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CHANGES OF ANTIOXIDANT ENZYME ACTIVITIES AND ISOENZYME PROFILES DURING *IN VITRO* SHOOT FORMATION IN SAFFRON (*CROCUS SATIVUS* L.)

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Among the different concentrations of Thidiazuron (TDZ) and between the two media Gamborg (B5) and Murashige and Skoog (MS), the highest frequency of shoot formation could be seen in the MS medium with TDZ concentration of 4.54 μM. Among the different concentrations of Naphtalene acetic acid (NAA) and Benzyl adenine (BA) in the two aforementioned media, the maximum proliferation and rooting of saffron shoots were obtained in a B5 medium containing 2.22 μM NAA and 2.68 μM BA. Peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), esterase (EST) and polyphenoloxidase (PPO) measurements proved that all the enzymes had a similar pattern of changes, according to which their concentrations increased in the first stages of development and then decreased. The same pattern was observed for polyphenoloxidase in a B5 medium while in the MS medium a reverse pattern was observed. The enzyme concentration decreased and then increased during shoot formation. The results show the principal role of antioxidant enzymes in the complicated process of organogenesis.

Keywords: Crocus sativus L. – saffron – shoot formation – antioxidative enzyme – protein

INTRODUCTION

Saffron (*Crocus sativus* L.) is one of the most valuable crop species worldwide and is the only plant whose product is sold in grams. The three-branched stigma of *Crocus sativus* flowers, economically the most important part of plant, is known as saffron [17]. Several hundreds of the flowers are needed to produce one gram of saffron. Cultivated saffron is of great value throughout the world. It is widely cultivated in Khorasan province of Iran. Historical evidences indicate that either in the past or present, Iran was the home of cultivated saffron [25]. Saffron is a sterile triploid plant and therefore, corms are used for its vegetative propagation. Bacterial, fungal and viral diseases usually infect corms and remain active after the harvest. Despite care and sanitation, these pathogens are the main cause of necrosis in corms and young leaves, and consequently decrease the flowering. Plants infected by fungal or bacterial pathogens could be treated with appropriate chemicals but such treatments

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are not effective in viral infections. Meristem tip-culture and plant regeneration from the cultured tissues is the only way to produce pathogen-free saffron.

Tissue culture technique, on the other hand, has been used for many geophytes including saffron. This technique is based on totipotency or the ability of plant's intact cells, tissues and organs to develop new organs or somatic embryos when grown in a specific culture medium [4]. A variety of gene expressions and protein syntheses are involved in the shoot organogenesis that are biologically complex developmental and differentiation processes [28]. At optimal concentrations, reactive oxygen species (ROS) play a positive role in the plant's normal development and response to the environmental stresses [5]. Isozymes have a number of roles in the growth and development of plants. Isozyme analyses at different cultural stages might throw light on the physiological, biochemical and genetic changes during differentiation. Thus, activity changes in some antioxidant enzymes and esterase during organogenesis were monitored.

Thidiazuron (TDZ), a non-purine phenylurea derivative, is widely used for plant organogenesis and somatic embryogenesis [27]. Peroxidase (POD) is a multifunctional enzyme and its activity has been suspected to occur in auxin catabolism. Different molecular forms of peroxidases take part in growth control, development, differentiation and morphogenesis. Superoxide dismutase (SOD) is a metaloprotein, catalyzing the dismutation of superoxide radicals to hydrogen peroxide and oxygen [30]. Under normal conditions, the resulting H_2O_2 is effectively scavenged by catalases (CAT) and peroxidases(POD). Superoxide radicals can be formed in most cellular compartments enzymatically by autoxidation of several substrates. The major sources of superoxide formation are the reducing side of PSI in chloroplasts, and the NADH-oxidoreductase complex as well as the autoxidation of reduced ubiquinone in mitochondria. Furthermore, superoxide radicals are known to be produced by an NAD(P)H-dependent microsomal and peroxisomal electron transport chains and by xanthine oxidases in peroxisomes [7]. Enzymes such as esterases, which hydrolyze ester bonds, generally have a broad spectrum of substrates and act on a wide variety of natural and xenobiotic compounds. Esterase (EST) isoform polymorphism in *Mammillaria gracillis* has been previously studied using 1- and 2-naphthyl acetate as substrates. Esterases are more suitable biochemical markers of developmental processes in the *Mammillaria gracillis* tissue culture than peroxidases, and have a greater number of expressed isoforms [6]. Polyphenoloxidase (PPO) is a nuclear-encoded copper-containing enzyme that is widely distributed in plant species and catalyzes the oxidation of phenols to *o*-quinones. Polyphenoloxidase is localized in the plastids while its phenolic substrates are mainly present in the vacuole.

Plant tissue cultures have been used to study the differentiation of plant cells, since they provide a system in which known growth conditions can be imposed upon a fixed population of cells. Having established the conditions necessary for the induction of differentiation in a callus, it is possible to develop quantitative methods for the estimation of differentiation, using biochemical rather than histological techniques. General metabolic changes have been found to occur in differentiating callus

and some, such as increased enzyme activity, concerned with sugar utilization. These methods, however, indicate a change in the growth pattern of the whole callus rather than the production of a specific cell type [20]. A variety of gene expression and protein synthesis procedures are involved in the shoot organogenesis, which is a biologically complex developmental and differentiation process [28].

