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EFFECT OF INDOLE-3-ACETIC ACID ON PEA ROOT GROWTH, PEROXIDASE PROFILES AND HYDROXYL RADICAL FORMATION

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Abstract — Changes in growth, peroxidase profiles, and hydroxyl radical formation were examined in IAA (0.5 – 10 mg/l) treated pea plants grown hydroponically and in isolated roots in liquid in vitro culture. IAA inhibited root elongation, both in hydroponically grown pea plants and in isolated roots in vitro. A remarkable increase in the number of POD isoforms was noticed in isolated roots grown in vitro, compared to the roots from plants grown hydroponically. IAA induced both disappearance of several root POD isoforms and hydroxyl radical formation in the root and the root cell wall.

Key words: Indole-3-acetic acid, peroxidase, *Pisum sativum*, root, cell wall, hydroxyl radical

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INTRODUCTION

Auxins are phytohormones involved in mediating a number of essential plant growth and developmental processes, such as cell elongation and division, induction of root growth and flower and fruit development. Indole-3-acetic acid (IAA) is considered to be the most important native auxin in higher plants and is therefore the most studied one (Normanly et al., 1995). Exogenous IAA usually inhibits root growth (Pilet and Elliot, 1981), but in some cases it can promote root elongation (Pilet, 1961).

To function as a regulator of cell expansion, IAA concentration in the target tissue must be controlled (Pilet and Saugy, 1985). Phytotoxicity of some bacteria (Barazani and Friedman, 1999) and fungi (Ditengou and Lapeyrie, 2000) is based on suppression of root growth by secretion of a high concentration of IAA or an indolic compound (hypaphorine). Free IAA can be modulated via several pathways, including IAA metabolism, synthesis and breakdown of conjugated IAA, and transport of IAA (Kraepiel et al., 2001; Jensen et al., 1998).

Peroxidases (PODs) (EC 1.11.1.7), as heme enzymes, are involved in the metabolism of IAA by oxidizing IAA via two different mechanisms: (1) the conventional H_2O_2-dependent pathway (Rutter et al., 1983; Schulz et al., 1984); and (2) one that requires O_2, but not H_2O_2 (Gazarian and Lagrimini, 1996; Gazaryan et al., 1998; Savitsky et al., 1999). PODs also participate in various physiological processes, such as lignification, suberization, auxin catabolism, wound healing and defense mechanisms against pathogen infection (Hiraga et al., 2001). A key role for peroxidase was shown in rooting of different plant species cultured in vitro, which was correlated with auxin content in the media (Gaspar, 1992; Rivalet al., 1997; Rout et al., 2000; Arena et al., 2003; Molassiotis et al., 2004).

Studies on IAA-plant peroxidase interactions are therefore prerequisite to understanding the mechanism of IAA perception by plants. In this study, we report differences in peroxidase profiles induced by high concentrations of IAA in roots of pea plants grown hydroponically and in isolated roots grown in vitro in a liquid medium. Also, hydroxyl radical generation in pea roots and root cell walls is demonstrated and discussed in rela-

tion to the inhibition of root elongation induced by IAA.

MATERIAL AND METHODS

Plant material

Hydroponically grown *P. sativum* plants: Seeds were washed for 5 h under tap water and sown on (and covered with) moistened filter paper. Seed germination took place in darkness at 25°C for 4 days. Four-day-old pea seedlings were grown hydroponically in tap water supplemented with IAA (0.5 – 2 mg L^{-1}) for 14 days. The temperature in the growth chamber was 17–21 °C (night/day), and the photoperiod was 16 h/8 h (light/darkness). Irradiance of 10 mmol $m^{-1}s^{-1}$ (low) or 100 mmol $m^{-1}s^{-1}$ (high) was provided by white fluorescent tubes.

In vitro liquid culture of *P. sativum* isolated roots: Seeds were washed in mild detergent for 10 min and rinsed in tap water for 5 h. Surface sterilization was performed in 2% Na-hypochlorite for 1-2 min and washed with sterile distilled water three times. Sterilized seeds were aseptically sown on (and covered with) moistened filter paper in Petri dishes. Seed germination took place in darkness at 25°C. Root tips, 20 mm in length, were excised from 4-day-old seedlings. They were transferred to glass jars containing 100 ml of liquid MS (M u r a s h i g e and S k o o g, 1962) medium supplemented with 3% sucrose and IAA (0.5 – 10 mg L^{-1}), pH 5.7. The temperature in the growth chamber was 23 - 25°C, and the photoperiod was 16 h/8 h (light/darkness). Irradiance of 40 mmol $m^{-1}s^{-1}$ was provided by white fluorescent tubes.

