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Histological and biochemical parameters of *Crocus sativus* during *in vitro* root and shoot organogenesis

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Abstract

Content of malondialdehyde (MDA), proline, phenolics, and saccharides was analyzed during different developmental stages of *in vitro* root and shoot organogenesis in saffron. The highest content of MDA, proline, and phenolics was detected in nodular calli. Significant changes were also found in the content of polysaccharides, soluble saccharides, oligosaccharides, and reducing saccharides during developmental stages. Histological investigation of nodular calli showed meristematic zones with small and densely stained cells situated at peripheral zones of calli. The meristematic zones surrounded some vascular areas from which *de novo* organs originated. The parenchymatic cells of inner zones of calli converted to procambium cells that produced vascular tissues.

Additional key words: lipid peroxidation, malondialdehyde, nodular callus, phenolics, proline, saccharides, saffron.

Introduction

Crocus sativus L. (saffron) is cultivated in several countries of the world for its red stigmatic lobes that constitute the high value spice saffron. Saffron being triploid (2n=3x=24) is sterile and is propagated vegetatively through daughter corms. Under natural conditions, the generation rate of daughter corms is low (Jirage *et al*. 1994, Chahota *et al*. 2003). Micropropagation can be used to produce large quantities of saffron regenerants (Sharma *et al.* 2008). The *in vitro* regeneration of shoot buds from undifferentiated callus cells is an important technique (Hare and Cress 1997) and provides a useful model system for the investigation of the morphological, biochemical, and molecular events taking place during early developmental stages (De Klerk 1996). Saccharides play a pivotal role in plant growth and development (Romano *et al.* 1995) not only by functioning as substrates for growth, but also by affecting cell differentiation and cell cycle (Koch 1996). Considerable evidence supports an important role for proline in regulating cell morphology and differentiation (Nanjo *et al*. 1999) even in the absence of a stress (Hare and Cress 1997). Growth promotion by proline may be fostered by enhanced cell division in meristems (Mattiolia *et al.* 2009). Phenolics constitute a group of substances with a broad spectrum of physiological activities. They participate in redox processes and in plant defense reaction. Indeed, they intervene in the regulation of developmental processes, such as organogenesis through their interaction with growth regulators. Phenolics are the main substrates of peroxidases. The preferential oxidation of phenolics by peroxidase prevents peroxidase catalyzed oxidation of auxin (Kanmegne and Omokolo 2003).

According to De Klerk *et al.* (1997), the number of cells involved in the regeneration process is almost always a very small fraction of the total number of cells of explants or cell suspensions. In molecular and biochemical studies, usually all of the cell material is analyzed. Thus, to establish the relevance for regeneration, the molecular and biochemical studies should be accompanied by appropriate analysis at the microscopic level, preferably supplemented by histological studies. Therefore, the present study was initiated to decipher the changes of phenolics, proline, malondialdehyde (MDA), and saccharide content during *in vitro* development and root and shoot organogenesis from meristem explants of *C. sativus*. The experiments were completed by histological studies.

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Abbreviations: BAP - benzylaminopurine; DMRT - Duncan multiple range test; MDA - malondialdehyde; MS - Murashige and Skoog; NAA - α-naphthalenacetic acid; OS - oligosaccharide; PS - polysaccharide; RS - reducing saccharide; SS - soluble saccharide; TBA - thiobarbituric acid; TCA - trichloroacetic acid; TDZ - thidiazuron.
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Materials and methods

The *de novo* shoots and roots were developed from cultured corms of saffron as described previously (Vatankhah *et al.* 2010). Healthy daughter corms of *Crocus sativus* L. were collected from the research farm of the College of Science, the University of Tehran (Mardabad, Karaj, Iran), in February 2010. Corms were washed in running water for 30 min. After surface disinfection and sterilization, a rectangular section from the central meristematic region was cut and placed on a Murashige and Skoog (1962; MS) basal medium containing 0.7 % (m/v) agar, 1 mg dm^{-3} α-naphthalene-acetic acid (NAA) and 1 mg dm^{-3} thidiazuron (TDZ) for shoot regeneration, and 1 mg dm^{-3} NAA and 2 mg dm^{-3} kinetin for root regeneration. The dishes were incubated at temperature of 25 ± 3 °C in the dark. All parameters were studied at three different developmental stages: corm explants, organogenic calli (after 8 - 10 weeks), and calli with *de novo* organs (after 14 - 16 weeks). The samples from several explants or calli were pooled together and stored at -80°C for subsequent analyses.

