African Journal of Plant Science Vol. 4(12), pp. 479-487, December 2010
Available online at http://www.academicjournals.org/ajps
ISSN 1996-0824 ©2010 Academic Journals

Full Length Research Paper

Effect of endogenous phenols and some antioxidant enzyme activities on rooting of Persian walnut (*Juglans regia* L.)

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Accepted 18 November, 2010

***In-vitro* cultured microshoots of two Persian walnut cultivars differed in rooting response following induction with auxin (3 mg dm^{-3} IBA). Microcuttings of the cultivar 'Sunland' rooted well (66%) with a mean of 2.81 roots per shoot, while 'Howard' microshoots rooted poorly (29%) and produced only 1.6 roots per shoot. Because no exclusive order for the progress of phenolic changes during root emerging response was seen, no exact relationship was found between phenolic contents and rooting ability of these cultivars. The activity and isoenzymatic patterns of antioxidant enzymes were estimated using spectrophotometery and native PAGE. The differing activity patterns for peroxidase and polyphenol oxidase during root development may be useful as reliable and successful markers for the physiological processes of rooting. Maximum peroxidase activity was observed on day 35 of shoot culture during the initiation phase of rooting, and was correlated with the rooting ability of genotypes. 'Sunland', the most easily rooted cultivar, exhibited a greater increase than the more difficult to root 'Howard' cultivar. Polyphenol oxidase activity maximized on the 28th day and then declined to a minimum on day 49 in both cultivars, but the magnitude of change in 'Sunland' was consistently greater throughout the root development period. These results show that peroxidase and polyphenol oxidase activities are involved in defining the beginning and the length of particular phases of walnut rooting.**

Key words: Persian walnut, peroxidase, polyphenol oxidase, phenolic compounds, root response, *in-vitro* culture.

INTRODUCTION

An important aspect of walnut micropropagation is the induction of adventitious rooting of microshoots. A relatively high cytokinin/auxin ratio is needed for walnut shoot multiplication, but treatment with low concentrations concentrations of IBA in the absence of cytokinin is preferable for root induction (Saadat et al., 2002). Studies of adventitious rhizogenesis of a number of woody species, particularly using *Malus* as a model, have led to a general recognition of three phases of this process: (1) induction – defined as the period in which no morphological events are clearly observed; (2) initiation – in which cell divisions take place, root meristems are formed and root primordia are established; (3) expression – in which root growth and emergence from the cuttings occurs (Kevers et al., 1997). Knowledge of the

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Abbreviations: POX, Peroxidase; **PPO,** polyphenol oxidase; **IBA,** indolebutyric acid; **BAP,** benzylaminoporine; **ID,** induction medium; **EXP,** expressive medium; **PHE,** phenolic content.

biochemical and morphological events associated with root induction and formation may lead to improvements in rooting procedures, which could limit losses, particularly towards the final stages of production. Therefore, it would be useful to identify reliable biochemical marker(s) for rooting, which could be applied to commercial *in-vitro* systems (Schwambach et al., 2008).

In walnut, as in many recalcitrant species, rooting is the principal barrier to successful vegetative propagation. This is due to both the limited rhizogenic capacity of the species and the complexity of the rooting process (Rios et al., 1999). Considerable work has been directed towards the optimization of walnut rooting, both in microplants and in field cuttings (McGranahan et al., 1987; Rodriguez et al., 1989; Gruselle and Boxus, 1990; Dolcet-Sanjuan et al. 2004), but finding the factors that affect the rooting capacity of walnut, especially identification of any genetic determinates, are important in this species.

Classical plant peroxidases (POX) (E.C. 1.11.1.7) are heme-containing enzymes that catalyze the oxidation of a diverse group of organic compounds (Dawson, 1998). It has been suggested that peroxidases play significant roles in plant growth, differentiation and development (Gaspar et al., 1985), in hormone catabolism (Nag et al., 2001) and in lignin polymerization (Christensen et al., 1998).

