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Full Length Research Paper

Interaction of IBA and NAA with enzymes in root induction of *Crocus sativus* (L)

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Effect of auxin type on some antioxidative enzymes during *in vitro* root induction of saffron (*Crocus sativus* L.) was investigated to reveal the variation of activities and isoenzyme banding patterns during root induction of saffron (*C. sativus* L.). Indole-3- butyric acid (IBA) and naphtalene acetic acid (NAA) at different concentrations were added to the solidified MS or B5 medium. Corm explants of saffron were placed on root inducing media and enzyme levels in explants prior or after adventitious root formation were measured and compared. Percentage of root formation on B5 medium containing 2.46 µM IBA was highest; however the maximum number of root per explant and per cm^2 observed on MS medium with 19.6 µM IBA. Significant changes occurred in the activities of these enzymes. Some polymorphisms occurred in patterns of lactate dehydrogenase and esterase during root induction in different treatments.

Key words: *Crocus sativus* L., saffron, root formation, IBA, NAA.

INTRODUCTION

Saffron as the world's most valuable industrial/medicinal plant is an important export commodity and is of great significance in Iran's agricultural economy. Currently Iran produces about 65% of the world saffron and ranked as the largest producer worldwide. Production of saffron has several domestic advantages for the country beside the value gained through its export, including requirement of little water, increasing the rural employment rate, and the plant's high net profit compared to other crops (Kafi et al., 2006). About 95% of the Iranian saffron is cultivated in Khorasan province (Rashed-Mohassel, 2006). Saffron is propagated and cultivated in conventional manner using corms. Roots are generated from corms; three types of roots have been distinguished in saffron:

1.) Absorbing roots, which appear at the base of mother corms. These roots are fine and long.
2.) Short roots, which are thick and short and located individually in the base of the forming daughter corms. These types of roots are formed in corms adjacent to soil surface, and therefore, play an important role in connecting the daughter corms to deeper depths of soil for water and nutrient uptake. The stimulatory factor for formation of these roots is probably light and temperature varies.
3.) Connecting and uptaking roots are formed in mother corms, near the differentiating daughter corms. These roots are thinner and longer than short roots and their formation is not only dependent on light and temperature variations, but also on the presence of short roots (Kafi, 2006).

Direct shoot and root organogenesis are special ways of morphogenesis in plants (Tang and Newton, 2005). Several reports have suggested a link between the activities of certain enzymes and organogenic callus. Peroxidase activity, as a whole, is effective in the regulating the growth processes through the control of endogenous auxin levels and the precipitation in growth inhibition via regulation of cell wall rigidification and

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Abbreviations: **IBA,** Indole-3- butyric acid; **NAA,** α-naphthalene acetic acid; **NBT,** nitro blue tetrazolium; **PMS,** phenazine metho sulphate; **CAT,** catalase; **PPO,** polyphenoloxidase; **PRX,** peroxidase; **EST,** esterase; **LDH,** lactate dehydrogenase and **L-DOPA,** L-dihydroxy-phenyl-alanine.

lignification (Nieves et al. 2003). For lignification, specific cell wall peroxidases are required to generate H_2O_2 and monolignol radicals (Van Huystee, 1987).

Polyphenoloxidase (PPO) catalyzes the oxidation of phenols to o-quinones (Tang and Newton, 2004). One of the main enzymes in the catabolism of hydrogen peroxide is catalase. Catalase is one of the few enzymes that exhibit dual enzyme activity. This enzyme has a hyperoxidase activity when catalyzing the dismutation of hydrogen peroxide into water and oxygen. It plays a specific peroxidase role to protect cells from toxic effects of hydrogen peroxide (Luhová et al., 2003); however, the role of catalase in the rooting process has not been fully studied so far (Molassiotis et al., 2004). In the case of lactate dehydrogenase in roots, a function in the field of pH regulation has been proposed, but more emphasis has been put on malate dehydrogenase, phosphoenolpyruvate carboxylase and malic enzyme as possible pH stabilizers (Betsche, 1981).

The influence of endogenous growth factors, their transport and decomposition, in experiments using intact plants is unknown, and it is likely that these are important in any response to an applied hormone, which could alter the relative amounts of the growth factors within the tissue (Lindsay and Northcote, 1976). The greater efficiency of IBA versus IAA in root formation is probably due to its progressive conversion (β-oxidation) (Gaspar et al., 2002).

The present study showed the effects of NAA and IBA on root induction of saffron corm explants which possibly caused changes of activity of some enzymes. The objective is to reveal the variation of activities and isoenzyme banding patterns of catalase, polyphenoloxidase, peroxidase and also isoenzyme banding patterns of esterase and lactate dehydrogenase at different treatments and in the process of rhizogenesis.

MATERIALS AND METHODS

Plant materials

Healthy resting corms were collected between August to October 2006, from the research farm of Faculty of Science, University of Tehran, Mardabad, Karaj, Iran. Corms were washed in running water for 30 min and after they were surface disinfected with detergent (dish washing liquid) they were soaked in Hygen (Benzalconium Chloride 1%) for 10 min and were rinsed in tap water. Corms explants were transferred into a sterile laminar air flow cabinet and were incubated in 70% ethanol for 2 min, then in 20% (v/v) commercial bleach containing 1% sodium hypochlorite for 15 min and they were rinsed three times in distilled sterile water.