In order to get a better insight about differentiation stages, histological analyses have been carried out in organogenic calli (after 8 - 10 weeks). Nodular calli were fixed in 1.25 % (m/v) glutaraldehyde, 2 % (m/v) paraformaldehyde (PFA), and a 0.05 M piperazine-N,N'-bis-[2-ethanolsulphonic acid] (PIPES) buffer, rinsed twice in the buffer, then dehydrated in a gradient of ethanol (10 to 100 %) and embedded in Spurr's epoxy resin following a graded infiltration series (10 to 100 %) (O'Brien And McCully 1981). The sections of 3 µm were cut using a microtome and stained with 0.5 % (m/v) toluidine blue in 1 % (m/v) Na_2CO_3. Sections were observed by a *BX51 Olympus* (Tokyo, Japan) microscope and images were taken by a *DP12 Olympus* digital camera.

Free proline content was determined according to the method described by Bates *et al.* (1973). Dry plant material (0.1 g) was homogenized in 5 cm^3 of 3 % (m/v) sulfosalicylic acid. After filtration, 2 cm^3 of the solution was mixed with 2 cm^3 of 2.5 % acid-ninhydrin and 2 cm^3 of 96 % glacial acetic acid. Test tubes with the mixture were placed in a water bath at 100 °C for 1 h, and the reaction was finished in an ice bath. The reaction mixture was extracted with 4 cm^3 of toluene, after which the chromophore-containing toluene was aspirated and the absorbance was measured at 520 nm using a spectrophotometer (*Shimadzu UV-160*, Tokyo, Japan).

Malondialdehyde (MDA) content was determined according to Heath and Packer (1968). Fresh plant material (1 g) was homogenized in 5 cm^3 of 0.1 % (m/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000 *g* and 25 °C for 5 min. TCA (20 %, m/v; 4 cm^3) containing 0.5 % (m/v) thiobarbituric acid (TBA) was added to 1 cm^3 of the supernatant. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. The content was centrifuged at 10 000 *g* and 25 °C for 10 min. The absorbance of the supernatant was measured at 532 nm. The value for a nonspecific absorption at 600 nm was subtracted. The content of MDA-TBA complex was calculated using the coefficient of absorbance of 155 $mM^{-1}cm^{-1}$.

Phenolics were extracted with 80 % (v/v) methanol at 70 °C for 3 h (Niknam and Ebrahimzadeh 2002). The suspensions of methanolic extraction were filtered; the methanol was removed by vacuum distillation. Then the residues were dissolved in 3 cm^3 of distilled water and the aqueous solutions were used for quantitative determinations. Phenolics content was assayed using the Folin-Ciocalteu reagent according to Dewanto *et al.* 2002. An aliquot (0.125 cm^3) of aqueous extract was added to 0.5 cm^3 of distilled water and 0.125 cm^3 of the Folin-Ciocalteu reagent. After 3 min, 1.25 cm^3 of 7 % (m/v) Na_2CO_3 solution was added and the final volume was adjusted to 3 cm^3 with distilled water. After incubation at 23 °C in dark for 90 min, the absorbance was measured at 760 nm. Phenolics content was determined using a calibration curve (0 - 400 mg cm^{-3} gallic acid).

For determination of sugar content, 0.2 g of dry powder was extracted using 10 cm^3 of 80 % (v/v) ethanol and the supernatant was collected after two centrifugations at 1 480 *g* for 15 min. The residue from ethanol extraction was subsequently used for polysaccharide (PS) extraction with boiling water (Niknam *et al*. 2004). Soluble saccharides (SS) content was estimated by the method of Dubois *et al*. (1956). Reducing saccharides (RS) were quantified according to Nelson (1944). Oligosaccharides (OS) content was obtained from difference between soluble and reducing saccharides content.

