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*Full Length Research Paper*

# Antioxidant enzymes activities during secondary somatic embryogenesis in Persian walnut (*Juglans regia* L.)

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**Somatic embryogenesis was achieved from immature cotyledon explants of Persian walnut (*Juglans regia* L.) *cave*. "Chandler" on DKW medium. Secondary somatic embryogenesis, the process by which adventitious embryos are formed from primary somatic embryos, is frequent during somatic embryogenesis in Persian walnut. It has certain advantages compared with primary somatic embryogenesis such as high multiplication rate, independence to the explant source and repeatability. Proteins and activities of antioxidant enzymes including catalase (CAT), peroxidase (POX), superoxide dismutase (SOD) and polyphenol oxidase (PPO) were evaluated in two types of secondary embryogenic calli and secondary somatic embryos in Persian walnut. In dark brown calli from which their secondary somatic embryos were separated, CAT, POX and POX had the highest activities while the activity of SOD and also protein content showed the lowest level. High SOD activity, protein content and low activities of CAT, POX and PPO were determined in secondary somatic embryos and light brown calli containing secondary somatic embryos. According to the obtained results, some proteins and antioxidant enzymes have been regarded as markers for secondary somatic embryogenesis.**

**Key words:** Persian walnut, secondary somatic embryogenesis, antioxidant enzymes.

## INTRODUCTION

Somatic embryogenesis and particularly the secondary somatic embryogenesis provide an efficient regeneration system for vegetative propagation and genetic transformation of walnut (Mc Granahan et al., 1990). Secondary somatic embryogenesis has been reported in many tree species and is the basis of embryo cloning (Merkle et al., 1995; Daigny et al., 1996; Benelli et al., 2001; Agarwal et al., 2004). Secondary or repetitive somatic embryogenesis, the process by which adventitious embryos are derived from primary somatic embryos, is frequent during somatic embryogenesis in walnut. Secondary embryos are formed on the cotyledons and most frequently at the root tips of somatic embryos. If these adventitious embryos removed when their cotyledons began to expand, they often develop into normal plants. Failure to remove adventitious embryos at this stage of development led to the formation of more somatic embryos, abnormal growth or callus formation. Somatic embryo tissues often turn into dark brown masses after several months in culture. These brown tissues frequently produce globular somatic embryos which could be removed and used to propagate the embryogenic lines (Tulecke and Mc Granahan, 1985). Martinelli and Gribaudo, (2001) called the secondary embryogenesis as re-initiation of embryogenic calli from somatic embryos. The secondary somatic embryos of walnut can be efficiently multiplied by repetitive or recurrent embryo-

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**Abbreviations: CAT**, Catalase; **POX**, peroxidase; **PPO**, polyphenoloxidase; **SOD**, superoxide dismutase; **DBC**, dark brown callus; **LBC**, light brown callus; **SSE**, secondary somatic embryo; **DKW**, Driver and Kuniyuki Walnut medium.

genesis on hormone-free DKW medium (Deng and Cornu, 1992).

As an experimental system, secondary somatic embryogenesis has certain advantages compared with primary somatic embryogenesis such as high multiplication rate, independence to the explant source and repeatability (Karami et al., 2008). Furthermore, embryogenicity can be maintained for a long period of time by repeated cycles of secondary embryogenesis (Raemakers et al., 1995). On the other hand, it is thought that adventitious embryogenesis including somatic and anderogenetic ones might be induced by different kinds of stress treatments (Kamada et al., 1994). There is a variety of data that link somatic embryogenesis, DNA methylation and oxidative stress response. Also, an increasing number of publications link reactive oxygen species (ROS) and somatic embryogenesis (Zavattieri et al., 2010).

