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Phenol induced physiological stress in hydroponically grown lettuce (*Lactuca sativa* L.)— Part 2

Vojin Tadić[a], Jovan Tadić[b,*], Snežana Milošević[c], Aleksandar Cingel[c], Olivera Prodanović[d], Tatjana Ćosić[c], Zoran Vujčić[e]

[a] Minining and Metallurgy Institute Bor, Zeleni bulevar 35, 19210 Bor, Serbia
[b] Climate Science Division, Lawrence Berkeley National Lab, Berkeley, CA 94720, USA
[c] Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia
[d] Institute for Multidisciplinary Research, University of Belgrade, Kneza Višeslava 1, 11030 Belgrade, Serbia
[e] Department for Biochemistry, Faculty of Chemistry, University of Belgrade, Studentski trg 12-16, 11000 Belgrade, Serbia

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ABSTRACT

In this study we investigated physiological parameters of stress (enzymatic and non-enzymatic) in lettuce (*Lactuca sativa* L.) and its hairy roots induced by water solution of phenol. Two varieties of lettuce were examined, Ljubljanska ledenka and Nansen. Plants were grown in water with phenol concentration of 200 mgL^{-1} during 10 days. We monitored activity of peroxidases, catalases, polyphenol oxidase and superoxide dismutase, as well as proline and chlorophyll content. We observed a decrease in peroxidases, and increase in activity of catalase, polyphenol oxidase and superoxide dismutase compared to control plants. The concentration of proline was constantly increasing in both lettuce varieties over the course of the experiment. We detected an increase in activity of all monitored enzymes, except polyphenol oxidases, in hairy roots. The hydroponic system provides a useful framework for studying the effect of different harmful substances and its elimination. In such a system, as used in this work for the study of physiological processes in antioxidant protection activated when plant was exposed to phenol, lettuce and its hairy roots can be viewed as tools for water remediation.

1. Introduction

Lettuce (*Lactuca sativa* L.) is an annual plant, grown on nearly all continents. Apart from its nutritional value, due its high antioxidant activity lettuce has been reported to prevent chronic diseases related to oxidative stress, including cancer (Chu et al., 2002).

There are many reports about the uptake of heavy metals (Cd, Hg and Pb) from industrially contaminated soil and water, and their accumulation in leaves of lettuce (Zhenyan et al., 2005; Benzarti et al., 2008; Lamb et al., 2010). Plants, candidates for bioremediation, must generally be capable of growing on soils soaked with wastewater, and must have the ability to cope with hazardous substances. These plants must have developed enzymatic mechanisms of protection against the harmful effect of pollutants, or a mechanism for accumulation of these substances. In iceberg lettuce, one of the most noticeable physiological processes is spotting (visible on leaf surfaces) in which phenolic compounds are accumulated and oxidized by polyphenol oxidase (PPO) (Ke and Saltveit, 1988).

The enzymatic destruction of phenol is a typical oxidative process with molecular oxygen serving as the oxidant for oxidases and hydrogen peroxide as the oxidant for peroxidases (Mayer, 2006). Enzymes catalyzing these reactions are polyphenol oxidase and peroxidase, respectively. A few previous studies have detected antioxidant activity and phenolic components in lettuce (Chu et al., 2002; Caldwell, 2003). The data on the physiological function of these enzymes are sparse, while the biochemical reactions catalyzed by PPOs are well known (Tran et al., 2012). The amount of stress induced by phenols can be determined indirectly by measuring the activity of antioxidant enzymes that are part of the plant antioxidant defense system. For this reason, we monitored activity of several enzymes in our study such as peroxidases (POX), catalases (CAT), PPO and superoxide dismutase (SOD).

There is little information available on the effects of phenolic content in water on the antioxidant capacity of different cultivars of lettuce. Enzyme activity in lettuce seeds germinated on phenol, has been determined at early stages (Tadić et al., 2014a), but further growth of plants on phenol and their enzymatic profile are still unknown. To examine this, two varieties, Ljubljanska ledenka (LJL) and Nansen (N), were selected for further analysis because of their high germination

Abbreviations: LJL, Ljubljanska ledenka; N, Nansen; POX, peroxidase; CAT, catalase; PPO, polyphenol oxidase; SOD, superoxide dismutase

* Corresponding author.
E-mail address: jotadic@lycos.com (J. Tadić).

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efficiency (100%) on phenol (200 mgL^{-1}), according to our previous research (Tadić et al., 2014a).

Besides the whole lettuce plants, root cultures can be used for elimination of hazardous substances similar to hairy roots of carrots and *Brassica napus* which use peroxidases (Agostini et al., 2003). In this paper we examine the capability of lettuce and its hairy roots to give a physiological response to stress caused by phenol in water solutions. Phenol solutions with an 'initial concentration' of phenol were used as a model system for lakes and ponds or other bodies of standing water that have been polluted with phenol in the past, but have no active influx of phenol-containing wastewater. On the other hand, phenol solutions with "constant concentration" were used as a model system for water bodies where there is ongoing influx of phenol-containing wastewater. As a secondary goal we provided some insight into the activity of antioxidant enzymes that are the physiological basis of stress.