The data were analyzed by the one-way analysis of variance (*ANOVA*) using *SPSS v. 14.0*. The significance of differences was determined according to the Duncan's multiple range test (DMRT). Correlation between parameters was also calculated based on Pearson correlation (2-tailed) using the bivariate analysis. Differences at P values < 0.05 were considered to be significant.

Results and discussion

Nodular calli were produced form corm explants after 8 to 10 weeks on both shoot regeneration and root regeneration media (Fig. 1*A*,C). After four subcultures (four weeks interval), a nodular callus developed *de novo* organs and the roots grew downward and the shoots upward in response to gravity (Fig. 1*B*,*D*).

Histological analysis of a saffron nodular organogenic callus (Fig. 2*A*-*E*) showed an intensively colored

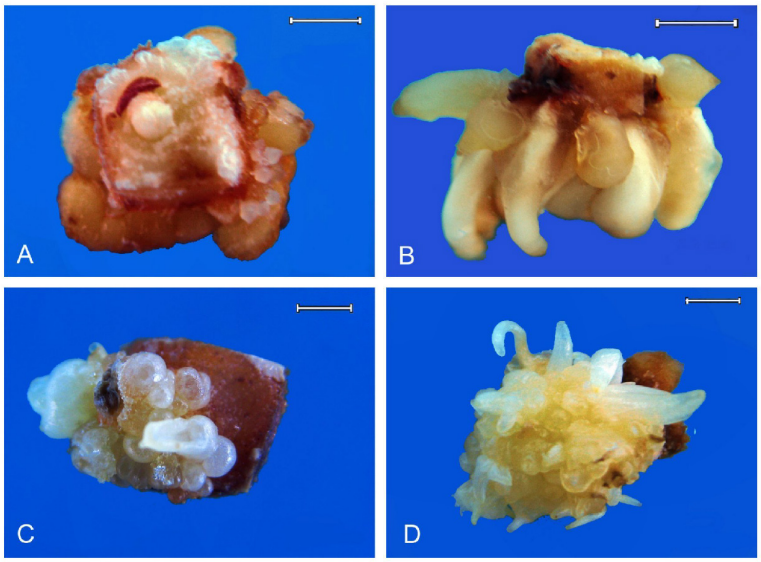

Fig. 1. Root and shoot organogenesis from a nodular callus of *C. sativus*. on MS medium: *A* - Nodular callus 8 to 10 weeks after transfer of corm explants to the MS medium with 1.0 mg dm^{-3} NAA + 2.0 mg dm^{-3} KIN (root organogenesis medium), *B* - *de novo* roots on the lower surface of explants on the root organogenesis medium, *C* - nodular callus 8 to 10 weeks after transfer of corm explants to the MS medium with 1.0 mg dm^{-3} NAA + 1.0 mg dm^{-3} TDZ (shoot organogenesis medium), *D* - *de de novo* shoots on the upper surface of a nodular callus after four subcultures on the shoot organogenesis medium. *Scale bars*: 2.1, 3.2, 2.2, and 2.5 mm in *A*, *B*, *C*, and *D*, respectively.