Root induction

Corm explants were cultured on MS (Murashige and Skoog, 1962) and B5 (Gamborg et al., 1968) media containing 2.46, 9.8 19.6 µM of IBA, 2.69, 10.74, and 21.48 µM of NAA, respectively. Explants on media free of growth regulators were also used as control tests. All plant media, growth regulators and DMSO were purchased from Duchefa (Haarlem, Netherlands) and Merck (Germany). All media contained 0.7% agar (BactoAgar®-Difco Laboratories) as a solidifying agent. The pH was adjusted to 5.8 with 1 N NaOH prior to autoclaving at 121ºC for 20 min. After autoclaving of media, plant hormones in a stock solution of DMSO (Dimethyl sulfoxide) were added. 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 root inducing media and were incubated away from light at 25 ± 3°C for 8 weeks.

Protein extraction

Fresh plant tissues was frozen in liquid nitrogen and preserved at -70ºC until extraction. When extracting the proteins, the plant tissues were grounded to a fine powder in a mortar using liquid nitrogen. Then 1 g of the ground tissue was suspended in 1 ml of the extraction buffer that includes a 50 mM Tris buffer plus 10 mM EDTA, 2 mM $MgSO_4$ and 20 mM cysteine or DTT (Dithiotreithol), 10%(v/v) glycerol (Jaaska, 1996). Protein extract was aliquoted in 2 ml Eppendorf tubes. The samples were centrifuged twice, each time at 13000 g for 30 min at 4ºC by a Heraeus 400R Lab fuge,. The resulting supernatant was used for the enzyme activity and protein assay. This extract was stored at -70 ºC for later use or direct analysis.

Protein quantification

Protein concentrations were measured according to Bradford (1976), using bovine serum albumin (BSA) as a standard. 5 ml of the Bradford reagent and 100 µl of the protein extract were mixed and the reaction mixture was incubated at room temperature for 20 min. The absorbance values were measured at 595 nm.

Enzyme assays

In all of the enzyme assays, each value is the mean of three replicates. Catalase (CAT) activity was determined spectrophotometrically at 240 nm, 60 s after addition of 40 µl protein extract to the mixture solution (Aebi, 1974). Polyphenoloxidase (PPO) assay was carried out as described by Raymond et al., (1993). After centrifugation, the supernatant was used for spectrophotometrically measuring the PPO activity at 430 nm and 28ºC. 80 µl of the protein extract was used in this experiment. The activity of the enzyme was defined as the change in absorbance per milligram of the protein per minute. Peroxidase (PRX) activity was determined spectrophotometrically at 530 nm using 50 µl of the protein extract (Abeles and Biles, 1991).

Electrophoretical separation of isoenzymes

Tissue extracts were analysed electrophoretically under non-denaturing conditions using vertical polyacrylamide slab gels with a Laemmli (1970) buffer system. Constant voltages of 200 V for stacking gel and 220 V for separating gel were applied for 6 - 7 h and the temperature was maintained at 4ºC. 11 µg of the protein were loaded onto each lane.

Enzyme activity staining

Staining solutions for the following enzymes were as follows: CAT: (Woodburry, 1971), PPO and PRX: (Van Loon, 1971), EST (Balen et al., 2004) and LDH (Wendel and Weeden, 1990).

