BIOLOGIA PLANTARUM 54 (3): 509-514, 2010

BRIEF COMMUNICATION

Activity of antioxidant enzyme during *in vitro* organogenesis in *Crocus sativus*

E. VATANKHAH, V. NIKNAM* and H. EBRAHIMZADEH

Department of Plant Sciences, School of Biology, College of Sciences, University of Tehran, Tehran 14155, I.R. Iran

Abstract

The effect of various hormonal combinations on regeneration of shoots and roots from meristem-derived callus of *Crocus sativus* L. and activities of antioxidant enzymes have been studied. The most efficient regeneration occurred with 1.0 mg dm^{-3} 1-naphthaleneacetic acid (NAA) + 1.0 mg dm^{-3} thidiazuron and 1.0 mg dm^{-3} NAA + 2.0 mg dm^{-3} kinetin. For sprouting, regenerated shoot were subcultured on Murashige and Skoog medium containing 1.0 mg dm^{-3} NAA + 1.0 mg dm^{-3} benzylaminopurine (BAP). Protein content and superoxide dismutase activity decreased in regenerated shoots and roots and increased in sprouting shoots, while catalase (CAT), peroxidase (POX) and polyphenol oxidase (PPO) activities increased during organogenesis and decreased in sprouting shoots. High CAT and PPO activities were detected in regenerated roots, whereas high POX activity was observed in regenerated shoot.

Additional key words: catalase, peroxidase, polyphenol oxidase, protein, saffron, superoxide dismutase.

Saffron (*Crocus sativus* L.) is native in Greece and Mediterranean regions. For many centuries, saffron has been cultivated for its stigmas, which not only comprise a highly valued spice but also have various therapeutic uses (Sampathu *et al.* 1984). The saffron plant is a geophyte and propagates through the formation of daughter corms from the mother corm. The autotriploid nature of the species renders improvement by breeding very difficult (Basker and Negbi 1983). Application of tissue culture for the large scale propagation and genetic improvement of saffron has been emphasized recently (Ding *et al.* 1981, Ilahi *et al.* 1987, Bhagyalakshmi 1999, Piqueras *et al.* 1999).

In plants, reactive oxygen species (ROS) are constantly produced, however, uncontrolled production of ROS can cause oxidative stress and cellular damage. The production of ROS has been associated with plant recalcitrance during *in vitro* culture (Benson 2000). Antioxidant enzymes stimulated improvement in growth and regeneration further support the relationship between oxidative stress and recalcitrance (Gupta and Datta 2003/4). 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 2003/4, Tang and Newton 2005, Libik *et al.* 2005). CAT and POX are known to play a role in growth and differentiation and their high activity could be correlated to the process of differentiation that occurred during shoot or root induction (Molassiotis *et al.* 2004). The role of ROS in plant growth and development is substantiated by the interplay of ROS with a number of phytohormones. Moreover, ROS have been implicated as second messenger in several plant hormone responses. Therefore the study of the ROS production, oxidative stress and the efficiency of antioxidants during different stages of organogenesis is of increasing interest (Baťková *et al.* 2008).

The objective of the present study was to establish a shoot and root organogenesis system from meristem-derived callus of saffron and compare the activities of

Received 5 October 2008, *accepted* 17 April 2009.

Abbreviations: BAP - N^6-benzylaminopurine; CAT - catalase; EDTA - ethylenediaminetetraacetic acid; KIN - kinetin; L-DOPA - 3,4-dihydroxy-L-phenylalanin; MS - Murashige and Skoog; NAA - 1-naphthaleneacetic acid; NBT - nitroblue tetrazolium; PAGE - polyacrylamide gel electrophoresis; PIC - picloram (4-amino-3,5-trichloropicolinic acid); POX - peroxidase; PPO - polyphenol oxidase; PVPP - polyvinylpolypyrrolidone; ROS - reactive oxygen species; SDS - sodium dodecyl sulfate; SOD - superoxide dismutase; TDZ - thidiazuron.

Acknowledgements: The financial support of this research was provided by University of Tehran. The authors are grateful to the anonymous reviewers for very helpful and valuable advices.

* Corresponding author; fax: (+98) 21 66405141, e-mail: vniknam@khayam.ut.ac.ir

antioxidative enzymes at various stages of shoot and root organogenesis.

