Chapter 14

# Plant Antioxidative Enzymes – Case Study: *In Vitro* Organogenesis of Saffron (*Crocus sativus* L.)

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Additional information is available at the end of the chapter

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## 1. Introduction

To tolerate environmental fluctuations and overcome the invasion by pathogens, plant metabolism must be flexible and dynamic. However, biotic and abiotic stresses disrupt the metabolic balance of cells, resulting in accumulation of reactive oxygen species (ROS) and oxidative burst [1]. ROS are produced as unavoidable byproducts of aerobic metabolism. they are known as mediators of various processes including programmed cell death, pathogen defense, and stomatal behavior[2, 3]. Plant cells normally produce ROS, particularly superoxide and $H_2O_2$ as signaling molecules in many processes associated with plant growth and development [4]. Change in steady-state levels of ROS in the cell is perceived by different proteins, enzymes and receptors which lead to the modulation of different developmental, metabolic, and defense pathways[1]. Although ROS are produced during normal metabolic processes but their formation is accelerated under stress conditions. In plant cells, most of these ROS are originated from chloroplasts or peroxisomes, but in non-green tissues or in the dark, mitochondria is the dominant site of ROS production.The lifetime of active oxygen species within the cellular environment is determined by numerous antioxidative systems, which provide crucial protection against oxidative stress imposed by these molecules. The antioxidative systems comprise numerous enzymes (superoxide dismutase, ascorbate peroxidase, catalase, gluthatione reductase, monodehydroascorbate reductase, dehydroascorbate reductase) and various compounds of low molecular weight (ascorbate, gluthatione, tocopherols, carotenoids, phenols) [3, 5,6].

Environmental factors such as low temperature, salinity, drought, high light, and heavy metals may affect the equilibrium between the production and removal of ROS in the cell. Generation of ROS during abiotic stresses is believed to be mediated by photorespiration

reaction and activity of NADPH oxidases[7].Enhanced generation of ROS during stress condition can be viewed as cellular indicators of stress and as signaling molecules involved in signal transduction for the stress response [7].Various studies have been conducted in different plants in order to evaluate the antioxidative systems under different abiotic stresses. In the following I will present the results of some of these studies.

Drought stress induces the generation of active oxygen species which their steady-state levels are tightly controlled in turn by increasing the activity of antioxidative systems[8]. In *Catharanthus roseus* for example it was shown that drought tolerance is mediated by enhanced antioxidant potentials and secondary metabolite accumulation[9]. In addition, It has been shown that some plant growth regulators like methyl jasmonate and uniconazole have inducing effects on the antioxidant system which causes higher drought tolerance in resistant cultivar of *Zea mays*[10].

Change in the activity of antioxidant enzymes such as superoxide dismutase (SOD: EC 1.15.1.1), peroxidase (POD: EC 1.11.1.7), catalase (CAT: EC 1.11.1.6), gluthatione reductase (GR: EC 1.6.4.2) and gluthatione S-transferase (GST: EC 2.5.1.18) were studied under salt stress in two susceptible and tolerant high yielding genotypes of mulberry under salt stress condition. Antioxidative enzymes activities were changed, but the extent of alteration varied between two geneotypes and higher amounts of antioxidative enzymes were observed in tolerant species[11].

Temperature is a key environmental factor that limits the productivity and geographical distribution of plant species. Studies have shown that cold stress changes the oxidative status and modulates the ROS production.Like the previous examples $H_2O_2$ content and activities of peroxidase, ascorbate peroxidase and gluthatione reductase were compared in cold acclimated and non-acclimated plants during freezing stress. It is supposed that cold acclimation induces $H_2O_2$ production, which in turn enhances the activities of antioxidative enzymes, resulting in alleviation of oxidative stress caused by freezing[6].

The effects of heavy metal stress on the activity of antioxidative enzymes superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) have also been studied. The increase in enzyme activities was accompanied with higher tolerance to heavy metal stress[11-13].The deficiency of some elements also causes the changes in the activities of some ROS scavenging enzymes, for example see [14].

Generally, plant antioxidative enzymes are important as a plant defense mechanism against reactive oxygen species. Besides different environmental stresses, reactive oxygen species play crucial roles in different stages of organogenesis and somatic embryogenesis.