Statistical analysis

Percentages were calculated by cross tabulation; chi square test

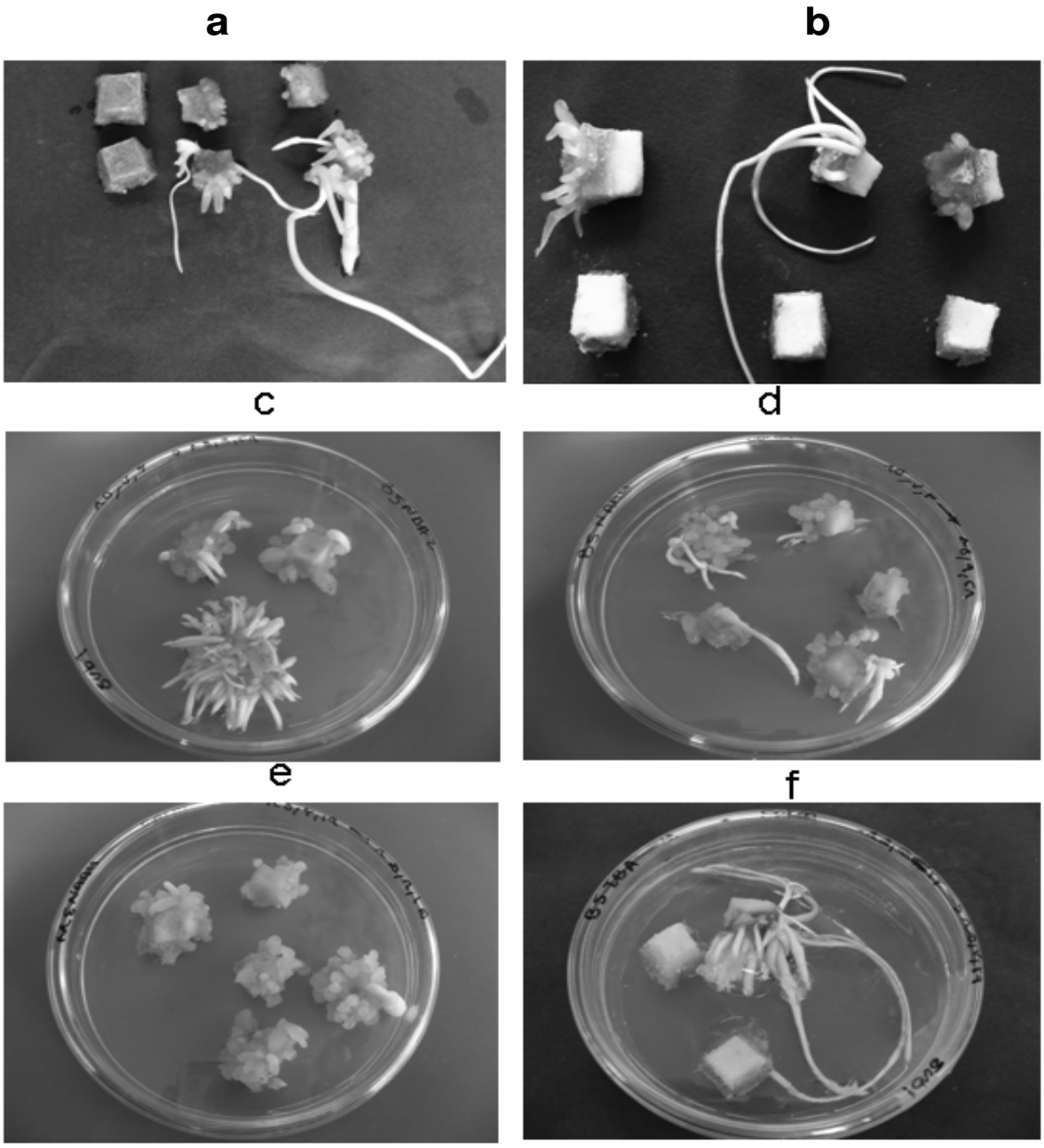

Figure 1. Morphology of root formation under different concentrations of IBA and NAA in B5 and MS media. (a) From left to right IBA 2.46, 9.8 and 19.6 µM on the MS medium. (b) From left to right IBA 2.46, 9.8 and 19.6 µM on B5 medium. (c) NAA 10.74 µM on B5 medium. (d) NAA 21.48 µM on B5 medium. (e) NAA 21.48 µM on MS medium. (f) IBA 19.6 µM on B5 medium.

and the significant differences were detected using Pearson chi square coefficient. Interaction of the factors was recognized by General Linear Model (GLM), Bonferroni test. Mean values of three replicates for the number of roots per explant and enzyme activities were calculated using one-way analysis of variance between averages (ANOVA), Duncan Multiple Range Test (DMRT). Correlation between root percentage, protein content and enzyme activities was calculated using bivariate analysis and based on Pearson correlation ($1^{\text{-tailed}}$). P values less than 0.05 were considered to be statistically significant. The analyses were performed by SPSS 14.0 for windows evaluation version.

RESULTS

Root induction

In Figure 1 some examples of root formation in the different treatments are shown. The effects of tested auxins

Table 1. Root induction in different concentrations of IBA and NAA on B5 and MS media.

Basal medium	Hormone (µM)	Root percentage	Number of root per explant	Number of root per cm^2
MS	0	0	0 a	0a
MS	NAA2.69	26.3	1.7±0.5 a,b	1.7±0.3a,b
MS	NAA10.74	20	2.2±0.5 a,b	3.1±0.8a,b
MS	NAA21.48	50	10.1±1.9 b,c	5.4±0.8b,c
MS	IBA2.46	0	0 a	0a
MS	IBA9.8	6.7	13.6±1.7c,d	17.7±1.9d
MS	IBA19.6	7.1	19.0±0.7d	18.6±1.6d
B5	0	0	0 a	0a
B5	NAA2.69	20	1.3±0.2 a,b	1.7±0.2a,b
B5	NAA10.74	59.5	8.2±2.8a,b,c	5.4±0.9b,c
B5	NAA21.48	26.7	1.3±0.2 a,b	1.5±0.2a,b
B5	IBA2.46	76.9	4.4±1.6 a,b	3.4±1.3a,b
B5	IBA9.8	31.3	6.0±3.3 a,b,c	4.8±2.4b
B5	IBA19.6	23.8	10.2±3.9b,c	9.1±3.9c

[a] Values followed by different letters are significantly different (α=0.05) by ANOVA. Values represent the means± SE. Each treatment was replicated three times.

