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EFFECT OF PHENOL ON GERMINATION CAPACITY AND POLYPHENOL OXIDASE, PEROXIDASE AND CATALASE ACTIVITIES IN LETTUCE

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Abstract – In this study we examined the activities of polyphenol oxidase (PPO) and antioxidant enzymes, peroxidase (POX) and catalase (CAT) during lettuce seed germination at different concentrations of phenol. Out of eleven varieties of lettuce, four were chosen according to their germination tolerance to phenol as follows: plants exhibiting high (Ljubljanska ledenka – LJL and Nansen – N) and low toleranace (Little Gem – LG and Majska kraljica – MK). A decrease in germination efficiency after exposure to LD_{50} of phenol was determined for these four varieties. The effects of phenol treatment on POX, CAT and PPO activities were determined after 4, 5, 6, 7 and 8 days of growth at LD_{50} concentrations. A trend of increased peroxidase activity was observed in seeds grown on LD_{50} of phenol compared to control seeds. A significant increase in CAT activity was observed at the beginning of treatment for MK, LG and N in seeds grown on phenol as well as in control seeds. A trend of increased PPO activity was observed in all control seeds. We also investigated the affinity of PPO for two different substrates that were used for the determination of enzyme activity. Our results show that LJL and N are the varieties most tolerant to growth on phenol. Here we report on the activities of their antioxidant enzymes and PPO during seed germination.

Key words: Lettuce seeds; phenol; antioxidant enzymes; polyphenol oxidase

INTRODUCTION

Lettuce (*Lactuca sativa* L.) is an important leafy vegetable that is primarily consumed fresh (Liu et al., 2007). For example, in the USA, between 2001 and 2006, lettuce was cultivated on over 121 000 ha per year, which makes it the most important vegetable in the country (Contreras et al., 2008). There is little information available on the effects of phenolic content in water on the the antioxidant capacity of different cultivars of lettuce.

Phenols are highly toxic compounds that have adverse effects on living organisms. Phenols bind to sulfhydryl groups of vitally important enzymes and inhibit redox reactions in cells. Phenol vapors are dangerous at concentrations above 0.001 mg/dm^3 and the maximum permissible concentrations of phenols range from 0.1 to 0.001 mg/dm^3 (Davidenko et al., 2004). In addition, the oxidation of phenols in natural waters is accompanied by an intensive uptake of oxygen dissolved in water that is having a negative impact on metabolism of plants. Phenol is a waste

product of many technological processes and wastewater may contain different amounts of phenols. Methods that are currently used for the elimination of phenols from water have some disadvantages, such as high cost, incomplete purification and low efficiency (Davidenko et al., 2004). The enzymatic destruction of phenols is typically an oxidative process with molecular oxygen serving as the oxidant for oxidases and hydrogen peroxide as the oxidant for peroxidases (Mayer 2006).

Polyphenol oxidase (PPO) is a copper protein that catalyzes two different reactions by using molecular oxygen: the hydroxylation of monophenols to o-diphenols and the oxidation of the o-diphenols to o-quinones (Chazzara et al., 2001). In plants, PPO is located in the chloroplast thylakoid membranes and exists in an inactive or latent state (Mayer and Harel, 1979). This enzyme is widespread in nature and is interesting in the fruit and vegetable industry for being responsible for browning after tissue injury (Chazarra et al., 1997). Beside PPO, peroxidases (POX) might be important factors in the browning process (Martin-Diana et al., 2005). In iceberg lettuce, one of the most important physiological processes is russet spotting in which phenolic compounds are accumulated and oxidized by PPO (Ke and Saltveit, 1988). PPO can be released from their inactivated state by many agents including some substrates (Golbeck and Cammarata, 1981). Plant PPOs are often considered defense proteins for their herbivore-, pathogen- and wound-induced expression (Constabel et al., 1996). Previous studies have detected significant levels of antioxidant activity and phenolic components in lettuce (Caldwell, 2003; Chu et al., 2002). While the biochemical reactions catalyzed by PPOs are well known, data on the physiological function of these enzymes are rare (Tran et al., 2012).

Phenol diluted in water can be considered as a harmful stress factor during plant development. Factors that have negative effects on the growth and development of plants, reducing their productivity levels, often cause the release of large amounts of reactive oxygen species (ROS; Bowler et al., 1994). Oxidative stress has been studied in many plants, but the mechanisms of its action are still not completely understood (Mittler 2002).

Plants have a very efficient enzymatic antioxidant system that catalyzes the removal of ROS (Inzé and Montagu, 1995). Enzymes such as POX and catalase (CAT) are part of plant enzymatic antioxidative defense systems (Apel and Hirt, 2004). The amount of stress can be measured indirectly by measuring the activity of antioxidant enzymes that are part of the plant antioxidant defense system.