Corms of *Crocus sativus* L. were collected from the University of Tehran farm located in Karaj, near Tehran, in February 2007. After careful washing, the corms were surface sterilized by 70 % ethanol for 2 min, 0.1 % $HgCl_2$ for 5 min, 20 % commercial bleach containing *Tween 20* for 10 min and rinsed three times with sterile, distilled water. Shoot meristems were dissected and cultured on Murashige and Skoog (1962; MS) medium containing 3 % sucrose and different concentrations of cytokinins (N^6-benzylaminopurine, BAP; kinetin, KIN; thidiazuron, TDZ) and auxins (1-naphthalene acetic acid, NAA; picloram, PIC) (Table 1, Fig 1). All media were solidified by 0.7 % agar after adjusting the pH to 5.7 and autoclaved at 121 °C for 20 min. Cultures were incubated at 20/25 °C in darkness. The experiments were repeated twice using 20 explants per treatment. The data for shoot and root formation were scored after 6 months of culture. For sprouting, regenerated shoots in MS medium supplemented with 1.0 mg dm^{-3} TDZ and 1.0 mg dm^{-3} NAA, were subcultured on MS medium containing 1.0 mg dm^{-3} NAA and 1.0 mg dm^{-3} BAP and grown under 16-h photoperiod (white fluorescent lamps; irradiance of 46 μmol m^{-2} s^{-1}) and day/night temperature of 20/25 °C.

For estimation of total protein content and enzyme activity, plant material (meristem explant, regenerated shoot, sprouted shoot and regenerated root) was homogenized at 4 °C with a mortar in 50 mM Tris-HCl buffer (pH 7.2) containing 10 mM EDTA, 2 mM $MgSO_4$, 20 mM L-cystein, 10 % (v/v) glycerol and 4 % (m/v) polyvinylpolypyrrolidone (PVPP) (Jasska 1996). The homogenates were centrifuged at 13 000 *g* for 45 min at 4 °C and resulting supernatants were kept at -70 °C and used for protein determination and enzyme assays. A high-speed centrifuge (*J2-21M, Beckman,* Palo Alto, USA) and UV-visible recording spectrophotometer (*UV-160, Shimadzu*, Tokyo, Japan) were used. The protein content was determined according to the method of Bradford (1976) using bovine serum albumin as a standard. Superoxide dismutase (SOD; EC 1.15.1.1) activity was estimated by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) as described by Giannopolitis and Ries (1977) in a reaction mixture containing 50 mM sodium phosphate buffer (pH 7.5), 13 mM methionine, 75 μM NBT, 75 μM riboflavin, 0.1 mM EDTA and 0.1 cm^3 of enzyme extract. The reaction mixture was irradiated for 14 min and absorbance was read at 560 nm against the non-irradiated blank. Catalase (CAT; EC 1.11.1.6) activity was assayed from the rate of H_2O_2 decomposition as measured by the decrease of absorbance at 240 nm, following the procedure of Aebi (1974). The reaction mixture contained 0.625 cm^3 50 mM sodium phosphate buffer (pH 7.0), 0.075 cm^3 H_2O_2 (3 %) and 0.01 cm^3 enzyme extract. Peroxidase (POX; EC 1.11.1.7) activity was measured according to the method of Abeles and Biles (1991). The reaction mixture contained 2 cm^3 of 0.2 M acetate buffer (pH 4.8), 0.2 cm^3 H_2O_2 (3 %), 0.1 cm^3 20 mM benzidine and 0.1 cm^3 enzyme extract. The increase of absorbance was recorded at 530 nm. Polyphenol oxidase (PPO; EC 1.14.18.1) activity was determined according to the method of Raymond *et al.* (1993) at 40 °C. The reaction mixture contained 2.5 cm^3 of 0.2 M sodium phosphate buffer (pH 6.8), 0.2 cm^3 20 mM pyrogallol and 0.02 cm^3 enzyme extract. The increase of absorbance was recorded at 430 nm.