In previous study[15] we focused on adventitious shoot induction from corm explants of saffron and reported the relationship between total protein content, peroxidase, polyphenoloxidase, catalase, superoxide dismutase, esterase activities and shoot formation.

Saffron is one of the most valuable crop species world-wide and is the only plant whose product is sold in grams. The three-branched stigma of *Crocus sativus* flower, economically the most important part of plant, is known as saffron [16]. 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 times, Iran has been the home of cultivated saffron [17]. 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 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.

*In vitro* propagation using tissue culture techniques has been used for the production of disease free plants and mass production of many geophytes including saffron. This technique is based on totipotency or the ability of plant cells to develop new organs or somatic embryos when grown in a specific culture medium [18]. A variety of gene and protein expression signatures are involved in the shoot organogenesis that is biologically and developmentally complex differentiation process[19]. At optimal concentrations, ROS play a critical role in the plant's normal development and response to the environmental stresses [20].Isozymes, or isoenzymes, are enzymes that catalyze the same reaction, but exist in multiple molecular forms, possess different properties, and show different tissue distributions[21]. Isozymes are the different gene products. They are usually recognized by the different electrophoretic mobilities they possess. Oxidative enzyme isozymes have a number of roles in the growth and development of plants. Isozyme analysis of some ROS scavenging enzymes during different cultural stages might throw light on the physiological, biochemical and genetic changes throughout differentiation. Thus, changes in activities of some antioxidant enzymes and esterase during organogenesis were monitored.

TDZ, a non-purine phenylurea derivative, is widely used for plant organogenesis and somatic embryogenesis [22]. Peroxidase (POD) is a multifunctional enzyme which known to be involved auxin catabolism. Different molecular forms of peroxidases participate in growth control, development, differentiation and morphogenesis. Superoxide dismutase (SOD) is a metaloprotein, catalyzing the dismutation of superoxide radicals to hydrogen peroxide and oxygen [23]. Under normal conditions, the resulting $H_2O_2$ is effectively scavenged by catalases (CAT) and peroxidases (POD). Superoxide radicals can be formed in the most cellular compartments enzymatically by autoxidation of several substrates. The major sources of superoxide formation are the reducing side of photosystem I (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 [24].

## 2. Plant media

The basal salts including vitamins of MS [25] and B5[26] media were used in this study. Plant media were enriched with 30 g/l (3% w/v) sucrose and 7g/l (0.7% w/v) agar (BactoAgar®-Difco Laboratories), as the solidifying agent, *p*H 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 the induction of organogenic callus 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.

### 2.1. Plant materials

Healthy resting corms were collected between August and October, from the research farm of the faculty of sciences, university of Tehran, Mardabad, Karaj, Iran. Corms were washed under running tap water for 30 minutes; surface disinfected with detergent (dish washing liquid), soaked in Hygen (Benzalkonium chloride1%) for 10 minutes and rinsed under tap water. Corm explants were transferred into a sterile laminar air flow cabinet. They first incubated in 70% ethanol for 2 minutes and then in 20% v/v commercial bleach, containing 1% sodium hypochlorite, for 15 minutes then rinsed three timeswithsterile distilled water. A rectangular section, from the central meristematic region of corm, was isolated as a starting explant. 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 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 NAA 2.22 BA 2.68 μM, 6. Proliferated nodular callus from MS medium containing NAA 8.88 BA 10.74 μM, 7. Proliferated nodular callus from MS medium containing NAA 4.44 BAP 5.37 μM, 8. Proliferated nodular callus from B5 medium containing NAA 2.22 BA 2.68 μM, 9. Developed shoots from MS medium containing NAA 4.44 μM BAP 5.37 μM includes 5 different developmental stages (Stage 1: sample1; Stage 2: samples2 and 3; Stage 3: Sample4; Stage 4: samples5, 6, 7 and 8; Stage 5 Sample 9) were used for protein and enzyme studies.

