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Guaiacol Peroxidases in Carrot (*Daucus carota* L.) Root**

Hrvoje Lepeduš[1*], *Vera Cesar*[1] *and Marijana Krsnik-Rasol*[2]

[1]Department of Biology, Faculty of Education, University of J. J. Strossmayer, L. Jägera 9, HR-31000 Osijek, Croatia

[2]Department of Molecular Biology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, HR-10000 Zagreb, Croatia

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Summary

Carrot (*Daucus carota* L.) belongs to the group of common edible vegetables. The aim of the present study was to establish the distribution of guaiacol peroxidases in carrot root sections as well as to determine the enzymatic activity and the electrophoretic pattern of isoperoxidases in tissue extracts of particular root regions: central cylinder, inner cortex and epidermis, including the peripheral region of the cortex. This is a preliminary study for further investigations of stress influence on the expression of peroxidases in carrot root. In the central cylinder and inner cortex region peroxidase activity was localised in tracheary elements. The most intensive enzyme-linked colouration was noticed in the epidermal region of the cortex. The lowest activity was measured in the extracts of primary xylem area, while the area containing inner cortex, secondary xylem and phloem had an almost double activity. The highest activity was measured in the epidermal region. Electrophoretic analysis of anionic peroxidases revealed one isoenzyme in the central cylinder, and two other isoenzymes in the epidermal region.

Key words: *Daucus carota*, guaiacol peroxidases, root

Introduction

Peroxidases are haem-containing glycoproteins found in animal and plant tissues, as well as in microorganisms. There is a family of class III plant peroxidases (POX, EC 1.11.1.7) encoded by a large multigene family that comprises a number of peroxidase isoenzymes. Generally, peroxidases catalyse the oxidoreduction between hydrogen peroxide and reductants (*1*). Catalytic mechanism involves the formation of two intermediates called compounds I and II, which can react with organic co-substrates, finally producing the radical product (*2*). Since a variety of organic and inorganic molecules can be oxidised by peroxidases it has been suggested that peroxidases play an important role in a wide range of biochemical processes. As reported by Gaspar *et al*. (*3*) peroxidases are involved in auxin and ethylene metabolism, redox reactions in plasma membranes, cell wall modifications (lignification and suberinization) as well as in developmental and defence processes. Numerous investigations (*4–10*) were performed using changes in peroxidase activity and their electrophoretic patterns as stress screening parameters. All these studies revealed that, in spite of low substrate specificity, peroxidases could be employed as sensitive and accurate stress markers.

Carrot (*Daucus carota* L.) belongs to the group of common edible vegetables exposed to different stress factors during cultivation. Herbicides as well as heavy metals are considered to be the main candidates for provoking such stress situation. The analysis of guaiacol peroxidases, as a convenient, cheap and simple method, could be employed for early detection of stressful influ-

* Corresponding author; Phone: ++385 31 211 400; Fax: ++385 31 212 514; E-mail: hlepedus@yahoo.com

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ences. The aim of the present study was to establish the distribution of guaiacol peroxidases in carrot root tissues as well as to investigate the enzyme activity and electrophoretic pattern of isoperoxidases in particular regions of carrot root: central cylinder, cortex and epidermal region. This is a preliminary study to test if peroxidases could be accurate stress markers in carrot root.

Materials and Methods

Fully developed carrot (*Daucus carota* L.) roots purchased from the local market were studied. Three tissue regions were selected: I – a part of central cylinder containing primary xylem, II – a part of central cylinder containing secondary xylem and neighbouring (inner) cortex region, III – epidermal region comprising the epidermis and outer part of the cortex.

For enzyme localisation fresh transversal sections and tissue printings were used (*11*). The sections were either hand-made or cut by using the rotary microtome (Leica RM 2155) with razor blade (60 μm thick sections). Peroxidase activity was detected by immersing tissue sections or nitrocellulose printings in the reaction mixture containing guaiacol and H_2O_2 as substrates. Tissue sections were also stained with 0.05 % Toluidine blue O in the benzoate buffer, pH=4.4 (*12*) and with phloroglucinol-HCl (*13*) for polyphenols and lignin detection, respectively.

