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Plant regeneration and corm formation of saffron (*Crocus sativus* L.) *in vitro*

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Abstract

In vitro plant regeneration and daughter corm formation of saffron (Crocus sativus L.) from corm parts consisting of meristematic region via direct organogenesis were performed. In the first experiment, corm explants were treated with five different sterilization procedures in order to overcome contamination difficulties as being encountered in all the geophyte. In the second experiment on direct shoot regeneration and providing the foliation, sterile meristematic node-containing corm explants were cultured on ½ Murashige and Skoog (MS) Medium or MS with several dose of BAP. According to the data MS+6 mg/l BAP had the best results in shoot initiation (96.7 %) and the foliation rate (93.3 %) while MS+1 mg/l BAP gave the least result in shoot initiation (16.7 %); MS+1 mg/l BAP and MS+10 mg/l BAP gave the least result in foliation from the initiated shoots in 90 days.

In the third experiment, the daughter corm formation and rooting were achived on MS supplemented with IBA or IAA. The data showed that MS+1 mg/l IAA have the best results on daughter corm formation rate (76.7 %) and daughter corm number per corm formed explants (1.74 corms/corm formed explants). On the other hand rooting rate (46.7 %) and root number per root formed explant (1.5 roots/ root formed explant) were highest on MS with 2 mg/l IBA in 120 days. The protocol reported in this study may help in the growth and manupulation of agriculture and biotechnology of saffron which is increasingly conspicuous and demanding species.

Keywords: *Crocus sativus*, saffron, node culture, micropropagation, corm.

Introduction

Saffron (*Crocus sativus* L.) is one of the world's highest priced medicinal and aromatic plants which belongs to the Iridaceae family from the stigmas of hermaphrodite flowers that also called saffron were edible and used for different purposes. Stigma is mostly used as spice and food colorant and less extensively as a dye or perfume[12]. It also has many analgesic and sedative medicinal properties and has been used in traditional folk herbal medicine of many culture for the treatment of some illness for centuries[1,3,12,24]. Flowering and corm production occur only once in a year that cause difficulties in saffron stigma and corm production. Because of being triploid and being seedless, production of the plants is possible only via corm multiplication in natural way[18,23,25]. Only 1.52-4.01 corms per mother corm are produced in one growing season in the field through conventional methods[6].

Low corm multiplication rates and fungal infestation of corms reduce the productivity and quality, thereby restraining the availability of planting material[8]. Mass production of pathogen-free corms would also be needed for modernization of saffron cultivation[20]. *In vitro* tissue culture and micropropagation techniques can solve the problems about multiplication rate, pathogen-free stock corms. Numerous studies have been made on *in vitro* regeneration or multiplication of saffron using different explants. A few research were carried out on direct and indirect organogenesis[9,13,14,15,19,23,27,28,30,31] while a small amount of studies were on direct and indirect somatic embryogenesis[2,4,7,21,22,29].

The study established a reliable sterilization procedures for saffron corms that can be an important problem like many other geophytes. In addition, the purpose of this study was to investigate the feasibility of plant regeneration from corms and corm multiplication using corm parts via direct organogenesis.

Material and Methods

Plant material, culture media and incubation condition: The initial dormant corms of 10-45 mm in diameter of *Crocus sativus* were provided by one of the main saffron farming village (Davutobası) in Safranbolu district of Turkey in harvest period at the beginning of summer. Dormant corms were kept under normal laboratory conditions in dakness in the same season until culture establishment in September. Fibrous bark was peeled from the corms by hand before sterilization experiments in September when studies began. Murashige and Skoog's Medium[17] (MS) and ½ MS (half-strength MS) media supplemented with 30 g/l (w/v) sucrose and solidified with 7 g/l (w/v) agar were used in all the experiments and pH was adjusted 5.7 before autoclave. All established *in vitro* cultures were incubated in a growth chamber at 25±2 ^{0}C with a 16 h light/8 h dark photoperiod provided by cool white fluorescent light at 50 μmol $s^{-2}m^{-1}$ at 70 % humidity throughout the experiments.