## 3. Protein extraction and protein assay

Samples were frozen in liquid nitrogen, crushed and homogenized with an extraction buffer containing 50 mMTris, 10 mM EDTA, 2 mM $MgSO_4$ and 20 mM DTT or Cysteine [27]

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 collected and stored at –70 °C until use. Samples for enzyme analysis were prepared from the same samples as for the protein analysis. Protein contents were determined according to the Bradford method [28]. Eleven micro gram of extracts weremixed with equal volume of sample buffer containing 2.5 ml of 0.5 mMTris-HCl buffer (pH 6.8), 4 ml of 10% SDS solution, 2 ml glycerol, 0.5 ml 2-mercaptoethanol and 1 ml distilled water and heated at 100 °C for 3 min then loadedin each lane of SDS-PAGE gels.SDS-PAGEs were run using single percentage (12%) gels. After electrophoresis the gels were stained by coomassie Brilliant Blue R250.

## 4. Enzyme activity

Superoxide dismutase (SOD) activity was measured as described previously [29]. 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 of tissues.

Peroxidase activity was determined according to [30]. The reaction buffer 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 addition of the protein extract to the reaction buffer. The activity was calculated from change in absorbance 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.

Catalase activity was measured according to the [31]. 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-naphthyl-acetate) 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-naphtylacetate or 30 μl of 2-naphtylacetate, dissolved in absolute methanol. Crude extract (100μl) was used throughout the experiment [29].

### 4.1. Enzyme electrophoresis

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

### 4.2. 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 (1990). 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 [24].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 a substrate[29]. 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. 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[32] . For visualization of isoesterases, 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)[33].

## 5. Results

### 5.1. Tissue culture

According to our previous experiments (data not shown), TDZ was more active during shoot induction than BA. MS medium containing 4.54 μMTDZ, 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. All the stages of shoot formation except the last stage which is a complete seedling were used for biochemical studies.

### 5.2. Total protein content

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 (sample1), while the lowest rate was found at sample3, 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).

### 5.3. Enzyme activities

As shown in Table 1, the SOD activity in both B5 and MS media increased at theearly stages. Based on the Duncan Multiple Range Test (DMRT) there is a significant difference between the proliferated nodular callus grown on the MS medium, containing NAA and BA (2.22 μM and 2.68 μM, respectively), and the proliferated nodular calli on the MS medium containing NAA and BAP (4.44 μM and 5.37 μM, respectively) 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

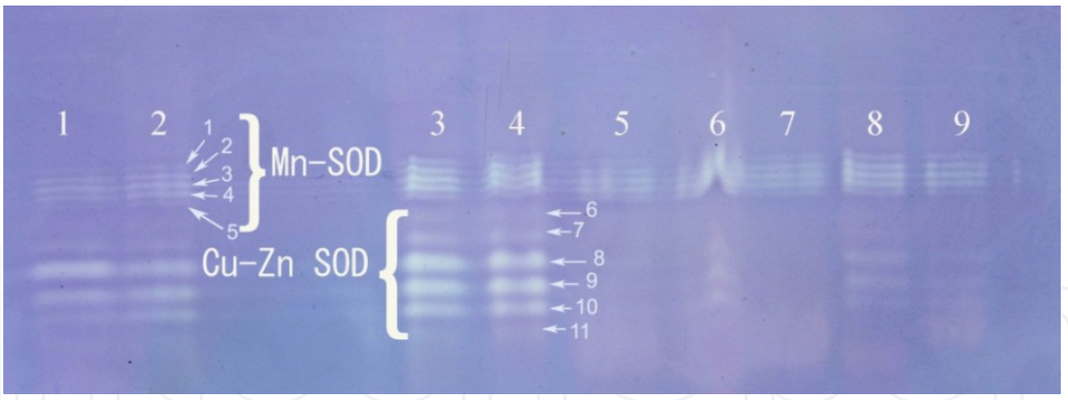
calli in the stages 5, 6 and 7 with different combinations of NAA and BA in the MS medium (Table 1).Polyphenoloxidase (PPO) activity showed two different patterns during shoot formation. In the B5 medium, the activity decreased and then increased during developmental stages, while in the case of MS medium, the activity increased and then decreased during this period (Table1). As shown in table1, 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 combinationsof NAA and BA in the MS medium for callus proliferation and shoot growth (Table 1).