Many studies of adventitious root formation have shown that peroxidase isoenzymes play a fundamental role in the rooting of cuttings (Gaspar et al., 1992; Metaxas et al., 2004; Molassiotis et al., 2004; Syros et al., 2004; Hatzilazarou et al., 2006). Therefore, changes in the activity of this enzyme could be used as analytical measures of the root initiation process (Gaspar et al., 1992, 1994; Rout et al., 2000; Syros et al., 2004).

Polyphenol oxidase (PPO) (E.C. 1.14.18.1) is a copper-containing enzyme localized in the thylakoids of plastids, which catalyses the oxidation of phenolics into quinones (Vaughn et al., 1988). It is also involved in the defense mechanism of plants against environmental stresses (Thipyapong et al., 1995). Furthermore, PPO plays a key role in rhizogenesis (Gonzalez et al., 1991; Gaspar et al., 1997) where it is involved in regulating the synthesis of phenolic precursors needed for lignin biosynthesis during root differentiation (Haissig, 1986).

Phenolic compounds occur in abundance as secondary metabolites in all plant organisms (Kefeli et al., 2003). They belong to a large and heterogeneous group of biologically active and non-nutrient compounds (Jay-Allemand et al., 2001). These are involved in physiological processes of fruit tree growth and development and they affect different aspects of pre- and post-harvest fruit life. Natural phenols are active as defense factors against various types of stresses caused by pathogens, adverse environmental conditions or wounding (Solar et al., 2006). In addition, correlations have been observed between phenolic content and root formation in cuttings of many species (Mato et al., 1998; Caboni et al., 1997; Qaddoury and Amssa, 2003).

The goals of this study were to: (1) investigate the correlation between changes in POX and PPO activities and in tissue phenolic content with the rooting ability of walnut cultivars, (2) evaluate the potential to use antioxidant enzymes as markers to define the beginning and length of the walnut rooting phases, and (3) study the activity of these enzymes under different in-vitro treatments in order to improve rooting procedures for walnut, particularly in the later stages of production, and to limit losses under both *in-vitro* and *in-vivo* conditions.

MATERIALS AND METHODS

Plant materials and culture

Microshoot cultures of two Persian walnut (*Juglans regia* L.) cultivars, 'Sunland' and 'Howard', which had been cultured on DKW medium (Driver and Kuniyuki, 1984) for more than 10 years by the University of Tehran, were used as a source of explants. Axillary proliferation was maintained through regular subcultures every three weeks. At each transfer, the apical bud and upper most two leaves of shoots were kept and the basal callus was removed. The culture conditions were according to Vahdati et al. (2004). Shoots of 3 cm in length were cultured on DKW medium supplemented with 2.1 g dm^{-3} gellan gum (Phytagel, Sigma Co.), 10^{-2} mg dm^{-3} IBA, and 1 mg dm^{-3} BAP and pH was adjusted to 5.5 (Vahdati et al., 2004). These cultures were kept at 26 ± 1°C in 500 cm^3 cylindrical jars covered with a plastic lid (four shoots each on 38 cm^3 medium) and exposed to a 16/8 h-photoperiod (standard cool white fluorescent bulbs, 75m^{-2} s^{-1}).

At the end of the multiplication cycle, suitable shoots were excised and transferred to a root induction medium (Id) containing MS salts (Murashige and Skoog 1962), 3% sucrose, 3 mg dm^{-3} IBA, and no cytokinins. This was adjusted to pH 5.7, solidified using 0.7% agar, and autoclaved at 121°C for 20 min. Explants for rooting were kept on this induction medium for seven days in the dark (Vahdati et al., 2001) because absence of light is often essential for stimulation of rooting in woody species (Rugini and Verma, 1983; Nemeth, 1986; Rugini et al., 1993). Induced shoot cuttings were then sub-cultured for four weeks on an expression medium (Exp) composed of quarter strength DKW basal medium gelled with gellan gum and mixed with coarse vermiculite (1:1.25 v/v) and grown under a 16 h-photoperiod as described (Jay-Allemand et al., 1992; Ripetti et al., 1994). To obtain an appropriate number of shoots for root analysis, microcuttings were subcultured on multiplication medium repeatedly and shoot explants for rooting were taken several times.