Some antioxidant enzymes have been introduced as indicators for somatic embryogenesis. According to Kairong et al. (1999) the differentiation and development of embryogenic cells in the somatic embryogenesis of *Lycium barbarum* L. are regulated by three antioxidant enzymes including CAT, POX and SOD. Somatic embryogenesis from somatic tissues of walnut, rather than zygotic embryos needs to be further investigated (Vahdati et al., 2006). Thus, study of antioxidant enzymes activities during secondary somatic embryogenesis can help to improve primary somatic embryogenesis and reduce problems in recalcitrant explants. For this purpose, two different types of secondary embryogenic calli, light and dark brown calli containing secondary somatic embryos at different stages of development and separated secondary somatic embryos were compared. Proteins and activities of some antioxidant enzymes including CAT, POX, SOD and PPO were studied during secondary somatic embryogenesis in Persian walnut (*Juglans regia* L.) *cv*. "Chandler".

## MATERIALS AND METHODS

### Plant material

Ten fruits of walnut cultivar "Chandler" were harvested 9 weeks after pollination and stored at 4°C for 5 days. They were surface sterilized and dissected immediately. Immature cotyledons were cultured on DKW solid medium (Driver and Kuniyuki, 1984) containing 0.01 mg/l IBA, 2 mg/l Kin, 1 mg/l BA and 250 mg/l glutamine (Tulecke and Mc Granahan, 1985). Cultures were grown in the dark at room temperature.

The repetitive somatic embryogenic line which initiated from immature cotyledons of walnut cultivar "Chandler" was used in the present study. This line has been maintained by subculturing every two weeks at 25°C in the dark for over 2 years. Somatic embryos were subcultured on basal DKW medium solidified with 0.21% Gelrite (w/v). Dark brown calli with secondary somatic embryos at different developmental stages on their surfaces (DBC+SSE), dark brown calli without secondary somatic embryo (DBC-SSE), secondary somatic embryos at different developmental stages which were separated from the surface of dark brown calli (SSE) and light brown calli with secondary somatic embryos at different developmental stages (LBC+SSE) were used for all the analyses.

### Protein extraction

For estimation of total protein content and enzymes activity, plant materials were homogenized at 4°C with a mortar in 1M Tris-HCl (pH 6.8) and 2.5 % (w/v) polyvinylpolypyrolidone (PVPP) to avoid phenol oxidative effects. The homogenates were centrifuged at 13000 g for 30 min two times at 4°C using a Heraeus 400R microfuge. Supernatants were kept at -70°C and used for protein determination and enzyme assay. An UV-visible spectrophotometer (UV-160, Shimadzu, Tokyo, Japan) were used for the determination of the absorbance.

### Protein quantification

Protein concentration was measured according to Bradford (1976), using bovine serum albumin (BSA) as standard. Five milliliters of the Bradford reagent and 100 µl of the each protein extract were mixed and then, reaction mixtures were incubated at room temperature for 20 min. The absorbance values were measured at 595 nm.

### Antioxidant enzymes assays

Peroxidase (POX; E.C. 1.11.1.7) activity was measured according to the method of Abeles and Biles (1991). The assay mixture consisted of 4 ml of 0.2 M acetate buffer (pH 4.8), 0.4 ml $H_2O_2$ (3 %), 0.2 ml 20 mM benzidine and 0.2 ml enzyme extract. The increase of absorbance was recorded at 530 nm. The POX activity was defined as 1 µM of benzidine oxidated per min per mg protein (Unit $mg^{-1}$ (protein)).

Superoxide dismutase (SOD; E.C. 1.15.1.1) activity was estimated by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm as described by Giannopolitis and Ries (1977) in a reaction mixture consisted of 0.1 ml enzyme extract, 50 mM sodium phosphate buffer (pH 7.5), 13 mM L-methionine, 75 µM NBT, 0.1 mM EDTA and 75 µM riboflavin. The reaction mixture was irradiated for 14 min and absorbance was recorded at 560 nm against the non-irradiated blank. One unit of SOD activity was defined as the amount of enzyme which caused 50% inhibition in NBT reduction.

