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CALLUS INDUCTION AND HIGH FREQUENCY ORGANOGENESIS IN SAFFRON (*Crocus sativus* L.)

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ABSTRACT

Saffron (*Crocus sativus* L.) is an economically important plant with a very slow natural multiplication rate and plant tissue culture is expected to enhance its reproductive capacity. In present study, corm explants were used for micro-propagation. Auxins and cytokinin were found important for *in vitro* propagation of explants. Overall, 93% calli were induced from meristematic region of corm explants propagated on Murashige and Skoog (MS) medium supplemented with 2 mg L^{-1} 2,4-dichlorophenoxy-acetic acid (2,4-D), 0.5 mg L^{-1} kinetin (Kn), 2% sucrose and 100 mg L^{-1} ascorbic acid after 6 weeks inoculation. Somatic embryogenesis was observed on half strength MS medium supplemented with 2 mg L^{-1} indole-3-acetic acid, 2 mg L^{-1} Kn and 100 mg L^{-1} ascorbic acid. Organogenesis from calli was observed with 89.6% shooting on MS medium containing 2 mg L^{-1} Kn and 2 mg L^{-1} IAA. Adventitious rooting of 91.6% calli was obtained on MS medium supplemented with 2 mg L^{-1} indole-3-butyric acid. There are plenty of protocols available for tissue culture of saffron but this protocol provides an easy and time efficient method for micropropagation of saffron *in vitro*.

Keywords: Auxin, cytokinin, micropropagation, saffron, tissue culture

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; BAP: 6-benzyl aminopurine, NAA: 1-naphthalene acetic acid; $HgCl_2$: mercuric chloride; NaOCl: sodium hypochlorite, $KMnO_4$: potassium permangnate; PPM: plant preservation mixture; TDZ: thidiazuron; MS: Murashige and Skoog medium; Kn: kinetin; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid.

INTRODUCTION

Saffron {*Crocus sativus* L.}, also known as "Golden condiment", is an important heritage crop of Jammu & Kashmir which has immense medicinal and cosmetic properties (Christodoulou *et al.*, 2015; Petrakis *et al.*, 2017). Saffron produces a spice derived from the stigmas of plant which also goes by the same name as saffron. Saffron is an auto-triploid (2n = 3x = 24), male sterile herb that belongs to family Iridaceae. Saffron is a low growing herb and a slow propagating geophyte, where one mother corm produces upto 10 daughter cormlets and out of which only 50-70% may survive to bear the next cycle (Poggi *et al*., 2016). Worldwide, Iran is the largest producer of saffron contributing around 94% of world production with annual production of 230 tons in 2018 (Abbaspour *et al.*, 2018). Jammu and Kashmir is the 3rd largest producer of saffron after Iran and Morocco with annual production of 9.2 tons in 2018; where it is primarily cultivated in Pampore and Pulwama districts

(34°01'12.00" N 74°55'48.00" E; 1574 m masl) [Golmohammadi *et al.,* 2014] with Kishtwar district (33˚19'0"N 75˚46'0") contributing a small part (Tundup *et al.,* 2015).

Saffron is a unique plant which flowers once in a year (Small *et al.,* 2016). The purple flowers blossom in autumn (November-April), followed by the maturation of daughter corms. Corms are essential for the propagation of crop since *C. sativus* is an auto-triploid and sterile. The corms are needed to be physically dug up, split and replanted. The auto-triploid nature of saffron bars the advancement of breeding practices making it extremely challenging. Conventional propagation system is very torpid thus propagation by tissue culture presents as an alternate to corm propagation. *In vitro* tissue culture technique are desired in propagation of many other important plants (Firoozi *et al.,* 2018).

Currently the production of saffron is on a decline due to various biotic and abiotic reasons with yearly dip of 68% production in 2018 from 2017 (*https://www.dailypioneer.com/2018/page1/kashmirs-prized-saffron-bears-brunt-of-dry-spell.html*). So, to meet the increasing demand of saffron as well as cover the yield loses, micropropagation is a viable option. Recently, there has been a rising interest in tissue culture and genetic engineering techniques for the propagation and genetic improvement of saffron (Yasmin and Nahvi, 2017; Kashtwari *et al.,* 2018). Many protocols are available for saffron tissue culture utilizing different concentrations of auxins and cytokinin for the propagation of plant such as few groups used thin layer explants for callus induction using hormones such as 2,4-D, BAP and NAA (Yasmin *et al.* 2013; Bagheri *et al.,* 2017). In present study, an improved protocol for plant regeneration through callus mediated somatic embryogenesis has been provided for saffron.