Rooting experiment

In order to determine the rooting response of each walnut cultivar, the rooting percentage, number of roots per microcutting and the length of roots were recorded at the end of the root expression phase. A total of 15 jars (60 microshoots in total) were used for scoring. The whole experiment was repeated twice.

Protein extraction and determination

For protein extraction, 500 mg of fresh mass (random samples of

Figure 1. Morphology of 'Sunland' (a) and 'Howard' (b) explants on DKW medium with 10^{-2} mg dm^{-3} IBA and 1 mg dm^{-3} BAP (multiplication phase). Morphology of 'Sunland' (c) and 'Howard' (d) explants on a DKW basal medium :vermiculite mixture gelled with gellan gum (expression phase).

whole shoots) was frozen in liquid nitrogen immediately after removal from culture jars and then ground with a mortar and pestle in 0.06 M phosphate buffer, pH 6.1, containing 5% (m/v) polyvinyl polypyrrolidone (PVPP). The homogenate was centrifuged twice (13000 rpm at 4℃) for 30 min each time. After the final centrifugation, the pellet was discarded and the supernatant was collected and stored at -20℃ until it was used for analysis. Protein concentrations were determined according to the Bradford method (Bradford, 1976) and bovine serum albumin (BSA) was used as a standard for establishing known concentrations.

Peroxidase assay

Peroxidase activity was determined spectrophotometrically (Shimadtzu, UV-160) using an assay system consisting of 80 mM guaiacol (0.37 ml), 0.1 M acetate buffer, pH 5.4 (2.3 ml), 80 mM H_2O_2 (0.275 ml) and enzyme extract (6 × 10^{-2} ml) set to a final volume of 3 ml (Chance and Maehly, 1955).

Polyphenol oxidase assay

Polyphenol oxidase activity was estimated by following the oxidation of pyrogallol at 430 nm and expressed as ΔA_{430} min^{-1} mg^{-1} protein as previously described by Raymond et al. (1993).

Polyacrylamide gel electrophoresis

Non-denaturating gel electrophoresis (PAGE) was performed according to the modified method of Davis (1964) using vertical slab gels (1 mm thick) and was set up to form a discontinuous system of two layers: (i) a resolving gel: An 8 cm layer of 10% polyacrylamide and (ii) a stacking gel: A 3 cm layer of 3.75% polyacrylamide. Then 200 µg of protein per well was loaded and electrophoresis was carried out using two constant voltages. Samples were run first at 120 mV until the color reached the dividing line between the two gel types, then at 140 mV. POX bands were detected by immersing the gels in a solution of 0.2 M acetate buffer, pH 4.8 containing 0.04 M benzidine and 0.3% H_2O_2 (Van Loon, 1971). PPO bands were stained in a solution of 0.2 M phosphate buffer, pH 6.8 containing 3,4-dihydroxyphenylalanine (L-DOPA) and calcium chloride (Van Loon, 1971). Data were collected after gel staining and only clear and consistent isoenzyme bands were scored.

Phenolic compounds extraction and assay

Phenolic compounds were extracted from 0.5 g samples of whole microshoots using the Waterman and Mole method (1994) and quantities were estimated using Folin Ciocalteu reagent at 760 nm. Phenolic content (PHE) expressed as mg phenolics (g Fresh weight)$^{-1}$ was calculated from a standard curve established using known concentrations of gallic acid.

Statistical analysis

Data were collected during the multiplication phase (every five days), at the end of the root induction phase (the 28th day), after the root initiation (the 35th day), and during the rooting expression phase (from the 35th to the 56th day- every seven days).

Data were evaluated statistically by analysis of variance (ANOVA) followed, when appropriate, by Duncan's multiple range test (DMRT) using the SPSS data analysis software package (Version 16 for Windows). All results presented are means ± standard error of at least three independent replicates from three separate experiments. Correlations between root percentage, phenolic content and enzyme activities were calculated using bivariate analysis and based on Pearson correlation (2 $^{-tailed}$).