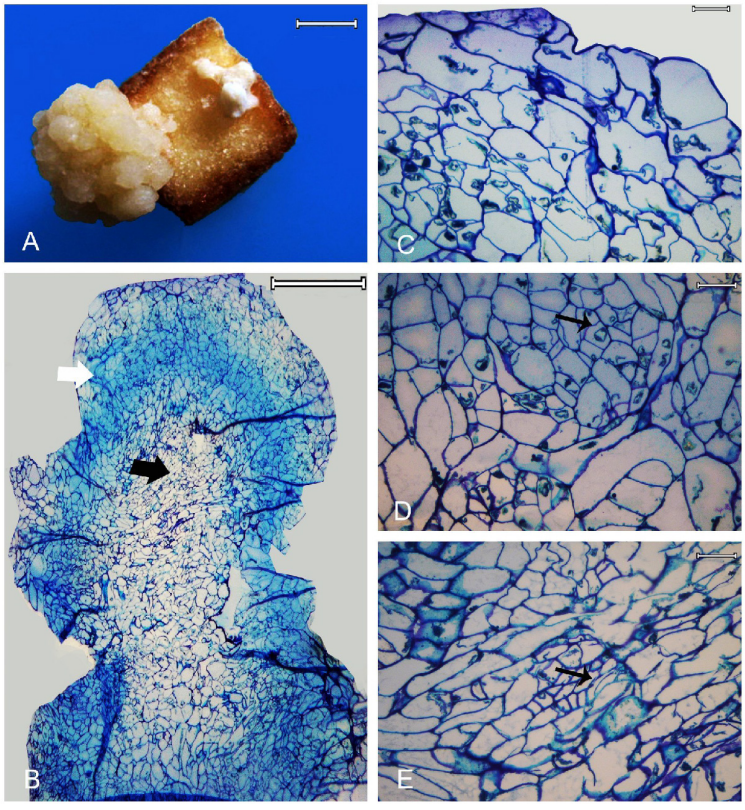

Fig. 2. Longitudinal sections of a nodular callus at various stages of *de novo* shoot development. *A* - Appearance of a nodular callus on upper side of corm explants, *B* - longitudinal section of nodular callus showing a densely stained meristematic zone (*white arrow*) situated at the peripheral zone of the callus and some vascular tissue (*black arrow*), C - vacuolated and large cells of a callus near the meristematic zone, *D* - dividing cells in the meristematic zone, *arrow* shows a meristematic cell in late metaphase, *E* - differentiation of vascular elements near dividing cells (*arrow*). *Scale bars*: 4.3 mm, 200 µm, and 300 µm, in *A*, *B*, and *C* - *E*, respectively.

meristematic zone at its peripheral area. This zone contained small and densely stained cells with small vacuoles and numerous starch grains surrounded with some vascular tissue (Fig. 2*B*). It appeared that the dividing meristematic cells retained the competence to form new shoot primordia in each nodule. Vatankhah *et al.* (2010) reported that regenerated shoot after transfer to MS medium containing 1.0 mg dm^{-3} NAA + 1.0 mg dm^{-3} benzylaminopurine (BAP) under a 16-h photoperiod continued to increase in size, turned green, and formed leaf structures but not roots. Organogenesis has been studied histologically in many plant species (*e.g.* Christianson and Warnick 1983, Reynolds 1989, Burritt and Leung 1996). Organogenesis can occur either directly from the cells of the original explant, or indirectly *via* callus formation. Often, cells associated with the vascular tissues are the origins of meristemoids which give rise to organ primordia. However, according to Tran Thanh Van (1973), even specialized cells, like those of the epidermis, can, if exposed to the right conditions, undergo organogenesis.

During root regeneration in a nodular callus (second stage), some areas of vascular tissues were observed (Fig. 3*A-H*). In other words, each rooting nodule contained a vascular center. Gutmann *et al.* (1996) reported the presence of some areas of vascular tissues in wounded walnut cotyledon during *in vitro* adventitious root formation. It appears that parenchymatic cells in the inner part of the callus were converted to procambium like cells generating the vascular tissues. Bobák *et al.* (1995) reported that dividing parenchymatic cells formed a layer resembling procambium around a vascular bundle in the inner part of the leaf explants of *Drosera rotundifolia* after 72 h of culture. Moreover, Glovannell *et al*. (2004) suggested that groups of dense meristematic cells are located near or along the vascular central tissue during *in vitro* organogenesis from chestnut cotyledon explants. The longitudinal and cross sections of *de novo*

