Progress in Biological Sciences
Vol. 5, Number 2, Summer / Autumn 2015/273-286

Effects of soil conductivity on properties of saffron corms and *in vitro* production of its style explants

Received: February 20, 2015; Accepted: June 15, 2015

Sepideh Mashayekhi, Monir Hosseinzadeh Namin*

Faculty of Science, Alzahra University, Tehran, Iran, P.O. Box: 1993891176

ABSTRACT

Saffron is the dried stigmas of *Crocus sativus* L., a member of the Iridaceae family which is propagated by corms. Corms are faced with many stresses in soil. Therefore, it is important to reduce these stresses and improve the quantity of saffron production. Biotic and abiotic stresses disrupt the metabolic balance of cells; thereby, resulting in accumulation of reactive oxygen species (ROS) which cause oxidative damage. In this study, the effect of soil electrical conductivity (EC) on biochemical indicators of corms, the percentages of callus formation and stigma-like structures (SLSs) on calli were investigated. In order to obtain calli and SLSs, immature style explants from floral buds of corms were collected from three regions (Shahroud, Mardabad and Torbat-e Heydarieh) and used for tissue culture. Style explants were separated first from the immature floral buds, then sterilized and used for tissue culture. Biochemical analysis of calli with SLSs including malondialdehyde (MDA) and proline contents, antioxidant enzymes activities and polysaccharides and reducing sugars contents were investigated and compared. Moreover, sodium and potassium ions content and EC of soils of the three regions were investigated. The results indicated that corms from Shahroud with the highest level of EC soil showed more imposed stress than that from Torbat-e Heidariye and Mardabad but the calli percentage and number of SLSs of Mardabad's were higher than those of the other two respectively. In this study, a close relationship between soil EC and *in vitro* production of saffron with a short glance on epigenetic modification was postulated.

Keywords: *Crocus sativus*, electrical conductivity, epigenetic modification, stigma-like structures, tissue culture

* **Corresponding author:** monirhosseinzade@yahoo.com

Introduction

Saffron (*Crocus sativus* L.) is the world's most valuable industrial and medicinal plant which belongs to the Iridaceae family. It is propagated by means of corms, because it is a sterile plant and cannot produce seeds. Each year, one corm produces 3-10 cormlets. Saffron's purple flower consists of three sepals and three petals which are similar to each other. The flower has three red stigmas and three distinct yellow stamens (1). The soil surrounding the corms influences the biochemical and physiological properties of corms. Salinity is an abiotic stress that affects the plant growth, development and productivity, so it causes a wide variety of physiological and biochemical changes in plant (2). The water available in the salt-contaminated soil increases reactive oxygen species (ROS) production and osmotic stress (2). Growing evidence suggests that ROS at low concentrations act as second messengers (3), but at higher concentrations, lead to a process that is often referred to as "oxidative stress" which, in turn, disturbs cell metabolism (4). Plants use several mechanisms such as production and accumulation of proline and sugars to tolerate stress. Proline accumulation is primarily due to *de novo* synthesis associated with decreased oxidation and utilization (5). In addition, proline plays a role in H_2O_2 scavenging and response to stresses(6). Sugars are compatible solutes which accumulate in plant tissues which are exposed to abiotic stresses such as water deficient, extreme temperatures and salt stress. It was reported that accumulation of sugars under stress conditions might be involved in osmoregulation and energy preservation (7).

Malondialdehyde (MDA) is the natural occurring product of lipid peroxidation due to oxidative damage. Severe stress may cause damage to the structure of unsaturated fatty acids which lead to increases in the permeability of the membrane. Therefore, MDA content resulting from lipid peroxidation is a reasonable indicator to estimate oxidative damage level on membrane which is caused by ROS (8).

ROS are formed in biological systems as part of normal metabolism. To avoid the damage caused by excess ROS, plants have developed antioxidants systems in cells which eliminate or reduce ROS levels. These systems include enzymatic and non-enzymatic systems. Enzymatic systems include superoxide dismutase, peroxidase, catalase, etc. (2).

Due to the widespread medical applications of saffron, traditional production techniques cannot meet its increasing demand. Therefore, biotechnological methods such as tissue culture can be used for its propagation. Stigma-like structures (SLSs) were reported to be induced from almost every part of the floral organs. Sarma et al. (1990) investigated the formation of SLSs as an important step in saffron production by tissue culture, and the effect of factors such as age, explant type and exogenous hormones on the quantity of SLS production (9). They also reported on the culture of saffron style explants on MS medium supplemented with naphthaleneacetic acid (NAA) (10 mg/l) and benzyleadenine (BA) (10 mg/l) to induce the optimum response for producing SLSs. The developmental stages of SLSs on calli have been shown by scanning electron microscopy (SEM) and transmittance electron microscopy (TEM) techniques (10).

Soil EC correlates very well with several physical and chemical properties of soil. Studies have indicated that higher yield of saffron coincides with soil EC which is between 0.09 and 0.30 ds/m^{-1} (11). Researchers showed that low EC is

appropriate for increasing the yield of saffron (12). They demonstrated that a decrease in soil EC level could lead to positive and significant influence on stigma production yield.

The aim of this study was to investigate the effect of soil EC on properties of corms and its attached immature floral buds and subsequently on immature styles which are used as explants for tissue culture, and to evaluate the percent production of calli and SLSs as final yield.

Materials and Methods

Plant materials. Saffron corm with attached immature flora bud and also their surrounding soils were collected from saffron field of three different distinct regions (Torbat-e Haidariye, Mardabad and Shahroud). Then, flora buds were excised from corms and used for tissue culture.