MATERIALS AND METHODS

Sample collection

Saffron corms were collected in sterilized bags from Pampore district of Jammu and Kashmir (34°01'12.00" N 74°55'48.00" E) in the month of July 2017. The samples were brought to the laboratory and stored at -20ºC till further use.

Surface sterilization

The corm explants were collected in beakers and outer layer (corm sheath) removed (Fig. 1). The explants were first washed 3-4 times with tap water with 2-3 drops of Tween 20 and 1% Savlon and then again washed 3-4 times with distilled water (Rutala, 1996). A total of thirteen different sterilization treatments were carried out singly or in combinations. The single sterilant treatment of corm explants comprised of 70% ethanol (for 30 sec), 1 ppm $KMnO_4$ (for 3 min) 5 or 10% NaOCl (5 min), 1% benomyl (30 min) and 1% gentamycin (30 min). Beside, some corms were treated with 70% ethanol for 30 sec duration followed by sterilization with $HgCl_2$ (variable concentrations, 0.1-0.3%) for 30 min. Some corms were sequentially treated with 1% PPM^{TM} (for 5 min), 70% ethanol (30 sec), 0.25% $HgCl_2$ (30 min), 1% benomyl (3 min) and 1% gentamycin (30 min). Similarly, some corms were sequentially treated with 1% PPM^{TM} (for 5 min), 70% ethanol (30 sec), 0.3% $HgCl_2$ (15 min) and 1% benomyl (30 min). PPM^{TM} is a plant préservation mixture which is a broad spectrum antibiotic with 5-chloro-2-methyl3(2H)-isothiazolone and 2-methyl-3(2H)-isothiazolone as active components. All the chemicals were procured from Sigma (USA). The different surface sterilants were used for variable time durations (Table 1). For all the experiments, the sample size taken was 25 and all the experiments were done in triplicate.

Establishment of explants

For bud sprouting, Murashige and Skoog (1962) basal MS medium (Murashige and Skoog, 1962) obtained from Hi-Media was prepared and pH adjusted to 5.8 then 0.7% (0.7 g in 100 mL) plant

tissue culture agar obtained from Hi-Media was added to the medium prior to autoclaving. The surface sterilized corms were then inoculated on the basal medium for the establishment of cultures. The cultures were maintained at 25±2˚C at a photoperiod of 16 h light with 8 h dark using a standard cool white light adjusted to a flux rate of 35 µMol s^{-1} m^{-2} provided by Philips LED lamps. The explants were regularly checked for contamination and subsequently sub-cultured after every 14 days. The sample size taken was 25 and experiment was done in triplicate.

Callus induction and somatic embryogenesis

The basal plate of corms was taken as explants for callus induction. The surface sterilized corms were taken and the basal plate was excised using scalpel. The basal plates were then inoculated on MS medium supplemented with different concentrations and combinations of hormones such as 2,4-D (1, 2, 3, 4 and 5 mg L^{-1}), Kn (0.5 mg L^{-1}), BAP (0.5, 1.0, 1.5 and 2.0 mg L^{-1}), TDZ (2 mg L^{-1}) sucrose (2%) and ascorbic acid (100 mg L^{-1}) as per the method described by (Blázquez *et al.*, 2003). The cultures were stored at 25±2˚C in dark. TDZ stands for thidiazuron which is N-phenyl-1,2,3-thidiazole-5-y-lurea, it is a cytokinin-like pseudo-hormone extensively used for shoot proliferation and regeneration in different plants (Deep *et al.*, 2018). For callus induction, 25 explants were taken and the experiment was performed in triplicate. The cultures were regularly checked for any contamination and subsequently sub-cultured for proliferation. For somatic embryogenesis, non-embryogenic calli were sub-cultured on half strength MS medium supplemented with 1AA (0.5, 1.0, 1.5 and 2.0 mg L^{-1}), Kn (1 and 2 mg L^{-1}) and ascorbic acid (100 mg L^{-1}) maintained at 25±2˚C with a photoperiod of 16 h light and 8 h dark. The proliferated calli after four weeks were then sub-cultured on MS medium supplemented with different concentrations and combinations of auxins and cytokinin for organogenesis.

