Research article

A robust workflow for indirect somatic embryogenesis and cormlet production in saffron (*Crocus sativus* L.) and its wild allies; *C. caspius* and *C. speciosus*

Ayat Taheri-Dehkordi [a], Roohangiz Naderi [a], Federico Martinelli [b], Seyed Alireza Salami [a,*]

[a] Department of Horticultural Science, Faculty of Agricultural Science and Engineering, University of Tehran, Karaj, Iran
[b] Department of Biology, University of Florence, Sesto Fiorentino, Italy

ARTICLE INFO

ABSTRACT

Saffron (*Crocus sativus* L.) and its wild relatives, *Crocus caspius* and *Crocus speciosus* are of considerable significance in the pharmaceutical, nutraceutical, and ornamental bulbs industry. Towards the ultimate goal of the conservation of wild *Crocus* species and establishment of an efficient workflow for *in vitro* production of Crocuses, efficient protocols were developed for disinfection and *in vitro* production of cormlets in *C. sativus* and its wild allies *C. caspius* and *C. speciosus*. Moreover, the differential expression of the Somatic Embryogenesis Receptor-like Kinase (*SERK*) gene was evaluated as a potential molecular marker during embryogenesis between embryogenic and non-embryogenic calli. A highly efficient disinfection recipe and a low-cost TDZ-free protocol have been successfully developed for *in vitro* cormlet production in three *Crocus* species. MS medium containing 10.18 μM 2, 4-D + 4.44 μM BAP was most efficiently induced callus and somatic embryo formation. The highest conversion frequency and maximum cormlet weight were achieved in MS containing 5.37 μM NAA + 8.88 μM BAP. The *SERK* expression was significantly much higher in embryogenic calli than non-embryogenic in all *Crocus* species. The current low-cost and easy-to-use recipe suggests a promising *in vitro* propagation workflow for mass production of uniform pathogen-free cormlets of *Crocus* species, as well as a platform to better conservation of wild *Crocus* species and effective gene and genome editing using CRISPR-Cas9 in future studies.

1. Introduction

The Iridaceae family with more than 2,000 species and 70 genera is divided into seven subfamilies based on DNA sequence data (Goldblatt et al., 2006). Iridaceae is one of the most important families from ornamental and medicinal points of view. The secondary metabolites produced in this family are responsible as insecticidal, antifungal and antibacterial and are involved in defenses against herbivores and microbial infections, and anti-inflammatory and antioxidant activities which are important in medicine (Li and Vederas 2009). The genus *Crocus* currently consists of about 100 corm-bearing, perennial species distributed from central Europe, North Africa to Western China; the center of species diversity is in Asia Minor and on the Balkan Peninsula (Harpke et al., 2013). Many crocuses are well-known as ornamentals; however, *Crocus sativus* L. (saffron), the most valuable spice in the world, is the most renowned member of the *Crocus* genus (Negbi 1999). Saffron is a sterile triploid (2n = 3x = 24) plant with a large genome size and a

unique source of specific apocarotenoids, including crocin, picrocrocin, and safranal, which contribute to its color, flavor, and aroma, respectively (Tarantilis et al., 1995). These molecules have exceptional therapeutic characteristics such as anti-cancer and anti-tumor effects (Rezaee-Khorasany et al., 2019).

Iran accounts for over 90% of the world's saffron production (Vahedi et al., 2018). The relative advantages of saffron cultivation, such as higher prices, low water requirements, adaptation to low-input systems, and the opportunity of long-term exploitation with one-time cultivation, have drawn farmers' attention to this precious crop. There is a growing demand for saffron cultivation around the world, however, due to the sterile nature of saffron, the only technique of propagation is clasically by daughter corms forming below or on the sides of mother corms; the mother corm thrives only for one growing season and produces 4–5 cormlets; therefore, the rate of propagation is relatively low. Moreover, saffron sterility culminated in poor genetic variation and as a result, limited the use of conventional breeding approaches; so, researchers are

now trying to use alternative techniques such as biotechnological approaches. Recently, a successful CRISPR/Cas9 system was reported for gene editing in saffron (Chib et al., 2020). Altogether, the use of tissue culture for mass production of cormlets is inevitable; it also provides the requisite platform for the improvement of saffron traits through molecular breeding.

Besides *Crocus sativus* L., eight other *Crocus* species are growing wild in Iran (Figure 1), including *C.* caspius, *C. speciosus, C. cancellatus, C. pallasii, C. almehensis, C. gilanicus, C. biflorus,* and *C. michelsonii* (Mathew 1983). Wild *Crocus* species are also of considerable importance due to the following reasons: (1) their relevance to *C. sativus* (Fernández 2004), (2) theories of saffron origin and being considered as the saffron parental species (Schmidt et al., 2019), (3) alternative sources for extraction of apocarotenoids (Ordoudi et al., 2019), (4) many of these species are on the verge of extinction in different countries (Fernández 2007), and (5) as ornamental bulbs as well as (6) pharmaceutical properties (Zengin et al., 2020). One of the primary objectives of the convention on biological diversity is to achieve a significant reduction of the current rate of biodiversity loss, such as plant taxa closely related to species of specific socio-economic significance including food, condiments, pharmaceutical products, and ornamentals (Maxted et al., 2007). Improving attempts to systematically conserve plant genetic resources to guarantee appropriate and representative diversity for future uses, including breeding programs, is an essential step towards attaining this objective (Fernández et al., 2011), and plant tissue culture has long been considered as one of these approaches for *ex situ* plant conservation (Niazian 2019).

A wide variety of microorganisms (bacteria, yeasts, filamentous fungi, etc.), were considered as contaminants *in vitro* (Altan et al., 2010). As one of the major considerations in plant tissue culture, successful disinfection of explants would guarantee the establishment and maintenance of plants. It should be noted that the contamination of corms and other underground organs has been reported to be up to 100% (Yasmin et al., 2013), so the development of an efficient disinfection protocol is more crucial and a major concern for geophytes, especially *Crocus* species.

Different plant growth regulators (PGRs) are used in plant tissue culture, and effective callogenesis relies on the appropriate combination of these compounds. Owing to their pervasive role in cell cycle regulation
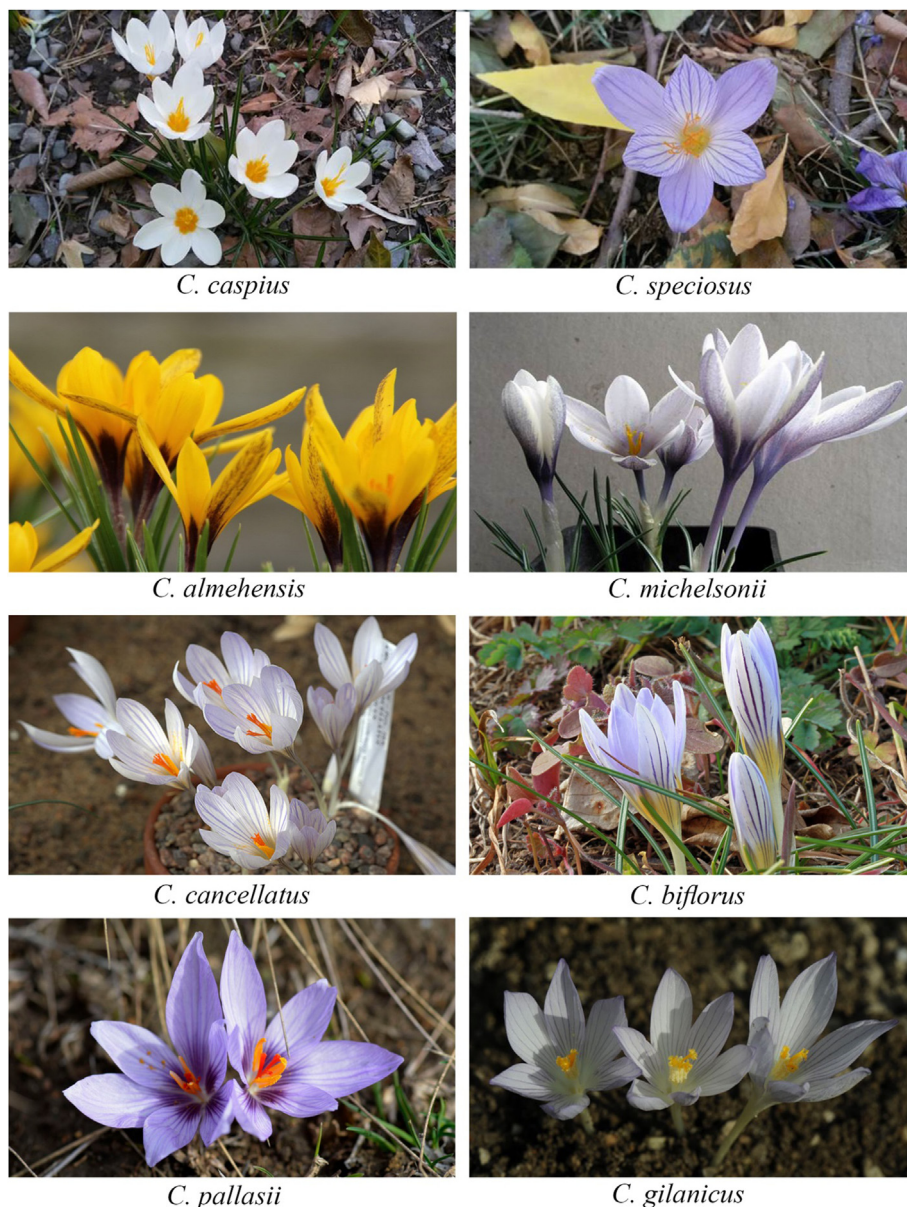