Sterilization of samples. Separated immature flora buds were first kept under running tap water for 30 min and sterilized according to the method of (10). Briefly, the buds were sterilized with 0.5% benzalkonium chloride solution for 15 min, treated first with 70% ethanol for 2 min and followed with 5% sodium hypochlorite solution with few drops of Tween 80 for 20 min. Finally, the buds were washed three times with sterile distilled water. The last two steps were carried out under laminar air flow.

Tissue culture. Under sterile conditions, style explants were separated from immature flora buds, and then cultured on MS medium supplemented with NAA (10 mg/L), BAP (10mg/L) and 30% sucrose (13). Petri dishes which contained six style explants each were kept in continuous darkness at 20±2°C temperature. Samples were sub-cultured on fresh MS medium every 28 days.

Callus and SLSs measurement. The number of induced calli and SLSs were measured during a period of 3-6 months since tissue culture was initiated. Then calli with SLSs were collected, after being frozen in liquid nitrogen, they were kept at -80°C for further analysis. The images of samples were taken by G6 Cannon digital camera from selected samples.

Analysis of exchangeable sodium and potassium of soils. The analysis of exchangeable Na^{+1} and K^{+1} content of soils were carried out according to Mutscher's method (14). Briefly, ammonium acetate (50 ml 1 N) was added to 2.5 g of air-dried and sieved (through 2 mm screen) soils which were collected from three regions, shacked slowly then filtered through a Whatman No.5 filter paper. The filtered solutions of soil samples were kept for 24 h at room temperature before being tested. The exchangeable Na^{+1} and K^{+1} ions in solutions of soils were measured by flame photometer (JENWAY ENGLAND model PEP7).

Soil electrical conductivity (EC). To determine the EC of soils, initially the soil sample (200 g) was saturated with distilled water and mixed to a consistence paste. The sample dish was sealed with parafilm and incubated at room temperature for one hour in order to diffuse water from paste. The electrical conductivity of diffused water from paste was measured by electrical conductivity meter MODEL FE20– FiveEasy™ (15).

Proline assay. For the determination of corms proline content, the method of Bates et al. (16) was used. For proline assay, 0.1 g of fresh corm tissue of three regions were homogenized in 10 mL of 3% sulphosalycylic acid, then the homogenized solution was filtrated and used for assay with Ninhydrin reagent. The absorbance was measured at 520 nm by spectrophotometer.

Lipid peroxidation assay. Lipid peroxidation (MDA) was determined according to the method of Heath and Packer (17). The basis of this method is to estimate the thiobarbituric acid content as reactive substances (TBARS). The MDA content of corm samples were measured at 525 and 600 nm by a spectrophotometer.

Assay of reducing sugars. Reducing sugars content of the corms were extracted by the modified method of Nelson (18). For the preparation of the standard curve, 0.3 µg/mL glucose was used in concentration ranges of 0 to 300 µg/mL. Reducing sugars contents of sample was measured at λ = 500 nm by a spectrophotometer.

Assay of polysaccharides. Polysaccharide contents of the corms were quantified by using phenol/sulfuric acid reagent (19). Also, for standard curve, 80 µg/mL glucose was used for concentrations ranging from 0 to 80 µg/mL. Polysaccharide contents of corm samples were measure at λ = 485 nm by a spectrophotometer.

Protein extraction and determination. Corms were homogenized with sodium phosphate buffer (0.2 M, pH = 6.8) as extraction buffer. Total protein content of samples was determined by the Bradford (20) method. Bovine serum albumin (1 mg/mL) was used to make a standard curve. For loading, 50 µg protein of each sample was used.

Antioxidant enzymes gel electrophoresis assay. To study isozymes in corms, polyacrylamide gel electrophoresis (PAGE) was performed as described by Davis (21). The percent acrylamide gels were for SOD (10%) and for CAT and POX (7.5%).

Peroxidase gel assay. Briefly, according to Van Loon's method (22), the gel was incubated in reaction solution that consist of sodium acetate buffer (0.05 M, pH = 4.8), fresh hydrogen peroxide (0.01%) and benzidine (0.04 M in methanol 50%) for 30 min and kept at room temperature under continuous darkness for 2-3 h, until all isoforms of POX appeared. Then, the stained gel was rinsed with distilled water several times to remove any trace of reaction solution (22).

Catalase gel assay. CAT activity was detected on gel electrophoresis by using the method of Woodbury et al. (23). First, the gel was incubated in 10 mM H_2O_2 for 5 min, washed thoroughly with distilled water and then the gel was transferred to the reaction solution which consists of $FeCl_3$ and $K3[FeCN]_6$ (1%) and kept at room temperature until CAT isozymes were revealed. To remove any excess dye, the gel was washed several times with distilled water.

Superoxide dismutase gel assay. SOD isoforms were determined by the method of Wendel and Weeden (24). Briefly, after incubation, the gel in the reaction solution which consists of 0.2 M Tris-HCl (pH 8.0) buffer, riboflavin, EDTA and NBT was kept for 30 min at room temperature under continues darkness, and transferred to continuous light while slowly shaking till the SOD isoforms appeared. The reaction solution was discarded and the gel was washed thoroughly with distilled water to remove any excess dye.

Note. The model of spectrophotometer that was used for all the assays was the SHIMAZDA model UV-160.

Statistical analyses. All analyses were conducted with SPSS software (Version 15) and the means were compared by the Tukey method (95% confidence interval).