Catalase (CAT; E.C. 1.11.1.6) activity was assayed from the rate of $H_2O_2$ decomposition as measured by decrease of absorbance at 240 nm (Aebi, 1974). The reaction mixture contained 0.625 ml of 50 mM sodium phosphate buffer (pH 7.0), 0.075 ml $H_2O_2$ (3%) and 0.05 ml enzyme extract.

Polyphenol oxidase (PPO; E.C. 1.14.18.1) activity was estimated following the method of Raymond et al. (1993) at 40°C. The reaction mixture contained 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.8), 0.2 $cm^3$ pyrogallol 20 mM and 0.02 $cm^3$ enzyme extract. The increase in absorbance was recorded at 430 nm. The PPO activity was defined as 1µM of pyrogallol oxidated per min per mg protein (Unit $mg^{-1}$ (protein)).

### Gel electrophoresis

For determination of protein patterns, discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970), using 12% acrylamide. For detection of proteins, gels were stained with 0.03% comassie brilliant blue G250. For isoenzyme assay, native PAGE was carried out according to a modified method of Davis (1964)

with a 12% (SOD) and 10% (POX, CAT and PPO) acrylamide gels at 4°C.

SOD bands were visualized using the activity staining procedure described by Wendel and Weeden (1989). Gels were incubated in 0.2 M Tris-HCl (pH 8.0) containing 4% riboflavin, 4% EDTA and 20% NBT for 40 min in the dark at room temperature and then, exposed to white light until white bands appeared in violet background. For SOD isoform identification, assays were performed in the presence of selective inhibitors. Application of KCN (3 mM) inhibits only Cu/Zn-SOD; $H_2O_2$ (5 mM) inhibits both Cu/Zn-SOD and Fe-SOD. Mn-SOD was not inhibited by KCN or $H_2O_2$ (Lee et al., 2001).

CAT activity was detected using the method of Woodbury et al. (1971). The gels were incubated in 5 mM $H_2O_2$. After 10 min, the gels were washed with distilled water and stained with a reaction mixture containing 2% (w/v) ferricyanide ($K_3Fe(CN)_6$) and 2% ferric chloride till yellow bands appeared on dark green background.

Electrophoresis pattern of POX was obtained by staining of the gels with benzidine according to Van Loon and Geelen, (1971). The gels were immersed in 0.2 M acetate buffer (pH 4.8) containing 3% $H_2O_2$ and 4% benzidine in 50% methanol in the dark at room temperature till the brown bands appeared.

PPO bands were visualized using the method of Van Loon and Geelen, (1971). The gels were incubated in 0.2 M potassium phosphate buffer (pH 6.8) containing 0.5% 3,4- dihydroxy L-phenylalanine (L-DOPA) and 3.5% calcium chloride in the dark at room temperature till brown bands appeared.

#### Statistical analysis

The data determined in triplicate were analyzed by analysis of variance (ANOVA) using SAS Software (SAS Institute, Inc., 2002). Significance of mean differences was determined by the Duncan multiple range test (DMRT) at $P < 0.05$.

## RESULTS

### Tissue culture

Most of the immature cotyledon explants of Chandler formed callus but only low percentage of them were embryogenic and the number of somatic embryo per callus ranged from 1 to 4 (data not shown). The embryos loosely attached without a vascular connection to the maternal tissues. They were removed from the embryogenic calli and cultured on basal medium. When walnut somatic embryos especially at the primary stages of development including globular and heart shape were cultured on basal DKW medium, two distinct responses were observed; (a) development, maturation and conversion to whole plant; and (b) secondary callus induction and repetitive somatic embryogenesis.

In some cases, mature embryos produced new individual embryos on their cotyledons or root tips directly. Cotyledon-derived primary embryogenic calli were compact, hard, yellowish and grow slowly with a few number of somatic embryos on the surface but secondary calli were fast growing, very friable and light brown at early stages of formation and gradually became dark brown while produced a large number of embryos (Figure 1).

