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Improved *in vitro* micropropagation method with adventitious corms and roots for endangered saffron

Research Article

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Abstract: The objective of this study was to investigate development of an efficient *in vitro* tissue culture system for saffron (*Crocus sativus* L.) complete with roots and corms. In indirect organogenesis, Murashige and Skoog (MS) media with 3% (w/v) sucrose, 100 mg L^{-1} ascorbic acid, and the combination of 0.25 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 mg L^{-1} 6-benzylaminopurine (BAP) were best for callus initiation and growth while 1.5 mg L^{-1} BAP was excellent for high rate of adventitious shoot formation. 1 mg L^{-1} indole-3-butyric acid (IBA) was more preferable for adventitious corm and root initiation as well as growth. Overall, 64% rooting and 33% corm production rates were achieved in indirect organogenesis. In direct organogenesis, MS medium supplemented with 3 % sucrose, 100 mg L^{-1} ascorbic acid and 1 mg L^{-1} BAP was optimum for shoot growth. While 1 mg L^{-1} IBA was best for adventitious corm formation, 2 mg L^{-1} IBA promoted adventitious root initiation and growth. Overall, 36% and 57% of explants had corm and contractile root, respectively. The high rates suggest that efficient tissue culture system could be achieved for mass propagation and *ex situ* conservation of threatened saffron genetic resources.

Keywords: *Saffron • Corm • In vitro • Tissue culture • Organogenesis*

Abbreviations

BAP - 6-benzylaminopurine
2,4-D - 2,4-dichlorophenoxyacetic acid
IBA - indole-3-butyric acid
MS - Murashige and Skoog

1. Introduction

Saffron, *Crocus sativus* L., which belongs to the iris family (Iridaceae) is an important crop cultivated since ancient times for its red stigmatic lobes, which have aromatic, colouring, and medicinal properties [1-3]. Cytological studies indicate that saffron is a sterile triploid (2n=3x=24) plant. The triploid nature of the species creates problems for sexual reproduction, but vegetative propagation is possible. In fact, saffron is propagated only through annual renewal of corms and grown slowly as a geophyte [4,5].

Saffron is considered to be the most expensive spice in the world [6]. The main reason for its great cost is that saffron is still propagated by intensive labor. To ensure the future of saffron crop, it is necessary to improve cultivation techniques. Plant tissue culture techniques might offer a great potential for saffron production when compared to the traditional cultivation methods. *In vitro* propagation of saffron through direct and indirect organogenesis from callus cultures was investigated previously [7-10]. These studies tested the effects of factors such as temperature and phytohormones on the reproductive capacities of saffron, adventitious root, adventitious shoot and corm development. Although adventitious shoots and corms were obtained, successful root development was not achieved in these studies. Complete plantlets with adventitious roots and corm formation were reported at low levels in the literature [11-13]. Regeneration *via* somatic embryogenesis has also been described from corm-derived callus cultures [14-16], from leaf explants [9], and from protoplast

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cultures [17]. *In vitro* production of stigma-like structures of saffron for the purpose of obtaining crocin, picrocrocin, and safranal was investigated by using different parts of the saffron plant such as corms [18], half ovaries [19-21], stigmas [22,23], petals and styles [23], anthers [24], stamens [25] and calli [26,27]. It is clear that majority of the *in vitro* studies were focused on the production of secondary metabolites through direct and indirect organogenesis. Additionally, there were very few studies related to the achievement of a complete plant with roots and corms [11-13]. An efficient *in vitro* tissue culture system is currently in great need, since saffron species are threatened in many countries where they are cultivated [28]. Therefore, improvement of an *in vitro* tissue culture method for the species is of great importance for both germplasm conservation as well as commercial propagation. Thus, the objective of this study was to improve an *in vitro* tissue culture system for the endangered saffron species complete with corm and root production.

2. Experimental Procedures

2.1 Plant material

The corms of saffron plants (*Crocus sativus* L.) were purchased from a local farmer in Kastamonu Province, Turkey. This is the only place where saffron farming is practiced in the country.

Although the lateral buds of corms with 2-3 cm diameters were used for direct organogenesis, the remaining parts of the corms sliced into 5-6 mm sections were used for indirect organogenesis.