Peroxidases are heme-containing enzymes that oxidize a wide variety of organic and inorganic substrates by reducing hydrogen-peroxide and peroxides (Kvaratskhelia et al.,1997). Most of plants secretory peroxidases are glycosylated proteins (Veitch 2004). They play an important role in lignification, cell-wall metabolism, plant resistance, elimination of hydrogen peroxide, healing of injuries and auxin metabolism (Hu et al., 2012). There are three groups of peroxidase, but the most common is group III, which is also called the plant peroxidase (Passardi et al., 2005).

CAT are metalloenzymes that remove hydrogen peroxide from plant cells (Mizuno et al., 1998). Large amounts of hydrogen peroxide synthesized in photosynthetic tissues can be removed by CAT, which represents a first line of cell defense from this harmful molecule (Mhamdi et al., 2010).

The aim of this work was to study the ability of lettuce peroxidase and polyphenol oxidase to eliminate phenol from water. In addition, we examined the resistance of 11 commercially valuable lettuce cultivars to different concentrations of phenol with the goal of determining the best cultivar and investigating its antioxidant capacity, as no previous studies have been conducted on the properties of antioxidant enzymes in lettuce growing on phenol.

MATERIALS AND METHODS

Plant material

Seeds of *Lactuca sativa* (Iceberg, Red Yugoslavian

Butterhead, Mascara, Little Gem (LG), Ruby red, Red fire) were obtained from Trade Winds Fruit (Santa Rosa, CA, USA), Ljubljanska ledenka (LJL) and Nansen (N) from "Semenarna" (Ljubljana, Slovenia), Vera and Viola from "Institut za Povrtarstvo" (Smederevska Palanka, Serbia) and Majska kraljica (MK) from "Agro Market" (Belgrade, Serbia).

In order to investigate the influence of phenol on the process of germination, seeds were treated with 50, 100, 150, 200, 250, 300, 350 and 400 mgL^{-1} phenol. In all treatments, a population of 20 seeds was used. All seeds were grown in Petri dishes on filter paper with phenol solution at 24±2°C and 16 h light/8 h dark photoperiod with irradiance of 40 $\mu mol\ m^{-2}\ s^{-1}$. LD_{50} concentrations of phenol were selected for each lettuce variety as the concentrations in which 50% of seeds germinated. The percentage of germinated seeds was noted after the end of the 8^{th} day of treatment. All measurements were repeated three times.

Seeds were collected after 4, 5, 6, 7 and 8 days in order to study the effects of phenol treatment on the activities of antioxidant enzymes (POX and CAT) and PPO.

Enzyme extraction

Lettuce seeds that germinated on LD_{50} phenol concentrations specific for each variety, were used as a starting material for the analyses of antioxidant enzyme activities.

Frozen (-70°C) seeds (1 g) were homogenized in 1 mL of 0.1 M potassium phosphate (K-P) extraction buffer (pH 7, containing 1.5% insoluble polyvinylpyrrolidone (PVPP), 10 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The homogenate was centrifuged for 5 min at 12 000 g at 4°C. Quantification of peroxidase (POX, EC 1.11.1.x) and catalase (CAT, EC 1.11.1.6.) was performed spectrophotometrically (Agilent 8453, Life Science, USA).

Quantification of POX activity

Activity of POX was determined according to Furumo and Furutani (2008). The reaction mixture contained 2.7 mL of K-P buffer, 0.1 mL of 3% hydrogen peroxide and 0.15 mL of 4% guaiacol, pH 6.5. The final concentration of hydrogen peroxide and guaiacol were 0.1 and 0.2%, respectively, in a total volume of 3 mL. The assay mixture was incubated for 3 min at room temperature. After incubation, 50 µL of diluted extract was added and POX activity was monitored at 470 nm for 2 min at room temperature. Enzyme activity is indicated as $\mu mol\ min^{-1}\ mg^{-1}$ (U/g fresh weight).

Quantification of CAT activity

Catalase activity was determined spectrophotometrically according to Aebi (1984) by monitoring the kinetics of disappearance of hydrogen peroxide, which can be detected by measuring the decrease in absorbance at 240 nm of reaction mixture in 50 mM K-Na-P buffer (pH 7), 20 mM hydrogen peroxide and enzyme extract. Catalase activity was measured at 20°C, every 20 s for 3 min. A unit of catalase activity is defined as the amount of enzyme that degrades 1 µmol of hydrogen peroxide in 1 min and is expressed as $\mu mol\ min^{-1}\ mg^{-1}$ (U/g fresh weight).

Quantification of PPO activity
Polyphenol oxidase (PPO, EC 1.10.3.1.) assay I

The reaction mixture contained 1.45 mL of 100 mM K-P buffer, pH 6.8, 0.5 mL of 100 mM 4-methylcatechol (4-MC) prepared daily by dissolving 124.1 mg 4-MC in 10 mL of assay buffer. The final 4-MC concentration in 200 mL assay was 25 mM. The reaction mixture was incubated for 3 min at room temperature. Diluted extract (50 µL) was added to the cuvette containing the assay mixture. The reaction was monitored at 412 nm for 2 min at room temperature. Enzyme activity is indicated as $\mu molmin^{-1}mg^{-1}$ (U/g fresh weight) and represents the quantity of enzyme required to produce 1 µmol of product per minute (Waite 1976).