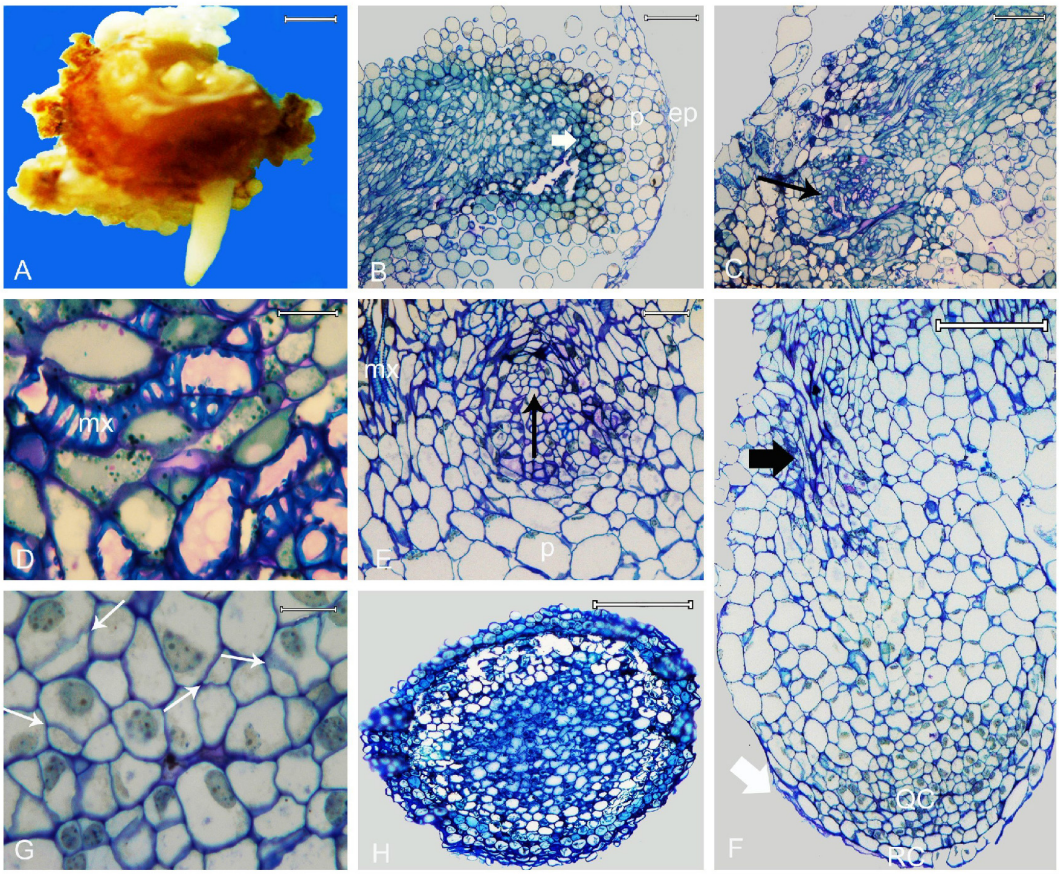

Fig. 3. A nodular callus at various differentiation stages of *de novo* root development. *A* - Appearance of a nodular callus and a newly formed root, *B* - longitudinal section of the apical region of rooting nodule showing a densely stained meristematic zone (*white arrow*), ep: epiderm; p: parenchyma, *C* - differentiation of a vascular center (*black arrow*), *D* - higher magnification of a vascular area and formation of tracheal elements with secondary walls, mx: metaxylem, *E* - vascular area in the cross section of nodular callus with nearby meristematic cells (*arrow*) and large parenchyma cells, *F* - longitudinal section of *de novo* root, *white arrow* shows *de novo* root apical meristem with a quiescent center (QC) and a root cap (RC) and *black arrow* shows newly differentiating vascular elements, *G* - actively dividing cells (*white arrows*) with amyloplasts in *de nove* root apical meristem, *H* - cross section of *de novo* root showing densely stained cells . *Scale bars*: *A* - 4.3 mm, *B*, *C*, *H* - 200 µm, *E* - 60 µm, *D*, *G* - 20 µm, *H* - 2 mm.

Table 1. Changes in the content of proline, phenolics, MDA, polysaccharides (PS), reducing saccharides (RS), soluble saccharides (SS), and oligosaccharides (OS) during root and shoot organogenesis in *C. sativus*. The parameters were measured in corm explants (S_0), nodular calli (S_1), and calli with *de novo* organs (S_2). Means ± SE from three repetitions. Different letters indicate significant differences ($P \leq 0.05$) among individual stages.