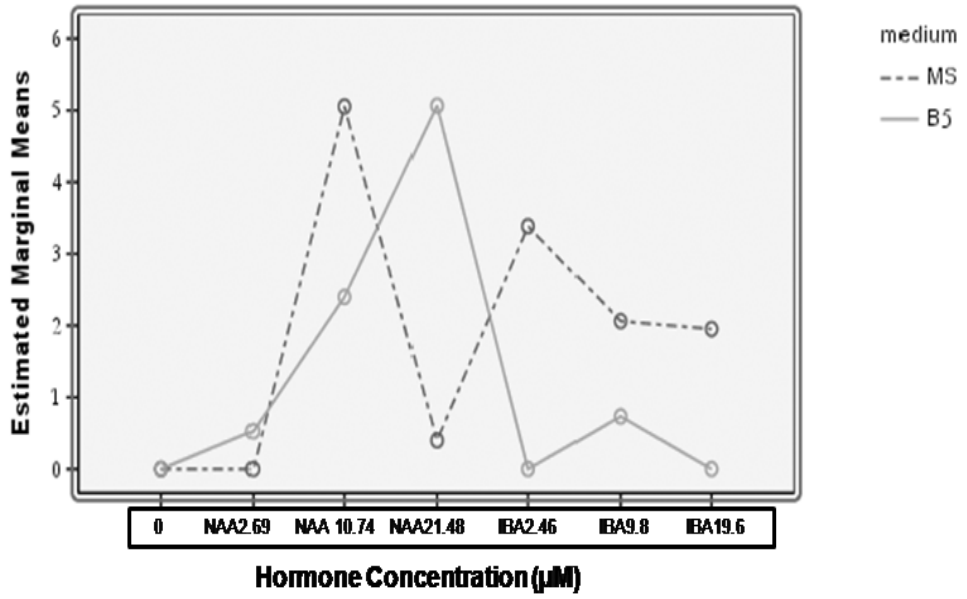

Figure 2. Interaction of medium and hormone and its effect on mean number of root per explant.

and media on root induction have been determined in corm explants of saffron (*Crocus sativus* L.). As shown in Table 1 the maximum percentage of root induction was observed in B5 medium containing 2.46 µM IBA. According to chi square test, there were significant differences between these percentages. According to Duncan Multiple Range Test (DMRT), the maximum average number of root per explant was 19 ± 0.7 and the maximum average number of root per cm^2 was 18.6 ± 1.6 in corm explants grown on MS medium containing 19.6 µM IBA. There were no significant differences between the number of root per explant and per cm^2 in 2.69 µM and 10.74 µM NAA on B5 and MS media but there are significant difference between different concentrations of IBA and 21.48 µM NAA on B5 and MS media (Table 1). The interaction of medium and Hormone type and concentration is shown in Figure 2. There were interactions between medium and hormone in 2.69 µM NAA, 21.48 µM

Table 2. Enzyme activities in different treatments of root formation.

S/N	Polyphenoloxidase (unit/mg protein)	Peroxidase (unit/mg protein)	Catalase (unit/mg protein)	Protein concentration (mg protein/g FW)
1	0.76±0.014$^{b, c, d}$	0.95±0.314^{a}	3.56±0.680$^{b, c, d}$	1.07±0.046 $^{c, d}$
2	0.83±0.015 $^{b, c, d}$	1.45±0.157$^{a, b}$	0.72±0.250^{a}	1.35±0.020 ^{e}
3	1.48±0.104$^{g, h}$	4.01±0.649$^{a, b, c}$	0.53±0.101^{a}	0.84±0.018 $^{b, c}$
4	1.56±0.170^{h}	7.13±2.100^{c}	1.57±0.250$^{a, b, c}$	0.43±0.037^{a}
5	2.87±0.017^{i}	4.83±0.333$^{b, c}$	11.86±1.990^{e}	0.49±0.121^{a}
6	1.36±0.003$^{f, g, h}$	1.85±0.104$^{a, b}$	0.98±0.435$^{a, b}$	0.91±0.258 $^{b, c}$
7	0.64±0.020$^{a, b, c}$	3.98±0.319$^{a, b, c}$	1.86±0.564$^{a, b, c, d}$	1.87±0.119^{f}
8	0.51±0.008$^{a, b}$	1.53±0.162$^{a, b}$	2.06±0.500$^{a, b, c, d}$	2.03±0.076^{f}
9	0.36±0.004^{a}	1.28±0.010$^{a, b}$	0.61±0.174^{a}	1.25±0.081$^{d, e}$
10	0.79±0.124 $^{b, c, d}$	1.47±0.077$^{a, b}$	3.73±0.480$^{c, d}$	0.45±0.027^{a}
11	0.85±0.077 $^{b, c, d}$	3.08±0.998$^{a, b}$	3.84±0.820$^{c, d}$	0.34±0.008^{a}
12	1.43±0.063$^{g, h}$	1.38±0.065$^{a, b}$	18.50±0.288^{f}	0.49±0.018^{a}
13	1.19±0.171$^{e, f, g}$	2.76±0.186$^{a, b}$	1.50±0.215$^{a, b, c}$	0.79±0.038^{b}
14	1.04±0.270$^{d, e, f}$	4.45±3.275$^{a, b,}$	4.24±1.477^{d}	0.53±0.021$^{a c}$
15	0.91±0.020$^{c, d, e}$	1.75±0.085$^{a, b}$	4.33±0.961^{d}	0.85±0.022 $^{b, c}$

1, Corm explant after sterilization and before exposure to the medium culture; 2, B5 control; 3, B5 NAA2.69 µM; 4, B5NAA 10.74µM; 5, B5NAA 21.48 µM; 6, B5IBA2.46µM; 7, B5IBA9.8µM; 8, B5IBA 19.6 µM; 9, MS control; 10, MSNAA 2.69 µM; 11, MSNAA 10.74 µM; 12, MSNAA 21.48 µM; 13, MSIBA 2.46 µM; 14, MSIBA 9.8 µM; 15, MSIBA 19.6 µM.
[a]Values followed by different letters are significantly different (α=0.05) by ANOVA. Values represent the means± SE. Each treatment was replicated three times.