Selected tissue regions (I, II, III) were isolated and cut into small pieces for protein extraction. The tissue was homogenised in an ice-cold 0.1 M Tris-HCl buffer, pH=8.0, with the addition of polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 20 000 x *g* at 4 °C for 20 min. The activity of guaiacol peroxidases in supernatant was determined spectrophotometrically by measuring the absorbance increase at 470 nm. The reaction mixture contained 5 mM guaiacol and 5 mM H_2O_2 in 0.2 M phosphate buffer, pH=5.8 (*14*). The activity in each selected region of the root was expressed as the percentage of the highest activity. The enzymatic reaction was started by adding 200 μL of the extract to 800 μL of reaction mixture. The protein content of the extracts was determined according to Bradford (*15*), using bovine albumin serum (BSA) as a standard.

Isoperoxidases were separated under native conditions by means of discontinuous vertical polyacrylamide gel electrophoresis (PAGE) as well as by isoelectric focusing (IEF). For PAGE the 1-mm stacking (2.5 %) and separating (5 %) polyacrylamide gels were prepared, according to the procedure of Laemmli (*16*), but without the addition of SDS. Electrophoresis was carried out at +4 °C for 5 h (2 h at 20 mA of constant current followed by 3 h at 150 V of constant voltage). Isoelectric focusing was performed in 5 % polyacrylamide gel with ampholytes in the pH range of 3.5–10, at +4 °C (*17*). The running conditions were: 0.5 h at 100 V, 1.5 h at 200 V followed by 3 h at 400 V. The bands of isoperoxidases were visualized by immersing the gel in the same reaction mixture as the one used for peroxidase activity determination.

Results and Discussion

The anatomy of carrot root is well established (*18*). The core of the central cylinder is structured as follows: the primary xylem is surrounded by mainly parenchymatous secondary xylem produced by vascular cambium, while the secondary phloem is produced centrifugally also by vascular cambium. The central cylinder is surrounded by a parenchymatous cortex. Distribution of peroxidases was monitored by the appearance of brown colouration after the incubation in reaction mixture containing guaiacol and H_2O_2 (Fig. 1). Brown colouration appeared in both central cylinder and cortex (Fig. 1A). Tissue printing technique was employed to avoid possible mistakes due to orange colouration of the carrot root itself. The same distribution of peroxidase reaction products was detected in tissue sections as well as in the prints (Fig. 1B). Closer examination revealed that peroxidase activity in the central cylinder occurred as a dotted pattern (Fig. 1C), while in the cortex it was distributed with increasing activity toward the epidermis (Fig. 1D). Staining of tissue sections with Toluidine blue O showed that cell walls of tracheary elements in primary and secondary xylem contained polyphenolic substances (Figs. 1E and 1H). Specific staining with phloroglucinol-HCl confirmed the presence of lignin in the cell walls (Figs. 1F and 1I). Peroxidase activity in the primary and secondary xylem was also localised in the cell walls of tracheary elements (Figs. 1G and 1J). The process of cell wall lignification includes the coupling of radicals produced by the oxidation of monolignols. Although the enzymes involved in the oxidation of monolignols have not been identified, peroxidases are considered to be the main candidates to perform the last step in the lignification process (*19*). Our results (Fig. 1) showed clear relationship between the localisation of guaiacol peroxidases and the lignification process in the xylem.

The results of guaiacol peroxidase activity measurements are shown in Fig. 2. The primary xylem area had the lowest activity. Almost double activity was measured in the tissue region containing secondary xylem and inner part of the cortex. The highest activity was determined in the epidermal region (the outer part of cortex with epidermis). Electrophoretic analysis revealed differences in the guaiacol isoperoxidases (Fig. 3). Protein samples from the central cylinder and the epidermal region were compared. PAGE electrophoresis showed one isoperoxidase band in the extracts of the central cylinder, while a wider activity zone of slightly higher mobility appeared in the extracts of the epidermal region (Fig. 3A). The IEF showed peroxidase activity in the neutral region of the gel. The epidermal region contained two peroxidase isoenzymes (Fig. 3B). Only one isoenzyme was present in the sample of the central cylinder, as in the native PAGE (Fig. 3B). The expression of different peroxidase isoenzymes we have observed (Fig. 3) could be associated with their different function in the carrot root. Nair and Showalter (*20*) have isolated a novel cell wall cationic guaiacol peroxidase (pI>9.3) from carrot plants. They showed that the enzyme was constitutively expressed in roots and wound-inducible in leaves and petioles.