2. Materials and methods

2.1. Plant material and growing system

Seeds of lettuce (*Lactuca sativa* L.) were used as starting material for hydroponic growing. Seeds of different lettuce varieties, Nansen (N) and Ljubljanska ledenka (LJL), were provided from Semenarna, Slovenia. 40 plants have been planted in two separate fields. All sample collections were repeated three times. The chamber for hydroponic cultivation (100 × 100 × 180 cm) was obtained from Hydroponics, Serbia.

Hairy roots were obtained using *Agrobacterium rhizogenes* A4M70GUS mediated genetic tranformation of lettuce LJL variety (Tadić et al., 2014b). Cultures of transformed roots were maintained on half-strength MS medium lacking plant growth regulators and subcultured on new medium every 30 days.

2.2. Plant growth and phenol treatments

A solution of phenol was added to the canister for hydroponic cultivation of plants (each canister contained deionized water with mineral solution HESI Hydro Growth mineral fertilizers, as described in Part 1), at the beginning of the experiment, to a concentration of 200 mgL^{-1}. The concentration of phenol used in the experiment was determined from the previous study in which we investigated the influence of phenol on the germination of lettuce (Tadić et al., 2014a).

In the "initial concentration of phenol (ICPh)" setup, plants were grown on phenol concentration of 200 mgL^{-1} for 10 days without further addition of phenol. In the "constant concentration of phenol (CCPh)" setup, phenol solution was replaced every two days by a "fresh" solution at 200 mgL^{-1}. Samples from all treatments were collected every two days during the 10 days of cultivation. Hairy roots of the LJL variety were grown at initial and constant concentration of phenol of 50 mgL^{-1}. The time interval (10 days) was chosen according to results of the previous study about phenol removal from water (see Part 1).

2.3. Protein extraction

Frozen (−70 °C) plants (1 g) were homogenized in 1 ml of 0.1 M potassium phosphate (K-P) extraction buffer (pH 7, containing 1.5% insoluble polyvinylpyrrolidon (PVPP), 10 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The homogenate was centrifuged for 5 min at 12000*g* at 4 °C. The protein content of supernatants was determined according to Bradford (Bradford, 1976). The quantification of enzymes was performed spectrophotometrically (Agilent 8453, Life Science, USA).

2.4. Quantification of POX activity

Activity of POX was determined according to Furumo and Furutani (Furumo and Furutani, 2008). The protocol for the separation of enzymes was the same as the protocol used by Petrić et al. (2015). After the run, one part of the gel was stained with Commassie Brilliant Blue (CBB) and the other was processed for enzyme activity staining. Isoelectric focusing was performed using a Multiphor II electrophoresis system (Pharmacia-LKB Biotechnology). Focusing was carried out on a gel with ampholytes in a pH range of 3.0–10.0, at 7 W of constant power for 1.5 h at 10 °C. A broad pI kit (GE Healthcare) for the isoelectric point (pI) markers was used.

Peroxidase isoforms were determined according to the modified method of Siegel and Galston (Siegel and Galston, 1967). Gel analysis was performed using the TotalLab TL 120 graphics package.

2.5. Quantification of CAT activity

Catalase activity was determined spectrophotometrically according to Aebi (Aebi, 1984).

2.6. Quantification of PPO activity

Polyphenol oxidase (PPO, EC 1.10.3.1.) activity was determined according to Waite, (1976) when 4-methylcatechol (4-MC) used as a substrate. When L-DOPA was used as a substrate, enzyme activity was monitored according to Behbahani et al. (1993).

For separation of PPO isoforms gel polyacrylamide gel containing 3.75 ml of acrylamide, 0.75 ml ampholyte pH range of 3–10 (Sigma), 4 ml of glycerol, 6.5 ml of water, 12 ml of a TEMED, 75 uL APS was used. PPO isoforms were determined by incubating the gel in 20 ml of 0.1 M Na-acetate buffer (pH 6) containing 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH) and 10 mM phenol as a substrate at 25 °C for 3–5 min. Isoelectric focusing was carried out at 10 °C on a polyacrylamide gel adding 15 ml of sample and 10 ml of the pI markers. Isoelectric focusing lasted for three hours at 1200 V, and the pH range used was from 3 to 10. Analysis of gels was done using the graphical package TotalLab TL 120.

2.7. Quantification of SOD activity

SOD activity was determined spectrophotometrically by a modified method of Beyer and Fridowich (Beyer and Fridowich, 1987).

2.8. Determination of chlorophyll and carotenoids

Plants that have been grown on an initial concentration of phenol and further grown in solution without phenol were subjected to analysis of chlorophyll and total pigment content. The analysis of these parameters was completed after 20 days, when the regeneration of new leaves ended. Chlorophyll (Chl) and carotenoid content were determined according to Lichtenthaler (1987). Total chlorophyll and carotenoid content were than calculated according to the formula below and has been expressed in mg/g fresh weight:

$$C_{(a+b)} = 5.24A_{664} + 22.24A_{648}$$

Content of chlorophyll a: $C_a = 13.36A_{664} - 5.19A_{648}$

Content of chlorophyll b: $C_b = 27.43A_{648} - 8.12A_{664}$

Carotenoid content: $C = (1000A_{470}–2.13C_a - 97.64C_b)/209$

The ratio of chlorophyll a and b was calculated according to the formula: C_a/C_b

All extractions were repeated three times.