Our study has focused on adventitious shoot induction from corm explants of saffron, for which the choice of growth regulators and media has been examined. Here, we report the relationship between total protein, peroxidase, polyphenoloxidase, catalase, superoxide dismutase, esterase activities and shoot formation. Different patterns of isozyme expression were also found for these enzymes.

MATERIALS AND METHODS

Plant media

The basal salts including vitamins of MS [22] and B5 [10] media were used in this study. Plant media were enriched with 30 g/l (3% w/v) sucrose and 7 g/l (0.7% w/v) agar (BactoAgar®-Difco Laboratories), as the solidifying agent, pH was adjusted to 5.7 and the plant hormones, in a stock solution of DMSO (dimethyl sulfoxide), were added to it. All plant media, growth regulators and DMSO were purchased from Duchefa (Haarlem, the Netherlands) and Merck (Germany). Depending on the experiment, MS and B5 media were supplemented with indicated amount of the plant growth regulators. For organogenic callus induction in MS and B5 media, 1.13, 4.54 and 9.08 μM TDZ, and 2.22, 8.87 and 17.75 μM BA were added as the growth regulators. For shoot growth and proliferation of calli, the following combinations of NAA and BA were used in MS or B5 media: 2.22 μM NAA and 2.68 μM BA, or 4.44 μM NAA and 5.37 μM BA, or 8.88 μM NAA and 10.74 μM BA.

Plant materials

Healthy resting corms were collected between August and October 2006, from the research farm of Faculty of Sciences, University of Tehran, Mardabad, Karaj, Iran. Corms were washed in the running water for 30 minutes; surface disinfected with detergent (dish washing liquid), soaked in Hygen (benzalconium chloride 1%) for 10 minutes and rinsed in tap water. Corms explants were transferred into a sterile laminar air flow cabinet. Incubation was done in 70% ethanol for 2 minutes and then in 20% v/v commercial bleach, containing 1% sodium hypochlorite, for 15 minutes and rinsed thrice in sterile distilled water. A rectangular section, from the central meristematic region of each corm, was isolated as a starting material. Experiments were done in two series. For each experiment, 25 corm explants, per treatment, were placed on shoot-inducing media and incubated in dark at 25 ± 3 °C for 14 weeks to allow callus induction. Explants with induced shoots were then transferred into jars,

containing shoot growth media, and maintained under 16/8 h photoperiod for further growth.

Nine different samples: 1. corm explant after sterilization and before exposure to the medium culture, 2. Nodular callus from B5 medium containing 4.54 μM TDZ, 3. Nodular callus from MS medium containing 4.54 μM TDZ, 4. Nodular callus with primary shoots from MS medium containing 4.54 μM TDZ, 5. Proliferated nodular callus from MS medium containing 2.22 μM NAA and 2.68 μM BA, 6. Proliferated nodular callus from MS medium containing 8.88 μM NAA and 10.74 μM BA, 7. Proliferated nodular callus from MS medium containing 4.44 μM NAA and 5.37 μM BAP, 8. Proliferated nodular callus from B5 medium containing 2.22 μM NAA and 2.68 μM BA, 9. Developed shoots from MS medium containing 4.44 μM NAA and 5.37 μM BAP includes 5 different developmental stages (Stage 1: sample 1; Stage 2: samples 2 and 3; Stage 3: Sample 4; Stage 4: samples 5, 6, 7 and 8; Stage 5 sample 9) for protein and enzyme studies.

Protein extraction, assay and electrophoresis

Samples were frozen in liquid nitrogen, crushed and homogenized with an extraction buffer, containing 50 mM Tris, 10 mM EDTA, 2 mM $MgSO_4$ and 20 mM DTT (dithiotreithol) or cysteine [15]. Glycerol (10% v/v) was added to increase the viscosity. Extraction buffer (1.5 ml) was poured on 1 g of the tissue. The samples were centrifuged twice for 30 min at 4 °C. The supernatants were stored at –70 °C for some months. Samples for enzyme analysis were prepared from the same samples as for the protein analysis. Protein contents were determined according to the Bradford method [8].

For uploading extracts (11 μg of the protein per sample) in each lane of electrophoresis, the protein samples were mixed with equal volumes of sample buffer, containing 2.5 ml of 0.5 mM tris-HCl buffer (pH 6.8), 4 ml of 10% SDS (sodium dodecyl sulfate) solution, 2 ml glycerol, 0.5 ml 2-mercaptoethanol and 1 ml distilled water. The mixture was heated at 100 °C for 3 min. before separating on SDS-PAGE (12%) using the method [19] and stained with Coomassie Brilliant Blue R250. Molecular sizes of the protein bands were estimated using Page Ruler^{TM} Prestained Protein Ladder of Fermentas.

Enzyme activity

Superoxide dismutase (SOD) activity was measured as described by Genkov and Ivanova [12]. The 3 ml reaction mixture consisted of 75 μM riboflavin, 75 μM Nitro Blue Tetrazolium (NBT), 13 mM methionine and 50 mM phosphate buffer (pH 7). SOD activity was expressed as unit per min per gram of fresh weight.

Peroxidase activity was determined according to Abeles and Biles [1]. The reaction buffer solution contained 0.2 M acetate buffer (pH 4.8), 0.3% H_2O_2 and 0.02 M benzidine in 50% methanol. The reaction started by the addition of the protein extract

to the reaction buffer solution. The activity was calculated from the absorbance changes at 530 nm.