Results

Tissue culture. The first sign of callus induction on style explants were identified as swollen structures at the cut edges of the explants and subsequently turned to colorless globular structures during the period of 1 to 2 months (Fig. 1a). During the period of 2 to 4 months, these colorless globular structures turned to yellow stigma-like structures (SLSs) (Fig. 1b). Finally, the yellow SLSs turned to trumpet-like red SLSs and matured during the period of 4 to 6 months when the explants were cultured (Fig. 1c and d).

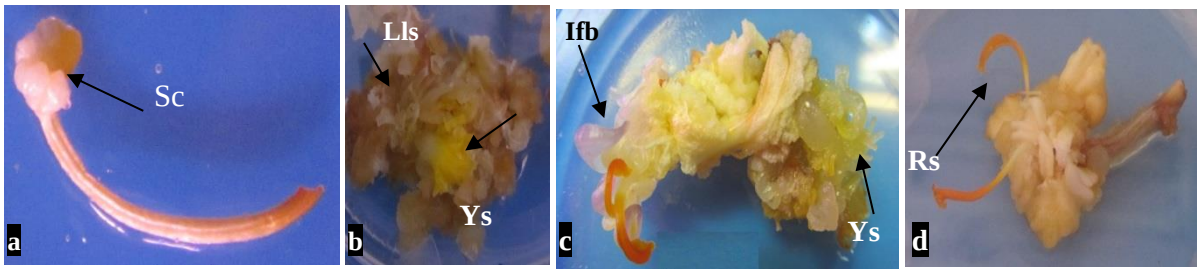

Figure 1. (a) Swollen cut edge of style (Sc), (b) Yellow stigma-like structures (SLSs) on callus of style (Ys) with leaf-like structures (Lls), (c) Incomplete flower bud (Ifb) and (d) Trumpet red stigma-like structures (SLS) on calli of style (Rs)

The percentage of callus and SLSs obtained from immature style explants of the three regions (Shahroud, Mardabad, and Torbat-e Heidariye) were measured for a duration of 6 months from the establishment of the cultures (Fig. 2a). The results showed that there was no statistically significant difference in callus percentage appearance on style explants between three areas during the period of 1 to 3 months, but significant differences ($P< 0.05$) of callus percentages were shown between Mardabad and Shahroud during the 4 months period from when tissue culture was initiated. From the 4^{th} to 6^{th} months, significant differences ($P< 0.05$) of callus percentage among the three samples were observed (Fig. 2a). The results obtained at the end of experiment showed that Mardabad's had the highest callus percentage (96%) in comparison with Torbat-e Heidariye (84%) and Sharood samples (78%).

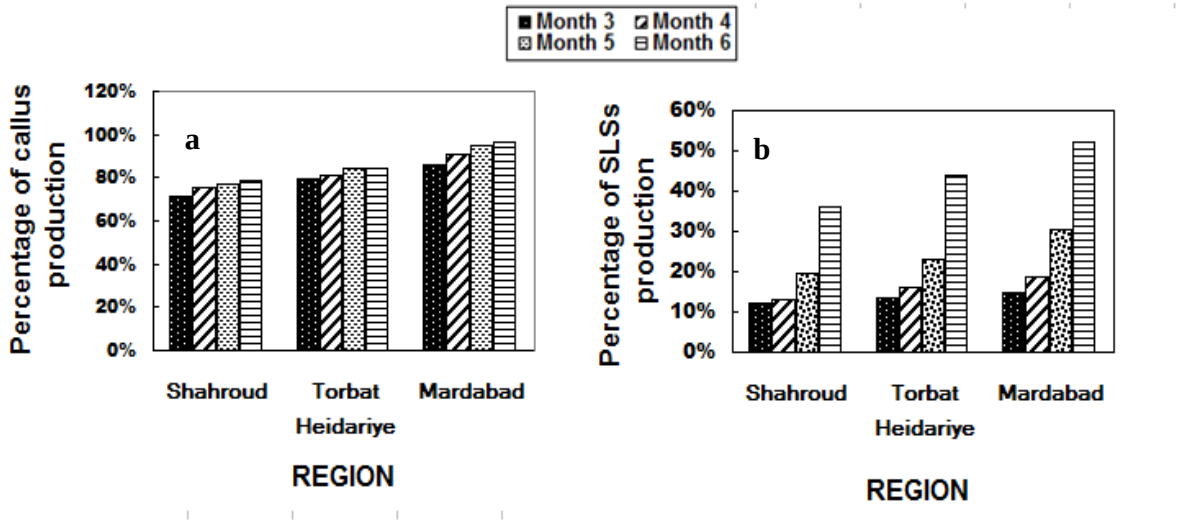

Figure 2. Percentage of (a) style callus formation and (b) stigma-like structures in Shahroud, Mardabad and Torbat-e Heidariye, after 6 monuth