Peroxydase isoform analysis

For analysis of peroxidase isoenzyme profiles, 14 day-old plants grown hydroponically (leaves and roots), 4-week-old isolated roots grown in vitro, and samples of culture media at the end of forth week of culturing were used. For extraction of soluble enzymes, frozen plant material was powdered in a mortar containing liquid N_2 and extracted in 100 mM K-phosphate buffer (pH 6.5). The homogenate was centrifuged at 10,000 g for 15 min at 4°C. The supernatant was used for analysis of soluble root PODs.

The cell wall was isolated from roots following a method described by V e l j o v i ć – J o v a n o v i ć et al. (2005). Roots were powdered in liquid N_2 and homogenized in buffer (50 mM Tris, 50 mM NaCl, 0.05% Tween 80, 1 mM PMSF). The homogenate was filtered through two layers of cloth, sonicated for 1 min and centrifuged at 1000g for 20 min. The cell wall pellet was washed four times in the above buffer without detergent and salt. After suspending of the pellet in 10 ml of 1 M NaCl, the suspension was incubated for 30 min at 4°C and then centrifuged at 1000g for 15 min. The pellet (cell wall isolates) was washed several times by centrifugation and suspended in 5 ml of 50 mM Tris/HCl (pH 7.2).

Protein content in solubile and cell wall isolates was measured according to B r a d f o r d (1976).

Isoelectric focusing was carried out in 7.5% polyacrylamide gel with 3% ampholite in a pH gradient from 3 to 9. To determine POD activity, the gel was incubated with 10% 4-chloro-α-naphthol and 0.03% H_2O_2 in 100 mM K-phosphate buffer (pH 6.5).

Markers for IEF in the pI range of 3.6-9.3 were purchased from Sigma (IEF-M1A).

Hydroxyl radical detection

EPR measurements using a DEPMPO, spin trap for OH and $O_2^{\cdot -}$ (M o j o v i ć et al. 2004) were performed using a custom-made Teflon flat cell (50 µl) with one cell side made of oxygen-permeable thin Teflon foil. Spectra were recorded at room temperature using a Varian E104-A EPR spectrometer operating at the X-band (9.51 GHz) under conditions of the following settings: modulation amplitude, 0.2 mT; modulation frequency, 100 kHz; microwave power, 10 mW; magnetic field center, 341 mT; and scan range, 20 mT; scan speed, 4 mT/min. Spectra were recorded and analyzed using EW software (Scientific Software). DEPMPO (final concentration, 42.5 mM) was added to the cell wall fraction.