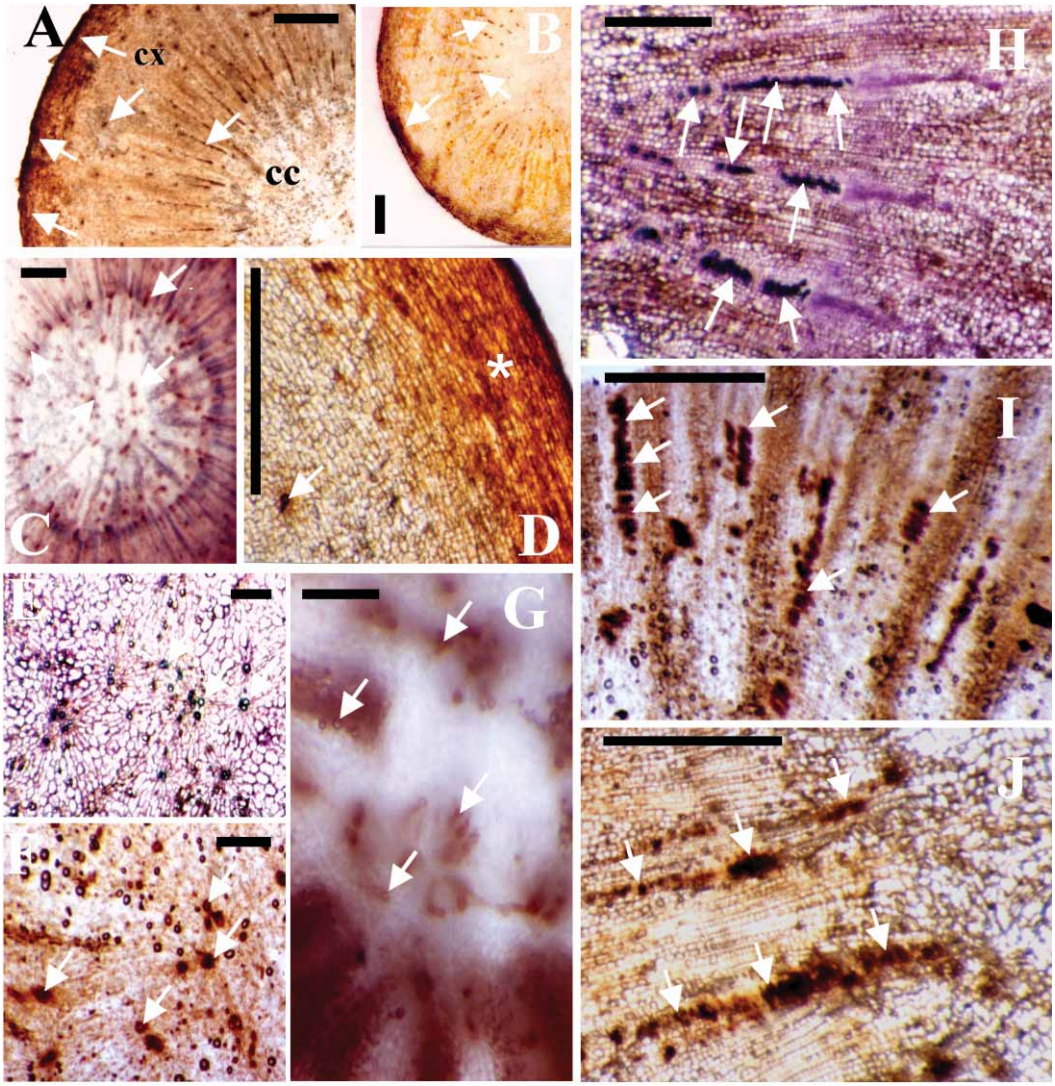

Fig. 1. Guaiacol peroxidase localisation in carrot (*Daucus carota* L.) root. Bar = 2 mm.
A. Peroxidase activity detected as brown colouration (arrows) on the root cross-section: cc – central cylinder, cx – cortex. **B.** Peroxidase activity (arrows) detection on the tissue prints. **C.** Detail of the central cylinder. Peroxidase activity detected as dotted spots (arrows). **D.** Detail of cortex. Peroxidase activity detected as dotted spots in the inner part of the cortex (arrow) and as the continuous colouration in the epidermal region (asterisk), with increasing tendency toward the epidermis. **E.** Staining with Toluidine blue O showed the presence of polyphenolic substances in the cell walls of tracheary elements in the primary xylem (arrows). **F.** Staining with phloroglucinol-HCl confirmed the presence of lignin in the cell walls of tracheary elements in the primary xylem (arrows). **G.** Peroxidase activity was located in the cell walls of tracheary elements in the primary xylem (arrows). **H.** Staining with Toluidine blue O showed the presence of polyphenolic substances in the cell walls of tracheary elements in the secondary xylem (arrows). **I.** Staining with phloroglucinol-HCl confirmed presence of lignin in the cell walls of tracheary elements in the secondary xylem (arrows). **J.** Peroxidase activity was located in the cell walls of tracheary elements in the secondary xylem (arrows)

