7, and 3.8) in samples of plants exposed to 17 days of CL could be linked with low POD production (Fig. 4) as effect of CL (Procházková and

Wilhelmová, 2004). This effect of CL is maintained even after plants of this group are transferred to a 14 h/10 h photoperiod (37th day, Fig. 5). But on the 65th day, the intensities of these bands increased. The unchanged intensities of the given three bands in samples of plants grown continuously under a 14 h/10 h photoperiod also argue in favor of a connection of these POD isoforms with the photoperiod plants were exposed to. We previously showed that the photoperiod during the life cycle of plants affects their growth and development to the end of ontogenesis (Mitrović et al., 2007) and even seed protein composition (unpublished data). A similar connection between POD isoforms and the photoperiod could also be presumed for other isoforms (pI 6.7, 6.8, and 6.9).

Results similar to ours were obtained on *Impatiens flanaganiae* leaves, where CAT, POD, and SOD activities depended on the phase of development and light intensities the plants were exposed to (Lall and Nikolova, 2003).

From our data, it can be concluded that flowering of *C. rubrum* is accompanied by increase in CAT activity; that PODs are involved in determination of growth and development of this species in keeping with the environment; and that the absence of some SOD isoforms can be an indicator of its senescence.

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ПРАЋЕЊЕ АКТИВНОСТИ АНТИОКСИДАТИВНИХ ЕНЗИМА У РАЗЛИЧИТИМ ФАЗАМА ОНТОГЕНЕЗЕ *CHENOPODIUM RUBRUM L. IN VITRO*

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За краткодневну биљку *C. rubrum*, фотопериод 14х/10х је индукциони за цветање, док је континуална светлост неиндукциона. Гајењем прве групе биљака 65 дана на индукционом фотопериоду, а друге групе биљака првих 17 дана на континуалној светлости, а потом на индукционом фотопериоду, раздвојили смо фазе цветања, заметања и сазревања семена биљака исте старости. Мерене су активности каталазе (CAT), супероксид дисмутаза (SOD) и пероксидаза (POD). Промена активности антиоксидативних ензима је уочена у зависности од фазе развића, и фотопериода. Код прве групе биљака, уочене су највише активности POD и SOD у време цветања, и снижавање активности током сазревања семена. Тренд промене активности ових ензима је различит код друге групе биљака, где је максимум уочен у време сазревања семена. Код обе групе биљака највиша CAT активност уочена је у време цветања.