Sterilization experiments: For surface sterilization of peeled corms with all apical and lateral meristematic node tips before cutting, were treated with 5 sterilization procedures. In this study distilled water, ethanol (Merck^{TM}- C_2H_5OH-Ethanol-96%), Na-

hypochlorite (Commercial bleach-ACE™), Tween-20 (Merck™-Polyoxyethylene sorbitan monolaurate-Tween®20), H_2O_2 (Merck™-Hydrogen peroxide-35%) and $HgCl_2$ (Merck™-Mercuric chloride) were used in different steps and in different times. In the first sterilization experiment, 70 % ethanol for 5 minute, 50 % Na-hypochlorite for 15 min., 7% H_2O_2 for 15 min. were applied. In the second sterilization experiment, 5 % Tween-20 in distilled water for 60 min., 70 % ethanol for 20 min., 50 % Na-hypochlorite for 5 min. and 7 % H_2O_2 for 10 min. were treated.

In the third sterilization experiment, 5 % Tween-20 in distilled water for 75 min., 70 % ethanol for 20 min. and 7 % H_2O_2 for 20 min. were applied. In the fourth sterilization experiment, 5 % Tween-20 was added in distilled water for 90 min., 70 % ethanol for 15 min. and 7 % H_2O_2 for 15 min. were applied. In the fifth sterilization experiment 5 % Tween-20 was added in 10 % ethanol for 10 min. and 0.15 % $HgCl_2$ for 10 min. to treat corm explants. All solutions used were prepeared with distilled water.

The sterilized corms after washing 3 times with autoclaved sterile distilled water were blot-dried with sterile paper and cut into 0.5-1 cm^3 corm parts including apikal or lateral meristematic nods before cultured *in vitro* in 9 cm diameter petri dishes containing 10 ml MS without any plant growth regulator. Contamination rate (%) was recorded after 30 days. The experiments were triplicated and each repeat consisted of 10 explants in sterilization.

Shoot initiation and foliation experiment: The naked corms were surface sterilized with 70 % ethanol for 5 minute, 50 % Na-hypochlorite for 15 min., 7 % H_2O_2 for 15 min. and then washed 3 times with sterilized distilled water. Sterile corms were blot-dried with sterile paper and cut into 0.5-1 cm^3 corm parts including at least one apikal or lateral meristematic nods before cultured *in vitro* in 9 cm in diameter disposable petri dishes containing 10 ml of ½ MS medium without any plant growth regulators (Fig. 4) or basal MS medium supplemented with N^6-Benzylaminopurine (BAP) (1, 2, 4, 6, 8 and 10 mg/l). For continuation of freshness and healty growth, the explants were transferred to same fresh medium in jar after 45 days. The shoot initiation rate (%) and in the same medium foliation rate from the initiated shoots (%) were recorded after 90 days. The experiments were triplicated and each repeat consisted of 10 plants in shoot initiation and foliation.

Corm formation, multiplication and rooting: Plants showing success in MS with 6 mg/l BAP during previous experiments were transferred to MS supplemented with 1 or 2 mg/l Indole-3-butyric acid (IBA) and MS supplemented with 1 or 2 mg/l Indole acetic acid (IAA). To ensure the continuity of freshness the explants were transferred to same fresh medium in jar or glass tube after 60 days. Totally after 120 days, daughter corm formation (%), daughter corm number per the corm formed explant, rooting rate (%), root number per the root formed explant were calculated. The experiments were triplicated and each repeat consisted of 10 plants in corm formation and rooting rate. Daugher corm number and root number were calculated via number in corm or root formed plants.

Experimental design and statistical analysis: The data was analysed using analysis of variance (ANOVA) of completely randomized design and the groups that showed variance were then subjected to Duncan's Multiple Range Test[10] with a significance value at $P<0.05$. The percentage data was transformed using angular transformation (Arc Sin √%) before carrying out ANOVA.