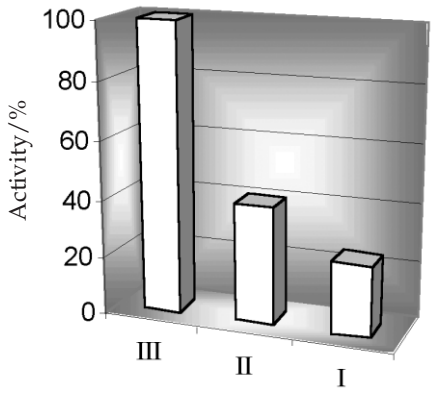

Fig. 2. Guaiacol peroxidase activity in different parts of the carrot root. The highest activity was measured in the epidermal region (III), while the lowest one was in the primary xylem area (I). The peroxidase activity in the region containing the secondary xylem and inner part of the cortex (II) was almost doubled in comparison with the part of central cylinder containing primary xylem

Previous reports showed the stimulation of guaiacol peroxidase activity and expression of new isoenzymes in different plant species in response to various influences such as the treatment with ozone and simulated acid rain (*8*), elevated deposition of fluorides (*4*), SO_2 treatment (*7*) or high sulphur and heavy metal loads

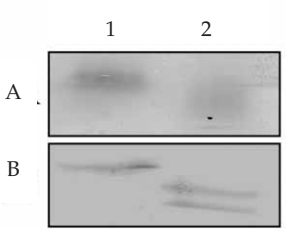

Fig. 3. The electrophoretic pattern of guaiacol peroxidases extracted from the central cylinder (1) and the epidermal region of cortex (2). **A.** Native PAGE revealed one band in the central cylinder, while in the sample from the epidermal region a wider activity band appeared. **B.** Isoelectric focusing also revealed one isoenzyme present in the central cylinder (1). The epidermal region (2) contained two isoenzymes

(*9,21*). During the tissue ageing the guaiacol peroxidase activity also increased (*3*). Our investigation demonstrated the expression of different guaiacol peroxidase isoenzymes in particular parts of the root. Also, the distribution of these isoenzymes was different. While the isoperoxidase in the central cylinder was shown to be related to the lignification process of tracheary elements, the isoperoxidases present in the epidermal region are probably involved in a defence mechanism against some stress conditions. Further studies will be concentrated on the effects of various stress factors (heavy metals, herbicides) on the peroxidase activity and electrophoretic pattern in carrot roots. This should reveal if altered situation would cause a functional difference among described peroxidase isoenzymes.

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Gvajakol-peroksidaze u korijenu mrkve (*Daucus carota* L.)

Sažetak

Mrkva (*Daucus carota* L.) vrsta je povrća koja se redovito koristi u prehrani. Svrha je rada bila istražiti distribuciju gvajakol-peroksidaza, te odrediti broj izoenzima i aktivnost u pojedinim dijelovima korijena: centralnom cilindru, korteksu i epidermalnom području. To je preliminarno istraživanje koje će poslužiti kao temelj za buduća istraživanja utjecaja stresa na ekspresiju peroksidaza u korijenu mrkve. U području centralnog cilindra i unutrašnjem dijelu korteksa peroksidazna se aktivnost odvija u trahealnim elementima. Najintenzivnije obojenje koje detektira aktivnost peroksidaza uočeno je u epidermalnom dijelu korteksa. Najmanja peroksidazna aktivnost izmjerena je u ekstraktima tkiva iz primarnog ksilema, dok je aktivnost u tkivu unutrašnjeg korteksa, sekundarnog ksilema i floema bila gotovo dvostruko veća. Najveća aktivnost izmjerena je u epidermalnom području. Elektroforetska analiza anionskih peroksidaza pokazala je jedan izoenzim u centralnom cilindru, te druga dva u epidermalnom području.