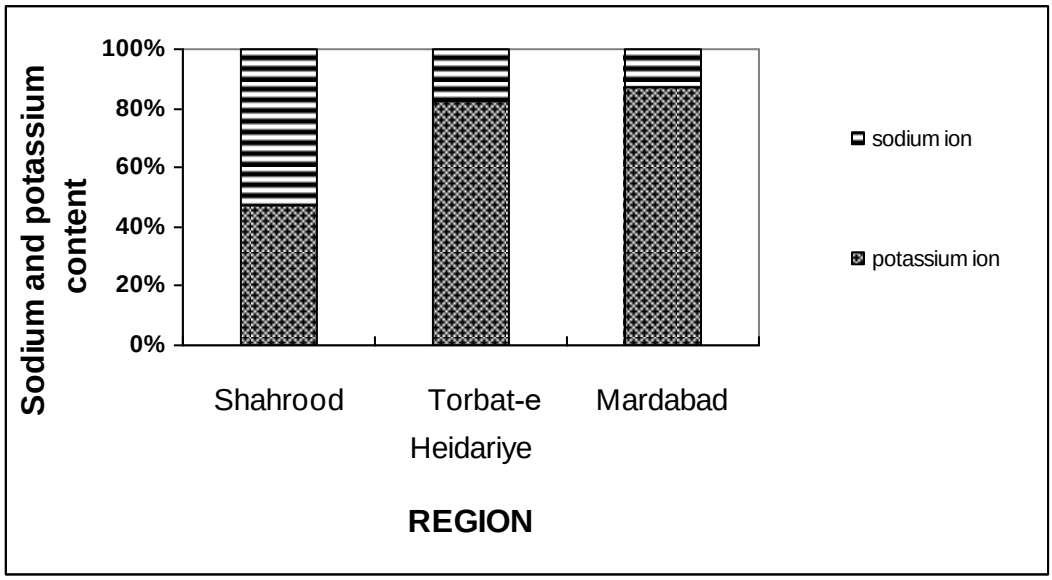

Figure 3. The content of exchangeable sodium and potassium in the soils of Shahrood, Mardabad and Torbat-e Heidariyeh

The number of SLSs production was statistically higher in Mardabad in comparison with the other two regions at the 0.05 level. The SLSs percentages were 52, 44 and 35% for Madabad, Torbat-e Heidariye and Shahroud, respectively at the end of the given period (6 months) (Fig. 2b).

Exchangeable sodium and potassium content. The results of exchangeable sodium and potassium ions contents in the three different soils showed that the Shahrood's soil had the highest level of exchangeable sodium ions (28 ppm) as compared to those of Torbat-e Heidariye (10 ppm) and Mardabad (9 ppm). Also, the results of exchangeable potassium ions in the soils of the three regions were: Mardabad (60 ppm), Torbat-e heidariye (48 ppm) and Shahrood's (25 ppm) (Fig. 3).

Soil electrical conductivity. The soils of the three regions were analyzed for electrical conductivity which showed that soil with EC=0.8 (non-saline), 2.2 (very slightly saline) and 4.1 (slightly saline) were obtained for the soils of Mardabad, Torbat Heidariye and Shahroud, respectively. The classes of salinity and EC from different types of soils according to Burt (25) are shown in Table 1.

Lipid peroxidation assay. The results indicated that the level of MDA in corms of Shahroud (0.015 $\mu molg^{-1}fw$) was higher than those of the other two samples: Torbat-e Heidariye (0.0083 $\mu molg^{-1}fw$) and Mardabad (0.0074 $\mu molg^{-1}fw$). The differences were significant at the 0.05 level (Fig. 4a).

Table 1. Classes of salinity and EC (1 dS/m = 1 mmhos/cm), adapted from NRCS Soil Survey Handbook (25)

Salinity Class	EC (dS/m)
Non-saline	0<2
Very slightly saline	2<4
Slightly saline	4<8
Moderately saline	8<16
Strongly saline	16≥

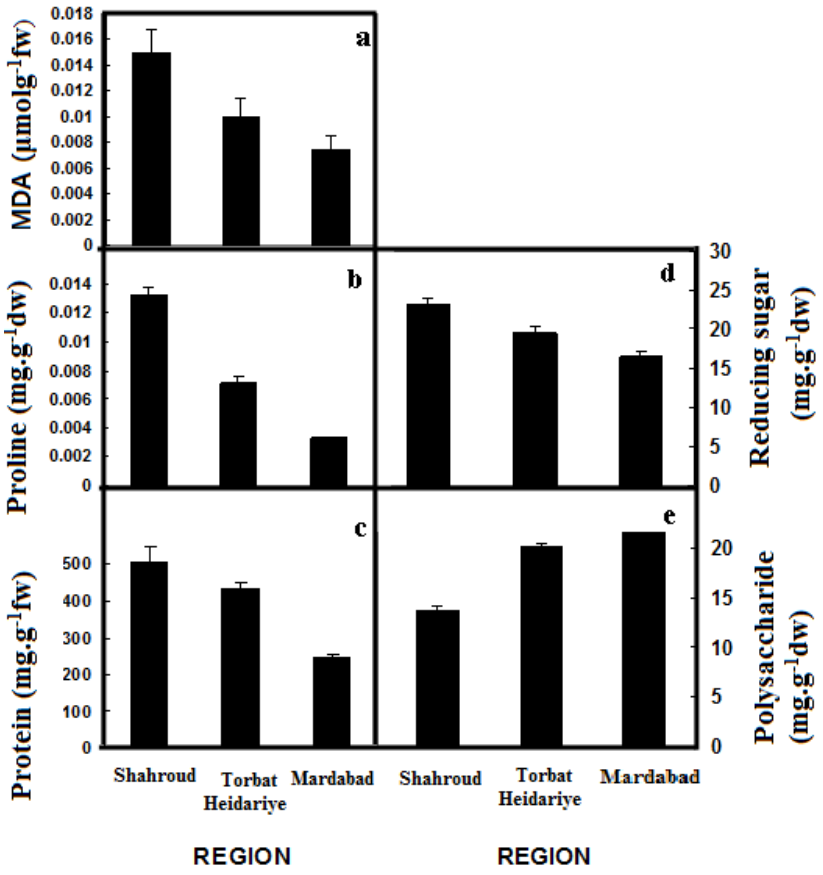

Figure 4. (a) Malondialdehyde concentration, (b) Proline content, (c) Protein content, (d) Reducing saccharides, (e) Polysaccharide content in corms of Shahroud, Torbat-e Heidariye and Mardabad

Proline content. The proline content of corms from three regions showed that the amount of proline content was highest in Shahroud (0.013 mg.g^{-1}dw) and lowest in Mardabad (0.0032 mg.g^{-1}dw). The proline content of Torbat-e Heidariye was 0.0070 mg.g^{-1} dw. The data showed significant differences at $P<0.05$ (Fig. 4b).