Polyphenol oxidase assay II

2.3-dihydroxy-L-phenylalanine (L-DOPA) was used

as substrate for the spectrophotometric assay of PPO activity. Fifty μL of extract was added to 2.95 mL of assay mix (5 mM L-DOPA in 50 mM MOPS buffer, pH 6.5) in a cuvette. Absorbance was measured at 475 nm, at which dopachrome has a molar absorptivity of 3 700 M^{-1} cm^{-1}. The linear rate of increase in absorbance for the first 60-90 s of the reaction time was proportional to the amount of the enzyme (Behbahani at al. 1993).

Statistical analysis of data

The results of all experiments are presented as mean values ± standard errors. Statistical analysis was performed using StatGrafics software version 4.2. Data were subjected to analysis of variance (ANOVA) and comparisons between the mean values of treatments were made by the least significant difference (LSD) test calculated at the confidence level of $P \leq 0.05$. In all treatments, a population of 20 seeds was used. All measurements were repeated three times.

RESULTS

Germination of lettuce seeds at different phenol concentrations

Eleven varieties of lettuce were grown on 8 different concentrations of phenol (Table). Two varieties of lettuce (LJL and Red Yugoslavian Butterhead) exhibited 100% germination on the two lowest concentrations of phenol. Seeds of LJL germinated 100% at a concentration of 200 mg L^{-1}. In addition, seeds of this variety germinated up to a phenol concentration of 350 mg L^{-1} on which no other variety could germinate. Therefore, it can be concluded that LJL is the most resistant cultivar during germination on phenol solution of all examined varieties. The lowest germination efficiency on all the concentrations of phenol except on 50 mg L^{-1}, was recorded for the variety MK. In three varieties, Iceberg, Ruby red and Vera, a total absence of germination occurred at 250 mg L^{-1} after a relatively high percentage of germination at 200 mg L^{-1}.

Two varieties (LJL and N) were selected for further analysis for their high germination efficiency on phenol, as well as two varieties (LG and MK) with a strong decline in germination efficiency on the initial concentrations of phenol. For three of these four varieties (LJL, LG and N) LD_{50} concentrations of phenol were chosen. All three LD_{50} concentrations of phenol (Table 1) allowed for 50-51% germination. The LD_{50} concentration for the remaining variety MK was determined separately by growing seeds in a range of concentrations between 50 and 150 mg L^{-1}, and was estimated to equal 75 mg L^{-1}.

Activity of antioxidant enzymes and polyphenol oxidase in lettuce seeds grown at different phenol concentrations

Activity of peroxidase

A trend of increased peroxidase activity was observed in seeds grown in the presence of LD_{50} concentrations of phenol compared to control seeds (Figs. 1a-d). In the lettuce variety MK, the activity of POX increased after six days of growth (Fig. 1a). Figs. 1b and d show a sharp increase in peroxidase activity after eight days of growth at LD_{50} concentrations of phenol.

Activity of catalase

Activity of CAT was also increased in seeds grown in the presence of LD_{50} concentrations of phenol compared to control seeds (Figs. 2a-d). A significant decrease in activity of CAT was observed after the fifth day of treatment for varieties LG and N for both seeds grown on phenol and for the control seeds (Figs. 2 b and c). In LJL, which has the highest tolerance for phenol, catalase activity increased until the sixth day of growth in the presence of phenol, and displayed a significant drop afterwards (Fig. 2d).

Activity of polyphenol oxidase

Fig. 4 shows that there was a trend of increased PPO activity in control seeds. It can also be seen that the enzyme activity significantly increased on the fifth