Figure 1. Schematic view of eight *Crocus* species grown wild in Iran.

Table 1. Disinfection protocols used for surface sterilization of *Crocus* corms.

Protocol 1	Protocol 2	Protocol 3
1 Removal of tunics and preliminary washing with tap water.	1 Removal of tunics and preliminary washing with tap water.	1 Removal of tunics and preliminary washing with tap water.
2 Washing under running tap water for 30 min.	2 Washing under running tap water for 30 min.	2 Washing under running tap water for 30 min.
3 Heat treatment (45 °C) for 15 min.	3 Submerge in 70% ethanol for 1 min.	3 Cleaning with Tween 20 and brush.
4 Submerge in 70% ethanol for 1 min.	4 Submerge in 1.5% Sodium hypochlorite for 15 min.	4 2,4-Dichloro Meta Xylenol (DCMX) 0.6% (V/V) for 30 min.
5 Submerge in 1.5% Sodium hypochlorite for 15 min.	5 Washing three times with sterilized double distilled water	5 Washing with sterilized double-distilled water.
6 Washing three times with sterilized double distilled water		6 Submerge in 70% ethanol for 45 s.
		7 Washing three times with sterilized double distilled water

Protocol 4	Protocol 5	Protocol 6
1 Removal of tunics and preliminary washing with tap water.	1 Removal of tunics and preliminary washing with tap water.	1 Removal of tunics and preliminary washing with tap water.
2 Shaking in Erlenmeyer flask with water and a few drops of washing detergent.	2 Soaking in water containing a few drops of washing detergent and Tween 20 for 15 min.	2 Soaking in water containing a few drops of Tween 20 and washing detergent.
3 Washing under running tap water for 20 min.	3 Washing under running tap water for 30 min.	3 Washing under running tap water for 30 min.
4 Submerge in 70% ethanol for 30 s.	4 Submerge in 70% ethanol for 30 s.	4 Submerge in 70% Ethanol for 30 s.
5 Washing three times with sterilized double-distilled water.	5 Submerge in 0.3% (W/V) Rovral-TS fungicide for 30 min.	5 Submerge in 0.3% (W/V) Rovral-TS fungicide for 30 min.
6 Submerge in Benomyl fungicide (2 mg/mL) for 15 min.	6 Submerge in 10% (V/V) Domestos ® for 30 min.	6 Submerge in 0.1% (W/V) HgCl2 for 15 min.
7 Submerge in 2.5% Sodium hypochlorite for 20 min.	7 Washing three times with sterilized double distilled water	7 Submerge in 0.05% (W/V) HgCl2 for 5 min.
8 Washing three times with sterilized double-distilled water.		8 Submerge in 10% (V/V) Domestos ® for 30 min.
		9 Washing three times with sterilized double-distilled water

and cell division control, auxins and cytokinins are essential factors in the determination of embryogenic response (Francis and Sorrell, 2001). To develop an efficient and practicable protocol with lower cost and due to their reported better results in *Crocus* species (Gantait and Vahedi 2015), 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA), and Picloram were selected as auxin sources and, cytokinins other than thidiazuron (TDZ), i.e. N^6-benzylaminopurine (BAP) and Kinetin (Kin) were used. It is noteworthy to mention that the prices of BAP and Kin compared with TDZ are around 30 times and 15 times lower, respectively.

Somatic embryogenesis (SE) is a process that offers a valuable tool for improving the genetics of distinct plant species and has considerable interest in biotechnological applications (Chugh and Khurana 2002). Data from several studies suggest that during plant embryogenesis, many genes are specifically activated or differently expressed (Kumar and Van Staden 2019). It has become apparent that a group of receptor-like kinases (RLKs) which contain leucine-rich repeats (LRRs) in their extracellular domain controls the molecular events leading to the development of somatic embryos. RLKs form a broad gene family responsible for diverse processes of signal transduction. The main group of RLKs includes leucine-rich-repeat RLKs (LRR-RLKs) (Tichtinsky et al., 2003). Somatic Embryogenesis Receptor-like Kinase (*SERK*) gene belongs to the group of highly conserved leucine-rich receptor-like kinase II (LRRII-RLK) (Hecht et al., 2001). *SERK* is reported to play a significant role among the genes engaged in plant embryogenesis and the early expression of the *SERK* gene is closely correlated to the formation of embryogenic cells in many plant species (Kumar and Van Staden 2019). *SERK* gene expression is considered a molecular marker of competent somatic embryogenic cells (Salaj et al., 2008). Elucidation of the essential molecular events that contribute to SE has the potential to improve the methods of propagation for *in vitro* culture specifically in recalcitrant plants (Mahdavi-Darvari et al., 2015).

Research to date has tended to focus on saffron tissue culture rather than *Crocus* wild species, and much less is known about *SERK* gene expression during somatic embryogenesis in saffron and its wild relatives. The primary objective of this paper was, therefore, to develop an efficient low-cost, and easy-to-use protocol for disinfection of corms as well as *in vitro* mass production of cormlets in saffron and its wild allies *C. caspius* and *C. speciosus*. Besides, the expression of *SERK* gene was assessed in embryogenic and non-embryogenic calli of three *Crocus* species to provide a preliminary view of the *SERK* expression as a probable molecular marker of embryogenesis in *Crocus* species. Our later objective was to develop a platform to better conservation of wild *Crocus* species and effective gene and genome editing using molecular tools such as CRISPR-Cas9 in future studies.

2. Materials and methods

2.1. Explants and surface sterilization

Corms of *C. sativus* L. (Ghaenat accession), *C. speciosus,* and *C. caspius* were collected from the research farm and Botanical Garden of the University of Tehran, Karaj, Iran, respectively. Six different protocols were utilized to optimize the sterilization procedure (Table 1). The experiment was carried out as a completely randomized design with five replicates per treatment and three corms in each replicate. The percentages of contamination (Eq. 1) and explant survival (Eq.2) were recorded after four weeks.

$$Contamination\ (\%) = \frac{number\ of\ contaminated\ explants}{total\ number\ of\ explants\ cultured} \times 100 \qquad (1)$$

$$Explant\ survival\ (\%) = \frac{number\ of\ survived\ explants}{total\ number\ of\ explants\ cultured} \times 100 \qquad (2)$$

Table 2. Effects of different combinations of PGRs on the frequencies of callus induction, embryogenesis, and the number of somatic embryos per explant in *Crocus* species.