Organogenesis from callus

For shoot induction, the proliferated calli were cut into small pieces with sterilized blade and sub-cultured on MS medium supplemented with different concentrations of Kn (0.5, 1.0, 1.5, 2.0 and 3.0 mg L^{-1}) [Majourhat *et al.*, 2006] in combination with auxins like IAA (1.0, 2.0 and 3.0 mg L^{-1}). For rooting, the calli were sub-cultured on MS medium supplemented with different concentrations of IBA (0.5, 1.0, 1.5 and 2.0 mg L^{-1}) [Wang *et al.*, 2011].

Statistical analysis

The data obtained for the *in vitro* organogenesis was checked for response variables. For other variables like explanatory variables, the data transformation wasn't required. Standard deviation from the mean was calculated using SPSS software (IBM Inc., New York, USA).

RESULTS AND DISCUSSION

Surface sterilization

A total of thirteen sterilization treatments were carried out singly or in combinations. The most effective treatment was found to be five-step sterilization method with 1% PPM™, 70% ethanol treatment for 30 sec, 0.25% $HgCl_2$ for 15 min, 1% benomyl for 30 min and 1% gentamycin for 30 min. This treatment showed 91% uncontaminated explants which is in accordance with the results of John *et al.* (2017). Karaoğlu *et al.* (2006) reported reduced contamination in saffron tissue culture with the use of PPM™ along with other fungicides. As and when the concentration of these sterilants was increase beyond a range, it resulted in browning and finally in the necrosis of explants (Daud *et al.*, 2012). The surface sterilization of explants was done using mercuric chloride, potassium permanganate, sodium hypochlorite, benomyl, gentamycin and ethanol. $HgCl_2$ at low concentration is an aphytotoxic chemical and kills both bacteria and fungi but at higher concentrations it affects

Table 1: Effect of different sterilant on surface sterilization of saffron explants

Treatments	Time duration	Percent survival of explants
70% ethanol	30 sec	Nil
$KMnO_4$ (1 ppm)	3 min	Nil
70% ethanol + 0.10% $HgCl_2$	30 sec + 30 min	11.3
70% ethanol + 0.15% $HgCl_2$	30 sec + 30 min	25.6
70% ethanol + 0.20% $HgCl_2$	30 sec + 30 min	30.0
70% ethanol + 0.25% $HgCl_2$	30 sec + 30 min	31.9
70% ethanol + 0.30% $HgCl_2$	30 sec +30 min	19.8
5% NaOCl	5 min	16.6
10% NaOCl	5 min	10.0
1% Benomyl	30 min	33.4
1% Gentamycin	30 min	45.0
1% PPM + 70% ethanol + 0.25% $HgCl_2$ + 1% benomyl +1% gentamycin	5 min + 30 sec + 30 min + 3 min + 30 min	91.0
1% PPM + 70% ethanol + 0.3% $HgCl_2$ + 1% benomyl	5 min + 30 sec + 15 min + 30 min	66.6

plant tissue as well. Sodium hypochlorite is a very strong oxidizing agent that contributes to its antimicrobial activity. PPM^{TM} is a broad-spectrum biocide. It acts by penetrating the microbial cell wall and disrupts many crucial enzymes of plant electron transport chain. The antimicrobial activity of potassium permanganate is due to its strong oxidizing property. Gentamycin is a bactericide which acts by binding to 30S subunit of bacterial ribosome (Mahmoud *et al.,* 2016). The various treatments applied in combination for surface sterilization of explants and the percentage survival has been indicated in Table 1.

Bud sprouting

In saffron, explants establishment is very slow on account of saffron being a geophyte. Saffron is a difficult plant, known to be recalcitrant towards adventitious shooting *in vitro* (Zeybek *et al.,* 2012). After 3 weeks, 15-20% of bud sprouting was observed on basal MS medium containing 3% (w/v) sucrose and 7 g L^{-1} agar. The cultures were maintained at 16/8 h photoperiod with photon flux density of 35 µMol s^{-1} m^{-2} which is in accordance with Mir *et al.* (2014).