### Biochemical assays

The highest and lowest content of proteins was determined in SSE and DBC-SSE, respectively. Protein content in LBC+SSE was significantly higher than that in DBC+SSE. The highest activity of CAT was measured in DBC-SSE and the lowest activity was detected in LBC+SSE. There was no significant difference in activity of CAT between DBC+SSE and SSE. The highest activity of POX was detected in DBC-SSE and POX activity decreased in the DBC+SSE, SSE and LBC+SSE, respectively. PPO showed the highest activity in DBC-SSE while DBC+SSE had lower activity of PPO. The lowest and highest activity of PPO was detected in SSE and LBC+SSE, respectively. The lowest activity of SOD was detected in DBC-SSE and no significant difference was determined in SOD activity among other samples (Table 1). According to the SDS-PAGE protein pattern (Figure 2), the strongest and weakest protein bands were detected in SSE and DBC-SSE, respectively. Among the proteins detected, the most abundant were those with molecular masses of 83, 53, 45, 40, 36, 30, 29 and 28 kDa, respectively. The 83, 53, 45 and 40-kDa proteins were present in all samples strongly except DBC-SSE. Also, the 30, 29 and 28-kDa proteins of light brown secondary somatic embryos (LBC+SSE) and SSE were expressed more than the corresponding fraction of dark brown callus with or without embryos (DBC+SSE and DBC-SSE).

According to non-denaturing PAGE, two CAT isoforms were detected in the DBC+SSE. CAT1 which was observed in SSE and LBC+SSE was stronger than CAT2 and was absent in DBC-SSE. In DBC-SSE only CAT2 was detected and this isoform was absent in SSE and LBC+SSE (Figure 3a). Thus, CAT 2 could be regarded as a marker for dark grown calli and embryos.

Five POX isoforms were determined in all samples examined. According to the activity staining, POX1 band was stronger than four other bands. This band in DBC+SSE was very strong compared with LBC+SSE and SSE. POX2 and POX3 isoforms were similar in all the samples. POX4 was present only in DBC+SSE. POX5 was weak in DBC-SSE and LBC+SSE in comparison with DBC+SSE and SSE (Figure 3b). Thus, POX4 could be regarded as a marker for dark grown calli containing embryos (DBC+SSE).

Five bands with PPO activity were detected on gels by native PAGE. PPO1 and PPO3 bands in DBC-SSE were stronger than the others. PPO2 was present only in DBC+SSE and DBC-SSE while PPO4 was present only in SSE and LBC+SSE. PPO5 band was common in all samples and is very strong in SSE and LBC+SSE in comparison with other samples (Figure 3c). Thus, PPO4 could be regarded as a specific marker for light grown calli and secondary somatic embryos.

Six bands of SOD were detected in the samples. The identities of the major SOD activity bands were tested by