Crocus species	Concentration of PGRs (μM)				Mean ± SE		
	2,4-D	Picloram	BAP	Kin	Frequency of Callus induction	Frequency of embryogenic callus	Number of somatic embryos per explant
C. sativus	0.0	0.0	0.0	0.0	0.00 e	0.00 f	0.00 f
	10.18	-	-	2.32	80.0 ± 4.5 b	65.0 ± 6.12 b	4.3 ± 1.92 b
	10.18	-	4.44	-	100.0 ± 0.00 a	80.0 ± 5.0 a	6.45 ± 2.88 a
	-	2.07	-	1.16	65.0 ± 5.5 c	55.0 ± 5.0 bcd	3.90 ± 1.74 c
C. caspius	0.0	0.0	0.0	0.0	0.00 e	0.00 f	0.00 f
	10.18	-	-	2.32	45.0 ± 5.00 d	40.0 ± 6.12 e	1.3 ± 0.12 e
	10.18	-	4.44	-	70.0 ± 5.00 bc	60.0 ± 5.16 bc	2.22 ± 0.09 d
	-	2.07	-	1.16	60.0 ± 6.12 c	50.0 ± 0.00 cde	1.7 ± 0.2 e
C. speciosus	0.0	0.0	0.0	0.0	0.00 e	0.00 f	0.00 f
	10.18	-	-	2.32	40.0 ± 6.12 d	42.66 ± 6.93 e	1.3 ± 0.2 e
	10.18	-	4.44	-	45.0 ± 5.00 d	48.06 ± 5.21 de	1.6 ± 0.41 e
	-	2.07	-	1.16	0.00 e	0.00 f	0.00 f

In each column, means with the same letters are not statistically different according to Duncan's multiple range test at $p < 0.05$.

2.2. Media preparation

The MS (Murashige and Skoog 1962) supplemented with vitamins (Duchefa Biochemie, Netherlands), 3% (w/v) sucrose (Duchefa Biochemie, Netherlands), and 0.8% (w/v) agar (Duchefa Biochemie, Netherlands), was used as the basic culture medium. The pH was adjusted to 5.8, before autoclaving at 121 °C for 20 min.

2.3. Callus induction and somatic embryogenesis

Rectangular slice of sterilized healthy corms (four slices per replicate and five replicates per treatment) were cultured on MS media supplemented with different combinations of plant growth regulators (PGRs) (Sigma-Aldrich, USA), as shown in Table 2. The cultures were maintained at 25 ± 2 °C in darkness and the frequency (%) of callus induction was recorded after 60 days of induction (doi). Clumps of calli (0.5 g each) were subcultured at 4-week intervals. Embryogenic calli were transferred to a PGR-free MS medium after 120 days. Non-embryogenic calli were spongy and amorphous, while the appearance of embryogenic calli was nodular and translucent (Sharifi et al., 2012). The frequency of embryogenic callus induction and the number of somatic embryos per explant were also determined after 160 doi using a binocular microscope (Zeiss Stemi SV6, Germany).

2.4. Somatic embryo germination and cormlet production

To estimate the conversion frequency of somatic embryos into cormlets, well-developed somatic embryos were transferred to MS medium containing different PGR combinations (Table 3). Explants were maintained at 25 ± 3 °C in 16 h light: 8 h dark photoperiod under 50 μmol m^{-2} S^{-1} cool white fluorescent light. To ensure the proper growth of the embryos, regular sub-culturing was performed at intervals of four weeks. The conversion frequency of somatic embryos into cormlets and the weight of cormlets per explant were recorded at the end.

2.5. SERK gene expression

To investigate the *SERK* expression among different *Crocus* species, both non-embryogenic and embryogenic callus samples were collected from the culture media supplemented with 10.18 μM 2,4-D + 4.44 μM BAP after 120 doi. Samples were quickly frizzed in liquid nitrogen and stored at -80 °C before RNA extraction. Total RNA was extracted (18 samples) using the NucleoSpin® RNA Plant Kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. cDNAs were synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) after DNase I (Thermo Fisher Scientific, USA). To check the cDNAs, a PCR reaction was performed using *rbcL* reference

Table 3. Effects of PGRs on cormlet production and cormlet weight in *Crocus* species.

Crocus species	Concentration of PGRs (μM)			Mean ± SE	
	NAA	BAP	2,4-D	Conversion frequency of somatic embryos into cormlets	Cormlet weight (g)
C. sativus	0.0	0.0	0.0	0.00 c	0.00 f
	2.69	9.99	-	69.09 ± 2.04 a	1.69 ± 0.07 b
	5.37	8.88	-	72.1 ± 2.42 a	1.87 ± 0.06 a
	-	8.88	2.26	67.63 ± 4.69 a	1.62 ± 0.02 b
C. caspius	0.0	0.0	0.0	0.00 c	0.00 f
	2.69	9.99	-	50.0 ± 0.00 b	0.86 ± 0.05 cd
	5.37	8.88	-	64.85 ± 4.17 a	0.96 ± 0.1 c
	-	8.88	2.26	53.33 ± 3.33 b	0.83 ± 0.03 d
C. speciosus	0.0	0.0	0.0	0.00 c	0.00 f
	2.69	9.99	-	53.33 ± 3.33 b	0.68 ± 0.03 e
	5.37	8.88	-	55.43 ± 6.33 b	0.79 ± 0.12 de
	-	8.88	2.26	0.00 c	0.00 f

