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Effects of Osmolytic Agents on Somatic Embryogenesis of Saffron (*Crocus sativus* L.)

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Abstract

A protocol for callus induction from meristem tissues and subsequent somatic embryo formation were established in this study. Explants were taken from apical and lateral meristems of saffron and these explants were cultured on MS medium supplemented with combinations of 2.4-dichlorophenoxyacetic acid (2.4-D) and Kinetin (Kn). The effects of osmotic agents such as abscisic acid (ABA), polyethylene glycol (PEG) and Gelrite on somatic embryogenesis were also investigated. After 45 and 60 days of culture, calli were induced from apical and lateral meristems, respectively. The apical meristems yielded higher quality calli when compared to the lateral meristems. The highest frequency of callogenesis and the growth rate of callus were achieved from apical meristems on Murashige and Skoogs (MS) medium supplemented with 2.4-D (2 mg/l) and Kinetin (0.5 mg/l). After 45 days of subculture, the segments of nodular calli were transferred to plant growth regulator (PGR)- free media for induction of pre-embryogenesis embryo formation. Pre-matured embryos were cultured on MS medium supplemented with different osmotic agents such as Gelrite, ABA and PEG to study their effects on embryo maturation. Both PEG and ABA proved more effective for somatic embryo maturation as compared to Gelrite.

Keywords: abscisic acid, gelrite, polyethylene glycol, meristem tip, micropropagation

Introduction

Saffron (*Crocus sativus* L.) belongs to the family Iridaceae (Angiosperms) and is one of the most important spices used and cultivated in Iran. Its valuable dried spice accumulates in the stigma; is widely used for its odoriferous, coloring and medicinal properties (Abdullaev, 2002). Saffron is a triploid and conventionally propagated asexually through corm. Corm regeneration rate under natural conditions is very low, resulting into a limited supply of planting material for field cultivation (Chahota *et al*., 2003). Plant tissue cultures are widely used for clonal propagation of many plants (Inamdar *et al*., 1990; Kher and Nataraj, 2012; Kher *et al*., 2014; Varghese *et al*., 1993; Verghese *et al*., 1993). Like many other asexually propagated plants, meristem culture followed by regeneration through tissue culture is the most effective method for mass production of healthy and pathogen-free saffron plant materials (Sheibani *et al*., 2006). Saffron propagation through somatic embryogenesis is cost effective and efficient. Saffron breeding is hampered mainly due to sterility caused by triploidy (Agayev, 2002). Somatic embryogenesis is an *in vitro* method of plant regeneration widely used as a suitable start for breeding superior cultivars with high quality individuals and high yielding, through mutation, somaclonal variation and genetic engineering (Hussain *et al*., 2012).

Somatic embryogenesis is a process by which somatic cells are differentiated into somatic embryos when cultured on suitable growth regulator enriched medium (Feher *et al*., 2003). For callus induction and somatic embryogenesis in saffron, different explants like corm tissues, auxillary and terminal buds, lateral and apical meristems, leaves, nodal tissues and various floral organs have been used (Ahmad *et al*., 2014a; Ahmad *et al*., 2014b). Initially, somatic embryogenesis in saffron reported by culturing explants on various media fortified with different combinations of growth regulators e.g. auxins and cytokinin supplemented with different osmo-regulators. There are few studies in the literature about the regeneration of saffron via somatic embryogenesis and effects of osmo-regulators on the induction and maturation of somatic embryo. George *et al*. (1992) reported for the first time successful somatic embryogenesis by using corm meristems cultured on medium enriched with 2.4-D for callus formation. Subsequent globular embryo redifferentiation was achieved in the presence of IAA, Kinetin and with the addition of ascorbic acid in the medium.

Ebrahimzadeh *et al*. (2000) and Blázquez *et al*. (2004) reported embryogenesis in saffron with a different combination of growth regulators and maturation without any additive. But Raja *et al*. (2007) combined steps of maturation and germination by using medium supplemented with abscisic acid (ABA) along with GA_3.