Parameters	Units	Root organogenesis			Shoot organogenesis		
		S_0	S_1	S_2	S_0	S_1	S_2
MDA	[nmol g^{-1}(f.m.)]	2.29±0.12c	3.79±0.06a	3.15±0.01b	2.43±0.07b	3.48±0.17a	3.42±0.20a
Proline	[mg g^{-1}(d.m.)]	0.16±0.01c	4.16±0.03b	4.87±0.09a	0.25±0.04b	2.97±0.19a	2.68±0.01a
Phenolics	[mg g^{-1}(f.m.)]	0.76±0.01b	5.00±0.11a	5.01±0.14a	0.92±0.01c	5.41±0.04a	4.52±0.06b
PS	[% (d.m.)]	19.92±0.14b	17.75±0.07c	25.34±0.14a	16.92±0.11b	21.69±0.56a	17.78±0.10b
RS	[% (d.m.)]	3.59±0.08a	0.85±0.02c	1.88±0.03b	3.87±0.07a	1.66±0.01b	1.49±0.04c
SS	[% (d.m.)]	9.41±0.16b	9.89±0.20b	11.59±0.31a	11.20±0.42a	8.41±0.30b	6.61±0.06c
OS	[% (d.m.)]	5.82±0.08b	9.04±0.18a	9.70±0.29a	7.33±0.38a	6.75±0.31a	5.13±0.01b

root are presented in Fig. 3*F* and *H*), respectively, and the root cap, quiescent center, and vascular elements can be seen.

A comparison of the content of metabolites during root *versus* shoot regeneration may identify characteristic compounds specific for shoot or root regeneration. Our results show that the content of all the metabolites changed during organ differentiation (Table 1). Lipid peroxidation during various stages of development was assessed as a MDA content. The MDA content increased continuously from 2.43 to 3.42 nmol g^{-1}(FM) during shoot regeneration. However, the MDA content during nodular callus formation increased from 2.29 to 3.79 nmol g^{-1}(FM) and decreased to 3.15 nmol g^{-1}(FM) at final stages of root development (Table 1). Increased lipid peroxidation found during organogenesis agrees with results reported for other plants (Deighton *et al.* 1997, Adams *et al.* 1999, Blazquez *et al.* 2009). Obert *et al.* (2005) showed that an application of 4-hydroxy-2-nonenal, a cytotoxic lipid peroxidation product, moderated morphogenetic responses and influenced hydroxyl radical formation during *in vitro* propagation from hypocotyl segments of *Linum usitatissimum* seedling. Benson and Roubelakis-Angelakis (1992) suggested that oxidative processes may affect the morphogenic responses of cells grown *in vitro*, and lipid peroxidation was associated with early dedifferentiation in *Vitis vinifera*. Also, Adams *et al.* (1999) expressed that free radical-mediated lipid peroxidation products synthesized by plant tissue cultures exerted both cytotoxic and cytoactive effects. The relative activity of each (toxic or active) may thus be dependent on physiological and developmental status, antioxidants, culture media, age, and stresses. It appears that an increasing MDA content in the differentiation stage is essential for organogenesis.

Accumulation of proline could play an important role in the differentiation and formation of root and shoot meristem during *in vitro* organogenesis in saffron. A significant increase in the proline content was seen during shoot as well as root development as compared to the explant. The proline content in second (S_1) and third (S_2) stages of root organoganesis was higher than that of shoot organogenesis. Positive correlation between the capacity for proline accumulation and *in vitro* morphogenetic processes has been reported by Rastogi *et al.* (2008) and Shriram *et al.* (2008). Moreover, exogenous proline accelerates organ growth and meristem formation and stimulates the expression of the cell cycle-related protein CYCB1 (Mattiolia *et al.* 2009). Proline is also considered to provide organic nitrogen and protect *in vitro* culture against possible stresses (Sen *et al.* 2002).