In each column, means with the same letters are not statistically different according to Duncan's multiple range test at $p < 0.05$.
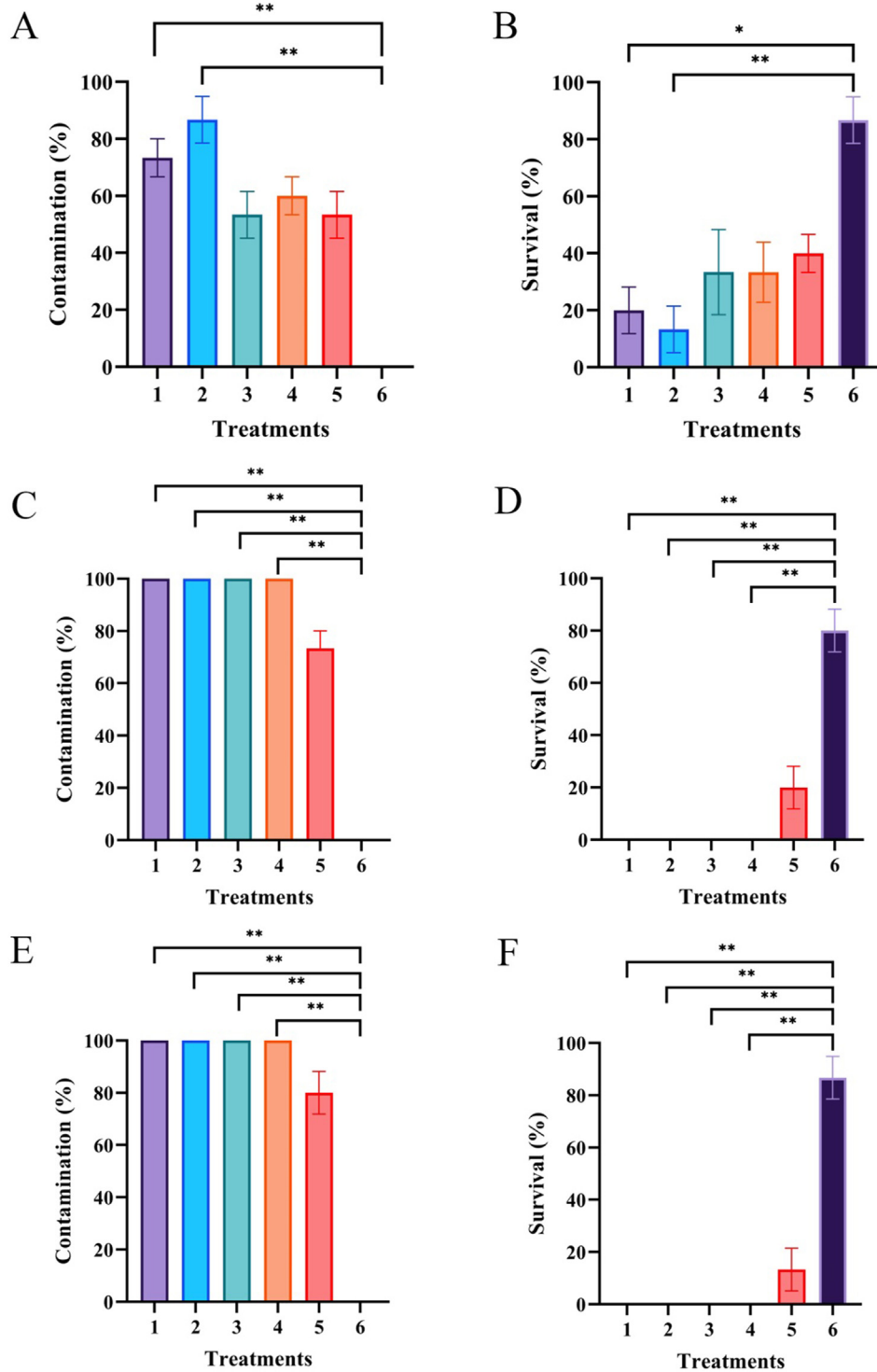
Figure 2. Contamination and survival rates of *Crocus* corms were sterilized with different disinfection protocols 1–6. (A, B) *C. sativus* (C, D) *C. caspius* (E, F) *C. speciosus*. Data are shown as means ± SE. Significant differences are indicated by horizontal capped bars. *: statistically significant with p-value <0.05; **: statistically significant with p-value <0.01.

gene primers (forward 5′-CTACTGGTACATGGACAACTG-3′ and reverse 5′- AATTGATTTTCTTCTCCAGCAACG-3′). Q-RT-PCR was performed in triplicate using RealQ Plus 2x Master Mix Green (Ampliqon, Denmark). The following *SERK* primer sequences were used to amplify the partial sequence of *SERK* gene: Forward: 5′-GCTAAAATGGAAGGTGATGC-3′ Reverse: 5′GCATTTCCAAGATCAACTC-3′ in three *Crocus* species. Q-RT-PCR was performed using the Step One Real-Time PCR system (Applied

Biosystems, USA) as the following PCR profile: an initial activation step at 95 °C for 15 min, and 40 PCR cycles of denaturing at 95 °C for 15 s, annealing at 58 °C for 30 s, and extension at 72 °C for 20 s followed by melting curve stage of 95 °C and 70 °C. The relative gene expression was calculated using the ΔΔCT method. All data were normalized against *rbcL* (as an internal control) and then the fold-changes in the *SERK* gene expression were measured.
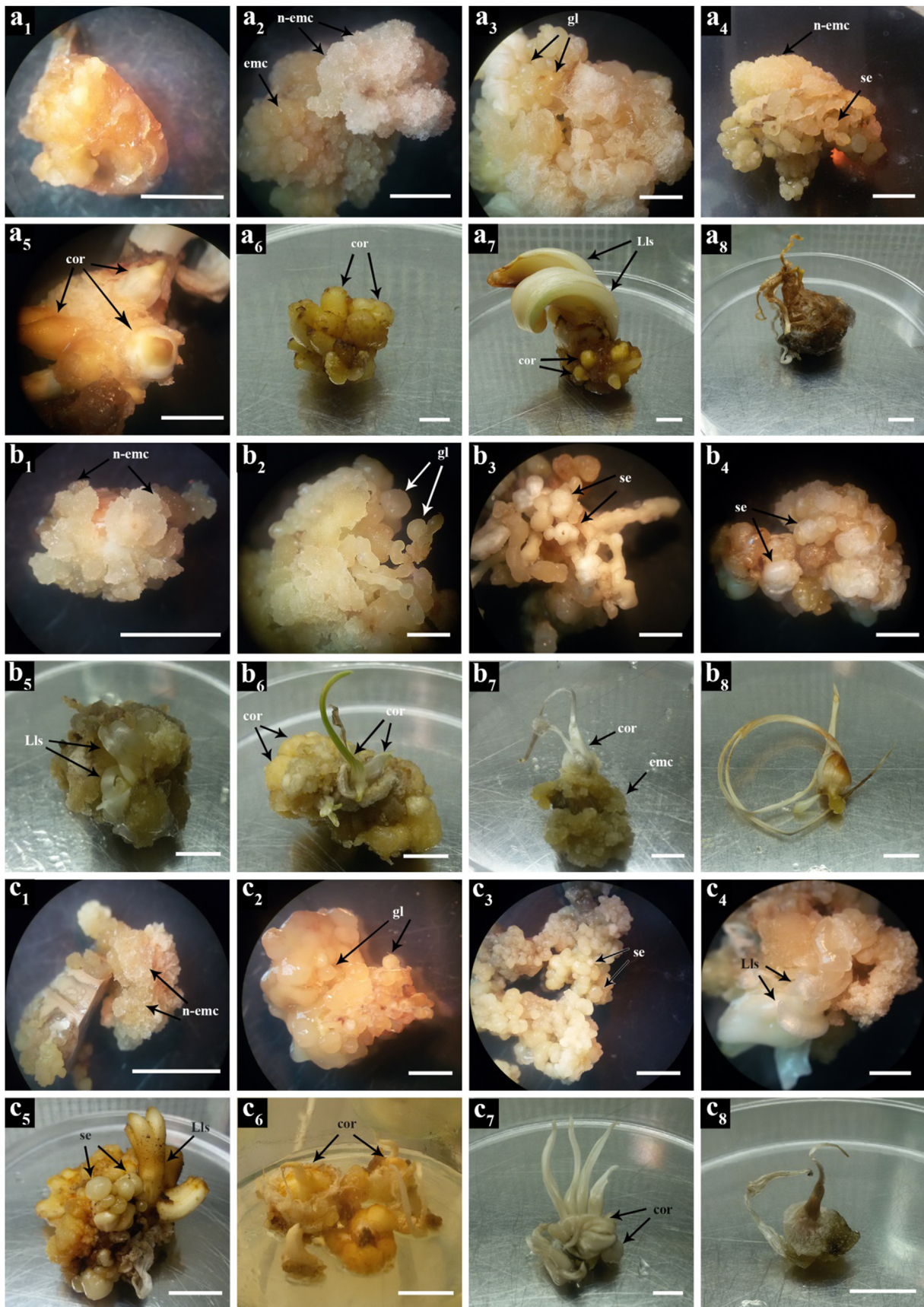
Figure 3. Schematic view of different developmental stages of somatic embryogenesis and cormlet production in *C. sativus* (a_1-a_8), *Crocus caspius* (b_1-b_8), and *Crocus speciosus* (c_1-c_8) in MS medium supplemented with 10.18 μM 2,4-D + 4.44 μM BAP. emc; embryogenic callus, n-emc; non-embryogenic callus, gl; globular embryo, se; somatic embryo, cor; cormlet, Lls; leaf-like structure. (Bar = 10 mm).
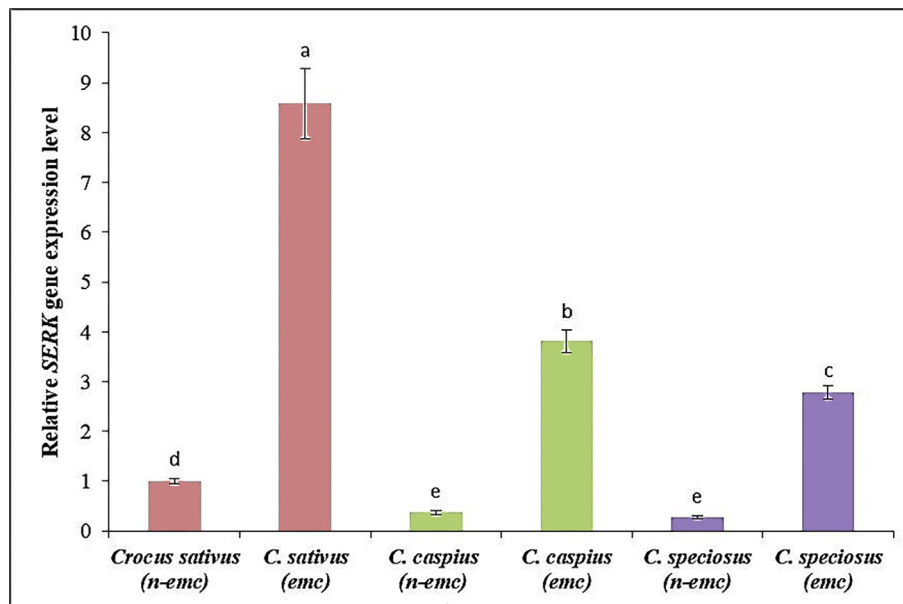
Figure 4. Relative expression of *SERK* gene in embryogenic (emc) and non-embryogenic (n-emc) calli of different *Crocus* species. Each point represents the mean of three replicates with error bars representing the SE. The *rbcL* was used as internal control and *SERK* expression in *C. sativus* non-embryogenic calli was considered as the control. Different letters above bars indicate significant differences according to Duncan's multiple range test at $p < 0.05$.