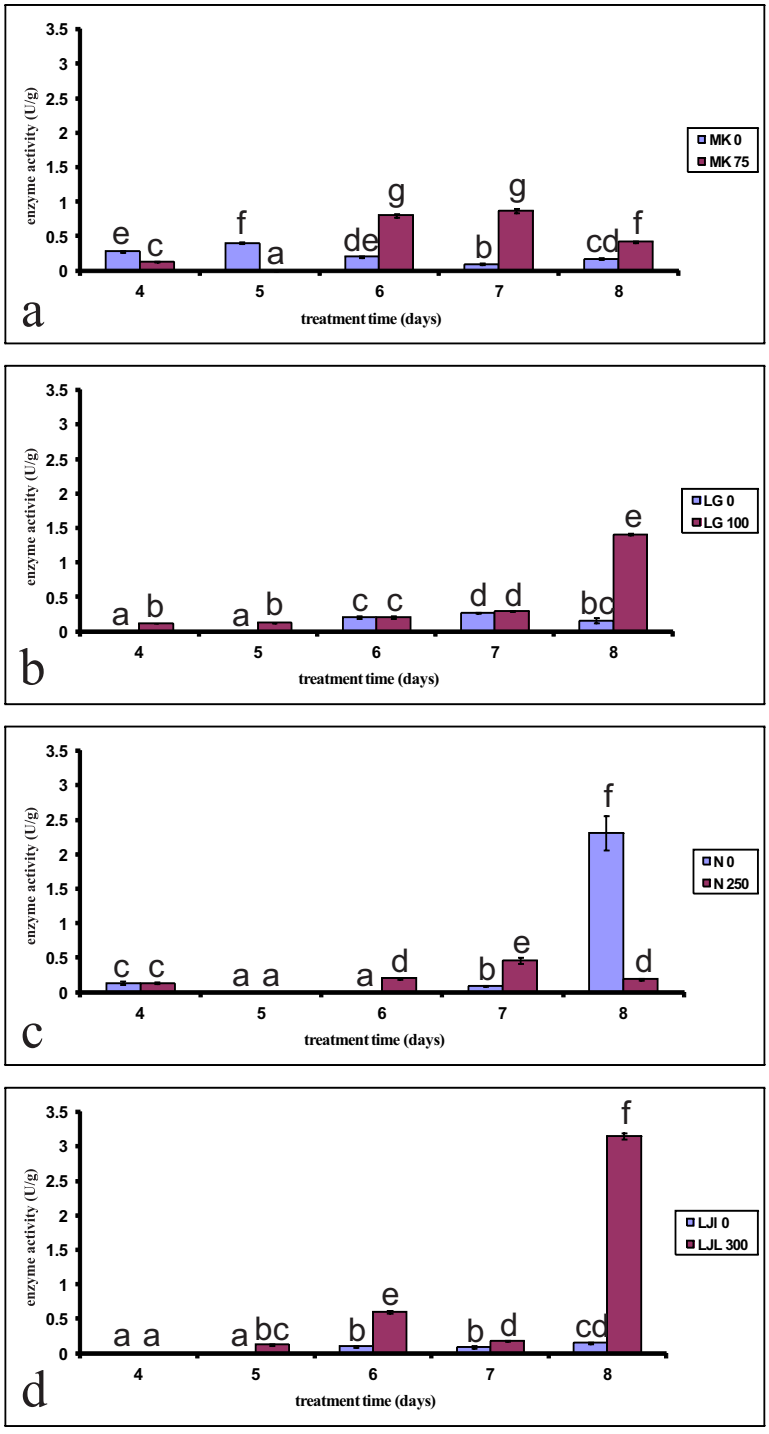

Fig. 1. The effect of LD_{50} concentration of phenol on peroxidase activity of four varieties of lettuce seeds during five days of germination. a) MK; b) LG; c) N; d) LJL.

Means followed by the same letters within one lettuce variety are not significantly different according to LSD test at $P \leq 0.05$ probability level. Varieties that are abbreviated are: MK, Majska kraljica; LG, Little Gem; N, Nansen and LJL, Ljubljanska ledenka. A value of 0 indicates that a particular variety has been grown without phenol, while the other values represent the LD_{50} concentration of phenol for a given variety.