For determination of protein patterns in the explant, regenerated shoot, sprouted shoot and regenerated root, discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) using 12 % acrylamide. For isoenzyme assay, native PAGE was carried out by a modified method of Davis (1964) with a 12 % (SOD and POX) and 10 % (CAT and PPO) acrylamide gels at 4 °C without SDS and mercaptoethanol. SOD bands were visualized using the activity staining procedure described by Wendel and Weeden (1989). The 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. KCN (3 mM) inhibited only Cu/ZnSOD and H_2O_2 (5 mM) inhibited both Cu/ZnSOD and Fe SOD. MnSOD was not inhibited by KCN nor 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 % (m/v) ferricyanide [$K_3Fe(CN)_6$] and 2 % ferric chloride till yellow bands on dark green background appeared. Electrophoretic pattern of POX was obtained by staining 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 brown bands appeared. PPO bands were visualized using the method of Van Loon and Geelen (1971). The gels were incubated in 0.2 M sodium phosphate buffer (pH 6.8) containing 0.5 % 3,4-dihydroxy-L-phenylalanin (L-DOPA) and 3.5 % calcium chloride in the dark at room temperature till brown bands appeared.

Each experiment was repeated at least three times. Analysis of variance was conducted using one-way *ANOVA* test using *SPSS 14* for *Microsoft Windows* and means were compared by Duncan tests at the 0.05 level of confidence.

Among the 12 hormonal treatments, three types of callus were induced from meristem explant. Type I was observed on MS medium containing NAA + KIN (Fig. 1*A*). Type II was induced on MS medium supplemented with NAA + TDZ (Fig. 1*B*). Type III was seen on MS medium containing picloram (PIC) + KIN and PIC + BAP (Fig. 1*C*). Type I and II calli are

organogenic and produce root and shoot, respectively while type III is not organogenic.

Shoot formation was induced by 3 out of 12 hormonal treatments (Table 1, Fig. 1*D*,E). Frequency of shoot formation varied between 30.5 - 64.4 %, the highest frequency was observed on medium supplemented with 1.0 mg dm^{-3} NAA and 1.0 mg dm^{-3} TDZ and the lowest with 1.0 mg dm^{-3} and 0.5 mg dm^{-3} TDZ. Maximum number of shoots per explant (18.4) was observed on medium with 1.0 mg dm^{-3} NAA and 1.0 mg dm^{-3} TDZ (Fig. 1*D*). These results are in agreement with the finding of Radhika *et al.* (2006). They obtained high frequency of shoot regeneration and high number of shoots per explant on a wide range of TDZ + NAA combinations. Thus, TDZ which is known for induction of adventitious shoots and proliferation of axillary shoots in woody species (Huetteman and Preece 1993) holds promise for a herbaceous species like saffron. Wang *et al.* (1991) found changes in key enzymes and other regulatory elements in response to TDZ. Wang and Faust (1988) observed that TDZ induces accumulation of unsaturated polar membrane fatty acids, which strongly propose the active involvement of TDZ in inducing metabolic changes and suggested that it is not a simple substitute for purine based cytokinins. Also Sharma *et al.* (2008) analyzed effect of BAP, indole-butyric acid (IBA) and sucrose on shoot regeneration in *C. sativus*.

Root formation was induced by 2 out of 12 treatments (Table 1, Fig 1*F*). Maximum percentage of root formation (83.3 %) and root number per explant (3.5) was observed on medium supplemented with 1.0 mg dm^{-3} NAA and 2.0 mg dm^{-3} KIN (Fig. 1*F*). NAA + KIN combinations induced caulogenesis in *Cassia angustifolia* (Agrawal and Sardar 2006). In the present study, media