2.6. Statistical analysis

Statistical analysis was performed using SPSS, version 25 (IBM, New York, USA). The data were represented as the mean ± SE, and the values were subjected to analysis of variance (ANOVA) followed by Duncan Multiple Range Test as a post-hoc analysis. In the case of surface sterilization, data were analyzed using the Kruskal-Wallis test followed by Dunn's post-hoc test. A probability level of $P \le 0.05$ was considered significant.

3. Results

3.1. Disinfection efficiency

To investigate the efficiency of different disinfection protocols, corms were sterilized using six different recipes. No contamination was recorded while corms of *C. sativus* were sterilized using protocol 6; however, other protocols resulted in over 50% contaminations (Figure 2A). Treatments with protocols 1–5 resulted in 73.3%, 86.7%, 53.3%, 60%, and 53.3% contamination, respectively. There was a significant difference (Kruskal-Wallis H = 20.48, df = 5, p = 0.001) between protocol 6 and protocol 1 and 2. In *C. sativus* the highest survival rate (86.67%), was observed in protocol 6, while the lowest survival rate (13.33%) belonged to protocol 2 (Figure 2B). Results represented the efficiency of protocol 6 which effectively disinfected *C. sativus* corms and resulted in over 85% survival rate.

In *C. caspius*, and *C. speciosus*, protocols 1–4 failed to disinfect the corms which resulted in 100% contamination; however, the contamination was effectively controlled using protocol 6 (Figures 2C and 2E). The survival rate of *C. caspius* and *C. speciosus* was 80% and 86.67%, respectively using this protocol (Figures 2D and 2F).

3.2. Callus induction

The effects of different combinations of PGRs were studied on frequencies of indirect callus induction and embryogenesis in three *Crocus* species (Table 2). Explants neither induced callus nor calli-like structures in MS medium without any PGRs and turned necrotic after 4 weeks in all three species. In *C. sativus,* after 4 weeks, calli were developed in all the

PGR combinations; however, the highest frequency of callus induction (100%) was observed in MS containing 10.18 μM 2, 4-D + 4.44 μM BAP (Figure 3 a_1-a_8). Callus induction was observed in *C. caspius* and *C. speciosus* within 6 and 8 weeks, respectively. Compared with *C. sativus,* in these species, a lower callus induction frequency (45–70%) was observed in the same medium (Figure 3 b1-b8; c1-c8). The minimum callus induction (40%) was observed in *C. speciosus* using MS supplemented with 10.18 μM 2, 4-D + 2.32 μM Kin. MS containing 2.07 μM Picloram + 1.16 μM Kin was unable to induce any callus in *C. speciosus.*

3.3. Somatic embryogenesis, germination, and cormlet production

As shown in Table 3, calli were subsequently developed into somatic embryos; the first embryogenesis response was observed in *C. sativus* within 4 weeks. The other species have shown a later response; the first response for *C. caspius* and *C. speciosus* was observed within 8 and 10 weeks, respectively. In all species, explants in the MS medium supplemented with 10.18 μM 2,4-D + 4.44 μM BAP showed the earliest response and the subsequent growth patterns of the explants were stronger than other PGR combinations. The highest average number of somatic embryos in *C. sativus* (6.45), *C. caspius* (2.22), and *C. speciosus* (1.6) was obtained using MS containing 10.18 μM 2,4-D + 4.44 μM BAP. In the present research, after the transition of the calli to a PGR-free medium, somatic embryos were further developed and increased in size. Globular embryos were prevalent; embryos, individually or as clusters, were white or pale yellow, small, and globular in shape. Globular somatic embryos with a soft and shiny appearance have further developed into somatic embryos. In the next stage, the somatic embryos were germinated and the shoots were developed within 4 weeks. After 2–3 weeks, the base of the shoots began to swell, which is an indicator of the development of the cormlet. Finally, after the formation of cormlets, the shoots were dried and well-developed cormlet tunics were observed after 10–12 weeks. As shown in Table 3, the conversion frequency of somatic embryos into cormlets in *C. sativus* ranged from 67.63 to 72.1%; however, there were no significant differences among three combinations of PGRs on cormlet production in *C. sativus*; although, the maximum cormlet weight (1.87 g) was obtained using 5.37 μM NAA + 8.88 μM BAP compared to 0.96 g in *C. caspius* and 0.79 g in *C. speciosus.*

3.4. SERK gene expression

The *SERK* relative expression was analyzed after 120 doi. As shown in Figure 4, the *SERK* expression level manifested a significant change in embryogenic calli compared to non-embryogenic calli. *SERK* expression level was much higher (8.5-fold) in embryogenic calli of *C. sativus* than in *C. caspius* (3.8-fold) and *C. speciosus* (2.8-fold).

4. Discussion

In the present study, the major aim was to develop a promising low-cost and easy-to-use recipe for disinfection of the *Crocus* corms as a crucial step of an *in vitro* propagation workflow, as well as a platform to better conservation of wild *Crocus* species and effective regeneration protocol towards gene and genome editing of Crocuses in future studies. Although a few studies reported bleach and ethanol as the only ingredients for the disinfection procedure (Freytag et al., 2017), according to our results, common surface sterilizers such as sodium hypochlorite and ethanol are not effective to control contamination of *Crocus* corms, particularly wild *C. caspius* and *C. speciosus*. The major problem with hypochlorites is their instability and sensibility to light (Connell 2006). It has also been reported that hot water (40 °C) could decrease the contamination of *Lilium* explants (Langens-Gerrits et al., 1998); however, although heat treatment in combination with sodium hypochlorite and ethanol slightly reduced the contamination in this study, there were no significant differences between protocol 1 using heat treatment and protocol 2. Due to the health hazards of heavy metals, it is recommended to use disinfection methods without HgCl$_2$, so we have tried different protocols without HgCl$_2$ but they have not been successful. In a similar study on *Crocus vernus*, 100% contamination-free corm explants have been obtained using 2.0% (v/v) NaOCl for 10 min followed by treatment with 0.01% (w/v) HgCl$_2$ for 15 min (Sivanesan et al., 2014). By raising the concentration of HgCl$_2$, the level of contamination would decrease; where the maximum disinfection rate was obtained by using 1.6% HgCl$_2$ (Yasmin et al., 2013); however, in the current study, the use of lower concentrations of HgCl$_2$ (0.1 % (w/v) for 15 min and 0.05 % (w/v) for 5 min) culminated in completely disinfected explants demonstrating the significance of concentration and exposure time which are reported in previous studies (Hesami et al., 2018).