Polyphenoloxidase (PPO) activity was determined spectrophotometrically by increasing the absorption at 430 nm. The reaction was performed in 200 mM phosphate buffer (pH 7.6), containing 20 mM pyrogallol and 90 µl extract at the final volume of 1 ml [26].

Catalase activity was measured according to the Aebi [3]. The reaction buffer solution consisted of 0.05 M phosphate buffer (pH 7) and 3% H_2O_2. The reaction initiated by the addition of 30 µl of the protein extract to the reaction buffer solution. The absorbance was measured at 240 nm and the activity was expressed in unit. mg protein^{-1} min^{-1}. The unit of activity was defined as 1 µmol of H_2O_2 decomposed per min.

The esterase activity was determined spectrophotometrically at room temperature (23 ± 1 °C) by measuring the increase in absorbance at 322 nm (for 1-naphthylacetate) and 313 nm (for 2-naphthylacetate). The reaction solution contained 750 µl of 0.1 M tris-HCl buffer (pH 7.4) and 15 µl of 100 mM 1-naphthylacetate or 30 µl of 2-naphthylacetate, dissolved in absolute methanol. Crude extract (100 µl) was used throughout the experiment [6].

Enzyme electrophoresis

Enzyme samples were loaded onto vertical PAGE gels: 12% separation gel and 4% stacking gels. Constant voltages of 200 V, for the stacking gel, and 220 V, for the separation gel, were applied.

Enzyme activity staining

For superoxide dismutase the incubation was performed for 30 min in a dark place in a mixture containing 20 mg NBT, 4 mg Na-EDTA, and 4 mg riboflavin in 100 ml of a 0.2 M tris-HCl at pH 8.0 Wendel and Weeden [33]. To discriminate between several isoforms of SOD, the gels were incubated prior to staining with a 5 mM solution of H_2O_2 to inhibit both Cu/Zn-SOD and Fe-SOD, or with a 3 mM solution of KCN for selective inhibition of Cu/Zn-SOD [7].

For peroxidase, the gel was incubated in 80 ml of a 0.2 M sodium acetate buffer (pH 4.8) in the presence of 4 ml benzidine (0.04 M at 50% methanol) for visualization and 8 ml of 8% H_2O_2 solution as the a substrate [31].

For polyphenoloxidase, incubation was performed in 50 ml of 0.2 M sodium phosphate buffer (pH 6.8), 20 ml of 0.5% L-DOPA, 0.7 ml of 3.5% (w/v) $CaCl_2$ solution [31].

For catalase the gel was incubated in 0.01% H_2O_2 for 10 min, followed by incubation in the mixture of 1% $FeCl_3$ and $K_3Fe(CN)_6$ for 15 min [33].

For visualization of iso-esterases, 50 mg 1-naphthylacetate, 50 mg of 2-naphthylacetate and 100 mg of Fast Blue RR were dissolved in a 0.1 M phosphate buffer (pH 7.6) [9].

Statistical analysis

Mean values of the three replicates of enzyme activities were calculated using the one-way analysis of variance (ANOVA) between averages and Duncan Multiple Range Test (DMRT).