2.2 Surface sterilization of saffron corms

The corms were thoroughly washed with running tap water for 30 minutes. The protective layer (*i.e.* tunics) were removed gently from the corms and then they were surface sterilized with 70% (v/v) ethanol for 30 seconds in a laminar flow-hood. The corms were then incubated in 0.15% (w/v) mercuric chloride ($HgCl_2$) solution for 20 minutes at room temperature. Finally, samples were rinsed with sterile distilled water for three times to remove residual $HgCl_2$.

2.3 Nutrient medium and culture conditions

The effects of different plant growth regulator combinations on *in vitro* regeneration capacity of saffron were investigated through indirect and direct organogenesis. The explants were cultured on MS medium [29] supplemented with 3% (w/v) sucrose, 100 mg L^{-1} ascorbic acid, and various plant growth regulators.

During indirect organogenesis, callus, adventitious shoot, and adventitious root-corm initiation experiments were performed. For initiating callus, twenty-five different treatment combinations including five levels of both 2,4-D (CAS RN: 94-75-7) as an auxin (absent, 0.25 mg L^{-1}, 0.5 mg L^{-1}, 1 mg $L^{-1}$and 2 mg L^{-1}) and BAP (CAS RN: 1214-39-7 (base)) as a cytokinin (absent, 0.25 mg L^{-1}, 0.5 mg L^{-1}, 1 mg L^{-1} and 2 mg L^{-1}) were applied to the corm slices with three replicates. For adventitious shoot initiation, selected explants were transferred to a culture medium supplemented with new combinations of 2,4-D (absent and 0.1 mg L^{-1}) and BAP (0.5 mg L^{-1}, 1 mg L^{-1}, 1.5 mg L^{-1} and 2 mg L^{-1}), with two replications. To obtain adventitious roots and corms, two levels of IBA (CAS RN: 133-32-4) (absent, 1 mg L^{-1} and 2 mg L^{-1}) and 5% (w/v) sucrose in three replicates were applied to the shoots obtained from the previous experiment.

During direct organogenesis, adventitious shoot initiation, improvement and root-corm initiation experiments were performed. In the adventitious shoot initiation experiment, three different levels of 2,4-D (absent, 0.1 mg L^{-1} and 1 mg L^{-1}) and five different levels of BAP (absent, 0.5 mg L^{-1}, 1 mg L^{-1}, 3 mg L^{-1} and 5 mg L^{-1}) were used with five replicates, constituting fifteen treatment combinations in total. As in indirect organogenesis, two levels of 2,4-D (absent and 0.1 mg L^{-1}) and four levels of BAP (0.5 mg L^{-1}, 1 mg L^{-1}, 1.5 mg L^{-1} and 2 mg L^{-1}) were used with two replications, which constituted eight treatment combinations. Finally, MS medium supplemented with two levels of IBA (absent, 1 mg L^{-1} and 2 mg L^{-1}) and 5% (w/v) sucrose was used for adventitious root and corm initiation with three replicates from the shoots obtained previously.

The pH of all the nutrient media was adjusted to 5.8±0.1 by the addition of NaOH or HCl in a drop-wise manner before autoclaving. The nutrient media were solidified with 0.8% (w/v) plant agar (Duchefa, The Netherlands). All tissue culture media were sterilized by autoclaving at 121ºC under the pressure of 1.1 kg/cm^2 for 20 minutes. The nutrient media were aseptically dispensed into sterile baby jars, test tubes, or Petri dishes, depending on the size of explants and type of experiments.

Explants in all experiments were subcultured every three weeks and were incubated in growth room at 24±1ºC. While the explants for indirect organogenesis and callus initiation were incubated continuously under darkness, in all other trials the explants were kept under 16 h light (2000 lux) and 8 h dark period.

2.4 Recorded parameters and statistical evaluation

Initiation (callus, adventitious shoot, corm and root), growth (callus and adventitious shoot), and number

of corms and roots were recorded after an 8-week incubation period. Although initiation trials were recorded as the presence or absence of a response, data on growth were visually scored on a scale from 1 to 10 where 1 being the minimal and 10 being the maximal growth. The number of corms and roots were determined by direct counting during the adventitious root-corm initiation trials.