The survival rate of explants is known to be a crucial factor in any disinfection process (Hesami et al., 2019). In the present study, the survival rate of explants ranged between 80-86.67%. Approximately 81% of survival rate was reported in a similar study utilizing 0.1% (w/v) carbendazim, 0.2% (w/v) mancozeb followed by 50% (v/v) sodium hypochlorite and 1.6% (w/v) mercuric chloride in *C. sativus* (Yasmin and Nehvi 2014). The use of 0.1% mercuric chloride followed by 4% sodium hypochlorite resulted in 86% survival rate in *C. sativus* (Chib et al., 2020). Results showed that the development of disinfection protocols for wild crocus species is more challenging than *C. sativus*, maybe because of their recalcitrant nature.

A variety of factors can affect the efficiency of disinfection including cultivation condition, the physiological condition of the mother plant, age, type and size of the explants as well as concentration of disinfectants, temperature, and exposure time (Teixeira da Silva 2016). While the higher concentration of disinfectants, as well as longer exposure time, can increase the asepsis condition, it has a detrimental impact on the survival of the explants and can raise the costs from a commercial point of view. Thus, in this research, we have tried to develop an inexpensive, effective, easy-to-use disinfection protocol for *Crocus* corms that can be carried out even by inexperienced tissue culture personnel.

Because of the recalcitrant nature of geophytic species such as Crocuses, it is of great importance to consider the conditions of *in vitro* culture including the concentration of PGRs. The first step towards indirect cormlet production is callogenesis. Due to ease of availability, several researchers have preferred the use of leaf and shoot explants. Many researchers have also tried to set up *in vitro* regeneration using the plant's reproductive parts such as stigma, style, ovary, and whole bud (Ahmad et al., 2014). In the present study, corm slices were used as explants due to their availability during the year, a good source of meristematic cells and vascular tissues that could lead to the formation of organ primordia (Blazquez et al., 2009). We have successfully induced the callus from corm slices in all three studied *Crocus* species using different combinations of auxins and cytokinins. These PGRs are responsible for the control of plant cell differentiation, and their ratio is important especially during the initial stages of morphogenesis (Jiménez 2005). In this study, the medium supplemented with 10.18 μM 2,4-D + 4.44 μM BAP was found to best support the callus induction and somatic embryogenesis. It was reported that 0.25 mg/L 2,4-D + 1 mg/L BAP showed the best results for callus initiation and callus growth in *Crocus* (Zeybek et al., 2012); while 4 mg/L NAA + 4 mg/L TDZ was proved to successfully induce callus in five *Crocus* species (Verma et al., 2016). In another report, over 70% callus induction was achieved using MS medium containing 0.5 mg/L 2,4-D, 1 mg/L BAP and 1 mg/L IAA (Chib et al., 2020). It is also reported that higher levels of auxin relative to cytokinin had increased somatic embryogenesis in the Ajowan calli (*Carum copticum* L.) (Niazian et al., 2017).

Our findings demonstrated that different *Crocus* species respond differently to the concentration and type of PGRs (Karamian and Ebrahimzadeh 2001). Although it was reported that Picloram in combination with other PGRs improved callus induction (Ahmed et al., 2011), however, by using 2.07 μM Picloram + 1.16 μM Kin the lowest or no callus induction was observed. Overall, the MS media supplemented with 10.18 μM 2,4-D + 4.44 μM BAP showed the best results followed by 10.18 μM 2,4-D + 2.32 μM Kin and 2.07 μM Picloram + 1.16 μM Kin.

In many plant species, the concentration of auxin is essential for the induction of somatic embryos. Moreover, the distribution of auxin in plant tissues is essential for multiple developmental aspects, including embryo development (Su et al., 2011). In *Crocus* tissue culture, embryogenic calli develop under different PGR concentrations; some papers mention the importance of IBA (Zeybek et al., 2012), whereas others emphasize the use of cytokinin without IBA (Sharma et al., 2008). In this research, we used different combinations of auxins (NAA and 2,4-D) and cytokinin (BAP) for somatic embryo development and cormlet production. The use of 5.37 μM NAA + 8.88 μM BAP successfully resulted in somatic embryo germination and cormlet production in all three species. Although the previous reports indicated that ABA is required for the development of somatic embryos (Verma et al., 2016), in this study the use of a medium without ABA for the development of embryos showed similar results; however, further research is needed to investigate the role of ABA in the development *Crocus* embryos. The morphology of embryogenic calli and somatic embryos developed in this study was consistent with previous studies on *C. sativus* (Blazquez et al., 2009). The maximum number of somatic embryos in all three species was observed in MS medium supplemented with 10.18 μM 2,4-D + 4.44 μM BAP. So it is apparent that an appropriate balance between auxin (2, 4-D) and cytokinin (BAP) is required for somatic embryogenesis in crocus species. Verma et al. (2016) reported that auxin (NAA), cytokinin (TDZ), and ABA are required for somatic embryo development in crocus species. In *C. sativus,* somatic embryo maturation was performed in a medium using ABA (Karamian 2003), and the germination of somatic embryos was performed in either a PGR-free medium (Sheibani et al., 2006) or a medium supplemented with GA$_3$ (Vatankhah et al., 2010). It is reported that auxin concentration has a direct effect on the conversion frequency of somatic embryos into plantlets (George and Capen 1993). In the present study, MS medium without PGRs was used for somatic embryo maturation and further somatic embryo germination was performed using different combinations of auxins (2,4-D and NAA) and cytokinin (BAP); however, in another study, researchers combined two stages using MS medium supplemented with 1.75 mg L^{-1} ABA + 0.5 mg L^{-1} BAP + 20 mg L^{-1} GA$_3$ (Raja et al., 2006). Half strength MS medium without PGRs was also used for somatic embryo maturation (Ebrahimzadeh et al., 2000). Mir et al. (2014) obtained the maximum number of cormlets (10)

and the maximum weight (1.54 g) in *C. sativus* using MS medium supplemented with 2 mg/L BAP + 0.5 mg/L NAA+ 1.5 mg/L paclobutrazol. In the present study by using 5.37 μM NAA + 8.88 μM BAP we obtained the higher cormlet weight (1.87 g). It has been shown that a high cytokinin/auxin ratio is crucial for plant regeneration and cormlet production in *Crocus* species; In their research, the maximum number of cormlets (6.1) in *Crocus vernus* was obtained using SH medium supplemented with 2.0 mg L^{-1} BA and 0.5 mg L^{-1} NAA (Sivanesan et al., 2014). Our results represented the successful conversion of somatic embryos into cormlets in *C. sativus* as well as *C. caspius* and *C. speciosus.*

SERK plays an important role amongst the genes involved in SE induction and it has great importance regarding somatic embryogenesis (Somleva et al., 2000). The first report of the *SERK* gene is related to embryogenic carrot cells (Schmidt et al., 1997) and its expression has been linked to SE in several species including *Helianthus annuus* (Thomas et al., 2004), and *Cedrela odorata* (Porras-Murillo et al., 2018). *SERK* overexpression is documented to improve the efficiency of somatic embryogenesis whereas its down-regulation has a negative impact (Hu et al., 2005). In a recent study, it has been reported that the *SERK* gene has a role in the somatic embryogenesis of the oil palm by triggering cellular reprogramming for the formation of callus (Lee et al., 2019).