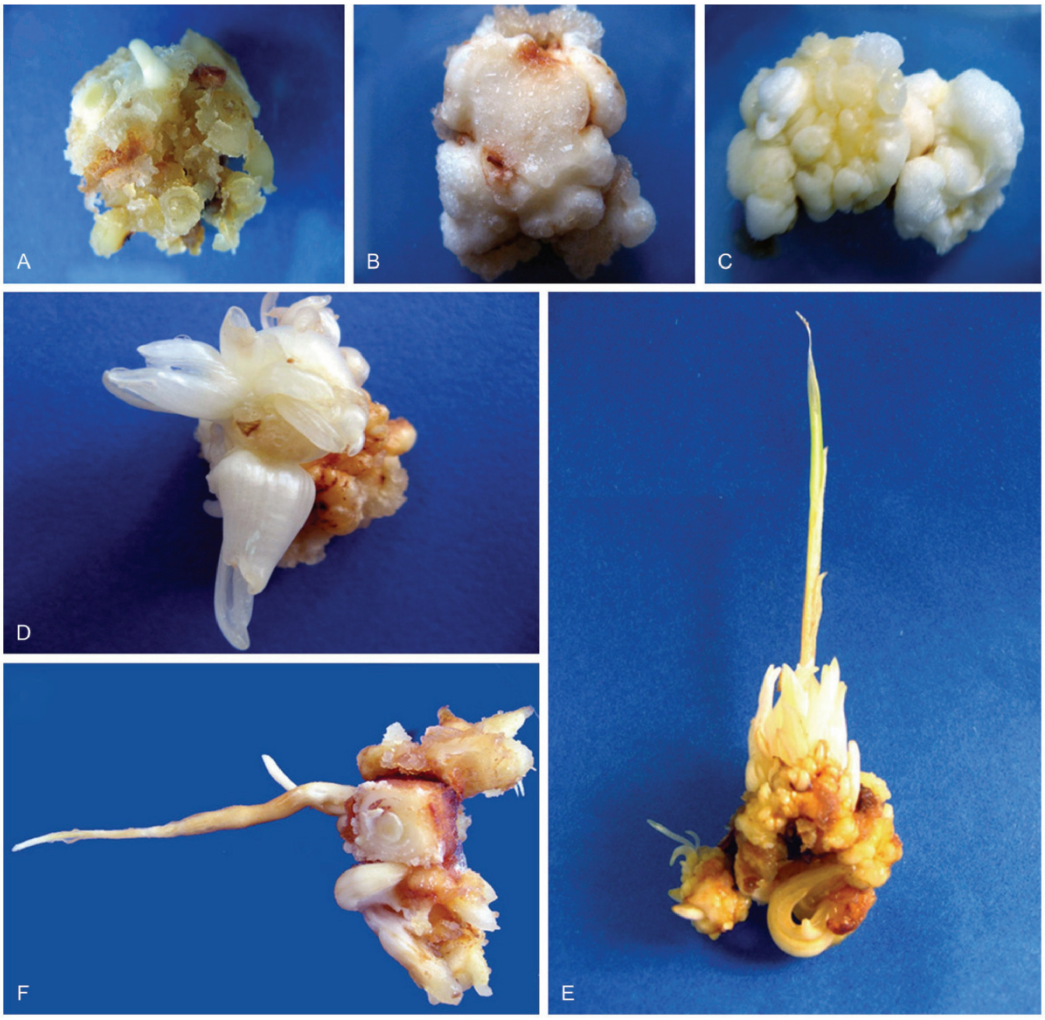

Fig 1. Shoot and root regeneration from callus of *C. sativus* on MS medium. Three types of meristem-derived callus on MS medium containing different combinations of growth regulators: *A* - 1.0 mg dm^{-3} NAA + 2.0 mg dm^{-3} KIN (type I); *B* - 1.0 mg dm^{-3} NAA + 2.0 mg dm^{-3} TDZ (type II); *C* - 10 mg dm^{-3} PIC + 4.0 mg dm^{-3} KIN (type III). *D* - shoot regeneration through callus on medium with 1.0 mg dm^{-3} TDZ + 1.0 mg dm^{-3} NAA; *E* - sprouted shoot after transfer of regenerated shoot to medium with 1.0 mg dm^{-3} BAP + 1.0 mg dm^{-3} NAA under 16-h photoperiod; *F* - root regeneration through callus on medium with 2.0 mg dm^{-3} KIN + 1.0 mg dm^{-3} NAA.

Table 1. Effect of various concentrations of cytokinins (CK) [mg dm^{-3}] on regeneration and number of shoot and root from meristem-derived callus of *C. sativus*. MS medium with 1.0 mg dm^{-3} NAA. Means ± SE, n = 3; values marked with different letters are significantly different at $P < 0.05$.