Data obtained from direct and indirect organogenesis were checked for normality and arcsine transformation applied for response variables. For other variables, data transformation was not necessary. To test the treatment effects on traits assessed in direct and indirect organogenesis, the General Linear Model (GLM) procedure of SAS software (9.1 Edition; SAS Institute Inc., Cary, NC, USA) was used with the mean test option to apply Duncan's Multiple Range Test [30] for treatment means for all traits in direct and indirect organogenesis.

3. Results

3.1 Indirect organogenesis

There were significant differences among the hormone combinations with respect to callus initiation and growth. The combination of 0.25 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP was observed to be the best for both callus initiation and callus growth (0.79 and 1.21, respectively), as shown in Table 1. Consequently, out of 280 explants tested in this experiment, 35% of the explants produced callus.

2,4-D (mg L^{-1})	BAP (mg L^{-1})	Number of Explants	Callus Initiation*	Callus Growth#
0	0	15	0.00 ± 0.00a	0.00 ± 0.00a
0.25	0	10	0.50 ± 0.17b	0.80 ± 0.29b
0.50	0	15	0.40 ± 0.13c	0.53 ± 0.19c
1.00	0	15	0.00 ± 0.00a	0.00 ± 0.00a
2.00	0	14	0.14 ± 0.10d	0.21 ± 0.15d
0	0.25	-	-	-
0.25	0.25	-	-	-
0.50	0.25	4	0.00 ± 0.00a	0.00 ± 0.00a
1.00	0.25	-	-	-
2.00	0.25	10	0.40 ± 0.16c	0.40 ± 0.16c
0	0.50	10	0.00 ± 0.00a	0.00 ± 0.00a
0.25	0.50	15	0.67 ± 0.13e	1.00 ± 0.22e
0.50	0.50	14	0.50 ± 0.14b	0.64 ± 0.20f
1.00	0.50	15	0.40 ± 0.13c	0.53 ± 0.19c
2.00	0.50	15	0.40 ± 0.13c	0.47 ± 0.17c
0	1.00	10	0.00 ± 0.00a	0.00 ± 0.00a
0.25	1.00	14	0.79 ± 0.11f	1.21 ± 0.21g
0.50	1.00	15	0.27 ± 0.12g	0.47 ± 0.22c
1.00	1.00	15	0.60 ± 0.13h	0.93 ± 0.23e
2.00	1.00	15	0.47 ± 0.13b	0.53 ± 0.17c
0	2.00	14	0.14 ± 0.10d	0.21 ± 0.15d
0.25	2.00	15	0.47 ± 0.13b	0.67 ± 0.21f
0.50	2.00	9	0.56 ± 0.18b	0.67 ± 0.24f
1.00	2.00	10	0.20 ± 0.65i	0.20 ± 0.65d
2.00	2.00	11	0.45 ± 0.16b	0.55 ± 0.21c

Table 1. Effects of different 2,4-D and BAP combinations on callus initiation and growth in indirect organogenesis.

*The values represent the means ± SE of three independent experiments. * Absent: 0 Present: 1, # Minimal: 0 Maximal: 10, '-' All explants in the treatment were lost due to contamination. Mean values followed with the different letters indicate significant differences at the P<0.01 level by Duncan's multiple range test.*

Although there were no significant differences among the hormone combinations designed for adventitious shoot development, 1.5 mg L^{-1} BAP alone was observed to promote the most adventitious shoot initiation (0.33) (Table 2). On the average, 19% of the explants produced adventitious shoots.

Saffron is a difficult geophyte species, and recalcitrant towards adventitious root induction under *in vitro* conditions. After preliminary experiments with several growth regulators (α-naphthaleneacetic acid (NAA) and BAP; data not shown), IBA was chosen to supplement the MS medium containing 5% (w/v) sucrose, and the 33 explants were incubated in this medium for adventitious root and corm formation. Although the tested IBA treatments did not differ significantly, 1 mg L^{-1} IBA seemed to be the best concentration for this purpose. Corm and root numbers were found to be 0.45, and 5.00, respectively (Table 3). As a result, 64% of the explants produced adventitious roots while 33% of the explants developed corms (Figure 1).