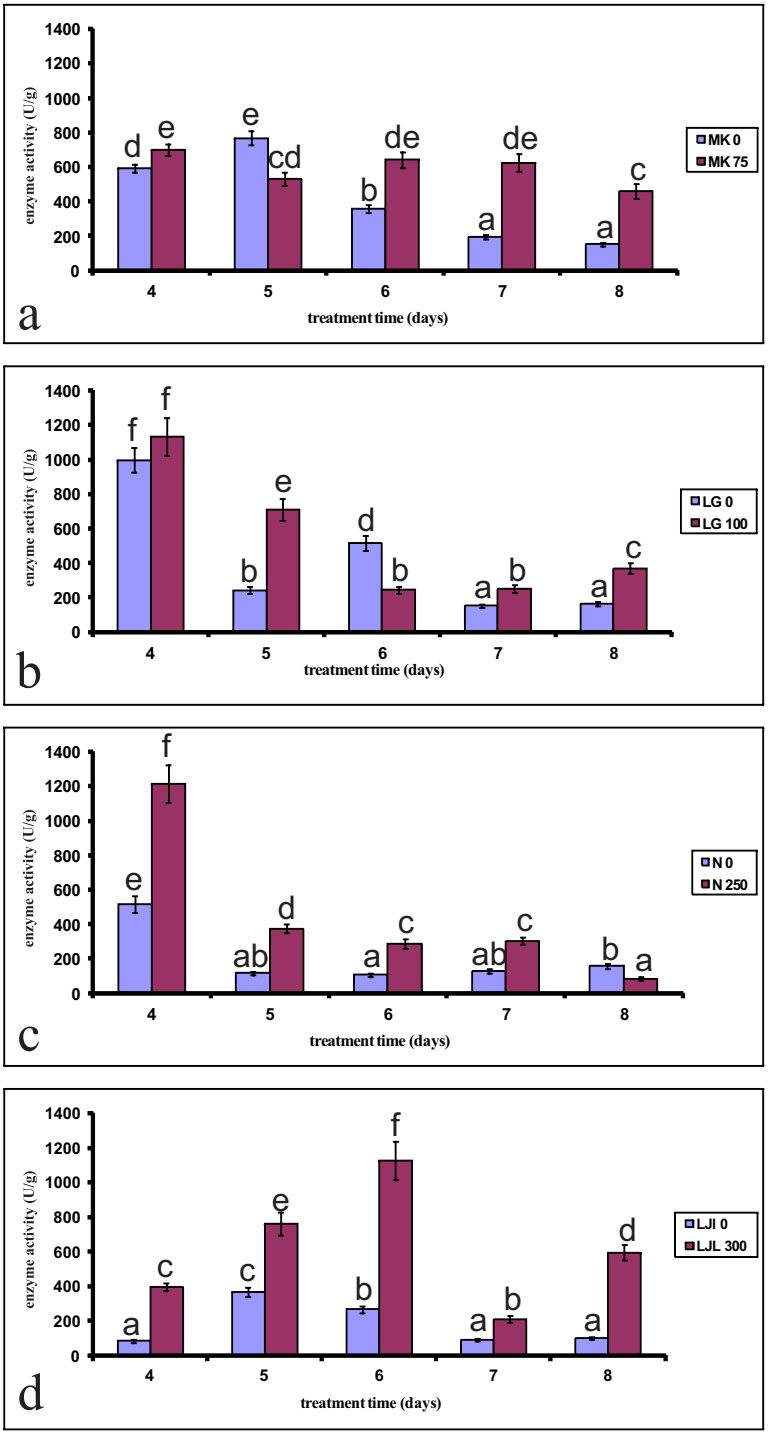

Fig. 2. The effect of LD_{50} concentration of phenol on catalase activity of four varieties of lettuce seeds during five days of germination. a) MK; b) LG; c) N; d) LJL

Means followed by the same letters within one lettuce variety are not significantly different according to LSD test at $P \leq 0.05$ probability level. Varieties that are abbreviated are: MK, Majska kraljica; LG, Little Gem; N, Nansen and LJL, Ljubljanska ledenka. A value of 0 indicates that a particular variety has been grown without phenol, while the other values represent the LD_{50} concentration of phenol for a given variety.