CK	Shooting [%]	Rooting [%]	Shoot number [explant^{-1}]	Root number [explant^{-1}]
TDZ 0.5	30.55±19.44a		4.60±3.80a	
TDZ 1.0	64.44±19.37a		18.40±5.07a	
TDZ 2.0	33.33±16.67a		9.40±6.35a	
KIN 1.0		66.67±33.33ab		2.67±1.02ab
KIN 2.0		83.33±16.67a		3.50±1.54a

supplemented with other growth regulator combinations (PIC + BAP and PIC + KIN) failed to induce organogenesis. PIC + BAP combinations induced somatic embryogenesis in *Arachis pintoi* (Rey and Mroginski 2006). This results show that diverse auxin and cytokinin concentrations and/or their combinations are required in different species.

Protein content was high in the explant and decreased in regenerated shoot and root, and then increased in sprouted shoot. Activities of CAT, POX and PPO increased in regenerated shoot and root while decreased in sprouted shoot (Table 2). The highest activity of CAT and PPO was observed in regenerated root while the highest activity of POX was detected in regenerated shoot. Activity of SOD was high in explant and decreased during shoot and root regeneration while increased in sprouted shoot. Increase in CAT and POX and decrease in SOD as observed during shoot organogenesis, were also reported in *Prunus* (Franck *et al.* 1995), *Solanum* (Kumar and Knowles 1993) and *Gladiolus* (Gupta and Datta 2003/4). CAT and POX are known to play important role in growth and differentiation (Gaspar 1995) and high activity could be correlated to the process of differentiation during organogenesis. Also the impact of oxidative stress in plant morphogenesis has been proved. Plant cells possess well-developed systems to regulate the level of ROS, and the concentration of ROS can be changed or affected by several antioxidant enzymes such as SOD, CAT and POX (Libik *et al.* 2005). Also PPO has been reported to function in oxygen scavenging by oxidation of phenols (Tang and Newton 2005).

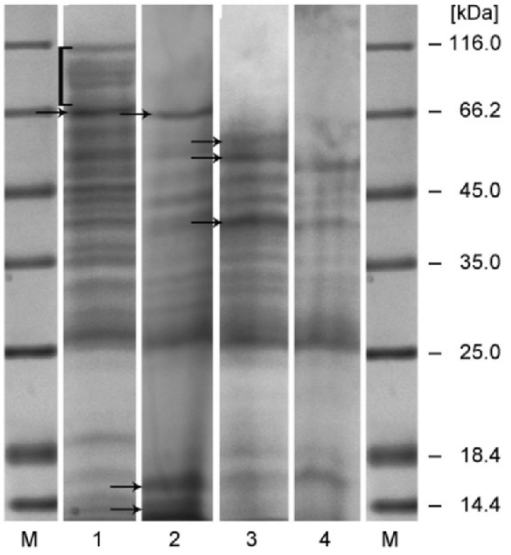

Fig. 2. SDS-PAGE patterns of proteins during shoot and root organogenesis of *C. sativus*: meristem explant (1), regenerated shoot (2), sprouted shoot (3), regenerated root (4) and molecular mass markers (M). *Arrows* indicate the new and changed bands. Equal amounts of protein (100 μg) were loaded in each lane.

According to the protein pattern (Fig. 2), several protein bands (*upper bracket*, *line 1*) with Mr ~ 111, 103, 94 and 87 kDa were observed only in meristem explant while they disappeared during organogenesis. A 66.2 kDa protein was detected only in meristem explant and regenerated shoot. Deletion of 66.2 kDa protein in regenerated root and sprouted shoot suggests that this protein may play an important role in root regeneration and shoot sprouting. Also, intensity of some protein bands with Mr ~ 63, 54 and 40 kDa decreased in regenerated shoot and root. Probably, decrease of these proteins play a role in organogenesis. Specifically, an accumulation of the 18 and 13 kDa proteins were noted only in sprouted shoot. The organogenesis from the callus is a very complex process, accompanied with a variety of genes expression and protein synthesis (Tian *et al.* 2003), where H_2O_2 can play a role (Libik *et al*. 2005). So, we suggest that the endogenous H_2O_2 could induce organogenesis by influence on the expression of genes and protein synthesis.

Table 2. Protein content [mg g^{-1}(f.m.)] and activity of CAT, SOD, POX and PPO [U mg^{-1} (protein)] in meristem, regenerated shoots, sprouted shoots and regenerated roots of *C. sativus*. Means ± SE, n = 3; values marked with different letters are significantly different according to DMRT at $P < 0.05$.