3.2 Direct organogenesis

Three different levels of 2,4-D (absent, 0.1 mg L^{-1} and 1 mg L^{-1}) and five different levels of BAP (absent, 0.5 mg L^{-1}, 1 mg L^{-1}, 3 mg L^{-1} and 5 mg L^{-1}) combinations did not yield significant differences in adventitious shoot initiation and growth. Due to this, different combinations of 2,4-D and BAP were applied to the explants. As shown in Table 4, significant differences in adventitious shoot initiation and growth were observed. Although the presence of 0.1mg L^{-1} 2,4-D in combination with 2 mg L^{-1} BAP was found to be promotional for shoot initiation (0.90), this requirement was vacated and lower levels of BAP (1 mg L^{-1}) alone produced the highest shoot growth (1.70). Overall, 72% of explants (out of 82 explants) produced adventitious shoots.

There were also significant differences among the treatments with respect to corm initiation and number. The treatment with 1 mg L^{-1} IBA yielded significantly higher corm initiation (0.52) and number of corms (1.04) than the others did (Table 5). Tested IBA treatments and 5% (w/v) sucrose did not differ significantly in their effects on adventitious root initiation, but 2 mg L^{-1} IBA had a significant effect on number of roots produced (4.96) (Table 5). Overall, the yields for adventitious root and corm productions were 57% and 36%, respectively (Figure 2).

4. Discussion

This study showed that choice and concentration of plant growth regulators and media composition are important factors influencing the *in vitro* response of saffron to organogenesis. Particularly, the combination of 2,4-D and BAP was crucial to induce callus and adventitious shoots. Our findings, as supported by the previous studies [7,11,27], reveal the positive effects of lower concentrations of 2,4-D (0.25 mg L^{-1}) and higher concentrations of BAP (1 mg L^{-1}) on callus initiation.

We showed that while 2,4-D was essential in early adventitious shoot induction, it can be excluded from the culture media to enable further shoot development, which is consistent with a previous study [8]. On the other hand, the requirement of BAP as cytokinin for direct organogenesis of saffron was still essential as it was suggested in the literature [19,20,22].

2,4-D (mg L^{-1})	BAP (mg L^{-1})	Number of Explants	Shoot Initiation*
0	0.5	12	0.25 ± 0.13a
0	1	12	0.25 ± 0.13a
0	1.5	12	0.33 ± 0.14a
0	2	12	0.17 ± 0.11a
0.1	0.5	12	0.25 ± 0.13a
0.1	1	11	0.18 ± 0.12a
0.1	1.5	12	0.00 ± 0.00a
0.1	2	12	0.08 ± 0.08a

Table 2. Effects of different 2,4-D and BAP combinations on shoot initiation in indirect organogenesis.

*The values represent the means ± SE of two independent experiments. * Absent: 0 Present: 1. Mean values followed with the same letter are not significantly different at P<0.05 by Duncan's multiple range test.*

IBA (mg L^{-1})	Number of Explants	Corm Initiation*	Corm Number	Root Initiation*	Root Number
1	11	0.36 ± 0.15a	0.45 ± 0.21a	0.82 ± 0.12a	5.00 ± 1.57a
2	11	0.27 ± 0.14a	0.36 ± 0.20a	0.45 ± 0.16a	3.55 ± 1.93a
0	11	0.36 ± 0.15a	0.36 ± 0.15a	0.64 ± 0.15a	3.46 ± 1.26a

Table 3. Effects of different IBA concentrations on corm initiation, corm number, root initiation and root number in indirect organogenesis.

*The values represent the means ± SE of three independent experiments. * Absent: 0 Present: 1. Mean values followed with the same letter are not significantly different at P<0.05 by Duncan's multiple range test.*

Figure 1. Callus, adventitious shoot, corm and root formation through indirect organogenesis. (a), Callus formation in MS media supplemented with 3% (w/v) sucrose, 0.25 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP. (b), Meristemoids. (c), Adventitious shoot initiation from callus tissue on MS media with 1.5 mg L^{-1} BAP. (d, e), Further growth of adventitious shoots. (f), Corm formation on MS media supplemented with 5% (w/v) sucrose and 1 mg L^{-1} IBA. (g), Contractile roots on the same media as in (f).