Results and Discussion

Preliminary experiments were conducted for selection of the best sterilization methods to sterile corm parts safely. The first procedure (70 % Ethanol for 5 minute, 50 % Na-hypochlorite for 15 min., 7 % H_2O_2 for 15 min.) was found to be more effective and contamination rate was 0 % (Table 1). According to the results Na-hypochlorite is an essential chemical for the corm sterilization. Similarly ethanol and Na-hypochlorite were used in several doses and durations in previous studies[5,11,15,22].

Because of having meristematic nodes, the usage of corms (Fig. 1) in saffron tissue culture studies is a common modality. Therefore the experiment was continued in this manner. The corm explants including meristematic nodes when cultured on MS medium containing 6 mg/l BAP showed the best condition for direct shoot initiation (96.7 %) and foliation from the initiated shoot (93.3 %) in 90 days (Table 2; Fig. 3).

Actually shoot initiation started in 2-3 weeks in all media but at the end, some of the shoots turned in fully leaves while some of the initiated shoots did not continue to foliation (Fig. 2). BAP, alone or in combination with other plant growth regulators, was used[9,23,28,31] for direct shoot development from corms. Sharma et al[28] found that duration has taken at least 3 months for shoot multiplication.

In the corm and root formation studies, explants that converted to whole plants excepting roots in MS medium containing 6 mg/l BAP, were cultured in MS supplemented with 1 or 2 mg/l IBA and MS supplemented with 1 or 2 mg/l IAA for direct corm and root formation. New corm formation began from the base of the developed plants attached to corm explants (Fig. 6). The best results for daughter corm formation rate (76.7 %) and daughter corm number per corm formed explant (1.74) were obtained from MS+ 1 mg/l IAA (Table 3; Fig. 7; Fig. 8).

According to data MS with 2 mg/l IBA gave the highest rooting rate (46.7 %) and root number per root formed explants (1.5 roots) (Fig. 5). Auxins alone or in combination with other plant growth regulators, have an important role on rooting[26,31] and

corm production[16,23,28,31] of saffron. It is well known that saffron is a recalcitrant species towards adventitious roots induction under *in vitro* conditions[31].

According to the other studies performed, Mir et al[16] found that when 27 µM NAA and 44.4 µM BA were added in G-5 medium, apical bud (25 % in response) produced microcorm 1-1.5 g in weight. Sharifi and Ebrahimzadeh[26] used different NAA and IBA doses in MS and B5 medium for root induction. According to the data B5 with 2.46 µM IBA gave the best root percentage (76.9 %) and MS with 19.6 µM IBA gave the highest number of root per explant. Zeybek et al[31] found that MS with 1 mg/l IBA yielded higher corm initiation (0.52) and number of corms (1.04) as well as MS with 2 mg/l IBA yielded higher root initiation (0.68) and root number (4.96). In our study MS with 1 mg/l IBA resulted in 33.3 % daugher corm formation and 1 corm/corm formed explant.

Direct tissue culture methods does not produce somaclonal variation in general. If difficulties in labor and cost can be eliminated, saffron corm and whole plant production will be feasible via the methodology. The present studies are expected to lead to attention of efficient methods of Crocus sativus L., saffron, sterilization, plant regeneration and corm production via in vitro direct tissue culture methods to increase the rate of multiplication of healty corms.

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Fig. 1: Sterilized and cultured corm explant with meristematic nods after 2 weeks in MS with 4 mg/l BAP

Fig. 2: Initiated but showed no-progress shoots in MS with 1 mg/l BAP after 90 days.

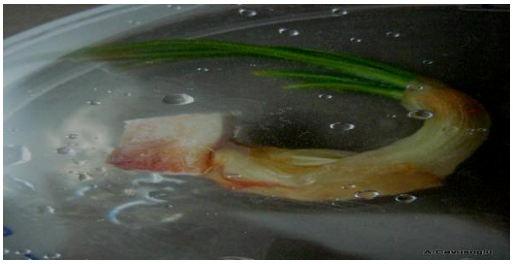

Fig. 3: Initiated and developed in leaves explant in MS with 6 mg/l BAP after 90 days.