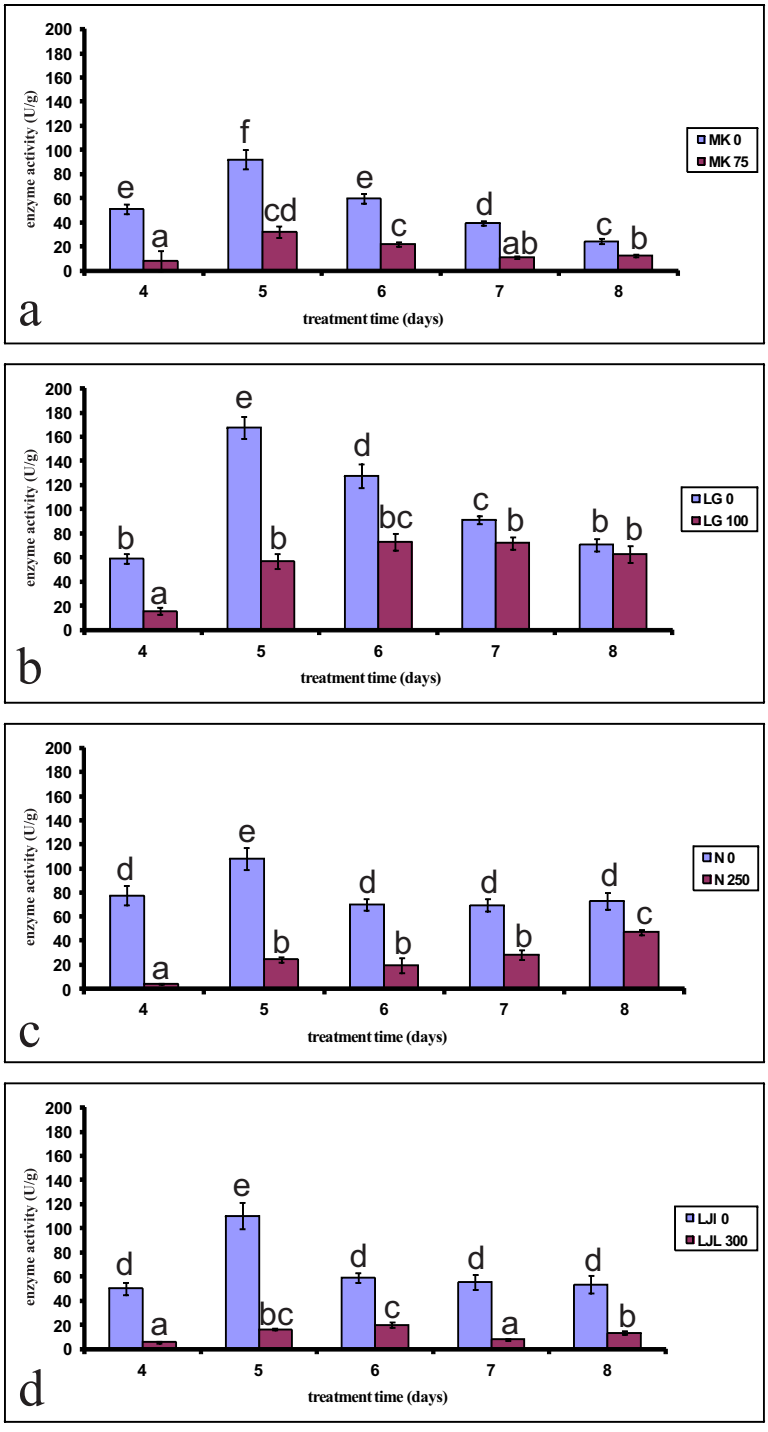

Fig. 3. The effect of LD_{50} concentration of phenol on polyphenol oxidase activity of four varieties of lettuce seeds during five days of germination. a) MK; b) LG; c) N; d) LJL

Means followed by the same letters within one lettuce variety are not significantly different according to LSD test at $P \leq 0.05$ probability level. The graph shows the value for PPO activity described as Polyphenol oxidase assay II. Varieties that are abbreviated are: MK, Majska kraljica; LG, Little Gem; N, Nansen and LJL, Ljubljanska ledenka. A value of 0 indicates that a particular variety has been grown without phenol, while the other values represent the LD_{50} concentration of phenol for a given variety.

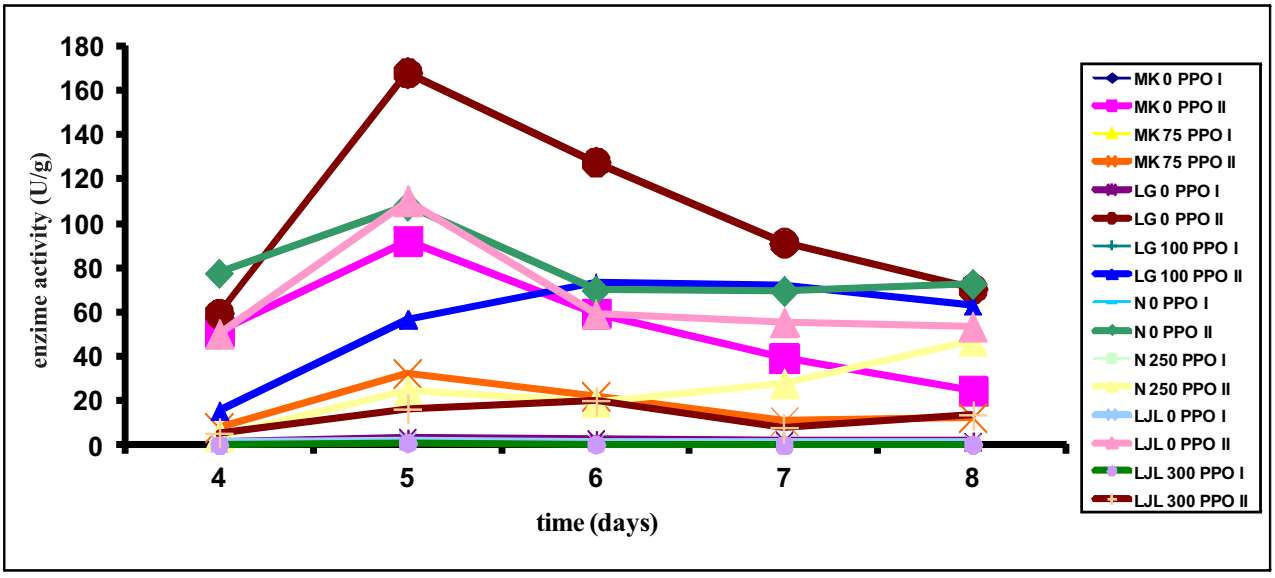

Fig. 4. Polyphenol oxidase activity in relation to the substrate that was used for determination. PPOI, Polyphenol oxidase assay I with 4-methylcatechol as substrate; PPOII, Polyphenol oxidase assay II with L-DOPA as substrate. Varieties that are abbreviated are: MK, Majska kraljica; LG, Little Gem; N, Nansen and LJL, Ljubljanska ledenka. A value of 0 indicates that a particular variety has been grown without phenol, while the other values represent the LD_{50} concentration of phenol for a given variety.

day from the beginning of germination in seeds that were not grown in the presence of phenol. After the fifth day of germination, the activity of the enzyme decreased constantly for MK and LG (Figs. 4a and b), while for N and LJL remained unchanged during the final three days of growth (Figs. 4c and d). LJL, which shows the highest tolerance for phenol, has the lowest PPO activity when seeds germinate in the presence of LD_{50} concentration of phenol (Fig. 4d).

Differences in polyphenol oxidase activity related to the substrate

We used two substrates for the determination of PPO activity: 4-MC (enzyme assay I) and L-DOPA (enzyme assay II). 4-MC proved to be a much better substrate for the determination of PPO activity, giving higher values for enzyme activity in all lettuce varieties (Fig. 5).