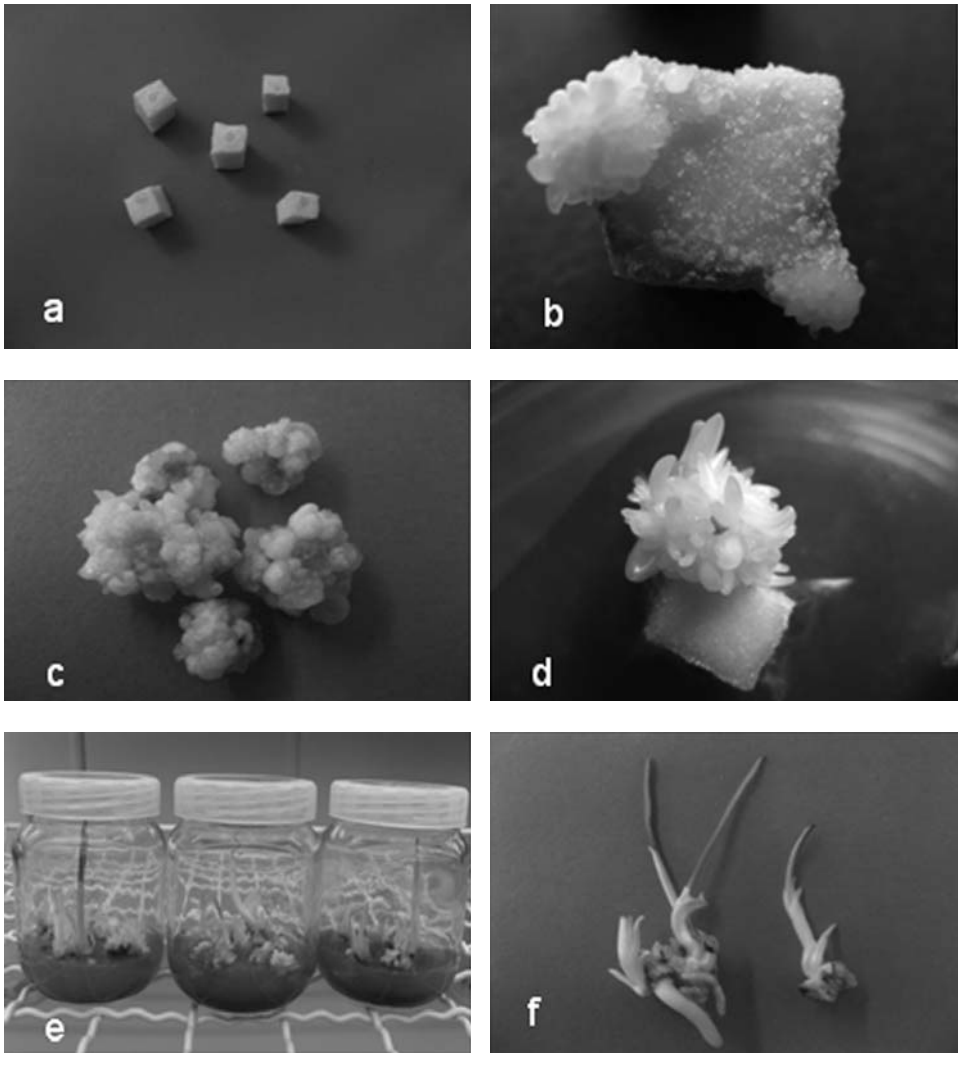

Fig. 1. Different developmental stages leading to regeneration: a – primary corm explants; b – corm explant with nodular callus; c – proliferated nodular callus; d – corm explant with primary shoot; e – developed shoots; f – regenerated plantlets

p values less than 0.05 were considered to be statistically significant. The analyses were performed by SPSS 14.0 for windows, evaluation version. Molecular weight of protein bands were computed using TotalLab version 10.

RESULTS

Tissue culture

According to our previous experiments (data not shown), thidiazuron (TDZ) was more active during shoot induction than BA. MS medium containing 4.54 μM TDZ, and B5 medium with NAA and BA (2.22 μM and 2.68 μM, respectively) were optimum for shoot induction as well as the proliferation and development of nodular calli. So we can see in Fig. 1 that the samples have been chosen from the media containing TDZ. This figure shows the different stages of plant regeneration. Except stage 6 which is a complete seedling and has not been used in biochemical experiments, we have used our samples for the present work from all of the other stages.

Total protein content

The total protein content has a tendency to decrease with the developmental stage of shoot. The highest protein content was observed in the primary explant before culture (sample 1), while the lowest rate was found at sample 3, i.e. nodular callus, from MS in the presence of 4.54 μM TDZ. In the late stages of shoot formation, the protein content increased again (Table 1).

Protein banding pattern

As shown in Fig. 2, among the different protein bands with different molecular weights, only 10 bands showed significant differences in different developmental stages. Two protein bands with molecular weights of 70 and 10 KDa were present in the first three developmental stages and disappeared during the next ones. A 12 KDa protein band was present at the first three developmental stages but diminished at the later stages while protein bands of 7, 22 and 25 KDa were present only in the first stage and then weakened in the next developmental stages. The protein bands with molecular weights of 18 and 19 KDa were significant at third and seventh stages while 22 and 25 KDa proteins were present in the first two stages and then disappeared in the next stages. The protein band of 112 KDa was present in the second developmental stage as well as in 3 and 7.

Table 1
Protein content and enzyme activities during different stages of shoot formation and between different treatments (Samples 1–9)

Sample	Mean concentration of protein (mg/g FW)±SE	Polyphenol-oxidase (μmol/mg protein/min)	Peroxidase (μmol/mg protein/min)	Esterase (μmol/mg protein/min)		Catalase (μmol/mg protein/min)	Superoxide dismutase (unit. mg protein^{-1} min^{-1})
				(1-naphthylacetate)	(2-naphthylacetate)		
1	2.39 ± 0.053^{d}	0.26 ± 0.022^{b,c}	0.14 ± 0.009^{a}	0.01 ± 0.002^{a}	0.10 ± 0.012^{a}	2.17 ± 0.014^{a}	1.21 ± 0.005^{b,c}
2	1.36 ± 0.144^{b,c}	0.16 ± 0.020^{a,b}	9.19 ± 0.427^{d}	0.04 ± 0.002^{a,b}	0.37 ± 0.036^{a}	8.98 ± 0.767^{c}	1.57 ± 0.003^{c}
3	0.88 ± 0.107^{a}	0.48 ± 0.057^{d}	1.11 ± 0.173^{a}	0.08 ± 0.006^{a,b}	2.43 ± 0.719^{b}	14.92 ± 1.044^{e}	1.39 ± 0.018^{b,c}
4	1.17 ± 0.027^{a,b}	0.48 ± 0.052^{d}	7.55 ± 1.012^{c}	0.16 ± 0.029^{b,c}	0.88 ± 0.052^{a}	12.33 ± 0.669^{d}	1.46 ± 0.332^{b,c}
5	1.55 ± 0.028	0.06 ± 0.004^{a}	4.86 ± 0.167^{b}	0.60 ± 0.017^{d}	0.25 ± 0.040^{a}	3.97 ± 0.391^{b}	1.10 ± 0.025^{b}
6	0.99 ± 0.151^{a}	0.40 ± 0.032^{c,d}	6.07 ± 0.430^{b}	1.78 ± 0.121^{f}	2.81 ± 0.203^{b}	8.30 ± 0.135^{c}	0.45 ± 0.003^{a}
7	1.44 ± 0.138^{b,c}	0.18 ± 0.025^{a,b}	4.85 ± 0.075^{b}	0.54 ± 0.023^{d}	0.36 ± 0.006^{a}	2.25 ± 0.314^{a}	0.52 ± 0.003^{a}
8	2.26 ± 0.031^{d}	0.45 ± 0.164^{d}	5.00 ± 0.560^{b}	0.23 ± 0.015^{c}	0.40 ± 0.005^{a}	4.48 ± 0.124^{b}	0.52 ± 0.003^{a}
9	1.47 ± 0.120^{b,c}	0.03 ± 0.009^{a}	1.03 ± 0.196^{a}	0.89 ± 0.032^{e}	0.83 ± 0.008^{a}	1.248 ± 0.47^{a}	0.74 ± 0.035^{a}

Data are means ± SE. Different letters denote a statistically significant difference at ($p < 0.05$), as determined by Duncan Multiple Range Test (DMRT).