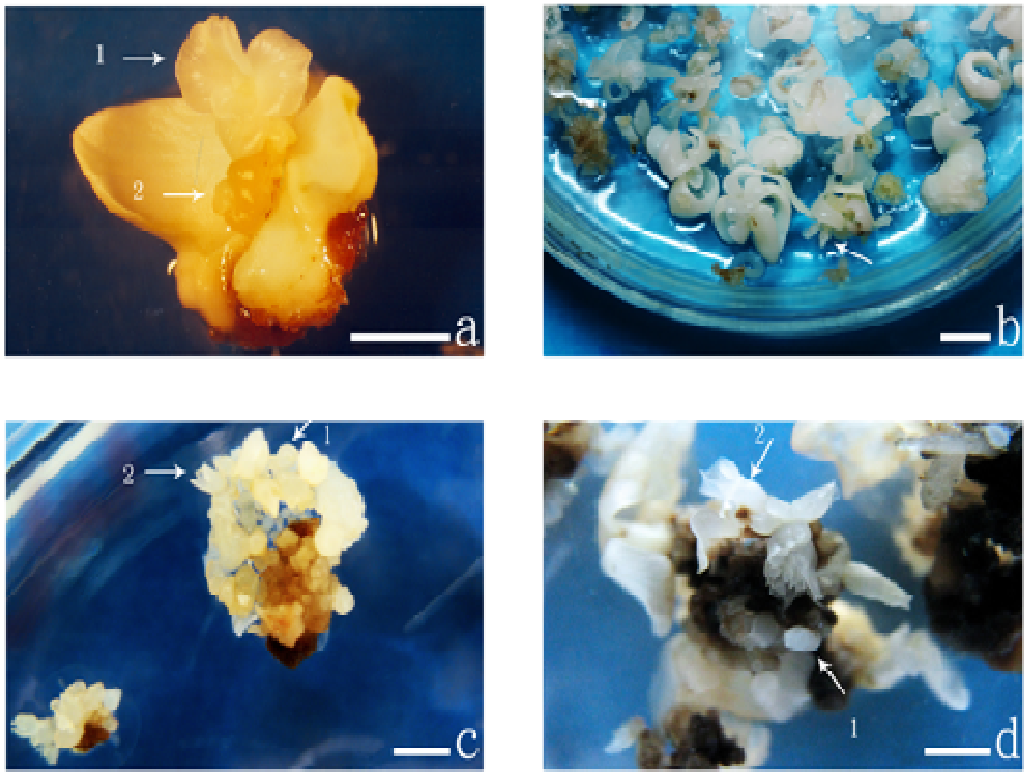


**Figure 1.** Secondary somatic embryogenesis in Persian walnut. (a) Primary embryogenic callus derived from immature cotyledons of Chandler cultivar with two somatic embryos; 1, arrowed = a well developed cotyledonary embryo; 2, arrowed = heart shape embryo (*bar* = 8 mm); (b) direct secondary somatic embryogenesis; arrowed = two torpedo embryos on the surface of the primary embryo's cotyledons (bar = 4 mm); (c) light brown secondary embryogenic callus with several secondary somatic embryos; 1, arrowed = globular secondary embryo, 2, arrowed = precotyledonary embryo *(*bar = 4 mm); (d) dark brown secondary embryogenic callus with several secondary somatic embryos; 1, arrowed = torpedo embryo; 2, arrowed = precotyledonary embryo (bar = 5 mm).

**Table 1.** Protein content (mg $g^{-1}$(F.W.)) and activity of antioxidant enzymes (U $mg^{-1}$ (protein)) in different stages of secondary somatic embryogenesis.

| Parameter | Total protein | CAT | POX | PPO | SOD |
|---|---|---|---|---|---|
| DBC+SSE | 2.52 ± 0.10c | 144.16 ± 4.56 b | 28.52 ± 1.30b | 0.28 ± 0.01b | 1.017 ± 0.001a |
| DBC-SSE | 0.55 ± 0.02d | 345.26 ± 10.73a | 135.51 ± 2.15a | 0.85 ± 0.04a | 0.980 ± 0.001b |
| SSE | 5.50 ± 0.28a | 125.30 ± 4.30b | 18.23 ± 1.10c | 0.11 ± 0.01c | 1.012 ± 0.001a |
| LBC+SSE | 4.26 ± 0.04b | 77.26 ± 5.17c | 9.24 ± 0.37d | 0.13 ± 0.01c | 1.015 ± 0.004a |

DBC+SSE, Dark brown calli with secondary somatic embryo; DBC-SSE, dark brown calli without secondary somatic embryo; SSE, secondary somatic embryo; LBC+SSE, light brown calli with secondary somatic embryo. Means ± SE; values marked with different letters are significantly different according to DMRT at $P < 0.05$.

preincubating the gels with well-characterized SOD inhibitors: KCN is an inhibitor of Cu/Zn-SOD, whereas, $H_2O_2$ inhibits both Cu/Zn-SOD and Fe-SOD. The upper band (Figure 3d), in all samples, represented a mitochondrial Mn-SOD. Five bands with higher mobility represented the cytosolic Cu/Zn-SOD isoenzymes. The band representing a chloroplastic Fe-SOD was not presented. No significant qualitative differences in the pattern of SOD isoforms were observed between tissues.