RESULTS AND DISCUSSION

Rooting experiments

Microshoots of Persian walnuts on DKW proliferation and rooting media are shown in Figure 1. Two walnut cultivars ('Sunland' and 'Howard') were maintained on shoot proliferation medium, then placed on induction medium containing IBA to initiate rooting, followed by a transfer of root expression medium without any auxin. Visible roots began to emerge on day 35 of shoot culture, one week after transfer from the root induction treatment to the root expression medium. This was consistent with preceding studies (Ripetti et al., 1994; Heloir et al., 1996; Kevers et al., 1997), which found the three rooting phases, (induction, initiation, and expression) began on the 21st, 28th and 35th days, respectively.

Table 1. Percent rooting, number of roots per rooted shoot, and length of roots for two Persian walnut cultivars induced in rooting medium[a].

Cultivar	Rooting (%)	Number of roots (explant^{-1})	Root length (cm)
Sunland	66 ± 0.5	2.81 ± 0.31 a	2.87 ± 0.21
Howard	29 ± 0.3	1.6 ± 0.4 b	1.62 ± 0.19

[a] Each value is the mean ± standard error (SE) of 60 explants ($p \leq 0.05$).

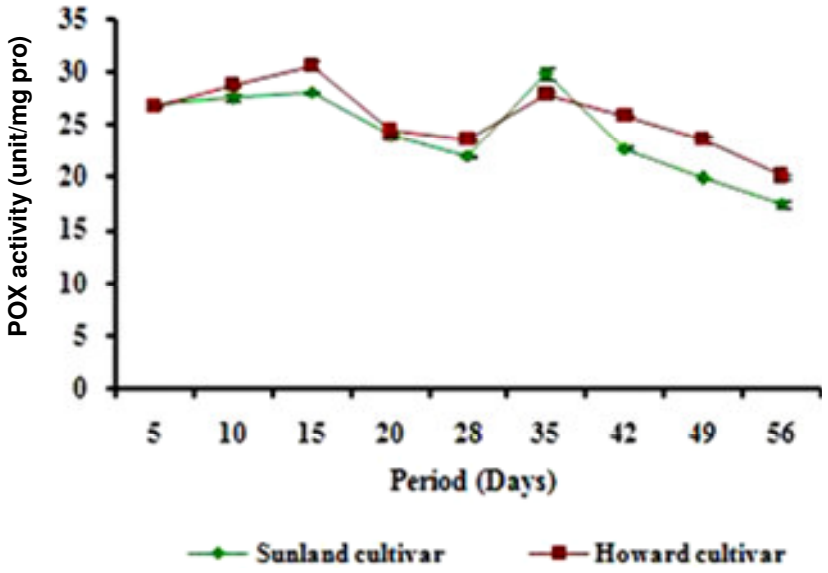

Figure 2. Changes in peroxidase specific activity at different time periods during development of adventitious roots from whole shoot microcuttings of two *J. regia* L. cultivars[a]. [a] One unit of enzyme activity is equivalent to the amount of enzyme required to increase the absorbance by 0.1/min. Each value is the mean (± SE) of three replicates.

Juglans regia L. is one of the most difficult-to-root plants (Rios et al., 1999), but genotypes can vary in root ability. In this study, the cultivar 'Sunland' exhibited a significantly better rooting percentage (66%) at the end of the root development process than 'Howard' (29%) (Table 1). Cultivars also differed in the number of adventitious roots produced per shoot with 'Sunland' significantly exceeding 'Howard' (Table 1). By both measures of rooting, 'Sunland' was the easiest to root of the two cultivars. Similar differences in the rooting ability of walnut genotypes were observed by Vahdati et al. (2001) and Dolcet-Sanjuan et al. (2004).

Enzyme activities

Total peroxidase (POX) was measured using guaiacol as a substrate for all samples (Figure 2). Total POX activity in both walnut cultivars peaked initially 15 days after starting shoot growth and then decreased gradually until the start of the initiation phase for rooting. According to Rajeswari et al. (2008), the overproduction of active oxygen species (AOS) in the cell is a sign of oxidative stress and is a common phenomenon in plants, which are exposed to stressful environmental conditions such as *in-vitro* culture. One explanation for the observed increase in POX activity in *in-vitro* cultures during shoot growth could be a role in protection of cells against reactive oxygen species (ROS). Hirage et al. (2001) described the participation of POX isoenzymes in various physiological processes such as lignification and suberization. A second explanation for high levels of POX activity during the early shoot growth period could play a role in the physiological processes of shoot organogenesis.