Total protein content. The results indicated that the highest level of protein content of corms was observed in Shahroud (504.98 mg.g^{-1} fw). The level of protein content of Mardabad and Torbat-e Heidariyeh corms were 247.27 and 432.04 mg.g^{-1}fw, respectively and significantly different at $P<0.05$ (Fig. 4c).

Sugar content. The results of comparison of sugar contents of corms which consist of reducing sugars and polysaccharides of the three region showed that the reducing sugar contents were as follows: Shahroud (22.29 mg.g^{-1}dw), Torbat-e Heidariye (19.47 mg. g^{-1} dw) and Mardabad (16.43 mg.g^{-1}dw). It has been revealed that higher reducing sugar content was observed in Shahroud corms (Fig. 4d). In contrast, the level of polysaccharide content of Mardabad (25.34 mg.g^{-1}dw) was higher than that of Torbat-e Heidarieh (20.08 mg.g^{-1}dw) and Shahroud (13.67 mg.g^{-1}dw), respectively. Statistical analysis showed significant differences between reducing sugars as well as polysaccharide contents in corms of the three regions ($P<0.05$) (Fig. 4e).

Peroxidase gel electrophoresis. The peroxidase (POX) electrogram of corms from the three regions showed two isoforms with Rm= 0.35 and 0.36, with highest intensity in the Mardabad corm (Fig. 5).

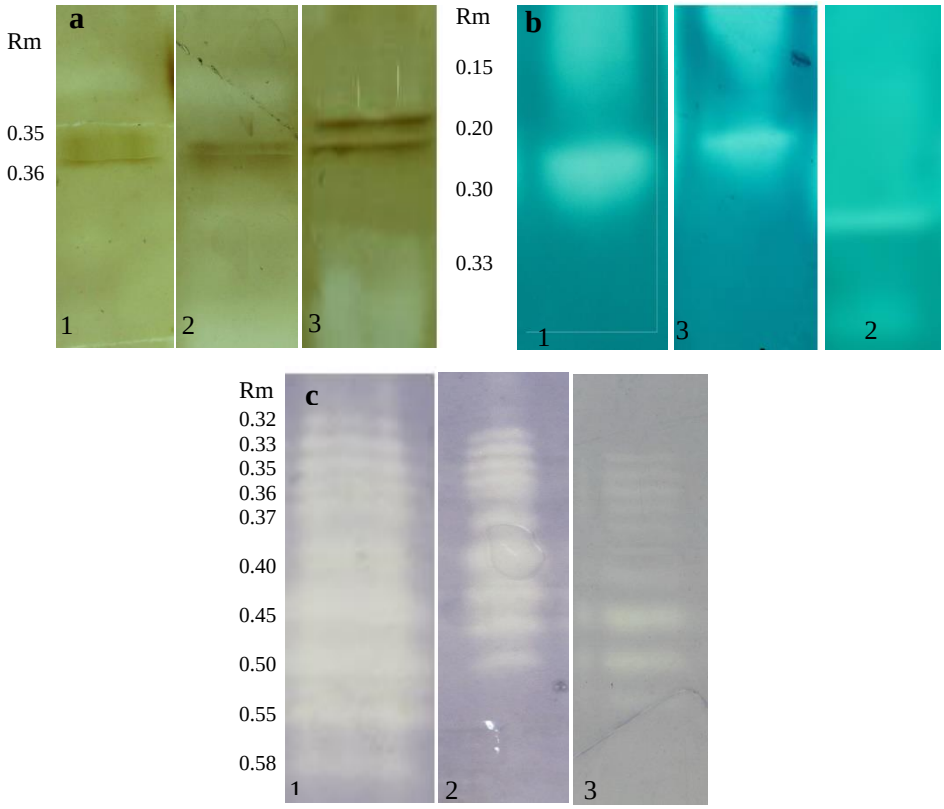

Figure 5. (a)Peroxidase electrophorogram with two isoforms by Rm=0.35 and Rm=0.36 as depicted in corms of (1) Shahroud, (2) Torbat-e Heidariye and (3) Mardabad. (b) Catalase electrophorogram with two isoforms by Rm=0.15 and 0.20 of corms from Shahroud (1), Mardabad (3), and Rm=0.30 and 0.33 of corms from Torbat-e Heidariye (2). (c) Superoxide dismutase electrophororogram with five isoforms of Mn-SOD and four isoforms of Cu/Zn SOD from corms of (1) Shahroud, (2) Torbat-e Heidariye and (3) Mardabad

Catalase gel electrophoresis. The corms of both Shahroud and Mardabad showed two catalase isoforms with Rm= 0.15 and 0.20, with high intensity in Shahroud. Also, two additional isoforms with Rm= 0.30 and 0.33 were observed only in the corm of Torbat-e Heidariyeh (Fig. 6).