In the present study, the *SERK* gene was induced in embryogenic calli but not in non-embryogenic calli which is in agreement with the previous report (Santos et al., 2018). The higher expression levels of the *SERK* gene in *C. sativus* may explain the observed higher callus induction frequencies in *C. sativus* and its overall *in vitro* culture efficiency. These findings could indicate the role of *SERK* in embryogenesis in *Crocus* species and are consistent with previous results in other species (Porras-Murillo et al., 2018). Zhang and coworkers (2011) stated that auxin, alone or in combination with cytokinin, upregulates *SERK* expression, but it depends on the species. Our results are in line with those of the previous studies on various species (Ma et al., 2012) and it provides additional evidence for the participation of the *SERK* gene in somatic embryogenesis in *Crocus* species. The *SERK* gene is known to be a conventional molecular marker for somatic embryogenesis (Talapatra et al., 2014) and, the observed higher expression of the *SERK* gene in the embryogenic callus of *Crocus* species suggests that it could be a good molecular marker for the embryogenesis potential in *Crocus* species.

5. Conclusion

Efficient protocols were developed for disinfection and *in vitro* production of cormlets in *C. sativus* and its wild allies *C. caspius* and *C. speciosus*. *SERK* gene expression profile was also proposed as a potential marker during embryogenesis in *Crocus* species. Due to the pharmaceutical and economic importance of the saffron and wild *Crocus* species, there is a high demand for the propagation of uniform pathogen-free corms. Our recipe suggests a promising robust *in vitro* propagation workflow not only for the production of uniform pathogen-free cormlets but also provides a platform to better conservation of wild *Crocus* species and effective gene and genome editing using CRISPR-Cas9 in the future, which requires proficient callus production and embryogenesis.

Declarations

Author contribution statement

Ayat Taheri-Dehkordi: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Roohangiz Naderi, Federico Martinelli: Conceived and designed the experiments; Wrote the paper.

Seyed Alireza Salami: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

Ahmad, M., Razvi, S.M., Zaffar, G., et al., 2014. Micropropagation of saffron (*Crocus sativus* L.): a review. J. Cell Tissue Res. 14, 4073.

Ahmed, A.B.A., Rao, A.S., Rao, M.V., Taha, R.M., 2011. Effect of picloram, additives and plant growth regulators on somatic embryogenesis of *Phyla nodiflora* (L.) Greene. Braz. Arch. Biol. Technol. 54, 7–13.

Altan, F., Bürün, B., Sahin, N., 2010. Fungal contaminants observed during micropropagation of *Lilium candidum* L. and the effect of chemotherapeutic substances applied after sterilization. Afr. J. Biotechnol. 9, 991–995.

Blazquez, S., Olmos, E., Hernández, J.A., et al., 2009. Somatic embryogenesis in saffron (*Crocus sativus* L.). Histological differentiation and implication of some components of the antioxidant enzymatic system. Plant Cell Tissue Organ Cult. 97, 49–57.

Chib, S., Thangaraj, A., Kaul, S., et al., 2020. Development of a system for efficient callus production, somatic embryogenesis and gene editing using CRISPR/Cas9 in Saffron (*Crocus sativus* L.). Plant Methods 16, 47.

Chugh, A., Khurana, P., 2002. Gene expression during somatic embryogenesis. Curr. Sci. 83, 715–730.

Connell, G.F., 2006. Key operating strategies for chlorine disinfection operating systems. Proc. Water Environ. Fed. 2006, 6329–6334.

Ebrahimzadeh, H., Karamian, R., Noori-Daloii, M.R., 2000. Somatic embryogenesis and plantlet regeneration in saffron. Crocus sativus 169–173.

Fernández, J.-A., 2004. Biology, biotechnology and biomedicine of saffron. Recent Res. Dev. Plant Sci. 2, 127–159.

Fernández, J.A., 2007. Genetic resources of saffron and allies (*crocus* SPP.). Acta Hortic. 167–185.

Fernández, J.-A., Santana, O., Guardiola, J.-L., et al., 2011. The World Saffron and Crocus collection: strategies for establishment, management, characterisation and utilisation. Genet. Resour. Crop Evol. 58, 125–137.

Freytag, C., Pabar, S.A., Demeter, Z., et al., 2017. Production and characterization of tissue cultures of four *crocus* species from the carpathian basin. Acta Biol. Cracoviensia s bot 59, 31–39.

Francis, Dennis, Sorrell, David A, 2001. The interface between the cell cycle and plant growth regulators: a mini review. Plant Growth Regul. 33, 1–12.

Gantait, S., Vahedi, M., 2015. In vitro regeneration of high value spice *Crocus sativus* L.: a concise appraisal. J. Appl. Res. Med. Aromat. Plants 2, 124–133.

George, L., Capen, S., 1993. Influence of genotype and explant source on somatic embryogenesis in peanut. Oleagineux 361–364.

Goldblatt, P., Davies, T.J., Manning, J.C., et al., 2006. Phylogeny of Iridaceae subfamily Crocoideae based on a combined multigene plastid DNA analysis. Aliso A J. Syst. Evol. Bot. 22, 399–411.

Harpke, D., Meng, S., Rutten, T., et al., 2013. Phylogeny of *Crocus* (Iridaceae) based on one chloroplast and two nuclear loci: ancient hybridization and chromosome number evolution. Mol. Phylogenet. Evol. 66, 617–627.

Hecht, V., Vielle-Calzada, J.-P., Hartog, M.V., et al., 2001. The arabidopsis somatic embryogenesis receptor kinase 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol. 127, 803–816.

Hesami, M., Naderi, R., Yoosefzadeh-Najafabadi, M., 2018. Optimizing sterilization conditions and growth regulator effects on *in vitro* shoot regeneration through direct organogenesis in *Chenopodium quinoa*. Biotechnologia 99, 49–57.

Hesami, M., Naderi, R., Tohidfar, M., 2019. Modeling and optimizing *in vitro* sterilization of Chrysanthemum via multilayer perceptron-non-dominated sorting genetic algorithm-II (MLP-NSGAII). Front. Plant Sci. 10, 282.

Hu, H., Xiong, L., Yang, Y., 2005. Rice *SERK1* gene positively regulates somatic embryogenesis of cultured cell and host defense response against fungal infection. Planta 222, 107–117.

Jiménez, V.M., 2005. Involvement of plant hormones and plant growth regulators on *in vitro* somatic embryogenesis. Plant Growth Regul. 47, 91–110.

Karamian, R., 2003. Plantlet regeneration via somatic embryogenesis in four species of *Crocus*. In: I International Symposium on Saffron Biology and Biotechnology, 650, pp. 253–259.

Karamian, R., Ebrahimzadeh, H., 2001. Plantlet regeneration from protoplast-derived embryogenic calli of *Crocus cancellatus*. Plant Cell Tissue Organ Cult. 65, 115–121.

Kumar, V., Van Staden, J., 2019. Multi-tasking of SERK-like kinases in plant embryogenesis, growth, and development: current advances and biotechnological applications. Acta Physiol. Plant. 41, 31.

Langens-Gerrits, M., Albers, M., De Klerk, G.-J., 1998. Hot-water treatment before tissue culture reduces initial contamination in Lilium and Acer. Plant Cell Tissue Organ Cult. 52, 75–77.

Lee, F.-C., Ong-Abdullah, M., Ooi, S.-E., et al., 2019. Cloning and characterization of Somatic Embryogenesis Receptor Kinase I (*EgSERK I*) and its association with callus initiation in oil palm. Vitro Cell Dev. Biol. Plant 55, 153–164.

Li, J.W.-H., Vederas, J.C., 2009. Drug discovery and natural products: end of an era or an endless frontier? Science (80-) 325, 161–165.

Ma, J., He, Y., Wu, C., et al., 2012. Cloning and molecular characterization of a *SERK* gene transcriptionally induced during somatic embryogenesis in *Ananas comosus* cv. Shenwan. Plant Mol Biol Report 30, 195–203.