	Protein	CAT	SOD	POX	PPO
Meristem	1.29 ± 0.02a	386.43 ± 13.41d	1.39 ± 0.16a	0.14 ± 0.01d	0.20 ± 0.01c
Regenerated shoots	0.27 ± 0.02c	3720.58 ± 221.55b	0.77 ± 0.15b	6.78 ± 0.10a	0.33 ± 0.02b
Sprouted shoots	0.60 ± 0.01b	1263.17 ± 28.62c	1.05 ± 0.04ab	2.13 ± 0.16c	0.24 ± 0.02c
Regenerated roots	0.22 ± 0.01d	6165.82 ± 277.12a	1.07 ± 0.10ab	4.01 ± 0.20b	0.70 ± 0.05a

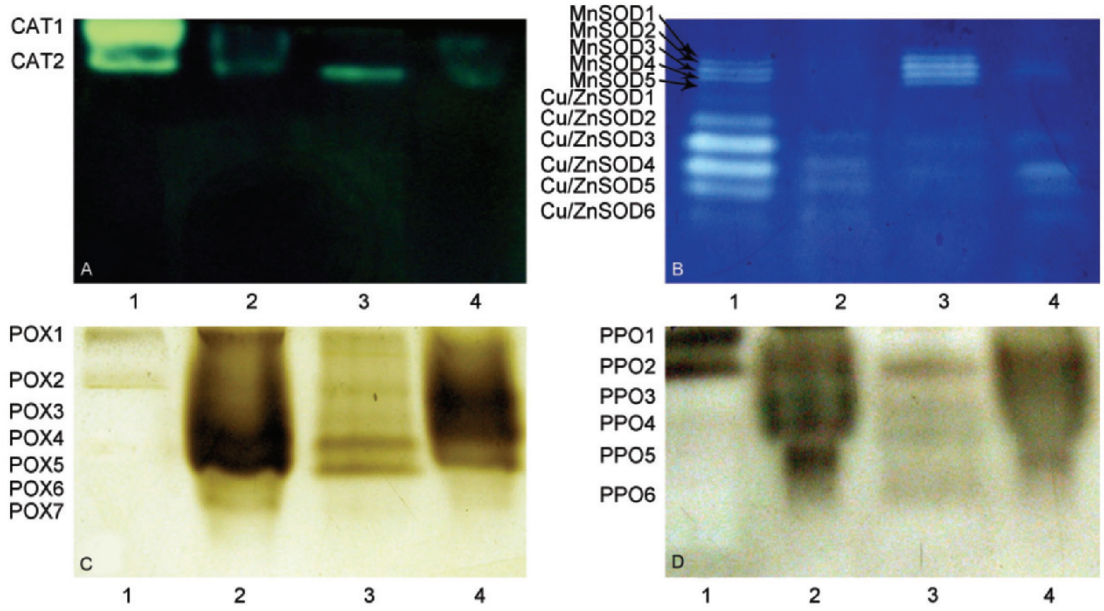

Fig. 3. Activity staining for CAT (*A*), SOD (*B*), POX (*C*) and PPO (*D*) during shoot and root organogenesis of *C. sativus*: Meristem explant (1), regenerated shoot (2), sprouted shoot (3) and regenerated root (4). Equal amounts of protein (100 μg) were loaded in each lane.

According to non-denaturing PAGE, two CAT isoforms were detected in the extracts of the specimens (Fig. 3*A*). The activity of CAT1 and CAT2 in meristem explant was high while decreased in regenerated root and shoot. Also CAT2 activity increased in sprouted shoot.

Application of inhibitors showed five MnSOD isoforms and six Cu/ZnSOD isoforms (Fig. 3*B*). MnSOD isoforms were not observed in regenerated shoot. All MnSOD isoforms were detected in meristem explant and sprouted shoot while three MnSOD (MnSOD2, MnSOD3 and MnSOD4) were detected in regenerated root. Cu/ZnSOD1 and Cu/ZnSOD2 were expressed only in meristem explant. Cu/ZnSOD3, Cu/ZnSOD4 and Cu/ZnSOD5 were detected in all the samples but with different activities. The activity of Cu/ZnSOD4 in regenerated root was higher than in regenerated shoot. Also Cu/ZnSOD4 could probably play a role in root regeneration. Cu/ZnSOD6 disappeared in sprouted shoot.