2,4-D (mg L^{-1})	BAP (mg L^{-1})	Number of Explants	Shoot Initiation*	Shoot Growth#
0	0.5	10	0.80 ± 0.13a	1.10 ± 0.28a
0	1.0	10	0.70 ± 0.15b	1.70 ± 0.42b
0	1.5	10	0.80 ± 0.13a	1.40 ± 0.27b
0	2.0	8	0.75 ± 0.16a	1.50 ± 0.42b
0.1	0.5	12	0.33 ± 0.14c	0.33 ± 0.14c
0.1	1.0	10	0.80 ± 0.13a	1.30 ± 0.30a
0.1	1.5	12	0.75 ± 0.13a	1.50 ± 0.42b
0.1	2.0	10	0.90 ± 0.10a	1.20 ± 0.25a

Table 4. Effects of different 2,4-D and BAP combinations on adventitious shoot initiation and growth in direct organogenesis.

*The values represent the means ± SE of two independent experiments. * Absent: 0 Present: 1, # Minimal: 0 Maximal: 10. Mean values followed with the different letters indicate significant differences at the $P<0.05$ level by Duncan's multiple range test.*

It is well known that saffron is a recalcitrant species towards adventitious roots induction under *in vitro* conditions. Limited numbers of studies are present in the literature concerning the *in vitro* root induction of saffron. One such study [11] described the morphogenesis of saffron in tissue culture. Shoot formation and development was enhanced with low levels of 2,4-D and root formation was increased with the addition of NAA. However, further growth of those roots was slow. Similar results were obtained with corms, where NAA induced the formation of *in vitro* shoots and kinetin induced root formations [12]. Complete plantlets with root systems