Osmotic agents such as ABA, polyethylene glycol (PEG) and Gelrite can provide low osmotic potential in the medium, therefore improve the quality of somatic embryos

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(Stasolla et *al*., 2003). PEG was used as a non-permeating osmoticum in maturation media and proved effective in increasing somatic embryo germination, normal development and conversion rate (Braybrook and Harada, 2008; Morcillo *et al*., 2001; Stasolla *et al*., 2003; Wasilewska *et al*., 2008). ABA used at low levels increased somatic embryo formation in early stages, whereas during the later developmental stages higher ABA levels seem to be beneficial (George *et al*., 2008). This study was started to develop an efficient protocol for somatic embryogenesis in saffron by testing different combinations of explants, growth regulators and osmotic agents.

Materials and methods

Healthy saffron corms were harvested from a local saffron accession (from a commercial farm located in Qaen, Khorasan province, Iran). Freshly harvested corms were thoroughly washed in running water for 30 min and disinfected with detergent. Disinfected corms were then soaked in Hygen (1% benzalconium chloride) for 10 min and subsequently rinsed with tap water. For surface sterilization, the scales were removed and the corms were sterilized using 70% ethanol for 4 min followed by 15 min wash with 20% v/v commercial bleach (containing 1% sodium hypochlorite) and finally rinsed with sterile distilled water thrice for 15 min.

Composition of the media

Cube sections both from apical and lateral corm regions were cultured on basal MS (Murashige and Skoog, 1962) medium supplemented with 0.7% agar, 3% sucrose and different combinations of 2.4-D and Kinetin (Table 1). After 2 months emerging calli were subcultured on MS medium supplemented with 2 mg/l 2.4-D and 0.5 mg/l Kinetin. After 10 months lateral segments of nodular calli (Fig. 1) were transferred to basal medium without plant growth regulators for pre-maturation. Three osmotic agents e.g. ABA (1 mg/l), PEG (5%) and Gelrite (0.5%) were used to study their effect on embryo maturation. All media pH was adjusted to 6.0 before sterilization by autoclaving at 121 °C for 20 min. All cultures were incubated and maintained in the dark at 25± 2 °C.

Data analysis

A completely randomized design was used for this experiment. Five replicates each with five meristems were used for each treatment. Percentage of callogenesis was recorded after 8 weeks of culture. Effects of 2-5 hormonal treatments on apical and lateral meristems callogenesis were investigated. Heights of calli were recorded on a scale of 0-2 (0: None, 1: Medium, 2: High). Average calli diameter was determined based on the average of the longest width and length of calli, as suggested by Taylor and Secor (1992). Finally, calli rate of growth index was calculated by multiplying the average diameter and height of calli.

To study the effect of different osmotic agents on embryo maturation, fifteen explants (five in each plate) were placed on MS medium including ABA, PEG and Gelrite. Experiment was replicated two times and the data were analyzed using SAS Version 9.0. Means with significant differences were compared using Duncan's Multiple Range Test (DMRT) at P≤ 0.05 probability level of significance.

Results and discussions

Callus initiation was observed from apical meristem after 45 days of inoculation and after 60 days from lateral meristem cultures respectively (Fig. 1). During initial stages of callus development, white to yellowish and soft calli grew from cultured explants, which after 6 sub-culturing could be characterized into two types: off-white, friable and nodular, secondly yellowish soft non-embryogenic calli (Fig. 1A). Significant differences were observed for different explants and growth regulators combinations.