In the plant material investigated seven POX isoforms were present (Fig. 3*C*). Seven POX isoforms were expressed in regenerated root and shoot while two isoforms (POX1 and POX2) were expressed in meristem explant. The intensity of POX isoforms increased in regenerated root and shoot while decreased in sprouted shoot.

Six PPO isoforms were detected in the plant materials (Fig. 3*D*). PPO1 and PPO2 were only observed in meristem explant. Activity of PPO3 and PPO4 increased in regenerated shoot and root then decreased in sprouted shoot. PPO5 was detected in regenerated root and shoot while was absent in sprouted shoot.

The organogenesis of callus is a complex process, accompanied with a variety of gene expression and protein synthesis (Tian *et al.* 2003). Results of PAGE analysis indicated different expressions of CAT, SOD, POX and PPO isoforms during organogenesis. The results obtained in the present study provided evidence that antioxidant enzymes are correlated with the morphogenetic process in safron callus.

In summary, there are differences in antioxidant metabolism between the calli showing different morphogenic potential. These differences might be linked to different metabolic pathways accompanying the formation of roots or shoots. Also, role of antioxidative enzymes during different stages of organogenesis is substantiated.

References

Aebi, H.: Catalases. - In: Bergmeyer, H.U. (ed.): Methods of Enzymatic Analysis. Vol. 2. Pp. 673-684. Academic Press, New York 1974.

Abeles, F.B., Biles, C.L.: Characterization of peroxidases in lignifying peach fruit endocarp. - Plant Physiol. **95**: 269-273, 1991.

Agrawal, V., Sardar, P.: *In vitro* propagation of *Cassia angustifolia* through leaflet and cotyledon derived calli. - Biol. Plant. **50**: 118-122, 2006.

Basker, D., Negbi, M.: Uses of saffron. - Econ. Bot. **37**: 228-236, 1983.

Benson, E.E.: Do free radicals have a role in plant tissue culture recalcitrance? - *In Vitro* cell. dev. Biol. Plant **36**: 163-170, 2000.

Baťková, P., Pospíšilová, J., Synková, H.: Production of reactive oxygen species and development of antioxidative systems during *in vitro* growth and *ex vitro* transfer. - Biol. Plant. **52**: 413-422, 2008.

Bhagyalakshmi, N.: Factors influencing direct shoot regeneration from ovary explants of saffron. - Plant Cell Tissue Organ Cult. **58**: 205-211, 1999.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Davis, B.J.: Disc electrophoresis. II. Method and application to human serum proteins. - Ann. N.Y. Acad. Sci. **121**: 404-427, 1964.

Ding, B.Z., Bai, S.H., Wu, Y., Fan, X.P.: Induction of callus and regeneration of plantlets from corm of *Crocus sativus* L. - Acta bot. sin. **23**: 434-440, 1981.

Franck, T., Kevers, C., Gaspar, T.: protective enzymatic systems against activated oxygen species compared in normal and vitrified shoots of *Prunus avium* L. raised *in vitro*. - Plant Growth Regul. **16**: 253-256, 1995.

Gaspar, T.: The concept of cancer in *in vitro* plant cultures and the implication of habituation to hormones and hyperhydricity. - Plant Tissue Cult. Biotechnol. **1**: 126-136, 1995.

Giannopolitis, C.N., Ries, S.K.: Superoxide dismutases II. purification and quantitative relationship with water-soluble protein in seedlings. - Plant Physiol. **59**: 315-318, 1977.

Gupta, S.D., Datta, S.: Antioxidant enzyme activities during *in vitro* morphogenesis of gladiolus and the effect of application of antioxidants on plant regeneration. - Biol. Plant. **47**: 179-183, 2003/4.

Huetteman, C.A., Preece, J.E.: Thidiazuron: a potent cytokinin for woody plant tissue culture. - Plant Cell Tissue Organ Cult. **33**: 105-119, 1993.

Ilahi, I., Jabeen, M., Firdous, N.: Morphogenesis with saffron tissue culture. - J. Plant Physiol. **128**: 227-232, 1987.