NAA, 2.46 µM IBA and 9.8 µM IBA. (Table 1, Figures 1 and 2)

Protein content

It is apparent in Table2 that the fluctuations in protein content were independent of root percentage. For example as it is shown in Table 2, the maximum protein content is in the sample 8 from induced roots on B5 medium containing 19.6 µM IBA. There were no significant differences in protein content between controls grown on B5 (sample 2) or MS (sample 9) medium. Among three concentrations of NAA, the significant difference between MS and B5 media was found at 2.69 µM of NAA where the protein content is higher in B5 medium. There were significant differences in protein content in all the samples grown on MS or B5 media. In all concentration levels of IBA, the protein accumulation was higher in B5 medium (Table 2).

Enzyme activities

Peroxidase activity in all treatments started to decline after reaching a peak in concentration of NAA at10.74 µM or IBA at 9.8 µM (Table 2). Polyphenoloxidase activity increased with enhanced level of NAA but in contrary declined with increasing IBA concentration (Table 2). All of the treatments led to increase in total activity of CAT, but a high maximum level was observed at 21.4 8 µM of NAA (Table 2).

Correlation between tested factors

There were no significant correlation between root induction and protein content or root induction and enzyme activities. There was a significant negative correlation (-0.53) between protein content and polyphenoloxidase activity. Among the different enzymes, there were two significant positive correlations: 1- between polyphenoloxidase and peroxidase (0.54), 2- between polyphenoloxidase and catalase (0.501).

Protein banding pattern

SDS- PAGE of polypeptides revealed that among the different bands only 20 bands showed differential expression in different treatments. These bands were between 3-71 KDa appeared only in some treatments but the 3 KDa polypeptide only observed in lanes 1, 2, 9 and 13 from the samples without any root formation (Figure 3a). The remarkable point in this gel was the similarity between the polypeptide pattern of lane 1 and lane 13 which were from explants before culture and the explant on MS medium containing 2.46 µM IBA. Possibly lack of root induction could be the reason of this pattern that showed the relation between root induction and pattern of polypeptide expression. The lanes 2 and 9 that were from control samples grown on B5 or MS also had the same patterns.

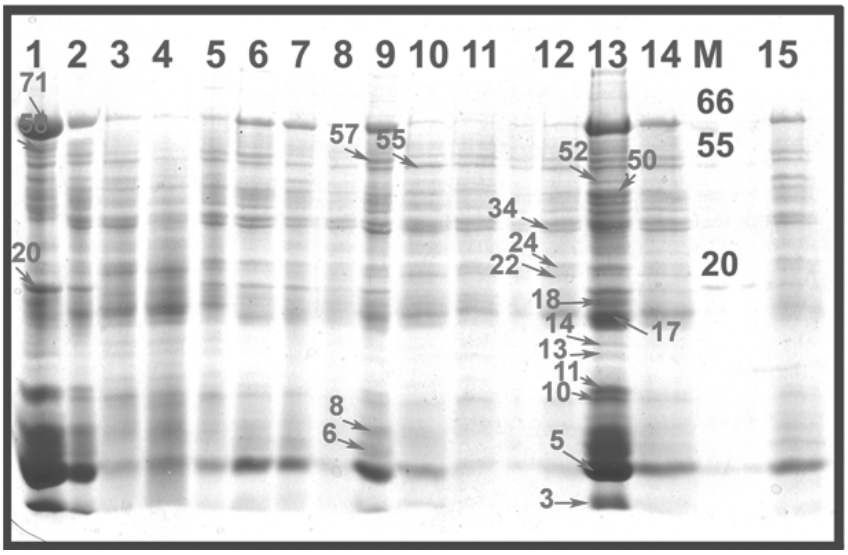

Figure 3. Banding pattern of protein content. 1, Corm explant after sterilization and before exposure to the medium culture; 2, B5 control; 3, B5 NAA2.69 µM; 4, B5NAA 10.74µM; 5, B5NAA 21.48 µM; 6, B5IBA2.46µM; 7, B5IBA9.8µM; 8, B5IBA 19.6 µM; 9, MS control; 10, MSNAA 2.69 µM; 11, MSNAA 10.74 µM; 12, MSNAA 21.48 µM; 13, MSIBA 2.46 µM; 14, MSIBA 9.8 µM; 15, MSIBA 19.6 µM.

Isoenzyme patterns

Esterase showed 7 isoenzymes. Isoenzyme number 1 is present only in the first four treatments. EST 2 and EST7 were observed in 1, 2, 3, 5, 6, 8, 9, 14 and 15 treatments. EST3 is present in all treatments except treatment 8. EST4 was only expressed in treatment 1 which is control. EST5 expressed in all of the treatments except treatment 1 and 8. EST6 expressed in the 3, 4, 8 and 10 treatments (Figure 3b and Table 3).

Peroxidase showed 5 isoenzymes, among them, isoenzymes 1 and 2 were present in all treatments while isoenzyme 3 expressed in treatments 1, 3, 4, 5, 6, 7, 8 and 13 and PRX 4 expressed in 1, 3, 4, 5, 6, 7 and 8 treatments. PRX 5 was observed at 1, 2, 7, 8, 9, 10, 12 and 14 (Table 3). As shown in Figure 3c, the minimum band intensity was observed in the lanes 1, 2 and 9 which were in accordance to Table 2.

Three bands with polyphenoloxidase activity were detected on gels by native PAGE (Table 2). PPO1 were present in all the treatments, while PPO 2 and 3 were only expressed in treatment 13 (Table 3 and Figure 3d).

In our study, 2 major bands with catalase activity were detected (Figure 3e). CAT1 was observed in the 1, 2, 4, 6, 7, 8 and 10 treatments while CAT2 was present in all the treatments (Table 3).