## DISCUSSION

*In vitro* culture conditions represent an unusual combination of stress factors that plant cells encounter (oxidative stress as a result of wounding at excision of the explant tissue, PGRs, low or high salt concentration in solution, low or high light intensities). The stress not only promotes dedifferentiation but also induce somatic embryo formation. The drastic changes in the cellular

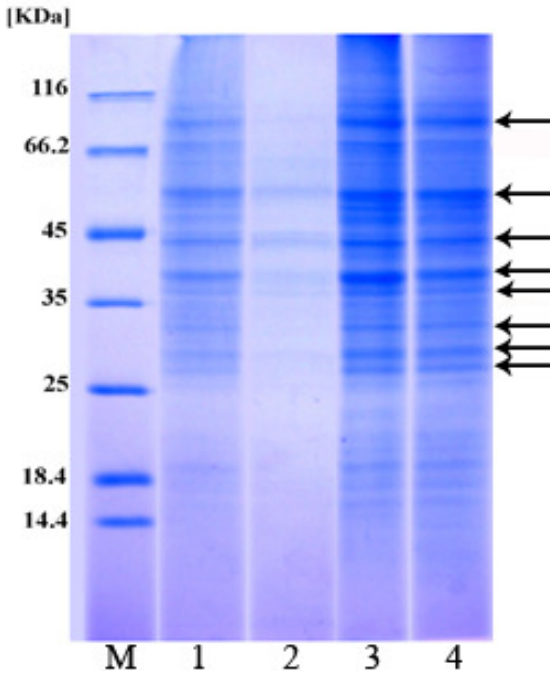


**Figure 2.** SDS-PAGE pattern of proteins during secondary somatic embryogenesis in Persian walnut; dark brown calli with secondary somatic embryo (DBC+SSE) (1), dark brown calli without secondary somatic embryo (DBC-SSE) (2), secondary somatic embryos (SSE) (3), light brown calli with secondary somatic embryo (LBC+SSE) (4) and molecular mass marker (M). Equal amounts of protein (40 µg) were loaded in each lane. Arrows indicate the affected and embryo- specific bands.

environment of the *in vitro* culture induced by a 'stressor' in the culture medium or in the physical environment of the culture, are responsible for reprogramming of gene expression (Zavattieri et al., 2010). However, secondary somatic embryogenesis from a dark brown calli in absence of any growth regulator might be induced by oxidative stress. The effect of stress during culture may help to improve induction of somatic embryogenesis in recalcitrant somatic tissues of Persian walnut.

Somatic embryos are formed from immature cotyledons of walnut cultivar and this result is in agreement with Tulecke and Mc Granahan (1985) and Vahdati et al. (2006). According to Tulecke and Mc Granahan (1985) somatic embryo tissues in walnut cultivars often turn into dark brown masses after several months in culture and they frequently produce globular somatic embryos which could be removed and used to propagate the embryogenic lines. It should be mentioned that, all of the developmental stages of embryos including globular, heart shape, torpedo and cotyledonary can be observed on the surface of secondary embrygenic calli. Although, all related articles have confirmed the secondary embryogenesis in walnut cultivars but there is no report on the alterations of antioxidant enzymes and proteins during somatic embryogenesis.