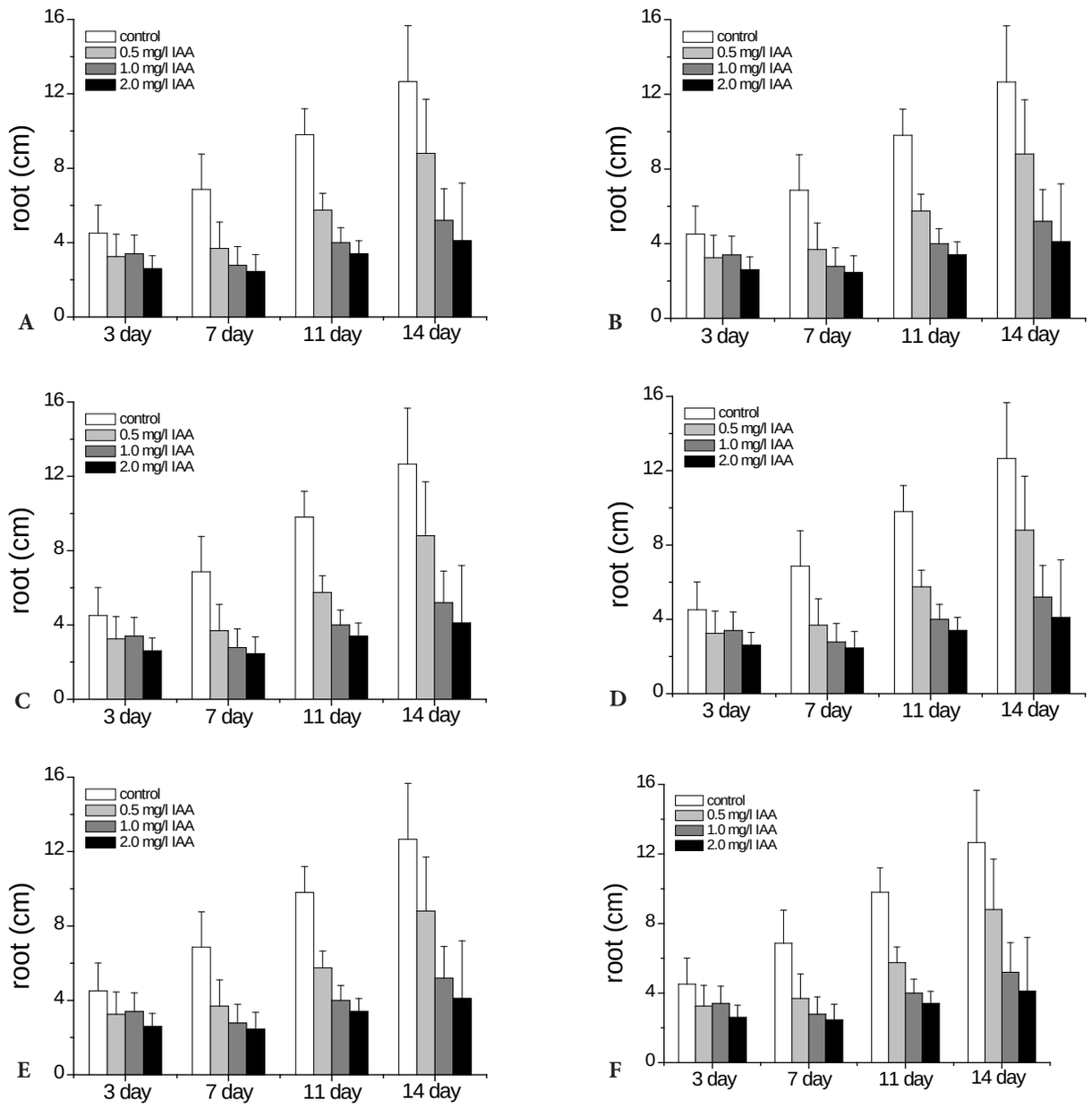

Fig. 1. Effect of IAA (0.5 – 2.0 mg L-1) on P. sativum root length (A, B), number of lateral roots (C, D), and stem length (E, F) during 2 weeks of culturing in tap water under low- (A, C, E) and high- (B, D, F) intensity light. Means ± SE, n = 20.

RESULTS AND DISCUSSION

Effects of IAA and light intensity on growth

Light regulates the metabolism, transport and binding of auxin to specific proteins and sugars (Kraepiel et al., 2001; Jensen et al., 1998). Figure 1 shows the effect of IAA (0.5 – 2 mg/l) on stem height, root length, and the number of lateral roots in 2-week-old pea plants grown hydroponically at different light intensities. IAA (0.5 - 2 mg L^{-1}) had no significant effect on stem height regardless of

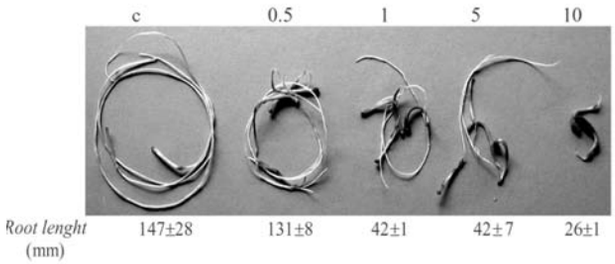

Fig. 2. Effect of IAA (0.5 – 10.0 mg L-1) on P. sativum root length at the end of the fourth week of in vitro culturing in liquid MS medium. Means ± SE, n = 10.

light intensity, while stem elongation was increased by low-intensity light (Fig. 1E, F). Light intensity did not influence root length or the number of lateral roots in the control plants (Fig. 1 A, B, C, D). IAA (0.5 – 2 mg L^{-1}) inhibited root elongation and stimulated lateral root formation in plants grown under low-intensity light (Fig. 1A, C), while in plants grown under high-intensity light, IAA (0.5