| Sample | Mean Concentration of protein (mg/g FW)±SE | Polyphenoloxidase (unit. mg protein-1.min-1) | Peroxidase (unit. mg protein-1.min-1) | Catalase(unit. mg protein-1.min-1) | Superoxide dismutase( unit. mg protein-1.min-1) |
|---|---|---|---|---|---|
| 1 | 2.39±0.053d | 0.26±0.022b,c | 0.14±0.009a | 2.17±0.014a | 1.21±0.005b,c |
| 2 | 1.36±0.144b,c | 0.16±0.020a,b | 9.19±0.427d | 8.98±0.767c | 1.57±0.003c |
| 3 | 0.88±0.107a | 0.48±0.057d | 1.11±0.173a | 4.92±1.044e | 1.39±0.018b,c |
| 4 | 1.17±0.027a,b | 0.48±0.052d | 7.55±1.012c | 12.33±0.669d | 1.46±0.332b,c |
| 5 | 1.55±0.028c | 0.06±0.004a | 4.86±0.167b | 3.97±0.391b | 1.10±0.025b |
| 6 | 0.99±0.151a | 0.40±0.032c,d | 6.07±0.430b | 8.30±0.135c | 0.45±0.003a |
| 7 | 1.44±0.138b,c | 0.18±0.025a,b | 4.85±0.075b | 2.25±0.314a | 0.52±0.003a |
| 8 | 2.26±0.031d | 0.45±0.164d | 5.00±0.560b | 4.48±0.124b | 0.52±0.003a |
| 9 | 1.47±0.120b,c | 0.03±0.009a | 1.03±0.196a | 1.248±0.47a | 0.74±0.035a |

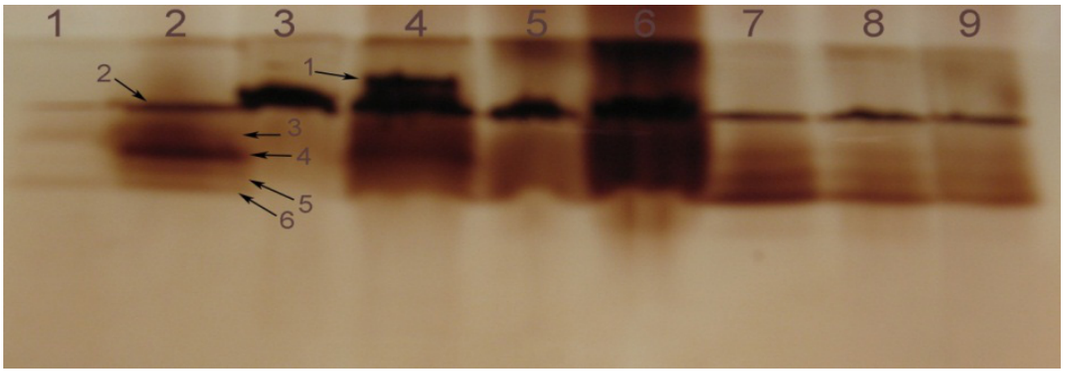
**Table 1.** Protein content and antioxidative enzymes activities during different stages of shoot formation and between different treatments.

## 6. Isozyme banding patterns

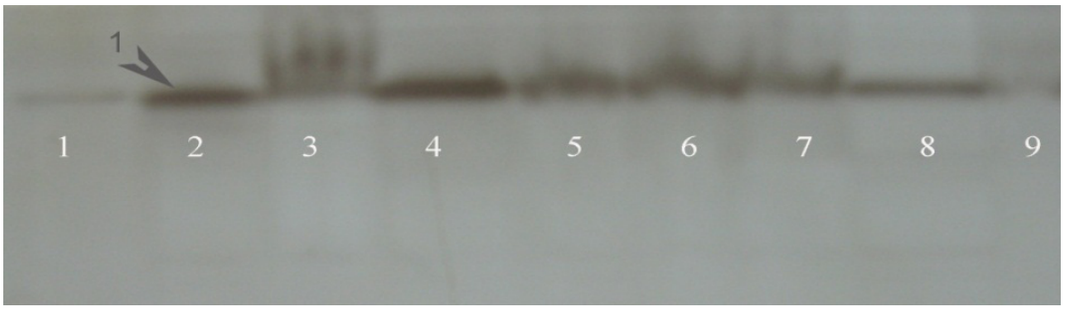
Superoxide dismutase: The isozymes 1 to 5 are present in all of the stages. We find in Fig. 1a these isozymes correspond to Mn-SOD. Isozymes 6 and 7 (Fe-SOD) and 11 (Cu-Zn SOD) are present in the first four stages and disappeared in the next five stages. Isozymes 8, 9 and 10 are present in all of the stages except for stage 7. 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 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. 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. 1b).Polyphenoloxidase showed only 1 isozyme and the intensity of this band was different among different developmental stages. This band is very faint in stage 1 and during the next stages; it increased significantly but disappeared in the last stage (Fig. 1c).