A second peak was observed on the 35th day of sampling, the end of the root initiation phase. Interestingly, 'Sunland' samples showed a significantly higher second peak at this stage than 'Howard'. Based on these results and similar observations in other plants, for example *Prunus* species, *Sequoiadendrom*

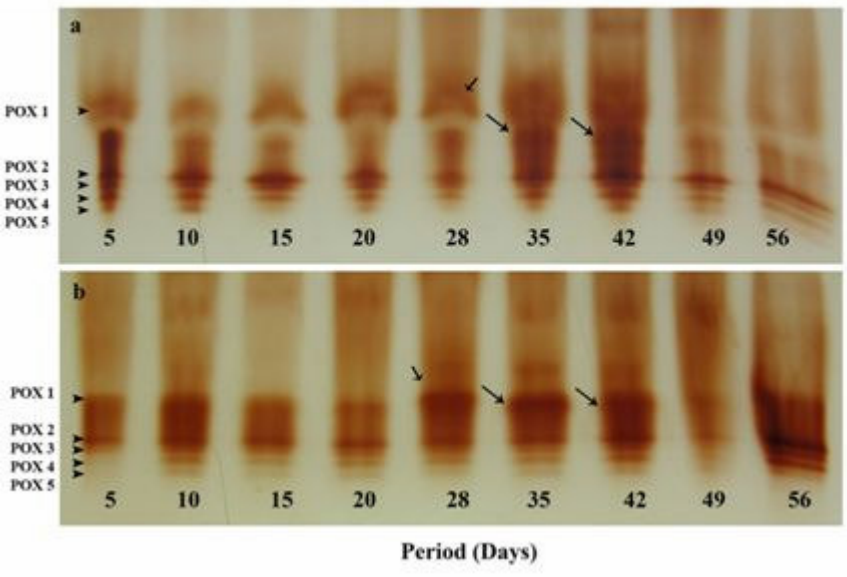

Figure 3. Peroxidase isoenzymatic profile of extracts taken from whole shoot cuttings of two walnut cultivars at different time periods during the rooting process (from days 5 to 56). Arrows indicate different isoforms. Numbers show different sampling times. Equal amounts of protein (200 µg) were loaded in each lane[a.]; [a] Letters a and b show the POX isoenzymatic pattern of 'Sunland' and 'Howard'.

giganteum, *Plumbungo zeylanica*, *Vinga radiata* and *Ebenus cretica*, it can be concluded that higher POX activity at the beginning of the root expression phase correlates with increased root formation.

The changes in POX activity after transferring microcuttings to root initiation medium on day 28, with the second peak observed on the 35th day, are in agreement with Berthon et al. (1990) and Gaspar et al. (1992). They observed that a sharp drop in POX activity beginning at day 21 corresponds to the root induction phase, which is characterized by numerous biochemical changes and cytological and histological events (Moncousin et al., 1990; Gaspar et al., 1997). The period of time between the POX minimum activity at day 28 and the POX maximum at day 35 is the initiation phase of rooting characterized by cell division and meristem formation. This is followed by the root expression, or growth and emergence stage, marked by a gradual drop in POX. This decreasing peroxidase activity is due to the inhibition of the *de novo* synthesis of the peroxidase isoenzymes during adventitious root formation (Chao et al., 2001).

POX activity has been linked to the oxidation of a wide variety of substrates including auxin (IAA) (Hiraga et al., 2001). Many basic POXs have indol-3-acetic acid (IAA) oxidase activity (Schwambach et al., 2008) and POXs have been shown to be effective in IAA oxidation *in-vitro*. For adventitious rooting, elevated IAA concentration is required during the induction phase, but during the root emergence phase, the phytohormone becomes inhibitory. This pattern has been observed in many plant species. High POX activity during the induction phase would deplete the high IAA concentrations necessary for rooting, so it is reasonable to have decreased POX activity initially. An inverse relationship between POX activity and of auxin concentration during the induction phase was reported previously by Heloir et al. (1996). Because IAA plays an inhibitory role during the subsequent root initiation phase, POX exhibits its highest activity during this meristem formation period in order to decrease the IAA concentration.

