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Evaluation of callus formation and embryogenesis in saffron (*Crocus sativus* L.) for flower harvesting

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Abstract

Crocus sativus is belonging to Iridaceae. Due to its triploid nature it is sterile and is not able to set seeds, so it is propagated only by corms. Breeding and producing corms with free pathogens especially viruses is generally difficult; although vegetative production of the plant does easily by new corms. The aim was optimizing medium, hormones, sucrose level and temperature for embryogenesis and shoot elongation of saffron. Therefore, an experimental study was done in 7 treatments and 4 replications in two temperatures. Benzyl Adenopurine and 2,4-Dichlorophenoxy acetic acid were plant growth regulators. Data analyzed by statistical software. Results showed that the highest callus formation was in T4 containing 1 mg/lit of 2,4-D and 2 mg/lit BAP with the mean of 44.2 %. Also, the treatment with 45 gm/lit of sucrose produced the most length of shoot. The highest embryogenesis occurred in 4°C.

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Introduction

Islamic Republic of Iran, with the annual production of 150 ton, is the first producer of saffron in the world. It uses as food and medicinal plant in Iran. Thus, the plant is very important in agriculture. Among the 85 species belonging to the genus *Crocus*, *C. sativus* L. (Saffron) is the most fascinating and intriguing species (Fernández, 2004). The species is a fall-flowering perennial, sterile, triploid and mutant of the eastern Mediterranean *Crocus cartwrightianus* (Deo, 2003). Due to its triploid nature it is sterile and is not able to set seeds, so it is propagated only by corms (Mir *et al.*, 2010). Therefore, stigma production in the laboratory is an ultimate will. The stigmas are dried for use in medicine, food seasoning and coloring for centuries and are characterized as the most expensive spice by weight. Saffron contains more than 150 volatile and aroma-yielding compounds. Saffron is belonging to Iridaceae and produces annual renewal corms which are indispensable for their propagation. This plant propagated vegetative easily by new corms, but breeding and producing corms with free pathogens especially viruses is generally difficult (Sampathu *et al.*, 1987). The successful tissue culture protocol was developed in saffron by several authors (Ding *et al.*, 1981; Homes *et al.*, 1987; Ilahi *et al.*, 1987).

The aim of this research was investigation on tissue culture, somatic embryogenesis and shoot meristem culture by optimizing medium, hormones and sucrose concentration and temperature on corm explants for producing plantlets with free pathogens and high potential in order to producing stigma like structures and flower harvesting.

Materials and methods

Saffron corms grown in the research farm of Agriculture Biotechnology Institute were selected by the weight of 3-6 grams and utilized as source of explants for callus formation and embryogenesis. After removing scales, corms were sterile using sodium hypochloride 5% for 8 minutes, ethyl alcohol 80% for one minute and nanosilver (37%) for 17 minutes respectively. Then, they rinsed three times with distilled water after each stage (Table 1). These stages used for shoot meristems too.

Table 1. Optimum substance and time that used for sterilizing corm and shoot meristems.

Sterilizer	Sodium Hypochloride 5%	Ethanol 80%	Nanosilver 37%
Time	8 min. with shaking	1 min.	17 min. with shaking

Corm explants dissected as 3 millimeters from core part of corms. They culture in sterile Petri dishes containing MS (Murashige and Skoog, 1962) as basic media and plant growth regulators in dark at 24±2°C. All media were adjusted to pH 5.7-5.8. Plant growth regulators were 2,4-D and BAP. Four concentrations in 2,4-D and 5 for BAP designed for study (Table 2). This experiment was included 7 treatments and 4 replicas (36 explants) were done as completely randomized design. Evaluations write down base on callus formation and embryogenesis. Subcultures were done after 4 weeks on the same media.

Table 2. plant growth regulator concentrations and treatments.

		BAP(mg/lit)				
		4	**2**	**1**	**0/5**	**0**
2,4-D (mg/lit)	**0**	T6	-	-	-	T5
	0/5	-	T3	-	-	-
	1	T2	T4	-	-	-
	2	-	-	T7	T1	-

Callus formation was evaluated by two temperatures (4 and 19°). Also, four level of sucrose (30, 35, 40, and 45 gr/lit) were studied for growth and increasing length of shoot in dark. Data were analyzed by SPSS and MSTATC software.

Results

a. *Callus formation and embryogenesis*: Callus formation was first observed about 2 months after incubation. Results obtained from analysis of variance showed that interact effect of plant regulators used in the study on the embryogenic calluses were significant at 0.01 levels. Also, results showed that the highest callus formation was in T4

containing 1 mg/lit of 2,4-D and 2 mg/lit BAP with the mean of 44.2 % and T3 with the concentration of 0.5 mg/lit of 2,4-D and 2 mg/lit BAP with the mean 40.4% at the second. Mean of embryogenic explants for them were 36.1% and 34.1% respectively. Lowest level for callus and embryogenesis observed at T6 with 0 mg/lit 2,4-D and 4 mg/lit BAP. We found that high levels of auxins and cytokinins are more suitable for callus formation. We, also, observed two type of callus. Some were soft and white, whereas others were crisp and yellowish that formed trace spherical embryos.

b. *Temperature*: Analysis of variance showed that temperature has significant effect on the embryo maturation at 0.01 levels. Mean of embryogenesis was 29. 4 for temperature at 4° C and 12.3% for 19° C.

c. *Effect of sucrose on the growth of shoot meristem*: Results showed that concentrations of sucrose have different effects on the growth of shoot apex, as the treatment with 45 gr/lit of sucrose produced the most length (8.2 cm) of shoot.