Super oxide dismutase gel electrophoresis. The electrogram of SOD corms showed five Mn-SOD isozymes with Rm= 0.32, 0.33, 0.35, 0.36, 0.37 and four Cu-Zn SOD isozymes with Rm= 0.40, 0.45, 0.50, 0.55, 0.58. The intensity of both Mn-SOD and Cu/Zn-SOD isoforms was higher in Shahroud than in Torbat-e Heidariye and Mardabad, respectively (Fig. 7).

Discussion

Exchangeable sodium and potassium content

The exchangeable sodium ions content was found to be higher in Shahroud soil in comparison with those of Torbat-e Heidariye and Mardabad. Exchangeable sodium ions at high levels lead to diffusion into the soil colloidal particles, so it destroys soil structure, breaks up the soil pores and limits the soil drainage ability (26); on the other hand, the soil from Shahroud had the lowest level of potassium ions. Potassium ions versus sodium ions activate at least 60 different enzymes in cells that are involved in plant growth and also associated with movement of water, nutrients and

carbohydrates in plant tissue (26). Potassium is critical for high yields,hence a decrease in potassium ions and increase in sodium ions lead to salinity as shown in soil from Shahroud in comparison with soil from Mardabad.

Soil EC. According to the results of EC measurement, the soil of Shahroud, Torbat-e Heidariye and Mardabad were categorized in the class of 'slightly saline', 'very slightly saline' and 'non-saline' soils, respectively. Saffron plant prefers very well drained soil. A neutral to slightly alkaline soil is suitable for its cultivation with pH ranging from 6.3 to 8.3 and with electrical conductivity between 0.009 and 0.30 dsm^{-1}(9 and 10). Thus, it seems that among the three soil samples, soil from Shahroud imposed a slight salt stress on the corms.

Proline content. proline, one of the important osmoprotective compounds has been reported to be accumulated during drought, with high salinity and oxidative stresses in response to biotic stresses (27). The high proline content in corms of Shahroud leads to a better control of undesirable soil EC than the other two samples.

Reducing sugars. The accumulation of reducing sugars at high level is one of the protective mechanisms in plants and a good indicator to tolerant salt stress, which occurred in Shahroud' corms. It has been reported that an increase in reducing sugars occurred in salt stress situations (7). During the stress conditions, starch as one of the main polysaccharides started to degrade as a rapid response to ease stress (28).

MDA content assay. Malondialdehyde (MDA) is a biomarker to estimate damage to cell membranes (8). The lipid peroxidation of membrane leads to release of MDA. It seems that both increased proline and reducing sugars contents were not sufficient to have impact on soil EC, therefore, MDA increased in corms of Shahroud. In contrast, Mardabad corms showed lower level of lipid peroxidation because its soil was non-saline.

Antioxidant enzymes activities. In response to salinity stress, the production of ROS, such as singlet oxygen, superoxide, hydroxyl radical and hydrogen peroxide, was enhanced (29). Antioxidant enzymes such as superoxide dismutase, peroxidase, catalase, etc., exist in plants to scavenge excess ROS. SOD is one of the first enzymatic protective mechanisms against the ROS that dismutase O^{-}_2 to H_2O_2, subsequently H_2O_2 through the action of CAT and POX or both converted to O_2 and H_2O (30). Superoxide dismutase is considered as a key player in the antioxidant defense system. Its isoforms are located in different compartments, for reasons such as excess superoxide, inadequate amounts of SOD, superoxides which have not been scavenged entirely; so leading to disturbed vital biomolecules (31). Furthermore, it has been reported that the activity of some SOD isoforms are halted due to sensitivity to high amounts of H_2O_2 (32). The results showed that activities of Mn-SOD and Cu-Zn SOD isoforms were higher in the corms of Shahroud than those of both Torbat-e Heidariye and Mardabad. It has been reported that under drought stress, SOD activity was increased in corms, leaves and roots of *C. sativus* (33). The activities of CAT and POX are altered during organogenesis and differentiation stages (34) Therefore, at low H_2O_2 concentrations, catalase can exert a peroxidatic activity (33). It seems that an increase in the level of CAT in the corms of Shahroud may have compensated for reduced POX activity in this tissue, because CAT can scavenge the H_2O_2 in low concentration and

complete the action of ROS elimination (35). It has been reported that the activities of catalase and SOD in dormant corms of saffron were stimulated under stressful conditions (36).

Song et al. (2006) investigated the effect of salt stress on activity of SOD in *Ulmus pumila* L. (37). They indicated that increased SOD activities may enhance the ability of *U. pumila* to scavenge ROS in salt stress, and activities of SOD and resistance of plant had a certain correlation. The increased SOD activity under stress condition has been reported in literatures (38).

Tian et al. (2003) reported that a high level of $O^{\bullet-}_2$ and low ratio of H_2O_2/ $O^{\bullet-}_2$ is indicative of meristemoid tissue with low potential for organogenesis. In contrast, high ratio of H_2O_2/ $O^{\bullet-}_2$ lead to organogenesis (32). The results obtained from activities of anti-oxidant enzymes indicated that Mardabad' samples impose non-saline soil with high POX and low SOD activities; on the other hand, high ratio of $H_2O_2/O^{\bullet-}_2$ (potential for high organogenesis) produced high percent SLS (52%) than Shahroud samples that possess slightly saline soil with low POX and high SOD activities, which means that low ratio of H_2O_2/ $O^{\bullet-}_2$ (with low potential of organogenesis) showed less production of SLS (35%) yield with significant difference at $P \leq 0.05$.