Callus induction and organogenesis

Calli were induced from the basal plates of surface sterilized saffron corms on different concentrations of 2,4-D and Kn six weeks post-inoculation [Table 2, Fig. 2]. Brownish-white friable

Table 2: Genotypic response of saffron on callus induction 6 weeks post-inoculation on MS medium supplemented with different concentrations of hormones

MS medium amended with different hormones	Genotypic response	Rate of callus induction	Percentage callus initiation
1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kn	No callus	-	0
2 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kn	Large Brown friable callus	++++	93.1
3 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kn	Small brown friable callus	+++	87.6
4 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kn	No callus	-	0
5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kn	No callus	-	0
2 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP	Smaller whitish callus	+++	59.2
2 mg L^{-1} 2,4-D + 1.0 mg L^{-1} BAP	Smaller whitish callus	+++	78.9
2 mg L^{-1} 2,4-D + 1.5 mg L^{-1} BAP	Smaller whitish callus	++	48.4
2 mg L^{-1} 2,4-D + 2.0 mg L^{-1} BAP	Smaller whitish callus	++	37.6
2 mg L^{-1} 2,4-D + 2.0 mg L^{-1} TDZ	Small callus	+	23.3

- = No callus induction, + = poor callus formation, ++ = moderate callus formation, +++ = good callus induction, and ++++ = very good callus induction

Fig. 1: Saffron corm explant; a. corm with corm sheath b. corm without sheath

Table 3: Somatic embryogenesis response of non-embryogenic calli on ½ MS supplemented with different hormones

½ × MS medium (+ 100 mg L^{-1} ascorbic acid) supplemented with	Rate of somatic embryogenesis	Somatic embryogenesis (%)
0.5 mg L^{-1} IAA + 1 mg L^{-1} Kn	-	0
1.0 mg L^{-1} IAA + 1 mg L^{-1} Kn	-	0
1.5 mg L^{-1} IAA + 2 mg L^{-1} Kn	+	11.2
2.0 mg L^{-1} IAA + 2 mg L^{-1} Kn	++	37.5

+ = Poor embryogenesis, ++ = moderate embryogenesis, and - = no embryogenesis

calli were observed on the medium amended with 2 mg L^{-1} 2,4-D, 0.5 mg L^{-1} Kn, 100 mg L^{-1} ascorbic acid and 2% sucrose with 93.1% incidence rate. Similar results have been reported by Vahedi *et al.* (2015) in case of callus induction in saffron. The exact mechanism of how auxins facilitate callus formation is unknown but it is reported that 2,4-D induces the cells to dedifferentiate to form undifferentiated mass of cells called as callus (Ikeuchi *et al.*, 2016). As the concentration of 2,4-D increases beyond 2 mg L^{-1} 2, 4-D, the explants start turning brown (Ryland *et al.*, 1948). To some extent the callus induction was seen in MS amended with 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP and 2 mg L^{-1} 2,4-D and 2 mg L^{-1} TDZ but in case of 2 mg L^{-1} 2,4-D and 2 mg L^{-1} TDZ no callus proliferation was observed even eight weeks after culturing. TDZ is known to cause hyperhydricity in cultures, so the use of TDZ in cultures was further restrained (Dewir *et al.*, 2018). For somatic embryogenesis, the non-embryogenic calli were sub-cultured on half strength MS medium supplemented with 100 mg L^{-1} ascorbic acid with different concentrations of IAA and Kn (Table 3). The globular somatic embryos were obtained on ½ MS medium with 2 mg L^{-1} IAA, 2 mg L^{-1} Kn and 100 mg L^{-1} ascorbic acid three weeks post-inoculation with an efficiency of 37.5% which is in agreement with the results of George *et al.* (1992). The molecular mechanism of somatic embryogenesis is not clear yet many