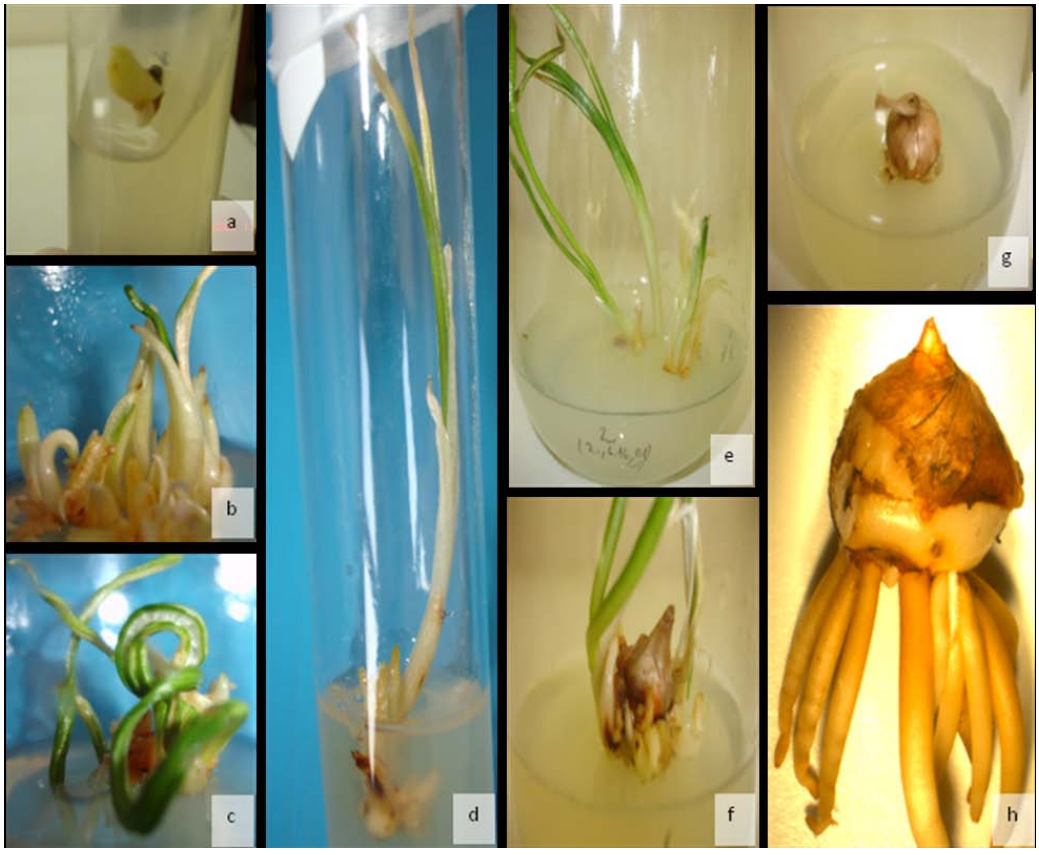

Figure 2. Adventitious shoot, corm and contractile root formation through direct organogenesis. (a), Buds cultured on MS medium with different combinations of 2,4-D and BAP. (b, c), White and green leaved shoots formed directly from explants cultured on MS medium with 3% (w/v) sucrose, 0.1 mg L^{-1} 2,4-D and 2 mg L^{-1} BAP. (d, e), Further developed adventitious shoots on MS media with 1 mg L^{-1} BAP alone. (f, g), Adventitious shoots with corm on MS media with 1 mg L^{-1} IBA and 5% sucrose. (h), The corm with contractile roots on MS medium with 2 mg L^{-1} IBA and 5% (w/v) sucrose.

IBA (mg L^{-1})	Number of Explants	Corm Initiation*	Corm Number	Root Initiation*	Root Number
1	25	0.52 ± 0.10a	1.04 ± 0.32a	0.60 ± 0.10a	4.36 ± 1.08a
2	25	0.36 ± 0.10b	0.52 ± 0.16b	0.68 ± 0.10a	4.96 ± 1.18b
0	25	0.20 ± 0.08c	0.28 ± 0.12c	0.44 ± 0.10a	1.84 ± 0.74c

Table 5. Effects of different IBA concentrations on corm initiation, corm number, root initiation and root number in direct organogenesis.

*The values represent the means ± SE of three independent experiments. * Absent: 0 Present: 1. Mean values followed with the different letters indicate significant differences at the P<0.05 level by Duncan's multiple range test.*

and corm formation, following somatic embryogenesis, were also reported [15] where germinated embryos were transferred to half-strength MS supplemented with 5 µM BA and 5 µM NAA. In another study [13], the effects of BA and NAA with different culture media, such as B5 and MS, were tested. The highest rate of plant regeneration complete with roots was observed in the B5 media containing 2.22 µM NAA and 2.68 µM BA. These studies lead us to believe that NAA might be a good candidate for root formation; therefore, we decided to try NAA in combination with BAP to induce adventitious root and corm formation. However, in our study, only limited corm production without roots was achieved and further shoot development was observed (data not presented).

Finally, we have considered changing NAA to IBA as the potential auxin source for root induction since this growth regulator is usually included in commercial powder preparations. In addition to IBA, sucrose level was increased to 5% in the growth media to induce

root formation and to increase corm number. A study [10] which was published after the termination of our experiments in 2007 on *Crocus sativus* [31] also emphasized the importance of elevated sucrose concentration for corm production. The results of the present study show that IBA and increased sucrose level are critical factors to initiate adventitious root formation and their further growth. Based on these findings, sucrose concentration was also identified as an important parameter for not only rooting, but also for the quality of roots formed.

In conclusion, high efficiency in the plantlet regeneration, complete with adventitious shoots, corms, and contractile roots, was obtained *in vitro* in this study. In indirect organogenesis, the frequencies of adventitious shoot, corm and contractile root were 19%, 33%, and 64%, respectively. In direct organogenesis, the frequencies were higher and found to be 72%, 36% and 57% for adventitious shoot, corm, and contractile root, respectively. Adventitious corm and root production in *in vitro* conditions is a rare event for saffron species and when compared with the previous studies, the values we obtained here are the highest. These high rates of adventitious corm and contractile root production suggest that efficient micropropagation of saffron could be achieved for mass propagation and *ex situ* conservation of threatened saffron genetic resources around the World.

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