The LDH isoenzymes differed depending on treatments. LDH showed 3 isoenzymes, among them LDH1 was present in all the treatments except lane 1 and 2 which were the primary explant and B5 control. LDH2 was not expressed in the treatments 1, 2, 8, 12, 14 and 15, which were the primary explant, B5 control, B5 with 19.6 µM IBA, MS with 21.48 µM NAA, MS with 9.8 µM IBA and MS with 19.6 µM IBA, respectively. LDH 3 was expressed in all the treatments (Figure 3f and Table 3).

DISCUSSION

Auxin as one of the major determinants for root formation evolves to other biochemical pathways causing various responses in plants (Sauer et al., 2006). We are extensively studying the effect of plant growth regulators on root induction of saffron. We also have studied the different stages of organogenesis and morphogenesis during shoot induction and regeneration of saffron (Data not published). Besides morphological changes in plant, regarding to some reports plant hormones in general and auxins in particular affect on enzymatic activity. Here in a set of experiments, rooting in saffron was induced by various levels of auxins (IBA, NAA) in MS or B5 medium and simultaneously activity of some enzymes were measured and compared to non-induced controls. In this way we can optimize the level of plant hormones and media to minimize unwanted side effects on enzymatic changes.

The response of antioxidant enzymes to different concentrations of IBA and NAA was examined in the rooting explants compared to the primary explants. Hormone metabolism and action cannot be dissociated from the primary metabolic pathways with reciprocal influences. This means that the effects of an externally applied hormone, or of an analogue, cannot be interpreted simply through an increase of its endogenous bulk but that changes in metabolism and the role as an

Table 3. Presence of isoenzymes in different treatments.

Enzyme	Treatment														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
LDH															
1	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
2	-	-	+	+	+	+	+	-	+	+	+	-	+	-	-
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
POD															
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	-	+	+	+	+	+	+	-	-	-	-	+	-	-
4	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-
5	+	+	-	-	-	-	+	+	+	+	-	+	-	+	-
CAT															
1	+	+	-	+	-	+	+	+	-	+	-	-	-	-	-
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PPO															
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
EST															
1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
2	+	+	+	-	+	+	-	+	+	-	-	-	-	+	+
3	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+
6	-	-	+	+	-	-	-	+	-	+	-	-	-	-	-
7	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+

1, Corm explant after sterilization and before exposure to the medium culture; 2, B5 control; 3, B5 NAA2.69 µM; 4, B5NAA 10.74µM; 5, B5NAA 21.48 µM; 6, B5IBA2.46µM; 7, B5IBA9.8µM; 8, B5IBA 19.6 µM; 9, MS control; 10, MSNAA 2.69 µM; 11, MSNAA 10.74 µM; 12, MSNAA 21.48 µM; 13, MSIBA 2.46 µM; 14, MSIBA 9.8 µM; 15, MSIBA 19.6 µM.

exterior signaling molecule have to be considered. Furthermore, experimental results strongly argue that phytohormone control is not only by concentration but also by changes in sensitivity of the cells to the compounds (Gaspar et al., 2002). Liu et al. (1996) studied the effect of exogenous naphtha-lene acetic acid (NAA) on the internal levels of indole-3-acetic acid (IAA) in rooting hypocotyls of *Glycine max.* They resulted that the increased IAA levels with a decrease of IAA oxidase activity accompanied a lower lignin content and a reduced peroxidase activity in NAA-treated tissues and suggested that the induction of adventitious roots by NAA in soybean cuttings may be due to the higher IAA levels accumulated in tissues (Liu et al.,1996).

According to some authors there is no relationship between peroxidase activity and the rooting process while, others stated that peroxidase plays an important role in the regulation of auxin content during the rooting of cuttings (Molassiotis et al., 2004).

The effect of exogenous indole-3-butyric acid (IBA) on the peroxidase activity during adventitious root formation in hypocotyls of *G. max* was investigated. The hypocotyls of IBA-treated cuttings grew significantly higher numbers of adventitious roots with a decline in lignin levels that corresponds with a decrease in peroxidase activity (Chao et al., 2001).

Similar effect was found in this study where the maximum root formation was induced by IBA at 2.46 µM in B5 medium and not by NAA. We found the peroxidase activity was increased when level of both NAA and IBA was increased in media but at higher concentration activity of peroxidase declined.

Li-Ming et al. (2002) showed that peroxidase patterns and transcription in NAA-treated hypocotyls demonstrated the specific effect of NAA on PRX isoenzymes and indicated that NAA suppresses PRX gene expression during the induction of adventitious root. The decline of PRX activity in NAA-treated hypocotyls was accompanied by a decrease in lignin content. They suggest that both anionic and cationic PRXs might be involved in lignin