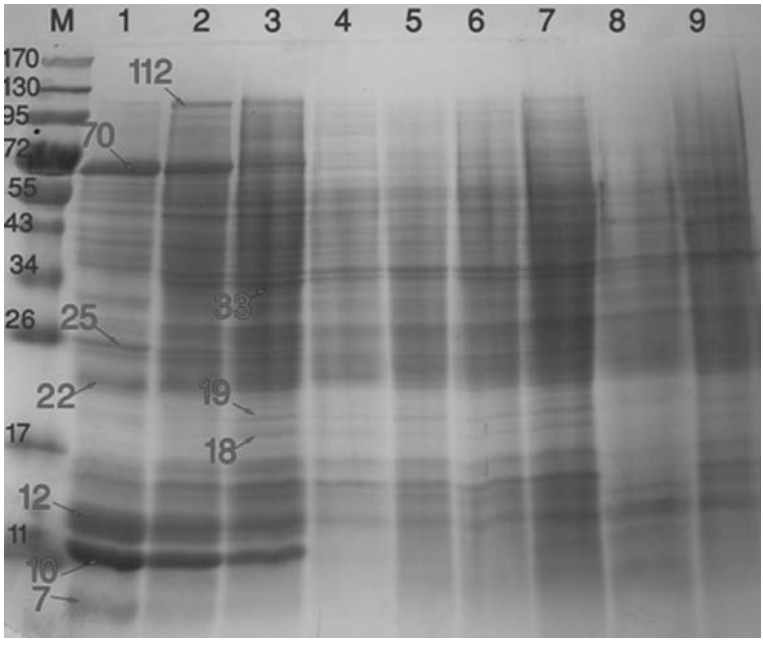

Fig. 2. Protein banding pattern during different different developmental stages of shoot formation (Lanes 1–9). 1. Corm explant after sterilization and before exposure to the medium culture; 2. Nodular callus from B5 medium containing TDZ 4.54 μM; 3. Nodular callus from MS medium containing TDZ 4.54 μM; 4. Nodular callus with primary shoots from MS medium containing TDZ 4.54 μM; 5. Proliferated nodular callus from MS medium containing 2.22 μM NAA and 2.68 μM BA; 6. Proliferated nodular callus from MS medium containing 8.88 μM NAA and 10.74 μM BA; 7. Proliferated nodular callus from MS medium containing 4.44 μM NAA and 5.37 μM BAP; 8. Proliferated nodular callus from B5 medium containing 2.22 μM NAA and 2.68 μM BA; 9. Developed shoots from MS medium containing 4.44 μM NAA and 5.37 μM BAP. includes 5 different developmental stages (Stage 1: Lane 1; Stage 2: Lanes 2 and 3; Stage 3: Lane 4; Stage 4: Lanes 5, 6, 7 and 8; Stage 5: Lane 9)

Enzyme activities

As shown in Table 1, the SOD activity in both B5 and MS media increased at the early stages. There is a significant difference between the proliferated nodular callus grown on the MS medium, containing NAA and BA 2.22 μM NAA and 2.68 μM BA, and the proliferated nodular calli on the MS medium containing NAA and BAP, 4.44 μM NAA and 5.37 μM BA and MS with 8.88 μM NAA and 10.74 μM BA. This significant difference shows the effect of different treatments.

An obvious correlation was observed between the developmental stages and changes in peroxidase activity. In both treatments, the peroxidase activity increased and then decreased during shoot formation. There were no significant differences between proliferated nodular calli in the stages 5, 6 and 7 with different combinations of NAA and BA in the MS medium (Table 1).

Polyphenoloxidase (PPO) activity showed two different patterns during shoot formation. In the B5 medium, the activity decreased and then increased during developmental stages, while in the case of MS medium, the activity increased and then decreased during this period (Table 1). As shown in Table 1, there are significant differences in the activity of polyphenoloxidase in the stages 5, 6 and 7, which are different combinations of BA and NAA in MS medium for callus proliferation and shoot growth.

The activity of catalase increased significantly in the first stages and then decreased. There are significant differences between stages 5, 6 and 7, which are different combinations of NAA and BA in the MS medium for callus proliferation and shoot growth (Table 1).

With 1-naphthylacetate as a substrate, the level of EST enzyme activity gradually increased, but there was a highly significant increase in the sixth developmental stage (Table 1). In the case of the other substrate, 2-naphthylacetate, there was a gradual increase during the shoot formation in the presence of B5 medium (Samples 1, 2 and 8). But in the case of MS medium, there was a significant increase in the first stage, i.e. stage 3 and then a decrease in the enzyme activity during next stages, i.e. stage 4 or stages 5, 7 and 9. For both isoesterases, there is a significant difference between stage 6 that involves proliferated nodular callus on the MS medium containing 8.88 μM NAA and 10.74 μM BA and the fifth and seventh stages, incorporating the proliferated nodular callus in the presence of MS medium with 2.22 μM NAA and 2.68 μM BA and, in MS containing 4.44 μM NAA and 5.37 μM BA, respectively (Table 1).