To test whether the increased POX activity could be attributed to specific isoforms, PAGE was conducted on plant extracts. Electrophoresis showed five clear bands indicating five POX isoforms present in each of the cultivars (POX1-5) (Figure 3). There are three periods at which it is most important to note correlations between POX isoform bands and the rooting ability of cultivars. The first is at the 28th day during the change from the induction phase to the initiation of meristems at which point 'Sunland' had weaker bands than 'Howard'. This corresponds with the lower total POX activity measured in 'Sunland' at this time. In both cultivars, the enzymatic bands show the strongest expression in 35th and 42nd day extracts, during the early root emergence period, and the isoform bands are more intense in 'Sunland' than 'Howard. This is in accordance with high total POX activity also measured at this stage. For the cultivars studied, more intense POX electrophoresis bands during

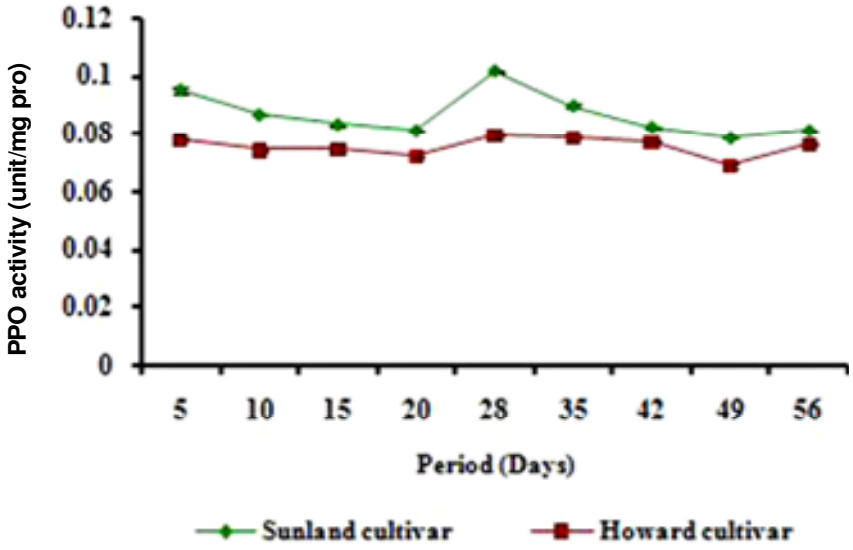

Figure 4. Changes in polyphenol oxidase specific activity at different time periods during development of adventitious roots from whole shoot microcuttings of two *J. regia* L. cultivars[a]. [a] One unit of enzyme activity is equivalent to the amount of enzyme required to increase the absorbance by 0.1/min. Each value is the mean (± SE) of three replicates.

the early transition from the root initiation to expression phase matched a higher ability to root.

The increase in soluble peroxidase activity could be due to a general increase in all isoenzymes to an increase in particular isoenzymes already present or to the appearance of new isoenzymes (Gardiner and Cleland, 1974). Based on our results, the increase in soluble peroxidase activity at days 35 and 42 was probably due to an increase in particular previously existing isoenzymes.

The PPO activity showed similar patterns of change in both 'Sunland' and 'Howard', with a maximum on day 28 and a minimum on day 49 (Figure 4), but in 'Howard' the activity changes were significantly less during the period of the root development. This suggests that PPO activity may be positively correlated with rooting ability in walnut. PPO is an important enzyme that plays a key role in adventitious root formation (Batis et al., 2008), and may be useful in defining the beginning and duration of the walnut rooting phases in microshoots as was observed in embryo-cotyledonary linking areas and cotyledon pieces by Rios et al. (1999) .