Protein content in SSE which contained embryos at different developmental stages was the highest, but DBC-SSE which lacks any embryos had the lowest level of protein. The difference in protein content was confirmed by gel electrophoresis profile. These results are in good agreement to Junaid et al. (2007), who reported that protein content in matured and proliferated somatic embryos were significantly higher than that in secondary embryogenic calli of *Catharanthus roseus* L. They showed that the protein content gradually increased with advancing stages of somatic embryogenesis. It has been reported that, the degradation of cell membranes and browning coincident with the loss of chlorophyll and browning is associated with cell disorganization (Laukkanen, et al., 2000). Among the protein bands, some were stronger than the others. Proteins with molecular weight of about 83, 53, 45, 40-kDa were expected to be involved in somatic embryogenesis. Sung and Okimoto (1983) in the studies on carrot have reported two somatic embryo-specific proteins (77 and 43 kDa). Similar studies performed by Chen and Luthe (1987) on rice revealed the presence of two major proteins of 54 and 24 kDa, which were regarded as somatic embryos-specific. According to Pakusch et al. (1991) a 24 kDa band may represent subunits of methyltransferase, an enzyme associated with lignin synthesis. The production of lignin compounds in callus of plants species may be as a result of stress reactions because several stresses are known to induce the synthesis of phenylpropanoids.

Activities of CAT, POX and PPO in DBC-SSE were significantly higher than those in the other samples but the lowest activity of SOD was detected in this tissue. According to these results, the differentiation and development of embryogenic tissues during secondary somatic embryogenesis of Persian walnut could be regulated by antioxidant enzymes (CAT and POX and PPO). The study analyses showed that activities of CAT, POX and PPO rapidly increased along with the secondary somatic embryogenesis. According to Kairong et al. (1999) the acquisition of competence, induction and development of somatic embryos in *L. barbarum* L. were associated with the gradual increase in SOD activity. POX and CAT activities were high in callus and rapidly decreased in the early days of the differentiation culture (Kairong et al., 1999). Laukkanen et al. (1999, 2000) reported that, tissue browning in Scot pine is associated with increased POX and PPO activities, as well as cell disorganization and eventually cell death. From the zymograms of CAT, it can be concluded that CAT1 was somatic embryo-specific and CAT2 was present only in dark brown calli and not in light brown ones. These differences might be related to tissue browning and thus, CAT could be an indicator for oxidative stress. Among five POX isoforms, POX1 band in DBC+SSE was very strong compared with LBC+SSE and SSE. POX4 band