Isozyme banding patterns

As shown in Table 2, the isozymes 1 to 5 are present in all of the stages. We find in Fig. 3a these isozymes correspond to Mn-SOD. Isozymes 6 and 7 (Fe-SOD) and 11 (Cu-Zn SOD) are present in the first four stages and disappeared in the next five stages. Isozymes 8, 9 and 10 are present in all of the stages except for stage 7 (Table 2 and Fig. 3a).

Seven isozymes for peroxidase were found during this study. Isozyme 1 was only present at stage 4 while isozyme 2 could be seen during all of the developmental stages. POD 3 was present in all of the stages except for 1 and 4 while POD 4 was seen at the stages 1, 2 and 4. POD 5 was observed at the stages 2, 4, 7, 8 and 9. POD 6 was observed at the stages 1, 2, 4, 7, 8 and 9. POD 7 was observed in all stages except for stage 3 (Table 2). The band intensities were low at first stage then increased at stages 2, 3, 4, 5 and 6, but decreased during the later steps of stage 3 (Fig. 3b).

Polyphenoloxidase showed only 1 isozyme (Table 2) and the intensity of this band was different among different developmental stages. As shown in Fig. 3c, this band is very faint in stage 1 and during the next stages; it increased significantly but disappeared in the last stage.

Table 2
Five enzyme multiple molecular forms during shoot formation (samples 1–9)

Isozyme	Rm	Sample								
Superoxide dismutase		1	2	3	4	5	6	7	8	9
1	0.24	+	+	+	+	+	+	+	+	+
2	0.25	+	+	+	+	+	+	+	+	+
3	0.26	+	+	+	+	+	+	+	+	+
4	0.27	+	+	+	+	+	+	+	+	+
5	0.28	+	+	+	+	+	+	+	+	+
6	0.31	+	+	+	+	–	–	–	–	–
7	0.34	+	+	+	+	–	–	–	–	–
8	0.36	+	+	+	+	+	+	–	+	+
9	0.41	+	+	+	+	+	+	–	+	+
10	0.43	+	+	+	+	+	+	–	+	+
11	0.46	+	+	+	+	–	–	–	–	–
Peroxidase										
1	0.05	–	–	–	+	–	–	–	–	–
2	0.08	+	+	+	+	+	+	+	+	+
3	0.09	–	+	+	–	+	+	+	+	+
4	0.10	+	+	–	+	–	–	–	–	–
5	0.13	–	+	–	+	–	–	+	+	+
6	0.15	+	+	–	+	–	–	+	+	+
7	0.16	+	+	–	+	+	+	+	+	+
Polyphenoloxidase										
1	0.12	+	+	+	+	+	+	+	+	–
Catalase										
1	0.06	–	+	+	+	–	–	–	–	–
2	0.12	+	+	+	+	+	+	+	+	+
Esterase										
1	0.02	+	+	–	–	–	–	–	–	–
2	0.04	+	+	–	–	–	–	–	–	–
3	0.09	+	+	–	–	–	–	–	–	+
4	0.10	+	+	–	+	+	+	–	+	+
5	0.15	+	+	+	–	+	+	–	+	+
6	0.18	+	+	+	–	+	–	–	–	+
7	0.20	–	–	–	+	+	+	–	+	+
8	0.37	–	+	+	+	+	+	+	+	+
9	0.40	–	+	–	–	–	+	–	–	–
10	0.46	–	–	+	+	–	–	–	–	–
11	0.47	+	+	+	+	+	+	+	+	–
12	0.48	–	+	–	–	+	+	+	+	–
13	0.49	–	+	–	–	–	–	–	–	–

(+: Isozyme expressed, –: no expression)