According to analysis of variance, all treatments and interact of them (temperature and hormone) was significant at 0.01 level for embryogenesis.

Table 3. Effect of sucrose level on the length of shoot (based on 10 measurements).

Sucrose (gr/lit)	30	35	40	45
Shoot length (cm)	3.33	4.16	6.33	8.25

Table 4. Analysis of variance for tissue culture of *Crocus sativus*.

Source of variation	Degree of freedom	Mean square	F
Sucrose	3	12.019	74.785**
Factor A (hormone)	6	1.713	29.541**
Factor B (temperature)	1	0.702	12.103**
A*B	6	0.817	14.078**
Error	42	0.058	
CV	24.35		

** significant at 0.01 *significant at 0.05
ns: nonsignificant

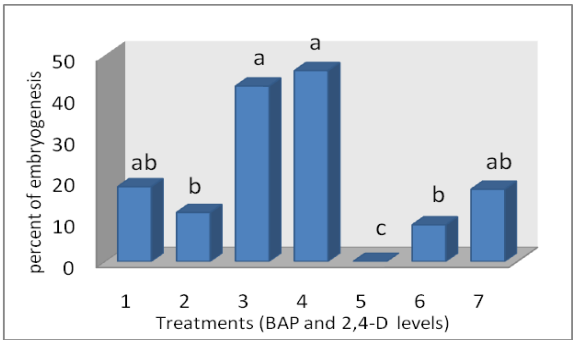

Fig. 1. Effect of plant growth regulators on embryogenesis.

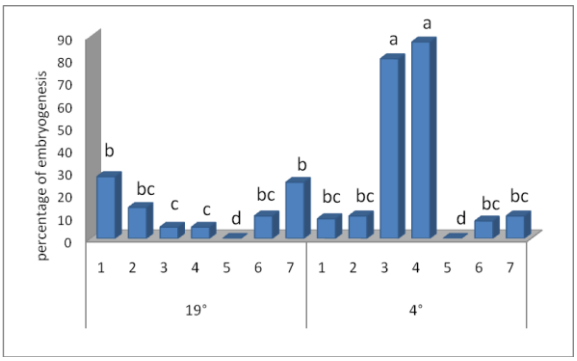

Fig. 2. Effect of BAP and 2,4-D (treatments) in different temperatures on the embryogenesis of *Crocus sativus*.

Discussion

According to Plessner and Ziv (1999), *Crocus sativus* has a low vegetative propagation rate. But there are several reports on tissue culture, embryogenesis and micro propagation (Ding *et al.*, 1979; Ilahi *et al.*, 1987; Aguero and Tizio, 1994; Ahuja *et al.*, 1994; Ebrahimzadeh *et al.*, 2000). Our findings about high concentrations of BAP and 2,4-D for callus formation and embryogenesis of crocus was confirmed by Ebrahimzadeh and coworkers (2000) suggested that LS medium supplemented with BAP (5×10^{-6} M) and NAA (5×10^{-6} M) could induce embryogenesis and growth of callus in saffron. Dhar and Sapru (1993) obtained callus on the medium MS from floral apices of sprouted corms by 2,4-D in 9.0 µM and KN 9.3µM. As, we obtained 1mg/lit for 2,4-D and 2mg/lit for BAP. Non-embryogenic callus formed best on media containing 10.8 M NAA and 8.9 M BA, while embryogenic callus formed most frequently with 4.5 M 2, 4-D and 4.4 M BA (Darvish *et al.*, 2007). This

seems high levels of auxins and cytokinins resulted in embryogenic callus and non-embryogenic obtained by lower levels.

Also, Ebrahimzadeh and coworkers (2000) reported that during initial stages of callus development, golden yellowish, soft, translucent calluses appeared from cultured explants (Ebrahimzadeh *et al*., 2000). Our results confirmed that crisp and yellowish callus were formed trace spherical embryos.

Plessner and Ziv (1999) said that several authors reported corm cultivation in media, however, most of these failed to give a detailed description of the tissue or zone from which these explants were isolated. But in our experiment, we described exactly isolating and sterilizing explants from corms.

The most common media used for the plant were MS, LS, N6, W and B5 (Plessner and Ziv, 1999). Sucrose utilization in past studies reported in 30, 40, 60 g/l (Aguero and Tizio, 1994). But, Otsuka and coworkers (1992) reported sucrose with the concentration about 50 – 120 g/l for obtaining stigma like tissues. These were used for micro corm induction which was promoted by a half-strength MS medium plus 9% sucrose (Raja *et al*., 2007). We optimized sucrose with the 45 g/l that lays in the range of last authors findings.

Under continuous darkness, many shoot primordial were formed. These elongated when placed in the light, and formed normal plantlets with corms (Bhagyalakshmi**,** 1999). Similar results were obtained with the same hormones by Karaoglu and coworkers (2007). We obtained elongated shoots in different levels of sucrose resulted in different length in dark and light respectively.

Study of temperature effect on the embryogenesis was first report in these experiments. We found that the temperature has significant effect on embryogenesis, as increases when the temperature falls to 4°.

Conclusion

Stigma production of saffron in the laboratory is an ultimate will, but our findings confirm that by balancing hormones and increasing additives such as sucrose and regulating temperature can promote callus formation, embryogenesis, shoot meristems and, finally, flowering in it.

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