Frequency of calli initiation ranged between 16-44%

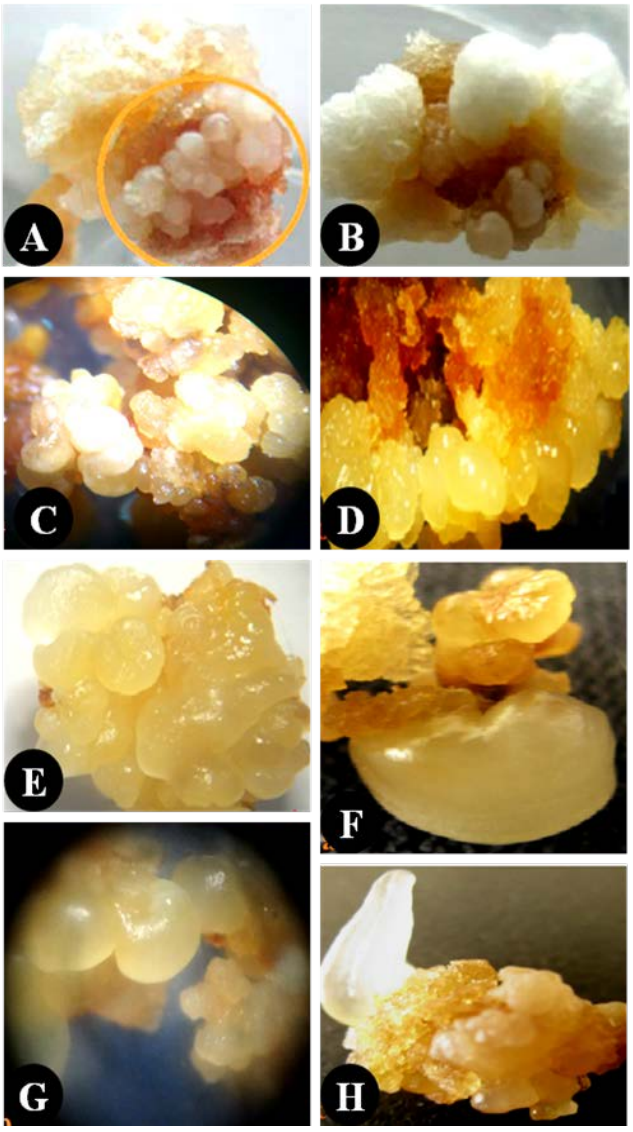

Fig. 1. Effect of explants on callus induction on MS medium supplemented with growth regulator (2mg/l, 2.4-D and 0.5 mg/l, Kinetin). A: from lateral meristem, B: apical meristem. C: Gelrite, D: ABA, E: PEG, F. Heart Shape SE, G: Globular SE, H: Torpido SE. Callus initiation was observed earlier from apical meristem (45 days) of inoculation and after 60 days from lateral meristem respectively. Somatic embryogenesis characterized by asymmetric callus induction. For embryo maturation nodular calli were transferred to a basal MS medium without growth regulators

(Table 1). The highest frequency of callus induction (44%) was observed on MS medium supplemented with 2.4-D (2 mg/l) and Kinetin (0.5 mg/l), culture from apical meristem. Similarly, different results for maximum calli height, diameter and area were observed from apical meristem when cultured on 2.4-D (2 mg/l) and Kinetin (0.5 mg/l) (Table 1). In case of lateral meristem, significant results among growth regulator concentrations were also achieved. All traits were observed to be superior and significantly different except callus height, which was slightly better at 2.4-D (4 mg/l) and Kinetin (1.0 mg/l) respectively (Table 1).

Embryo pre-maturation was achieved on growth regulator free medium after 45 days of culture. After pre-maturation stage, calli were transferred to different media containing ABA, PEG and Gelrite as osmo-regulators respectively (Fig. 2). The best results were obtained from medium supplemented with PEG and ABA (Table 2) which gave the highest number of somatic embryos. It was concluded that apical meristem proved better than lateral one when cultured on medium containing 2.4-D (2 mg/l) and Kinetin (0.5 mg/l) and supplemented with PEG or ABA osmotic agents.

In this study callus formation was successfully achieved by using a combination of 2.4-D and Kinetin, which agrees with some previous reports (Chaloushi *et al*., 2007; Dalila *et al*., 2013; Ebrahimzadeh *et al*., 2000; Inamdar, *et al*. 1990; Karamian and Ebrahimzadeh, 2001; Karamian and Ranjbar, 2010; Varghese *et al*., 1993, Verghese *et al*., 1993). Darvishi *et al*. (2006) reported a medium containing NAA and BAP which supported the induction of non-embryogenic callus, while medium containing 2.4-D and BAP had the best effect on induction of embryogenic callus. Highest growth rate and cell division have been reported when cultures were maintained in the dark (Karamian and Ebrahimzadeh, 2001). Light has been reported to be inhibitory for auxin production and this may offset the balance of growth hormones in the growing tissue (Asif *et al*., 2001).