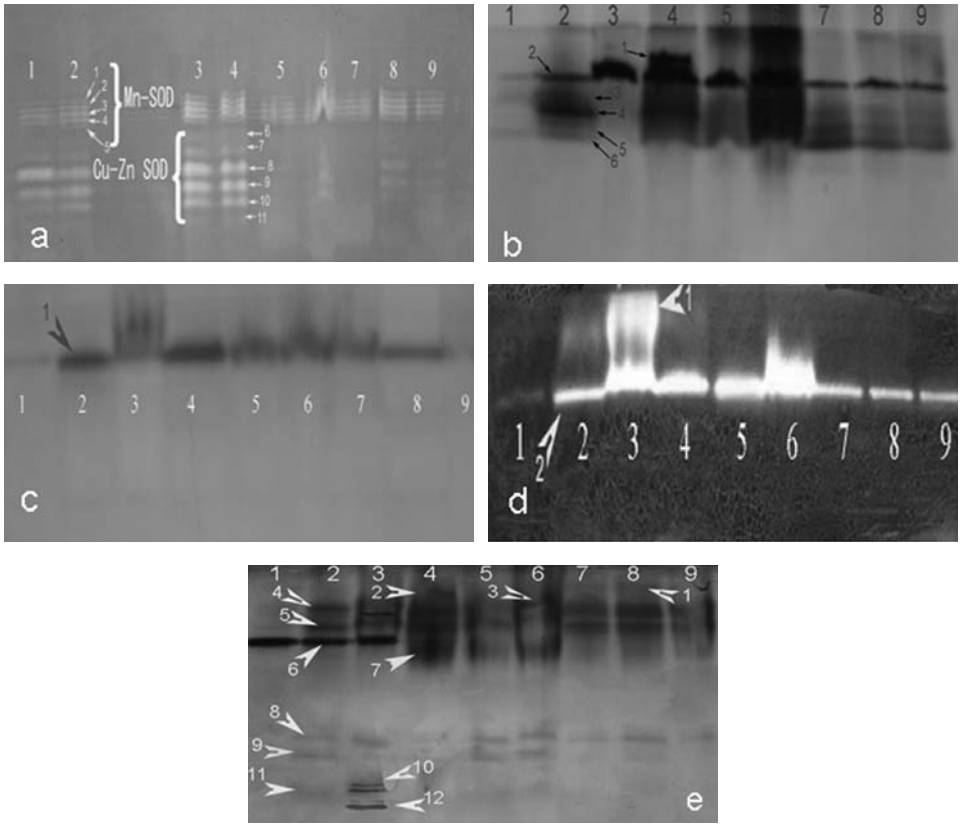

Fig. 3. Isozyme banding pattern during different developmental stages of shoot formation, a: Superoxide dismutase (SOD); b: Peroxidase (POD); c: Polyphenoloxidase (PPO); d: Catalase (CAT); e: Esterase (EST). (Lanes 1–9) 1. Corm explant after sterilization and before exposure to the medium culture; 2. Nodular callus from B5 medium containing TDZ 4.54 μM; 3. Nodular callus from MS medium containing TDZ 4.54 μM; 4. Nodular callus with primary shoots from MS medium containing TDZ 4.54 μM; 5. Proliferated nodular callus from MS medium containing 2.22 μM NAA and 2.68 μM BA; 6. Proliferated nodular callus from MS medium containing 8.88 μM NAA and 10.74 μM BA; 7. Proliferated nodular callus from MS medium containing 4.44 μM NAA and 5.37 μM BA; 8. Proliferated nodular callus from B5 medium containing 2.22 μM NAA and 2.68 μM BA; 9. Developed shoots from MS medium containing NAA 4.44 μM BAP 5.37 μM. includes 5 different developmental stages (Stage 1: Lane 1; Stage 2: Lanes 2 and 3; Stage 3: Lane 4; Stage 4: Lanes 5, 6, 7 and 8; Stage 5: Lane 9)

Zymogram analysis showed that CAT-2 is found in all of the developmental stages. CAT-1 was present only in stages 2, 3 and 4 (Table 2). CAT-2 was very weak in the fist stage and then increased in the stages 2, 3, 4, 5 and 6, but decreased in the last three stages (Fig. 3d).

According to the developmental stage, there are differences between esterase isozyme patterns. Of the total 13 isozymes, the first two were only observed in the

first two stages. EST-3 was present during the stages 1, 2 and 9. EST-4 was seen in all of the stages except the third and seventh ones. EST-5 was detected in all developmental stages except for stages 4 and 7 while EST-6 could be seen in the stages 1, 3, 5 and 9. EST-7 was seen in stages 4, 5, 6, 8 and 9. EST-8 was observed in all stages except the first stage. EST-9 was observed in the second and sixth stage. EST-10 was expressed only in the third and forth stage. EST-11 was present in all the developmental stages except stage 9. Except for stages 1, 3, 4 and 9, EST-12 was expressed in all other developmental stages. EST-13 was only expressed in the second stage (Table 2). The intensity of EST-6 was high in the first three stages and then declined in the next stages (Fig. 3e).

DISCUSSION

Thidiazuron is resistant to oxidases and is stable, but it is biologically more active at low concentrations than the adenine-type cytokinins and BAP [30]. Reports on the effect of different types of cytokinins on enzyme activities are limited. Miller [22] suggested that cytokinins might modify reactions that produce hydrogen peroxide or affect peroxidase directly [21]. Kuroda et al. [19] showed that C4 (R_f 0.4) chloroplast peroxidase was suppressed by kinetin treatment [18]. The growth-promoting activity of thidiazuron was accompanied by high acid phosphatase levels, shown by using polyacrylamide gel electrophoresis [22]. Thus, it can be concluded that the growth effect of cytokinins may be related to changes in the isozyme profiles. Cytokinin-active phenylureas, such as thidiazuron, show many biological properties that are qualitatively similar to those of adenine type cytokinins but also some quite different properties.

In *Solanum nigrum*, the initiation of shoot primordia occurred on B5 medium, supplemented with 0.5 mg/dm^{-3} benzylaminopurine (BAP) or kinetin but BAP was found to be more active. During shoot formation, the increased content and the expression of new isozymes of peroxidase (POD), esterase and malate dehydrogenase was found [14].

Tian et al. [30] showed that SOD activity gradually increased during the 10-day regeneration culture period and declined thereafter [29]. CAT activity constantly declined during the entire culture period, while the POD activity decreased during the 5-day culture period and gradually increased in the later stages of shoot bud formation. Our results, on the changes in SOD, are in accordance with this, regarding the fact that we had an increase followed by a decrease in SOD activity during different developmental stages.