Jasska, V.: Isoenzyme diversity and phylogenetic affinities among the *Phaseolus* beans (Fabaceae). - Plant Syst. Evol. **200**: 233-252, 1996.

Kumar, G.N.M., Knowles, N.R.: Changes in lipid peroxidation and lipolytic and free radical scavenging enzyme activities during aging and sprouting of potato (*Solanum tuberosum*) seed-tubers. - Plant Physiol. **102**: 115-124, 1993.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Lee, D.L., Kim, Y.S., Lee, C.B.: The inductive responses of the antioxidant enzymes by salt stress in the rice (*Oryza sativa* L.). - J. Plant Physiol. **158**: 737-745, 2001.

Libik, M., Konieczny, R., Pater, B., Ślesak, I., Miszalski, Z.: Differences in the activities of some antioxidant enzymes and in H_2O_2 content during rhizogenesis and somatic embryogenesis in callus cultures of the ice plant. - Plant Cell Rep. **23**: 834-841, 2005.

Molassiotis, A.N., Dimassi, K., Diamantidis, G., Therios, I.: Changes in peroxidases and catalase activity during *in vitro* rooting. - Biol. Plant. **48**: 1-5, 2004.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Piqueras, A., Han, B.H., Escribano, J., Rubio, C., Hellín, E., Fernández, J.A.: Development of cormgenic nodules and microcorms by tissue culture, a new tool for the multiplication and genetic improvement of saffron. - Agronomie **19**: 603-610, 1999.

Radhika, K., Sujatha, M., Nageshwar Rao, T.: Thidiazuron stimulates adventitious shoot regeneration in different sunflower explants. - Biol. Plant. **50**: 174-179, 2006.

Raymond, J., Rakariyatham, N., Azanza, J.L.: Purification and some properties of polyphenoloxidase from sunflower seeds. - Phytochemistry **34**: 927-931, 1993.

Rey, H.Y., Mroginski, L.A.: Somatic embryogenesis and plant regeneration in diploid and triploid *Arachis pintoi*. - Biol. Plant. **50**: 152-155, 2006.

Sampathu, S.R., Shivashankar, S., Lewis, Y.S.: Saffron (*Crocus sativus* L.) cultivation, processing, chemistry and standardization. - CRC Food Sci. Nutr. **20**: 123-157, 1984.

Sharma, K.D., Rathour, R., Sharma, R., Goel, S., Sharma, T.R., Singh, B.M.: *In vitro* development in *Crocus sativus*. - Biol. Plant. **52**: 709-712, 2008.

Tian, M., Gu, Q., Zhu, M.: The involvement of hydrogen peroxide and antioxidant enzymes in the process of shoot organogenesis of strawberry callus. - Plant Sci. **165**: 701-707, 2003.

Tang, W., Newton, R.J.: Peroxidase and catalase activities are involved in direct adventitious shoot formation induced by thidiazuron in eastern white pine (*Pinus strobes* L.) zygotic embryos. - Plant Physiol. Biochem. **43**: 760-769, 2005.

Van Loon, L.C., Geelen, J.L.M.C.: The relation of polyphenol oxidase and peroxidase to symptom expression in tobacco var. "Samsun NN" after infection with tobacco mosaic virus. - Acta phytopathol. Acad. Sci. hung. **6**: 9-20, 1971.

Wang, S.Y., Faust, M.: Changes of fatty acids and sterols in apple buds during the break induced by a plant bioregulator, thidiazuron. - Physiol. Plant. **72**: 115-120, 1988.

Wang, S.Y., Jiao, H. J., Faust, M.: Changes in activities of catalase, peroxidase and polyphenol oxidase in apple buds during bud break induced by thidiazuron. - J. Plant Growth Regul. **10**: 33-39, 1991.

Wendel, J.F., Weeden, N.F.: Visualization and interpretation of plant isozymes. - In: Soltis, D.E., Soltis, P.S. (ed.): Isozymes in Plant Biology. Pp. 4-45. Dioscorides Press, Portland 1989.

Woodbury, W., Spencer, A.K., Stahman, M.A.: An improved procedure using ferricyanide for detecting catalase isozymes. - Anal. Biochem. **44**: 301-305, 1971.