These results revealed that the salinity of soil was able to influence SLSs production obtained from tissue culture through corms as a main organ that directly challenge soil stresses and indirectly, immature styles which were used for tissue culture.

It has been shown that when plant experience stress, they respond in different ways, e.g., biosynthesis of osmoprotective compounds like proline and anti-oxidants enzymes (SOD, POD, etc.), accumulation of reducing sugars and MDA as biomarkers for cell membrane damage which also include epigenetic variation (33 and 35).

To regain totipotency and pluripotency (termed dedifferentiation), differences in regeneration capacity depended on how easy and fast epigenetic marks can be erased by cells. On the other hand, epigenetic mechanisms regulate gene expression during development by becoming methylated and switched off in some tissues, de-methylated and active in other tissues (39). These changes include alteration in DNA methylation, histones or both that influence gene transcription which are often temporary but some can be long lasting and even be transferred during sexual propagation (37). and stable from parents to offspring (34). Epigenetic variation was detected among plants from the different subcultures, between field plants and *in vitro* plants, differentiated and dedifferentiated tissues and also between juvenile and adult tissues (40). In the present study, when corms exposed to salinity and subsequently callus were formed from style explant in tissue culture and finally, when adventitious regeneration (SLSs) occurred, all the processes were revealed to impose stress and associated with different responses including epigenetic variation(40). In this experiment, because of the short period of tissue culture, it seems that the epigenetic modification of Shahroud corms was longer and was transferred in a stepwise manner upon short term, on SLSs production obtained *in vitro*.

It seems that soils from Sharhrood (high sodium content and high EC) had more influence on corms properties as a main organ which is directly challenged with soil stress. This stress may influence metabolite reservations of corms in many ways which

include epigenetic modification, and subsequently affect the quality and quantity reservoir of style which subsequently, affect production of calli and SLSs when these style explants are used for tissue culture. On the one hand, the SLSs percentage could be related to soil EC and ratio of $H2O2/O^{\bullet-}_2$ which lead to increase or decline in the organogenesis (SLSs). The importance of epigenetic mechanisms in the constitution of corms and its flora buds, and *in vitro* developmental processes of SLSs from style explant as the final yield, could be emphasized for more studies.

Acknowledgements

The authors are grateful to Jahan Saffron Organization (Naser and Taher Jahanshiri) for providing saffron corms from Torbat-e Heidariyeh. The authors express their appreciation to M.s Maryam Yousefi for her support and guidance.

.

REFERENCES

1. Kafi, M., Koocheki, A., Rashed, M.H., Nassiri, M. (eds.) (2006). Saffron (*Crocus sativus*) Production and Processing (1st ed.), Sci. Publishers.
2. Carillo, P., Annunziata, M. G., Pontecorvo, G., Fuggi, A., Woodrow, P. (2011). Salinity stress and salt tolerance. Abiotic Stress in plants-Mechanism and adaptations. Rijeka, Croatia: In Tech.
3. Finkel, T. (2011). Signal transduction by reactive oxygen species. *J. Cell Boil.* 194, 7-15.
4. Graves, D.B. (2012). The emerging role of reactive oxygen and nitrogen species in redox biology and some implications for plasma applications to medicine and biology. *J. Phys. D Appl. Phys.*, 45, 1-42.
5. Chiang, H.H. and Dandekar, A.M. (1995). Regulation of proline accumulation in *Arabidopsis thaliana* (L.) Heynh during development and in response to desiccation. *Plant. Cell. Environ.,* 18, 280–1290.
6. Moustakas, M., Sperdouli, I., Kouna, T., Antonopoulou, CI. and Therios, I. (2011). Exogenous proline induces soluble sugar accumulation and alleviates drought stress effects on photosystem II functioning of *Arabidopsis thaliana* leaves. *Plant Growth Regul.,* 65, 315–25.
7. Pérez-López, U., Robredo, A., Lacuesta, M., Muñoz-Rueda, A. and Mena-Petite, A. (2010). Atmospheric CO2 concentration influences the contributions of osmolytes accumulation and cell wall elasticity to salt tolerance in barley cultivars. *J. Plant Physiol*., 167, 15–22.
8. Miller, G., Suzuki, N., Ciftci-Yilma, S. and Mittler, R. (2010). Reactive oxygen species homeostasis and signaling during drought and salinity stresses. *Plant. Cell. Environ*., 33, 453–467.
9. Sarma, KS., Maesato, K., Hara, T. and Sonida, Y. (1990). *In vitro* production of stigma-like structures from stigma explants of *Crocus sativus* L. J. *Exp. Bot*. 41,745–748.
10. Hosseinzadeh Namin, M., Ebrahimzadeh, H., Ghareyazie, B., Radjabian, T., Gharavi, S. and Tafreshi, N. (2010). Initiation and origin of stigma-like-structures (SLS) on ovary and style explants of saffron in tissue culture. *Acta. Biol. Cracov. Bot*. 52, 1,55–60.
11. Ganai, MY. (2002). Corm root disease of saffron and its management. In proceeding of seminar-cum-workshop on saffron (*Crocus sativus*). *Skuast-k, India*, 107-112.
12. Paseban, M. and Rezaian, S. (2006). The Effect of Micronutrients and Manure Fertilizers on the Quantity and Quality of Khorasan Saffron. In *II International Symposium on Saffron Biology and Technology* 739 (pp. 155-158).
13. Murashing, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Plant physiol*., 15,473-497.
14. Mutscher, H. (1995). Measurement and Assessment of Soil potassium. Basel., Switzerland. *IPI*. Basel, Switzerland, 102 pp.
15. Bremner, J.M., Sparks, D.L., Page, A.L., Helmke, P.A., Loeppert, R.H., Soltanpour, P.N., Tabatabai, M.A., Johnston, C.T. and Sumner, M.E. (1996). Nitrogen-total. In *Methods of soil analysis.* Part 3-chemical methods. 1085-1121.