For successful callus initiation it is important to use auxin as an inducer. Among different auxins, 2.4-D is commonly used for somatic embryogenesis (alone or in combination with other growth regulators) (Gaj, 2001). However, decreasing concentration or complete removal of exogenous auxin has been reported to be necessary for embryo maturation and further development (Sharifi *et al*., 2012). Selection of embryogenic regions and transferring them into free regulator resulted in notable growth of embryogenic calli. It was suggested that division of the pre-embryogenic cells and their development into embryos is only resumed at lower auxin concentrations (Karamian and Ebrahimzadeh, 2001). Low osmotic potential in the maturation medium can significantly affect somatic embryo maturation (Walker and Parrott, 2001). Therefore, application of ABA, PEG and Gelrite can help to slow down the osmotic potential in the medium and thus improve the quality of somatic embryos, by promoting normal differentiation (Stasolla *et al*., 2003). When PEG was used as a non-permeating osmoticum in maturation media it proved effective in increasing somatic embryo germination and its conversion rate (Morcillo *et al*., 2001; Stasolla *et al*., 2003). ABA has shown to be important in both somatic embryogenesis and in maturation of somatic and zygotic embryos (Braybrook and Harada, 2008; Jimnez, 2005; Wasilewska *et al*., 2008). Comparison of the scores revealed the effective role of ABA in the maturation of the embryos and in the embryonic stages; it appears that ABA acts as an effective inducer of the expression of the genes, involved in maturation (Linacero, 2001; Chugh *et al*., 2002). Any significant difference between the effects of ABA and PEG on somatic embryo maturation was not observed. The morphology and structure of the embryogenic callus (Figs. 1 C, D and E) and somatic embryos development through various stages of globular, heart-shaped and torpedo-shaped (Figs. 1 F, G and H) are in agreement with those previously reported (Karamian and Ebrahimzadeh, 2001).

Table 1. Effects of 2.4-D and Kinetin concentrations on callusing, callus height, callus diameter and callus area of lateral and apical meristem as explants

Explants	PGRS (mg/l)		Callusing (%)	Callus height (Scoring)	Callus diameter (cm)	Callus area (diameter ×height)
	2.4-D	Kn				
Lateral meristem	2	0.5	32±4.89 abc	1±0 b	1.67±0.11 a	1.67 ± 0.11 b
	1	1	24.0±4 bcd	0.8±0.2 b	1.08±0.27 bc	1.08±0.27 b
	1	4	20±6.32 cd	1±0 b	0.98±0.12 c	0.98±0.12 b
	4	1	24.0±4 bcd	1.2±0.2 ab	1.17±0.07 bc	1.46 ± 0.36 b
	2	8	16.0±4 d	0.8±0.2 b	0.86±0.21 c	0.68 ± 0.21 b
Apical meristem	2	0.5	44.0±4 a	1.6±0.25 a	1.77±0.08 a	2.91±0.54 a
	2	8	36.0±4 ab	1.2±0.2 ab	1.47±0.07 ab	1.81±0.39 ab

Note: values are mean ± standard deviation; values followed by the same letter are not significantly different at P< 0.05

Table 2. Effects of osmo-regulators on somatic embryos number

Number of somatic embryos	Treatment
3.30 ± 0.54 a	ABA
3.35 ± 0.34 a	PEG
1.55 ± 0.09 b	Gelrite

Note: values are mean ± standard deviation; values followed by the same letter are not significantly different at P< 0.05

Conclusions

In this study, effects of different growth regulators as well as osmotic embryogenesis from apical and lateral meristems in saffron (*C. sativus* L.) were observed. Somatic embryos can be used to increase genetic variation in saffron germplasm through mutation breeding and by selection of superior quality within somaclonal variants exhibiting desired traits. Single cell origin of somatic embryos will be a great advantage for adding disease resistant and other desired genes through genetic engineering.

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