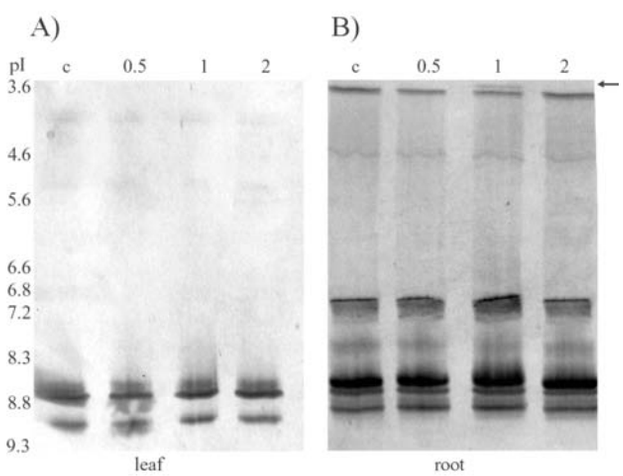

Fig. 3. Isoelectrofocusing of soluble peroxidases extracted from *P. sativum* leaves (L) and roots (R) of 2-week-old pea plants grown in tap water under low-intensity light: control (0), 0.5 mg L-1 IAA, 1 mg L-1 IAA, and 2 mg L-1 IAA. The arrow indicates a new isoform in IAA (1 mg L-1) treated roots.

mg L^{-1}) even slightly promoted root elongation (Fig. 1B) and inhibited lateral root formation (Fig. 1 D). It has been shown that auxin is required for both initiation of formation and emergence of lateral roots (C a s i m i r o et al., 2001; B h a l e r a o et al., 2002; B e n k o v a et al., 2003). On the other hand high IAA concentrations have an inhibitory

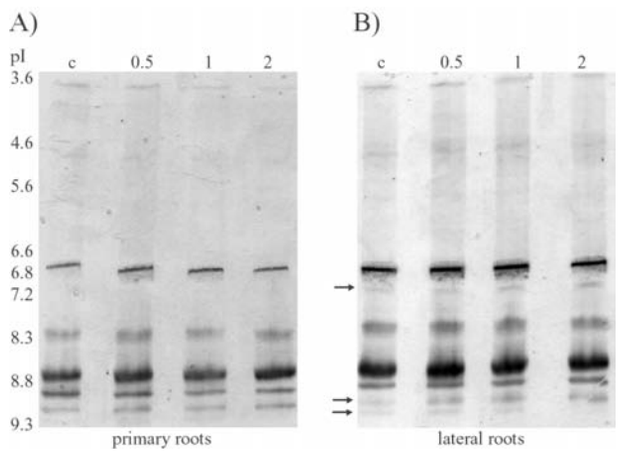

Fig. 4. Isoelectrofocusing of soluble peroxidases extracted from main *P. sativum* roots (A) and lateral roots (B) of 2-week-old plants grown in tap water under low-intensity light: control (0), 0.5 mg L-1 IAA, 1 mg L-1 IAA, and 2 mg L-1 IAA. Arrows indicate new isoforms in IAA (1 mg L-1) treated roots.

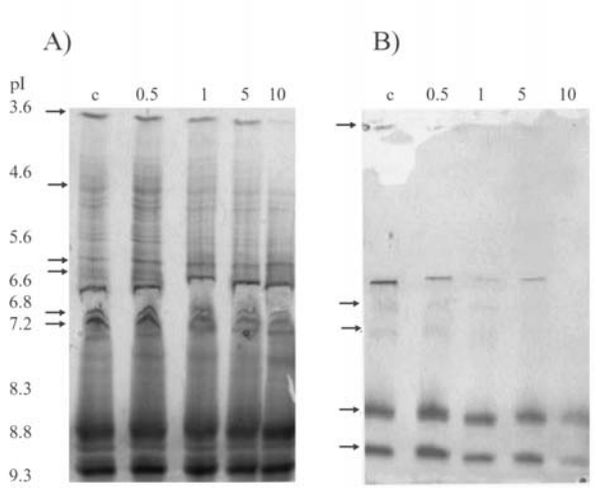

Fig. 5. IEF gel stained for POD activity in (A) soluble *P. sativum* root extracts and B) ambient liquid MS medium after 4 weeks of culturing in vitro: control (0), 0.5 mg L-1 IAA, 1 mg L-1 IAA, 5 mg L-1 IAA and 10 mg L-1 IAA. Arrows indicates different POD isoforms changing upon IAA treatment.

effect on root elongation (L a n e, 1936; P i l e t and S a u g y, 1987).