It has been found that monophenolics act generally as promoters of POX activity, while di- and polyphenolics act as POX inhibitors (Lee et al., 1982). It can be argued that fluctuations in PPO activity during root formation might lead to variations in abundance of modifiers of the activities of other enzymes like POX. During the induction phase of rooting, the increase in PPO activity can be expected to increase the products of the phenolic pathway, specially di- and polyphenolics, which then contribute to decreased POX activity. Alternatively, the decrease in PPO activity during the initiation phase of rhizogenesis should cause an accumulation of monophenolics that are stimulator of the POX enzyme. Thus, there should be an inverse relationship between POX and PPO activities. The same pattern of changes in PPO activity during root development was also observed in coteledonary walnut explants (*J. regia* L.) (Rios et al., 1999) and date palm (*Phoenix dactylifera* L.) (Qaddoury and Amssa, 2003). Based on PPO electrophoresis patterns (Figure 5), it is clear that there are five isoforms of PPO in both walnut cultivars (PPO1-5). These bands are more intense in 'Sunland' than in 'Howard', which indicates greater PPO activity in this cultivar at all phases of root development. The significantly higher PPO activity in 'Sunland' at the 28th day peak is also conspicuous in the electrophoresis patterns. Electrophoresis bands confirm that in these two cultivars, the higher capacity to root corresponds to more intense PPO electrophoresis bands during root emergence.

Phenolic content

The phenolic content (PHE) of both cultivars increased until day 21, then decreased through day 28 followed by a slight increase during the later stages of root development (Figure 6). The increase in PHE through the end of the multiplication phase is related to phenylalanine

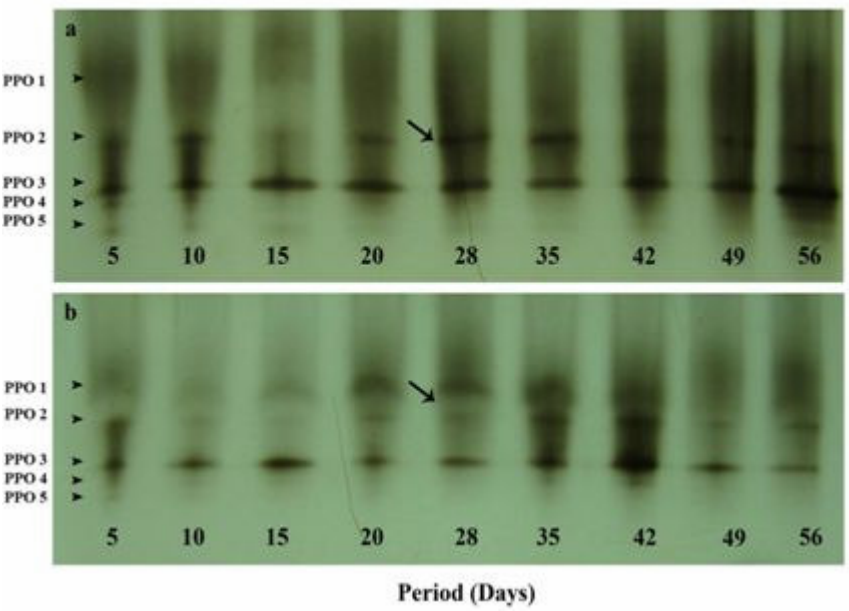

Figure 5. Polyphenol oxidase isoenzymatic profile of extracts taken from whole shoot cuttings of two walnut cultivars at different days during the rooting process (from days 5 to 56). Arrows indicate different isoforms. Numbers show different sampling times. Equal amounts of protein (200 µg) were loaded in each lane[a]. [a] Letters a and b shows the POX isoenzymatic pattern of 'Sunland' and 'Howard'.