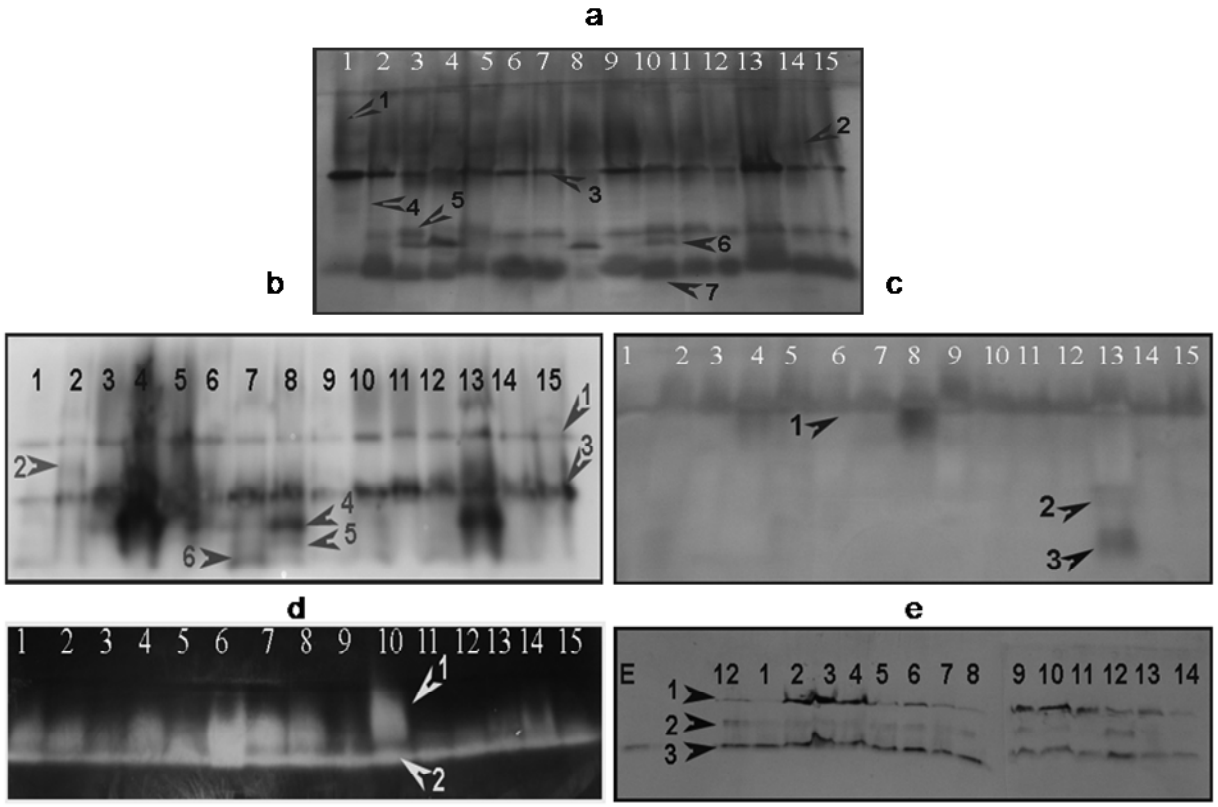

Figure 4. Isoenzyme banding pattern of (a) protein content, (a) esterase, (b) peroxidase, (c) polyphenoloxidase, (d) catalase and (e) lactate dehydrogenase in different concentrations of IBA and NAA in two different MS and B5 basic media. 1, Corm explant after sterilization and before exposure to the medium culture; 2, B5 control; 3, B5 NAA2.69 µM; 4, B5NAA 10.74µM; 5, B5NAA 21.48 µM; 6, B5IBA2.46µM; 7, B5IBA9.8µM; 8, B5IBA 19.6 µM; 9, MS control; 10, MSNAA 2.69 µM; 11, MSNAA 10.74 µM; 12, MSNAA 21.48 µM; 13, MSIBA 2.46 µM; 14, MSIBA 9.8 µM; 15, MSIBA 19.6 µM.

synthesis in soybean hypocotyls (Li-Ming et al., 2002). Such considerable changes in isoenzyme patterns were not observed in NAA treatments, but as mentioned before, its activity declined under high concentration. Gaspar et al. also stated that the variation of peroxidase activity and its secretion along roots are therefore not astonishing (Gaspar et al., 2002).

Habaguchi (1977) reported a significant increase in polyphenoloxidase activity occurs in carrot calli just before or simultaneously with root redifferentiation from the tissue culture cells. The enhancement of polyphenoloxidase activity inherent in redifferentiation (root formation) from calli is mainly dependent on the appearance of other kinds of polyphenoloxidase which are not detected in calli unless accompanying redifferentiation. Investigation using agents which have stimulatory, inhibitory, or repressive effects on redifferentiation shows that the elevation of oxidase activity is closely related to the organ forming capacity of carrot calli, (Habaguchi, 1977). Increasing polyphenoloxidase in accordance to NAA concentration increasing and decreasing it contrary to increase in IBA concentration show the stimulating effect of NAA and suppressing effect of IBA on polyphenoloxidase activity.

The present study has confirmed the role of auxins on induction of rooting ability of *C. sativus* L. and showed during root formation, catalase level increased in all the treatments but enhancement of polyphenoloxidase occurred under NAA treatments.

Increasing hormone concentration causes the accumulation of H_2O_2, which in turn may function in increasing the activity of catalase to prevent high accumulation of H_2O_2 and other ROS in plant cells.

The decreased peroxidase activity after its peak may be due to the decline of some kinds of ROS like peroxide ions.

Among the three isoenzymes for lactate dehydrogenase, only one is present in the first lane which is from the primary explant and the other appeared in the next stages under different concentrations of NAA and IBA.

Total number of isoenzymes in the case of esterase is 7 which in some treatments increased to 6 and in some treatments decreased to 2 isoenzymes. This variation in the number of EST isoenzymes shows the relationship between this enzyme and mechanism of root formation and application of hormones.