An inhibitory effect of IAA on root growth was also observed in 4-week-old isolated pea roots grown in liquid culture in vitro (Fig. 2). Contrary to hydroponically grown pea plants, isolated roots

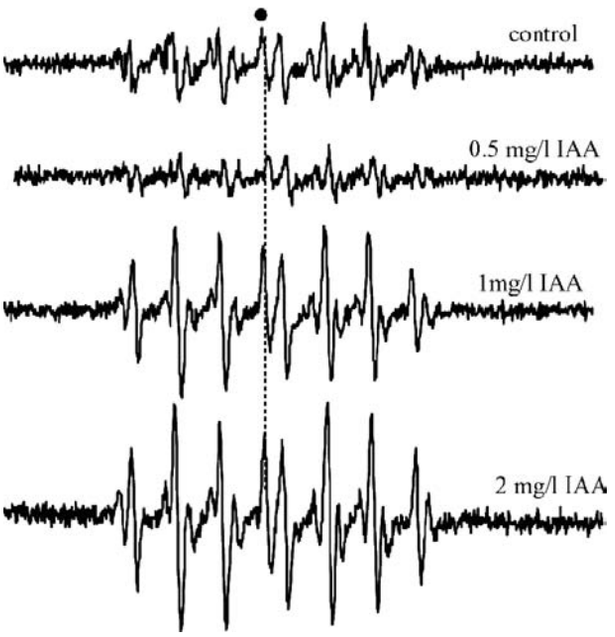

Fig. 6. EPR spectra of DEPMPO/OH adducts in *P. sativum* root cell wall isolated from 14-day-old plants grown in tap water under high-intensity light: control, 0.5 mg L-1 IAA, 1 mg L-1 IAA, and 2 mg L-1 IAA. The filled circle marks one characteristic peak of DEPMPO/OH adduct.

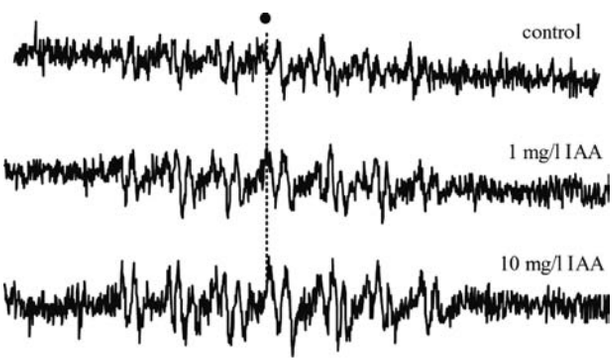

Fig. 7. EPR spectra of DEPMPO/OH adducts in root cell wall isolated from roots at the end of the fourth week of in vitro culturing in liquid MS medium: control (0), 0.5 mg L-1 IAA, 1 mg L-1 IAA, 5 mg L-1 IAA and 10 mg L-1 IAA. The filled circle marks one characteristic peak of DEPMPO/OH adduct.

grown in vitro did not produce lateral roots, regardless of IAA concentration in the medium. IAA (0.5 - 10 mg L^{-1}) inhibited root elongation (Fig. 2), produced root thickening, and increased lignification (data not shown).

Peroxydase isoform analysis

A combined role of auxin and photoperiod on in vitro rooting and root peroxidase activity was found in different plant species (R o u t et al., 2000; A r e n a et al., 2003). There are numerous studies that suggest inverse correlation between peroxidase activity and IAA content and/or root growth (T h a k e r et al., 1986; Z h e n g and v a n H u y s t e e, 1992; B o e u f et al., 1999). We analyzed the profile of isoperoxidases sorted by their pI values in leaves and roots of plants grown hydroponically (under low-intensity light) and in roots grown in liquid culture in vitro. In leaves, three POD isoforms – two cationic (with pI 8.8 and 9.0) and one anionic (with pI about 4.0) were present (Fig. 3A). The addition of IAA (1 and 2 mg L^{-1}) decreased intensity of the band with pI 9.0 (Fig. 3A). In whole root extracts of hydroponically grown pea plants, we detected five cationic POD isoforms (pI 7.2, 8.2, 8.8, 8.9 and 9.0) and two anionic ones (with pI 4.6 and 3.6). IAA (1 mg L^{-1}) induced the appearance of a new highly acidic POD isoform with pI 3.6 (Fig. 3B). We showed that cationic peroxidases with similar pI values are present in both leaves and roots, while anionic and neutral ones are characteristic of roots only (Fig. 3).

Analysis of peroxidase distribution between primary (Fig. 4A) and lateral (Fig. 4B) roots showed higher activity in lateral roots with two new bands (pI 4.6 and 7.2). IAA (0.5 and 1 mg L^{-1}) induced an increase in the activity of two POD isoforms (pI about 9.1 and 9.2) in lateral roots, while IAA (2 mg L^{-1}) caused the disappearance of the POD isoform with a pI value of about 9.2 (Fig. 4B).