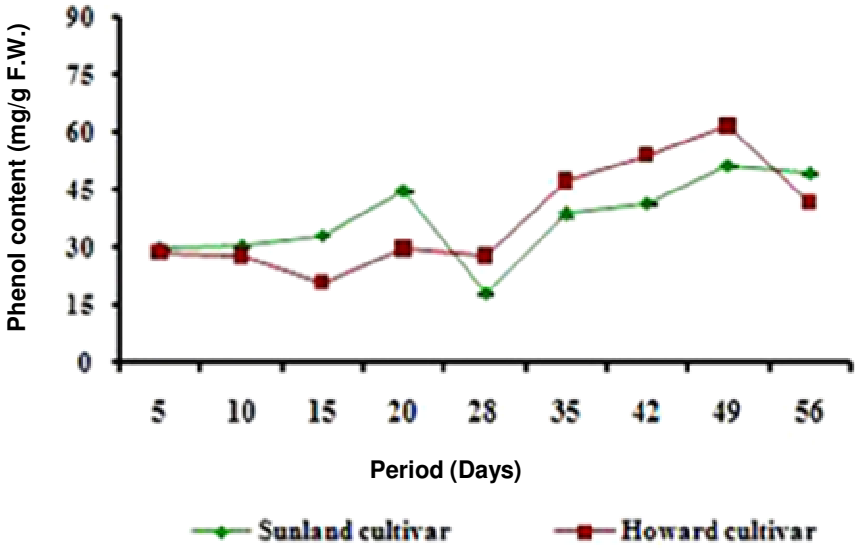

Figure 6. Changes in the total phenolic content of whole shoot microcuttings of two *Juglans regia L.* cultivars sampled at different times during the root development period[a]. [a] Each value is the mean (± SE) of three replicates.

ammonia-lyase (PAL) activity, a key enzyme in phenolic biosynthesis. Bisbis et al. (2003) showed that PAL activity in walnut microshoots (*J. regia* L.) increased until the end of the induction phase and then decreased. The observed accumulation of phenolic compounds during the multiplication phase corresponded with elevated PAL activity. He also confirmed that the lignin content in walnut increases during rooting, beginning with the root

induction phase. Phenolic compounds serve as precursors of the monolignols needed for lignin biosynthesis. This consumption of phenolics could account for their decreased concentration during this rooting phase. The inverse relationship between phenolic and lignin levels during root formation was further confirmed by analysis of rooted and non-rooted shoots (Bisbis et al., 2003).

Establishment of root primordia occurs during the initiation phase of the rooting process (Kevers et al., 1997) and phenolic compounds are thought to play a role in stimulating the organization and development of primordia (Vaughn et al., 1988; Gonzalez et al., 1991). The elevated PHE, as observed during this second phase of walnut root formation, is consistent with a role in primordia formation and supports the hypothesis that changes in phenolic metabolism play as crucial role in root formation of walnut cultivars.

Correlation between tested factors

Significant correlation of root induction with phenolic content and antioxidant enzyme activities were observed. Significant positive correlations were found between rooting percentage and PPO enzyme activity (0.699) and between POX and PPO enzyme activity (0.020). Negative correlations were seen between root induction and POX enzyme (-0.201), root induction and phenolic content (-0.06), phenolic content and POX enzyme activity (-0.396) and between phenolic content and PPO enzyme activity (-0.407).

Rhizogenesis is one of the most important steps in plant propagation. A better understanding of this process can be beneficial for future work to improve walnut propagation. This research examined the role of phenolic compounds and related enzyme activity during several aspects of rhizogenesis. More precisely, determining the role these enzyme systems play in rooting, is essential to understand the process of rooting and clarification of their activities, particularly of their electrophoretic isoforms and could be helpful in improving rooting methods and in selecting more easily rootable genotypes. Further study of the activity of these enzymes during *in vitro* culture could also help in developing methods to avoid detrimental effects of excess or deficient enzyme activities on root induction.

ACKNOWLEDGEMENTS

This paper represents a portion of the author's dissertation, which will be presented to the Faculty of Science, University of Tehran, in partial fulfillment of the requirements for the PhD degree. We are deeply grateful to Mr. Abbas Rahmani (Department of Plant Physiology, Faculty of Science, University of Tehran) for his research assistance. The authors also express sincere thanks to Dr. Hadi Zare (Faculty of Mathematics and Computer Science, Amirkabir University of Technology) for statistical suggestions and comments.

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