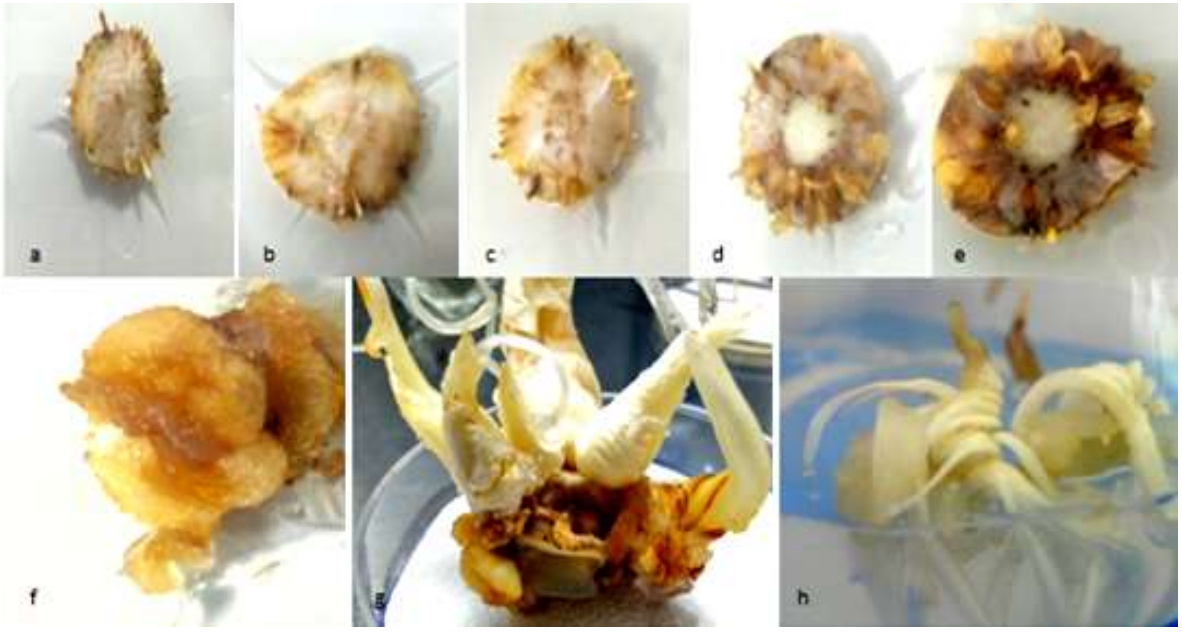

Fig. 2: Different stages of callus induction and organogenesis in saffron: a, b, c, d, e depicting initial stages of callus induction. f) shows the fully developed friable callus after six weeks post inoculation. The translucent patch forms the somatic embryogenic calli. g) shows the development of shoot primordial inside the cataphylls from the developing callus. h) shows the induction of the adventitious roots from the callus

Table 4: Effect of growth hormones on shooting from callus after four weeks post-transfer

MS medium with IAA + Kn (mg L^{-1})	Percentage shoot initiation (%)	Average No. of shoots explant^{-1} (mean ± SD)	Morphology of shoots
1.0 + 0.5	42.34	1.2 ± 0.43	Light green
1.0 + 1.0	55.90	1.9 ± 2.10	Light green
2.0 + 1.5	69.60	2.2 ± 0.00	Dark green
2.0 + 2.0	89.60	3.4 ± 0.22	Dark green
3.0 + 3.0	22.34	0.4 ± 0.76	Browning of shoots

Table 5: Effect of growth hormones on rooting from callus after three weeks post-transfer

MS medium with IBA (mg L^{-1})	Root initiation (%)	Average No. of roots explant^{-1} (mean ± SD)	Morphology of roots
0.5	-	-	-
1.0	34.00	1.2±0.32	Very few fragile roots
1.5	67.82	2.8±0.16	Developed roots
2.0	91.60	2.3±0.45	Well-developed adventitious roots

plants are reported to produce somatic embryos when grown in a high auxin media and then transferred to a low auxin media leading to the establishment of an auxin gradient, activating stress response genes and thus somatic embryogenesis (Ikeuchi *et al.*, 2016).

The eight weeks old friable calli were cut into smaller pieces and transferred to MS medium supplemented with different concentrations of auxins and cytokinins for shoot and root induction. After four weeks, maximum shoot regeneration (89.60%) was observed with an average of 3.4 ± 0.22 shoots explant^{-1} in medium amended with 2 mg L^{-1} IAA and 2 mg L^{-1} Kn (Table 4, Fig. 2 g). The apical shoots continued to grow under the cataphylls. Nevertheless, *in vitro* propagated callus was cut into small pieces and sub-cultured on MS medium supplemented with different concentrations of IBA resulted in root formation (Table 5, Fig. 2h). Adventitious roots were observed in MS medium with 2 mg L^{-1} IBA resulting in 91.60% root regeneration after three weeks of sub-culturing, similar results were also obtained by Zeybek *et al.* (2012).

Conclusion: The present study concludes a simple and time efficient protocol for high capacity *in vitro* organogenesis of saffron. The protocol for tissue culture of saffron would aid in the pilot scale propagation of the crop as compared to the plants produced by traditional methods.

Conflict of interest: The authors declare that they have no conflicts of interest.

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