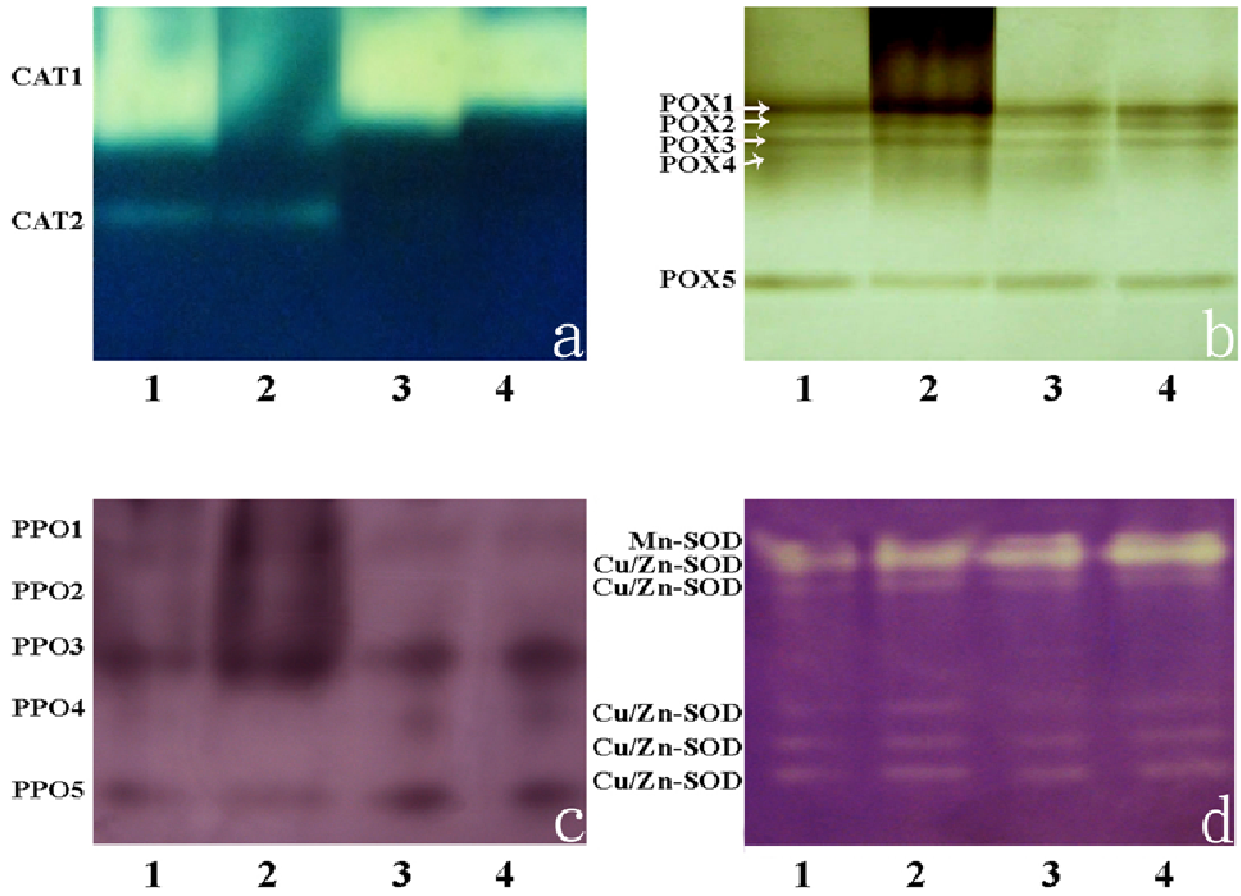


**Figure 3.** Activity staining for SOD; (a) CAT (b) PPO (c) and SOD (d) during secondary somatic embryogenesis in Persian walnut: dark brown calli with secondary somatic embryo (DBC+SSE) (1), dark brown calli without secondary somatic embryo (DBC-SSE) (2), secondary somatic embryos (SSE) (3), and light brown calli with secondary somatic embryo (LBC+SSE) (4). Equal amounts of protein (40 µg) were loaded in each lane.

was present only in DBC+SSE. Thus, POX4 could be related to tissue browning in these tissues. PPO1 and PPO3 bands were stronger in DBC-SSE and PPO2 which was only present in DBC+SSE could be related to tissue browning, while PPO4 was detected only in SSE and LBC+SSE and PPO5 could be related to somatic embryo development (Figure 3c). The contribution of individual SOD isoforms to total SOD activity was determined by performing SOD assays directly on protein extracts separated in native gels. The upper band (Figure 3d), in all samples, represented a mitochondrial Mn-SOD. Five bands with higher mobility represented the cytosolic Cu/Zn-SOD isoenzymes. The band representing a chloroplastic Fe-SOD was not detected in samples. The levels of superoxide dismutase activity were lower in browning tissues than that in non-browning tissues in culture condition in Scot pine (Laukkanen et al., 2000) and these results are in agreement with the latter research. In Virginia pine, tissue browning decreases the efficiency of *in vitro* regeneration through somatic embryogenesis (Tang and Newton, 2004). It was reported that, in non-browning callus cultures, PPO activity declined while in browning calli, PPO activity continued to increase. It is believed that the increased PPO activity in browning calli results from wounding or oxidative damage. Numerous experiments have recently been carried out on several plant species with the objective of explaining the role of oxidative stress in plant morphogenesis (Gupta and Datta, 2003, 2004; Libik et al., 2005). It is well known that somatic embryogenesis is one of the most useful approaches for understanding plant development. Higher levels of intracellular $H_2O_2$ induce and promote embryo-genesis of *L. barbarum* L. callus (Kairong et al., 1999). However, the relationship between the ROS and the callus differentiation and regeneration has not been well understood till now (Tian et al., 2003).

In summary, according to the present research, the differentiation and development of embryogenic tissues during secondary somatic embryogenesis of Persian walnut could be regulated by protein and antioxidant enzymes.

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