This research was multidimensional and follows some goals which are important from different points of view. Since rhizogenesis is one of the most important steps in plant transformation, its study can be beneficial for future work on *Crocus* transformation. On the other hand, each of the aforementioned enzyme systems is essential and their optimum activity is vital for plant life. Plant growth regulator, beside their effects on root formation can change the enzyme activities and thus can have some adverse effects. Studying their activity under different treatments can help to avoid detrimental effect of hyper or hypo activities.

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REFERENCES

Abeles FB, Biles CL (1991). Characterization of peroxidase in lignifying peach fruit endocarp. Plant. Physiol., 95: 269-273.

Aebi H (1974). Catalases. In: Bergmeyer HU, (ed). Methods of enzymatic analysis. Vol. 2. Academic Press, New York, pp. 673-684.

Balen B, Krsnik-Rasol M, Zadro I, Simeon-Rudolf V (2004). Esterase activity and isoenzymes in relation to morphogenesis in *Mammillaria gracillis* Pfeiff. Tissue culture. Acta. Bot. Croa. 63(2): 83-91.

Betsche T (1981). L-Lactate dehydrogenase from leaves of higher plants: kinetics and regulation of the enzyme from lettuce (*Lactuca sativa* L.). Biochem. J. 195: 615-622.

Bradford MM (1976). 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.

Chao IL, Cho CL, Chen LM, Liu ZH (2001). Effect of indole-3-butyric acid on the endogenous indole-3-acetic acid and lignin contents in soybean hypocotyl during adventitious root formation., J. Plant Phys. 158(10): 1257-1262.

Gaspar T, Hausman JF, Faivre-Rampant O, Kevers C, Dommes J (2002). Auxins in the Biology of Roots. In: Waisel Y., Eshel A., Kafkafi L., (eds). Plant roots: The hidden half. Third edition. NY, Dekker, pp. 383-404.

Habaguchi K (1977). Alterations in polyphenol oxidase activity during organ redifferentiation in carrot calluses cultured *in vitro*. Plant Cell Physiol. 18(1): 181-189.

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

Kafi M, Hemmati Kakhki A, Karbasi A (2006). Historical background, economy, acreage, production, yield and uses. In: Kafi M., Koocheki A., Rashed M.H. and Nassiri M (eds.), saffron (*Crocus sativus* L): Production and Processing. Science Publishers, USA, pp. 1-11.

Kafi M (2006). Saffron ecophysiology. In: Kafi M, Koocheki A, Rashed MH and Nassiri M (eds.), saffron (*Crocus sativus* L): Production and Processing. Science Publishers, USA, pp. 39-57.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nat. 227: 680-685.

Li-Ming Ch, Jiin-Tsuey Ch, Ei-Lu Ch, Tien-Joung Y, Zin-Huang L (2002) Naphtaleneacetic acid suppresses peroxidase activity during the induction of adventitious roots in soybean hypocotyls. J. Plant Physiol.159(12): 1349-1354.

Lindsay H, Northcote DH (1976). The influence of gibberellic acid and abscisic acid on cell and tissue differentiation of bean callus. J. cell Sci. 20: 47-55.

Liu ZH, Hsiao ICh, Pan YW (1996). Effect of naphthaleneacetic acid on endogenous indole-3-acetic acid, peroxidase and auxin oxidase in hypocotyl cuttings of soybean during root formation. Bot. Bull. Acad. Sin., 37(4): 247-253.

Luhová L, Lebeda A, Hedererová D, Peč P (2003). Activities of amine oxidase, peroxidase and catalase in seedlings of *Pisum sativum* L. under different light conditions. Plant Soil environ. 49(4): 151-157.

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

Nieves N, Segura-Nieto M, Blanco MA, Sánchez M, González A, González JL, Castillo R (2003). Biochemical characterization of embryogenic and non-embryogenic calluses of sugarcane. In Vitro: Cell. Dev. Plant, 39: 343-345.

Rashed-Mohassel MH (2006). Saffron Botany. In: Kafi M., Koocheki A., Rashed M.H. and Nassiri M (eds.), saffron (*Crocus sativus* L): Production and Processing. Science Publishers, USA, pp. 13-38.

Raymond J, Rakariyathem M, Azanze JL (1993). Purification and properties of polyphenoloxidase from sunflower seed. Phytochem. 34: 927-931.

Sauer M, Jakob A, Nordheim A, Hochholdinger F (2006). Proteomic analysis of shoot-borne root initiation in maize (*Zea mays* L.). Proteomics, 6: 2530-2541.

Tang W, Newton RJ (2004). Increase of polyphenoloxidase and decrease of polyamines correlate with tissue browning in Virginiana pine (*Pinus virginiana* Mill). Plant Sci. 167(3): 621-628.

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

Van Huystee RB (1987). Some molecular aspects of Plant Peroxidases: Biosynthetic studies. Annu. Rev. Plant Physiol. 38: 205-219.

Van Loon LC (1971). Tobacco polyphenol oxidase. A specific staining method indicating non-identify with peroxidase. Phytochem. 10: 503-507.

Wendel JF, Weeden NF (1990). In: Soltis DE and Soltis PS (eds.), Visualization and interpretation of plant isoenzymes, Dioscorides press, Portland OR, pp. 5-45.

Woodburry W, Spencer AK, Stahmann MA (1971). An improved procedure using Ferricyanide for detecting catalase isoenzymes. Anal. Biochem. 44: 301-305.