Compared to roots from plants grown hydroponically (Fig. 3B), soluble extracts of isolated roots grown in vitro contained numerous POD isoforms in the whole range of pI from 3.6 to 9.3 (Fig. 5A). IAA (1-10 mg L^{-1}) led to disappearance of the two POD isoforms with pI about 6.5 and decreased activity of the POD isoform with pI 4.6. IAA (10 mg L^{-1}) decreased activity of the POD isoform with pI about 3.6. Among all POD isoforms detected in isolated roots, only five (pI 9.0, 8.8, 8.2, 7.2, and 3.6) were present in the ambient medium after 4 weeks of culturing (Fig. 5B). Activity of the POD isoforms with pI 8.8 and 9.0 decreased in the ambient medium containing 10 mg L^{-1} IAA, while the POD

isoform with pI 3.6 was detected only in the control medium (Fig. 5B). Decrease in the activity of specific POD isoforms in the ambient medium (Fig. 5B), with increase of IAA concentration may be correlated with lower cell permeability due to cell wall thickening. Its has been shown in the literature that exogenous auxins can induce (L a g r i m i n i, 1996) or suppress (K l o t z and L a g r i m i n i, 1996) de novo peroxidase synthesis. Marked differences have been reported between acidic and basic cell wall isoperoxidases in relation to their efficiency in IAA oxidation (G o n z a l e et al. 1999).

Hydroxyl radical generation

Detection of oxygen-dependent hydroxyl radical production in isolated pea root cell wall using spin trap DEPMPO and EPR analysis was previously demonstrated by V e l j o v i ć – J o v a n o v i ć et al. (2005). In the present article, we obtained the signal of a DEPMPO/OH adduct in the root cell wall of control plants, which was increased by IAA (1 and 2 mg L^{-1}) treatment (2.3- and 2.6-folds, respectively) (Fig. 6). Similar results were obtained in the whole homogenate of isolated roots grown in vitro for 4 weeks: the relative peak intensity of DEPMPO/OH adducts increased with increase of IAA concentration (0.5 – 10 mg L^{-1}) in the medium (Fig. 7).

In the POD-dependent oxidizing reaction that requires O_2 but not H_2O_2, IAA-dependent generation of superoxide radical generation has been proposed (S a v i t s k y et al., 1999). S c h o p f e r et al. (2002) showed that exogenous IAA-induced coleoptile elongation was accompanied by 30% increase in the rate of $^{\bullet}O_2^-$ production (indirectly measured using a tetrazolium-based assay) and by 50% increase in the rate of $^{\bullet}$OH generation (measured by spin trap POBN). The authors concluded that IAA-induced cell elongation occurred through POD-dependent generation of $^{\bullet}$OH and its precursors $^{\bullet}O_2^-$ and H_2O_2, leading to cell wall loosening (C h e n and S c h o p f e r, 1999; S c h o p f e r, 2001). Contrary to the hypothesis of S c h o p f e r et al. (2002) that IAA induced $^{\bullet}$OH–dependent growth elongation, our finding suggests that $^{\bullet}$OH generation induced by IAA treatment was related to the inhibition of root growth, both in plants grown hydroponically and in isolated roots in vitro. From the results presented, we speculate that $^{\bullet}$OH generation may be also involved in the process of lignification and suberinization, leading to the cell wall stiffening and the inhibition of growth.

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УТИЦАЈ ИНДОЛ-3-СИРЋЕТНЕ КИСЕЛИНЕ НА РАСТ КОРЕНА ГРАШКА, ИЗОЕНЗИМСКИ ПРОФИЛ ПЕРОКСИДАЗА И НАСТАЈАЊЕ ХИДРОКСИЛ РАДИКАЛА

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Испитиван је утицај IAA (0.5 – 10 мг/л) на растење и изоензимски профил пероксидаза у листовима и корену грашка гајеног у воденој култури и у изолованим кореновима гајеним *in vitro.* IAA инхибира издуживање корена, како код биљака гајених у воденој култури, тако и изолованих коренова гајених *in vitro*. Значајно повећање броја POD изоформи је уочено код изолованих коренова гајених *in vitro* у односу на коренове биљака гајених у воденој култури. IAA је индуковала како нестајање неколико POD изоформи, тако и настајање хидроксил радикала у корену и ћелијском зиду корена, независно од услова гајења.