Morphological characteristics of lettuce seed during germination on phenol

As can be seen in Fig. 5, four varieties of lettuce seeds that had germinated for eight days on the lowest concentration of phenol (50 mg/L^{-1}) are shown. LJL and N, which have the highest tolerance for phenol, had green, well-developed leaves even eight days after germination (Figs. 5a and b). Also, all of the germinated seeds are on the same level of development and have the same morphological characteristics.

Varieties LG and MK are less resistant to phenol solution (Table 1), and, despite the less developed shoots, have light green and curled leaves (Figs. 5c and d).

DISCUSSION

Enzymes that oxidize phenolic compounds are unique in plants and most other organisms. The literature on phenol oxidase has been reviewed by Mayer (Mayer, 2006), who suggests that the function of most of these enzymes is yet to be studied. The same literature on POX is very scarce. Although phenol is primarily degraded by PPO, it can also be degraded by POX (Thypapong et al., 1996). In all varieties of lettuce, POX activity increases if the

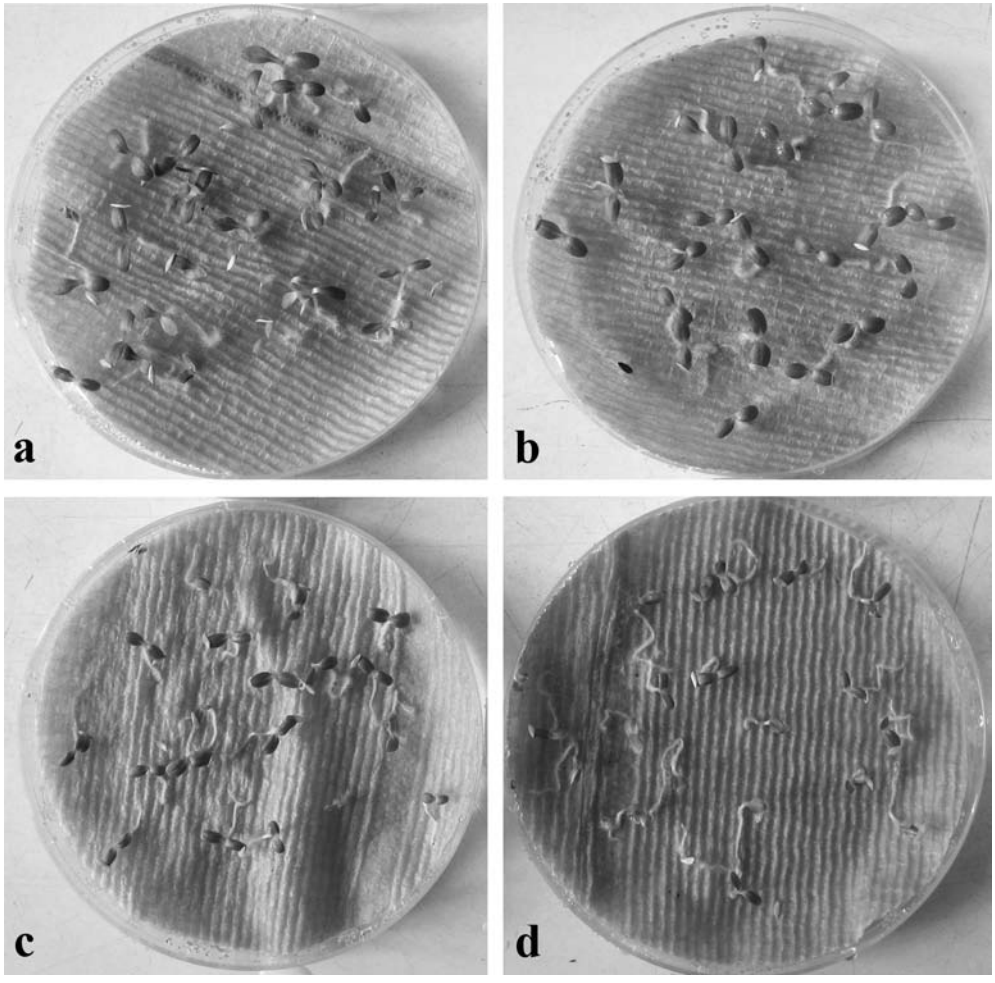

Fig.5. Germination of four varieties of lettuce seeds after eight days of growing on LD_{50} concentration of phenol: a) LJL; b) N; c) MK; d) LG

seeds grow on phenol, whereas the activity of PPO decreases at the same treatment. In sweet potato, the activities of both enzymes are increased in response to biotic and abiotic stresses (Kwak et al., 1996). The relatively low value of peroxidase activity in all varieties of lettuce can be attributed to the inhibition of enzyme synthesis, activity or from relocalization to an environment such as the cell wall, which might be recalcitrant to buffer extraction (Bestwick et al., 1998). Hu et al., (2012) reported that peroxidases from lettuce have a wide-range optimum pH due to the presence of several isoenzymes that are not purified or only partially purified. The same authors report that maximum activity was at pH 5, which was lower than in the standard procedure according to Furumo and Furutani (2008) that was used in our experiments. They also indicate that POX enzymes differ in optimum pH and temperature even when they are from the same species.