Mahdavi-Darvari, F., Noor, N.M., Ismanizan, I., 2015. Epigenetic regulation and gene markers as signals of early somatic embryogenesis. Plant Cell Tissue Organ Cult. 120, 407–422.

Mathew, B., 1983. The Crocus: a Revision of the Genus Crocus (Iridaceae). Timber Press.

Maxted, N., Scholten, M., Codd, R., Ford-Lloyd, B., 2007. Creation and use of a national inventory of crop wild relatives. Biol. Conserv. 140, 142–159.

Mir, J.I., Ahmed, N., Shafi, W., et al., 2014. *In vitro* development and regeneration of microcorms in saffron (*Crocus sativus* L). Afr. J. Biotechnol. 13, 2637–2640.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plantarum 15, 473–497.

Negbi, M., 1999. Saffron cultivation: past, present and future prospects. Saffron: *Crocus Sativus* L. Harwood Academic Publishers, Amsterdam, pp. 1–17.

Niazi, M., 2019. Application of genetics and biotechnology for improving medicinal plants. Planta 249, 953–973.

Niazian, Mohsen, Noori, Seyed Ahmad Sadat, Galuszka, Petr, Tohidfar, Masoud, Mortazavian, Seyed Mohammad Mahdi, 2017. Genetic stability of regenerated plants via indirect somatic embryogenesis and indirect shoot regeneration of *Carum copticum* L. Ind. Crop. Prod. 97, 330–337.

Ordoudi, S.A., Pástor-Férriz, M.T., Kyriakoudi, A., et al., 2019. Physicochemical characterization of crocus serotinus stigmas indicates their potential as a source of the bioactive apocarotenoid crocetin. Eur. J. Lipid Sci. Technol. 121, 1900011.

Porras-Murillo, R., Andrade-Torres, A., Solís-Ramos, L.Y., 2018. Expression analysis of two SOMATIC EMBRYOGENESIS RECEPTOR KINASE (*SERK*) genes during *in vitro* morphogenesis in Spanish cedar (*Cedrela odorata* L.). Biotech 8 3, 470.

Raja, W., Zaffer, G., Wani, S.A., 2006. *In vitro* microcorm formation in saffron (*Crocus sativus* L.). In: II International Symposium on Saffron Biology and Technology, 739, pp. 291–296.

Rezaee-Khorasany, A., Razavi, B.M., Taghiabadi, E., et al., 2019. Effect of saffron (stigma of *Crocus sativus* L.) aqueous extract on ethanol toxicity in rats: a biochemical, histopathological and molecular study. J. Ethnopharmacol. 237, 286–299.

Salaj, J., von Recklinghausen, I.R., Hecht, V., et al., 2008. *AtSERK1* expression precedes and coincides with early somatic embryogenesis in *Arabidopsis thaliana*. Plant Physiol. Biochem. 46, 709–714.

Santos, I.R., Maximiano, M.R., Almeida, R.F., et al., 2018. Genotype-dependent changes of gene expression during somatic embryogenesis in oil palm hybrids (*Elaeis oleifera* x *E. guineensis*). PloS One 13, e0209445.

Schmidt, E.D., Guzzo, F., Toonen, M.A., De Vries, S.C., 1997. A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development 124, 2049–2062.

Schmidt, T., Heitkam, T., Liedtke, S., et al., 2019. Adding color to a century-old enigma: multi-color chromosome identification unravels the autotriploid nature of saffron (*Crocus sativus*) as a hybrid of wild *Crocus cartwrightianus* cytotypes. New Phytol. 222, 1965–1980.

Sharifi, G., Ebrahimzadeh, H., Ghareyazie, B., et al., 2012. Identification of differentially accumulated proteins associated with embryogenic and non-embryogenic calli in saffron (*Crocus sativus* L.). Proteome Sci. 10, 3.

Sharma, K.D., Rathour, R., Sharma, R., et al., 2008. *In vitro* cormlet development in *Crocus sativus*. Biol. Plant. (Prague) 52, 709–712.

Sheibani, M., Nemati, S.H., Davarinejad, G.H., et al., 2006. Induction of somatic embryogenesis in saffron using thidiazuron (TDZ). In: II International Symposium on Saffron Biology and Technology, 739, pp. 259–267.

Sivanesan, I., Jana, S., Jeong, B.R., 2014. *In vitro* shoot regeneration and microcorm development in *Crocus vernus* (L.) Hill. Pakistan J. Bot. 46, 693–697.

Somleva, M.N., Schmidt, E.D.L., de Vries, S.C., 2000. Embryogenic cells in *Dactylis glomerata* L. (Poaceae) explants identified by cell tracking and by *SERK* expression. Plant Cell Rep. 19, 718–726.

Su, Y.-H., Liu, Y.-B., Zhang, X.-S., 2011. Auxin–cytokinin interaction regulates meristem development. Mol. Plant 4, 616–625.

Talapatra, S., Ghoshal, N., Sen, Raychaudhuri S., 2014. Molecular characterization, modeling and expression analysis of a somatic embryogenesis receptor kinase (*SERK*) gene in *Momordica charantia* L. during somatic embryogenesis. Plant Cell Tissue Organ Cult. 116, 271–283.

Tarantilis, P.A., Tsoupras, G., Polissiou, M., 1995. Determination of saffron (*Crocus sativus* L.) components in crude plant extract using high-performance liquid chromatography-UV-visible photodiode-array detection-mass spectrometry. J. Chromatogr. A 699, 107–118.

Teixeira da Silva, J.A., 2016. Disinfection of explants for saffron (*Crocus sativus*) tissue culture. Environ. Exp. Biol. 14, 183–198.

Thomas, C., Meyer, D., Himber, C., Steinmetz, A., 2004. Spatial expression of a sunflower *SERK* gene during induction of somatic embryogenesis and shoot organogenesis. Plant Physiol. Biochem. 42, 35–42.

Tichtinsky, G., Vanoosthuyse, V., Cock, J.M., Gaude, T., 2003. Making inroads into plant receptor kinase signalling pathways. Trends Plant Sci. 8, 231–237.

Vahedi, M., Kabiri, M., Salami, S.A., et al., 2018. Quantitative HPLC-based metabolomics of some Iranian saffron (*Crocus sativus* L.) accessions. Ind. Crop. Prod. 118, 26–29.

Vatankhah, E., Niknam, V., Ebrahimzadeh, H., 2010. Activity of antioxidant enzyme during *in vitro* organogenesis in *Crocus sativus*. Biol. Plant. (Prague) 54, 509–514.

Verma, S.K., Das, A.K., Cingoz, G.S., et al., 2016. Influence of nutrient media on callus induction, somatic embryogenesis and plant regeneration in selected Turkish crocus species. Biotechnol. Rep. 10, 66–74.

Yasmin, S., Nehvi, F., 2014. *In vitro* microcorm formation in saffron (*Crocus sativus* L.). J. Cell Tissue Res. 14, 4463–4470.

Yasmin, S., Nehvi, F.A., Wani, S.A., 2013. Tissue culture as an alternative for commercial corm production in saffron: a heritage crop of Kashmir. Afr. J. Biotechnol. 12, 3940–3946.

Zengin, G., Mahomoodally, M.F., Sinan, K.I., et al., 2020. Chemical characterization, antioxidant, enzyme inhibitory and cytotoxic properties of two geophytes: *Crocus pallasii* and *Cyclamen cilicium*. Food Res. Int. 133, 109129.

Zeybek, E., Önde, S., Kaya, Z., 2012. Improved *in vitro* micropropagation method with adventitious corms and roots for endangered saffron. Open Life Sci. 7, 138–145.

Zhang, S., Liu, X., Lin, Y., et al., 2011. Characterization of a *ZmSERK* gene and its relationship to somatic embryogenesis in a maize culture. Plant Cell Tissue Organ Cult. 105, 29–37.