16. Bates, L.S., Waldren, R.P. and Teare, I.D. (1973). Rapid determination of free proline for water-stress studies. *J. Plant and soil., 39*, 205-207.

17. Heath, R. L. and Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys*, 125, 189-198.

18. Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J. biol. Chem., 153*, 375-379.

19. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.T. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal. Chem*, 28, 350-356.

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

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

22. Van Loon, L.C. (1971). Tobacco polyphenoloxidases: a specific staining method indicating non-identity with peroxidases. *Phytochemistry*, 10, 503-507.

23. Woodbury, W., Spencer, A. K. and Stahmann, M.A. (1971). An improved procedure using ferricyanide for detecting catalase isozymes. *Anal. Biochem*. 44, 301-305.

24. Wendel, J. F. and Weeden, N.F. (1989). Visualization and interpretation of plant isozymes, In Soltis DE, Soltis PS (ed.), *Isozymes in plant biology*. 5-45.

25. Burt, R. (2009). Soil Survey Investigations Report No. 51, Version 1.0. In *Soil survey field and laboratory methods manual*. USDA-NRCS, National Soil Survey Center, Lincoln, NE.

26. Shaposhnik, V.A. (2007). History of the discovery of potassium and sodium (on the 200th anniversary of the discovery of potassium and sodium). *J. Anal. Chem.*, 62, 1100-1102.

27. Mohammadian, R., Khoyi, F.R., Rahimian, H., Moghaddam, M., Ghassemi-Golezani, K. and Sadeghian, S.Y. (2001). The effects of early season drought on stomatal conductance, leaf-air temperature different and proline accumulation in sugar beet genotypes. *J.Agric.Sci. Technol*., 3, 181-192.

28. Zeeman, SC., Thorneycroft, D., Schupp, N., Chapple, A., Weck, M., Dunstan, H., Haldimann, P., Bechtold, N., Smith, A.M. and Smith, S.M. (2004). Plastidial α-glucan phosphorylase is not required for starch degradation in Arabidopsis leaves but has a role in the tolerance of abiotic stress. *Plant Physiol.*, 135(2), 849-858.

29. Ahmad, P. and Prasad, M.N.V. (2012). Abiotic Stress Responses in Plants: Metabolism, Productivity and Sustainability, Springer, New York, NY, USA.

30. Martinez, C. A., Loureiro, M. E., Oliva, M.A. and Maestri, M. (2001). Differential responses of superoxide dismutase in freezing resistant *Solanum curtilobum* and freezing sensitive *Solanum tuberosum* subjected to oxidative and water stress. *Plant Sci*., 160(3), 505-515.

31. Mittler, R., Vanderauwera, S., Gollery, M. and Van Breusegem, F. (2004). Reactive oxygen gene network of plants. Trends. *Plant. Sci.,* 9, 490-498.

32. Maleki, M., Ebrahimzade, H., Gholami, M. and Niknam, V. (2013). The effect of drought stress

and exogenous abscisic acid on growth, protein content and antioxidative enzyme activity in saffron (*Crocus sativus* L.). *Afr. J. Biotechnol.*, *10*, 9068-9075.

33. Smirnoff, N. (2005). Ascorbate, tocopherol and carotenoids: metabolism, pathway engineering and functions. In Smirnoff, N. (ed.), *Antioxidants and Reactive Oxygen Species in Plants*, Blackwell Publishing Ltd., Oxford, UK, pp. 53-86.
34. Singh, N.D., Li, M., Lee, S.B., Schnell, D. and Daniell, H. (2008). Arabidopsis Tic40 expression in tobacco chloroplasts results in massive proliferation of the inner envelope membrane and upregulation of associated proteins. *The Plant Cell.*, 20, 3405-3417.
35. Keyhani, E., Ghamsari, L., Keyhani, J. and Hadizadeh, M. (2006). Antioxidant enzymes during hypoxia–anoxia signaling events in *Crocus sativus* L. corm. Ann. *N. Y. Acad. Sci.*, 1091(1), 65-75.
36. Tian, M., Gu, Q. and Zhu, M. (2003). The involvement of hydrogen peroxide and antioxidant enzymes in the process of shoot organogenesis of strawberry callus. *Plant Sci*., 165, 701-707.
37. Song, F.N., Yang, C.P., Liu, X.M. and Li, G.B. (2006). Effect of salt stress on activity of superoxide dismutase (SOD) in Ulmus pumila L. *Eur. J. For. Res.,* 17(1), 13-16.
38. Hermandwz, J.A. and Almansa, M.S. (2002). Short-term effects of salt stress on antioxidant systems and leaf water relations of pea leaves. *Physiol. Plant.*, 115(2), 251–257.
39. Smulders, M.J.M. and De Klerk, G.J. (2011). Epigenetics in plant tissue culture. *Plant Growth Regul.,* 63(2), 137-146.
40. Matzke, M., Kanno, T., Daxinger, L., Huettel, B, Matzke, AJM. (2009). RNA-mediated chromatin-based silencing in plants. Curr. Opin. Cell. Biol., 21, 367–376.