Environmental conditions as well as genotype may contribute to the antioxidant properties of lettuce. Varieties that have a high content of anthocyanins, which was not the case in our experiment, also have higher antioxidant capacity (Liu et al., 2007).

Low activity of PPO was also recorded in the leaves of silver birch seedlings, but increased with el-

evated temperatures (Tegelberg et al., 2008). Elevated activity of PPO in all lettuce seeds that have not germinated on phenol can be attributed to the normally increased activity of this enzyme at the temperature of 24°C at which seeds were grown. Both PPO and POX have increased activities at room temperature, which decrease if the temperature increases or decreases (Boo et al., 2011). It is also possible that the polyphenol oxidase is being released into the environment, whether by secretion or cell lysis, ending by degrading a broad spectrum of phenolic molecules outside (Sinsabaugh 2010).

Wounding of poplar leaves by herbivores causes a strong induction of PPO activity (Constabel and Ryan 1998), leading to covalent modifications of free amino and sulfhydryl groups and thus reducing the nutritive value of proteins. The low PPO activity in lettuce seedlings that were grown on phenol could be attributed to the toxic effects of the phenol on metabolic processes and biosynthesis of PPO, or to the release of PPO from the plant to the growth medium.

Reduced activity of polyphenol oxidase may be also the result of different substrates used for determination of the enzyme. Determination of phenolic compounds in plants may lead to the enormous differences in results related to different extraction procedures (Dinçer et al., 2013). The same authors suggest that results may be affected by the geographical location of plants, ecological conditions and climate. Polyphenol oxidase with monophenolase activity, isolated from iceberg lettuce, showed inhibition by high substrate concentration (Chazarra et al., 1999), which may be related to our experiment where LD_{50} phenol concentration inhibited enzyme activity.

One obvious hypothesis about PPO is that the enzyme serves as a signal molecule (Bais et al., 2004) that alerts the plant to the presence of phenols. In that case, PPO would not require large amounts of substrate. To approximately figure out the role of the lettuce PPO, the activity of the enzyme should be determined in the roots and other parts of plants. It is estimated that enzymes could have a role in signal transduction if localized in root tips.

Polyphenol oxidase activity was determined in the whole plant (upper part + the root), so we cannot say exactly which part of the plant is responsible for the production of this enzyme. Holzapfel et al. (2010) believe that a large part of the production of the PPO comes from the plant roots as well as from seedlings of *Bromus* spp. The same authors suggest that L-DOPA is not likely to be the naturally occurring substrate for the root enzyme, but they also noticed that catechol showed very weak results. This result does not correspond with our results, nor with those of Gawlik-Dziki et al. (2008) ,who reported that PPO had the greatest activity detected with catechol, followed by 4-MC.

Root elongation in lettuce showed signs of suppression two days after exposure to 0.1 mM L-DOPA, which indicates that lettuce roots have very low PPO activity (Hachinohe et al., 2004). In any case, an enzyme that functions to defend the plant against toxic compounds in soil would be expected to have broad substrate specificity.

Lettuce cultivars that were grown on the increased concentration of phenol showed increased CAT and POX activity compared to control seeds that were not exposed to this toxic agent. The varieties LJL (which shows the highest activity of POX) and N (with the highest activity of CAT), also have the greatest resistance to the harmful effects of phenol, which is proved by their morphological characteristics remaining unchanged. CAT as well as POX has been considered a defensive mechanism for plants against stress (Vamos-Vigyazo 1981; Apel and Hirt 2004). Increases in catalase activity at the beginning of treatment with an elevated concentration of phenol can be explained by a rapid increase in the concentration of hydrogen peroxide caused by the toxic effects of phenol. CAT normally have low affinity for hydrogen peroxide (Mizuno et al., 1998), i.e. hydrogen peroxide levels need to reach a certain concentration that would be sufficient to activate catalase, but in this case phenol can be a very powerful agent that leads to the production of large amounts of ROS. The very high level of CAT activity was observed for lettuce varieties grown on

an elevated concentration of NaCl at the start of the treatment (Mahmoudi et al., 2012). The origin of the ROS, especially hydrogen peroxide, produced during defense reactions in lettuce is intriguing. The largely apoplastic location suggests that either the plasma membrane or the cell wall is the primary site of the superoxide/H_2O_2 generator (Lamb and Dixon 1997).

We conclude that antioxidative enzyme activities can play a protective role against stress caused by phenol and these enzymes were effective in providing resistance to damage from the harmful influence of phenol at the stages of seed germination and growth of seedlings. The increase in the activity of antioxidant enzymes may be due to the higher levels of ROS produced under the stress. Further experiments should clarify whether PPO is released from the plant to the phenol solution. In that case, the most resistant varieties from our study can be used for cleaning water from phenol.

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