Tang and Newton [29] showed that the peroxidase activity is minimum in the 5th to 6th week of culture, while the catalase activity is lowest in the 7th to 8th week after culture on the medium containing TDZ [28]. These results showed that peroxidase and catalase activities are involved in direct adventitious shoot formation of *Pinus strobes* on the medium containing TDZ. Genkov and Ivanova [12] have also demon-

strated that in some cases, the changes in the peroxidase and superoxide dismutase activities go parallel [11]. During the present study, we found that the patterns of changes in peroxidase and superoxide dismutase are similar.

A new band was found in the multiplying shootlets of *Plantago ovata* Forssk when analyzed for superoxide dismutase [24]. Acevedo and Scandalios [2] examined the temporal and spatial patterns of expression in the *Cat* and *Sod* genes throughout the stem development in maize. They found that at stage 1, the catalase activity was low and internodes remained short while at stage 2, the catalase activity dramatically increased and internodes elongated rapidly. They showed that in fully-grown stems, the catalase is localized in the sclerenchyma beneath the epidermis and around the vascular bundles where it may possibly play a role in lignification. In the case of SOD, they observed that all the isozymes were present in the developing stem [2]. These two major antioxidant enzyme systems show differential patterns of expression during stem development in maize. During our study, we also observed an increase in the catalase activity in the first stages of shoot formation while in the case of SOD, only Mn-SOD isozymes were found to be present in all the stages and the expression patterns of the other isozymes are different depending on the developmental stage.

The multiplying shootlets of *Plantago ovata* Forssk showed two new bands for esterase, which were not found in either control or the regenerating plants [24]. In our control sample, esterase showed 7 isozymes and 6 new bands appeared in the next eight developmental stages.

SOD activity directly modulates the amount of ROS and higher SOD activity contributes to detoxification of superoxide [15]. The changes in the pattern of SOD activity in this research show an increase in the SOD activity during the first stages of shoot induction. This increase shows a high amount of ROS in these primary phases and under TDZ treatments, but the decrease of its activity in the next stages showed the reduced content of ROS in the proliferation of calli when treated with NAA, BA and also during the shoot growth.

Catalase and peroxidase also increased in the first stages and then declined in the proliferation stages and late stage of shoot growth. Table 1, Fig. 3b and Fig. 3d show that the patterns of these two enzyme activities as well as superoxide dismutase are similar. However, unlike the other two enzymes, POD showed a correlation between the activity and the medium type. In stages 2 and 3, in which nodular callus are formed under TDZ-treatment at 4.54 μM in the presence of B5 and MS media, the enzyme activity increased, but there was a significant difference between these two stages that showed a strong effect of B5 medium. In the proliferation stages of MS medium (stages 5–7), these activities increased as compared to the previous stage, i.e. stage 3, while in B5 medium, this activity declined as compared to the second developmental stage. This pattern of enzyme-changes showed the effects of plant growth regulators on enzyme activities. During the developmental stage 4, the POD activity also increased. All results prove the high content of ROS in the primary stages of shoot induction, which reduces during the shoot formation. Irrespective of

medium type, a primary increase in catalase activity during the second and third developmental stages and then a decline was observed (Table 1). Catalase and peroxidase play an important role in fine regulation of ROS concentrations in cells. By enhancing the activities of catalase and peroxidase in the primary stages of organogenesis, H_2O_2 is effectively detoxified.

Gupta and Datta [14] showed that the activity of superoxide dismutase (SOD) gradually increased during somatic embryogenesis, while activities of catalase (CAT) and peroxidase (POX) decreased. In contrast, increase in CAT and POX activity and a concomitant decrease in SOD activity were noted during shoot organogenesis. Exogenous application of antioxidants such as glutathione (GSH), α-tocopherol and ascorbate (AA) inhibited somatic embryogenesis but stimulated shoot organogenesis. The frequency of somatic embryogenesis increased with the addition of H_2O_2. However, H_2O_2 inhibited shoot organogenesis.

These results suggest a strict genetic control of saffron shoot formation and enable us to conclude that the developmental processes during organogenesis are finely tuned because certain isozyme bands disappear at different stages of shoot formation while others remain present or reappear during the course of organogenesis.

Tissue culture media and different plant growth regulators besides their positive effects on shoot formation act like oxidative stress factors. Our work suggests that tissue-cultured explants develop antioxidant enzymatic protective system which determine the ability to survive in oxidative stress and up regulation of these enzymes would help to reduce the built up of reactive oxygen species (ROS).

The next step in our experiments will be the transformation of *Crocus sativus* L. We know that browning and necrosis of transformed cells/tissues, and difficulty to regenerate transgenic plants from the transformed cells/tissues (recalcitrance) are common in *Agrobacterium*-mediated transformation process in many plant species. In addition, most crop transformation methods that use Neomycin Phosphotransferase (NPTII) selection produce a significant number of nontransgenic shoots, called "shoot escapes" even under stringent selection conditions. These common problems of plant transformation (browning and necrosis of transformed cells/tissues, recalcitrance, and the occurrence of shoot escapes) severely reduces transformation efficiency. Recent research indicates that reactive oxygen species (ROS) such as superoxide radical (O_2^-), the hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^\bullet$), and the peroxyl radical ($RO_2^\bullet$) which can cause growth inhibition, cell death, or alter plant metabolic pathways leading to poor regeneration of plants and the production of shoot escapes may be playing an important role in tissue browning and necrosis during transformation [9]. So we can examine the role of ROS and antioxidative enzyme activities in *in vitro* recalcitrance and genetic transformation and the opportunities to improve transformation efficiency using antioxidants.

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