The content of phenolics also increased significantly during shoot and root regeneration compared to that in the explant (Table 1). There are many reports on the growth regulating properties of phenolics in tissue cultures. The documented effects of exogenous phenols in culture media include an enhancement of callus growth, more effective adventitious shoot formation, improved rooting shoots, and a greater rate of shoot proliferation (Jordan *et al.* 1991, Machackova *et al.* 2008). The preferential oxidation of phenolics by peroxidase prevents peroxidase-catalyzed oxidation of auxin. Through their interaction with growth regulators, phenolics intervene in the regulation of developmental processes such as organogenesis (Kanmegne and Omokolo 2003). Adventitious shoot regeneration from leaflet explants of *Albizia procera* have been related to the changes in phenolic content (Sarkar *et al.* 2010). It seems that the increased content of phenolics during formation of nodular callus is associated with their growth regulating properties, and decreased content during organ formation is associated with lignification during cellular differentiation and organization into shoot primordia.

There were significant positive correlations among proline, phenolics, and MDA during *in vitro* shoot organogenesis (Table 1). Since cell wall phenolics and phenylpropanoids are derived from the erythrose-4-phosphate (the end product of oxidative pentose phosphate pathway; OPPP) *via* the shikimate pathway, they are anticipated to accumulate concomitantly with a proline-mediated increase in OPPP activity (Hare *et al.* 2001). Accordingly, an evidence has been presented for a

link between cycling proline and its precursors with increases in the content of rosmarinic acid and total phenolics in *Origanum vulgare* (Yang and Shetty 1998). The results obtained in the present study provided evidence that the proline and MDA content was correlated with the morphogenetic process in the saffron callus. Numerous experiments have recently been carried out in several plant species with the objective of explaining the role of oxidative stress in plant morphogenesis (*e.g.* Gupta and Datta 2003/4, Meratan *et al.* 2009, Vatankhah *et al*. 2010). The role of ROS and antioxidant enzymes, such as catalase, superoxide dismutase, and peroxidase during *in vitro* organogenesis and somatic embryogenesis in some species has been demonstrated (Vatankhah *et al*. 2010, Jana and Shekhawat 2012, Mitrović *et al*. 2012). According to Blazquez *et al.* (2009), a certain level of oxidative stress is required to promote and trigger morphogenic pathways.

The content of polysaccharides (PS) was higher during shoot regeneration compared to the explants. However, a PS content decreased at the second stage and then increased to the maximum value of 25.34 % (DM) during the final stage of root regeneration. Among the various forms of saccharides, the PS content was higher than that of the other saccharides (Table 1). The content of reducing saccharides (RS) decreased during both shoot and root organogeneses. The content of soluble saccharides (SS) and oligosaccharides (OS) increased significantly during root regeneration, whereas decreased during shoot regeneration (Table 1). The SS and OS content showed a positive correlation with the progression of root organogenesis and a negative correlation with the progression of shoot organogenesis. Many papers have reported an initial accumulation, followed by a decline of saccharides during *in vitro* organogenesis (Chatrath *et al.* 1996, Vaz *et al.* 1998, Wu *et al.* 1999, Kromer and Gamian 2000, Huang and Liu 2002, Panigrahi *et al.* 2007, Sarkar *et al.* 2010). The SS, RS, and OS content decreased during saffron shoot organogenesis and the sugars are important sources of energy and carbon skeletons (Sarkar *et al.* 2010) during shoot primordia differentiation and organization. The PS accumulation in a nodular callus undergoing shoot regeneration is a prerequisite for *in vitro* shoot development (Medina *et al.* 1998). The decline of the PS and RS content and the enhancement of the SS and OS content in the rooting nodular callus might be due to the hydrolysis of reserve PSs and the conversion of RSs to OSs. Also, *de novo* root might take up sucrose present in the medium. According to Huber and Huber (1996), there is evidence that sucrose regulates cellular metabolism, acting at the gene expression level.

The observed significant positive correlation between proline and OS indicates metabolic changes during saffron root organogenesis. Changes in glucose-6-phosphate dehydrogenase activity have been suggested as a reliable marker for determining the regenerability or recalcitrance of plant callus tissues (Gahan *et al.* 1997). Also, this enzyme activates OPPP which supplies NADPH for proline biosynthesis.

In conclusion, the present study revealed the association of biochemical changes with *in vitro* root and shoot organogenesis in saffron. According to the results, a higher content of MDA, proline, and phenolics could possibly explain that the onset and differentiation of organogenesis require a certain level of oxidative stress. Moreover, the present work demonstrates the involvement of saccharides in root and shoot differentiation.

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