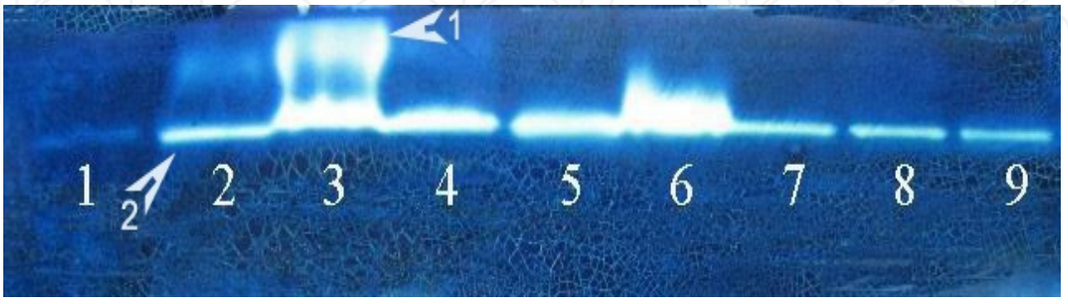

(a)




(b)




(c)




(d)

**Figure 1.** Antioxidative enzyme banding pattern during different developmental stages of shoot formation (samples1–9) 1. Corm explant after sterilization and before exposure to the culture medium; 2.Nodular callus from B5 medium containing TDZ 4.54 μM; 3.Nodular callus from MS medium containing TDZ 4.54μM; 4.Nodular callus with primary shoots from MS medium containing TDZ 4.54

μM; 5.Proliferated nodular callus from MS medium containing NAA 2.22 BA 2.68 μM; 6.Proliferated nodular callus from MS medium containing NAA 8.88 BA 10.74 μM; 7.Proliferated nodular callus from MS medium containingNAA 4.44 BAP 5.37 μM; 8.Proliferated nodular callus from B5 medium containing NAA 2.22BA 2.68 μM; 9. Developed shoots from MS medium containing NAA 4.44 μM BAP 5.37 μM. Includes 5 different developmental stages (Stage1: sample1; Stage2: samples 2 and 3; Stage3: Sample4; Stage4: samples 5, 6, 7 and 8; Stage 5 sample9). Similarly, activity of antioxidant enzyme during *in vitro* organogenesis in *Crocus sativus* L. was studied elsewhere[34].

In recent years, there have been several reports of antioxidative enzymes roles in various plant species in different stages of morphogenesis *in vitro*. For example in *Gladiolus* hybridus,*Acanthophylum sordidum* For more details see[35, 36].Another type of research on plant antioxidative enzymes is the study of subcellular compartments for the activity of these enzymes. For example, it was shown in tomato that the ascorbate-gluthatione cycle enzymes ascorbate peroxidase (APX), monodehydroascorbate reductase (DHAR), gluthatione reductase (GR) and superoxide dismutase (SOD) are present in chloroplast/plastids, mitochondria and peroxisomes of leaf and root cells of both tomato species [37].

## 7. Conclusion

As a whole, capacity and activity of the antioxidative defense systems are important in limiting photooxidative damage and in destroying active oxygen species that are produced in excess of those normally required for signal transduction or metabolism [38]. In addition this system plays crucial role in regulation of organogenesis, somatic embryogenesis and rhizogenesis in plant which is easier for study *in vitro.*

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