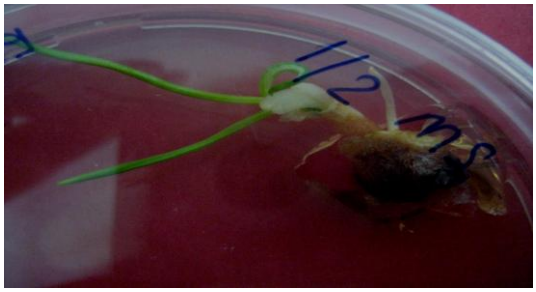

Fig. 4: Shoot initiation and foliation in ½ MS medium in 90 days.

Fig. 5: Root formation from the corm explant with fully growth plant in MS with 2 mg/l IBA after 120 days

Fig. 6: New corm forming at the base of plant in MS with 1 mg/l IAA

Fig. 7: Whole plants with new formed corms developed in MS with 1 mg/l IAA

Fig. 8: New formed corms attached to main corm explants in MS with 1 mg/l IAA in 120 days.

Table 1
***Crocus sativus* L. corm contamination rate after sterilization procedures at the end of the fourth week**

Sterilization number	Sterilization procedures	Treatment duration	Contamination rate
1	70 % Ethanol 50 % Na-hypochlorid 7 % H_2O_2	5 minute 15 minute 15 minute	0 % e*
2	5 % Tween-20+distilled water 70 % Ethanol 50 % Na-hypochlorid 7 % H_2O_2	60 minute 20 minute 5 minute 10 minute	16.67 % d
3	5 % Tween-20+distilled water 70 % Ethanol 7 % H_2O_2	75 minute 20 minute 20 minute	60 % c
4	5 % Tween-20+distilled water 70 % Ethanol 7 % H_2O_2	90 minute 15 minute 15 minute	83.3% b
5	10 % Ethanol+ 5 % tween-20 0.15 % $HgCl_2$	10 minute 10 minute	93.3 % a
			LSD: 11.27 $S\tilde{x}$: 3.578

*Means within the column having different letters were significantly different at $P < 0.05$.

Table 2
Direct shoot initiation from meristematic region of *Crocus sativus* corms and foliation rate from the proliferated shoots in 90 days

Media	Shoot initiation (%)	Foliation from the shoot (%)
½ MS	50 bc*	73.3 b*
MS+ 1 mg/l BAP	16.7 e	0 d
MS+ 2 mg/l BAP	33.3 cd	30.5 c
MS+ 4 mg/l BAP	63.3 b	57.1 b
MS+ 6 mg/l BAP	96.7 a	93.3 a
MS+ 8 mg/l BAP	66.7 b	25 c
MS+ 10 mg/l BAP	20 de	0 d
	LSD: 10.82 $S\tilde{x}$: 3.568	LSD: 14.46 $S\tilde{x}$: 4.768

*Means within the columns having different letters were significantly different at $P < 0.05$.

Table 3
Daughter corm formation and root occurance from the proliferated plants derived from MS+ 6 mg/l BAP culture medium in 120 days

Media	Daughter corm formation (%)	Daughter corm number/ the corm formed explant	Rooting rate (%)	Root number/ the root formed explant
MS+ 1 mg/l IBA	33.3 b*	1 b*	26.7 b*	1 ab*
MS+ 2 mg/l IBA	13.3 b	1 b	46.7 a	1.5 a
MS+ 1 mg/l IAA	76.7 a	1.74 a	10 c	1 ab
MS+ 2 mg/l IAA	26.7 b	1 b	6.67 c	0.67 b
	LSD: 14.39 $S\tilde{x}$: 4.413	LSD: 0.1141 $S\tilde{x}$: 0.03498	LSD: 11.11 $S\tilde{x}$: 3.406	LSD: 0.5516 $S\tilde{x}$: 0.1691

*Means within the columns having different letters were significantly different at $P < 0.05$.

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