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BRIEF COMMUNICATION

In vitro cormlet development in *Crocus sativus*

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Abstract

An improved protocol for generation of viable cormlets from tissue culture derived shoots of saffron has been developed. Multiple shoots were generated from apical buds, small corms and *in vitro* developed single shoots. Bunches of two to three shoots when cultured on half strength Murashige and Skoog (MS) medium containing 3 mg dm^{-3} benzyladenine (BA) and 80 g dm^{-3} sucrose developed 1.89 cormlets per shoot bunch with an average fresh mass of 1.18 g. It took nine months from culture of apical buds to the harvest of cormlets but under field conditions 22 months. Sucrose appeared to be essential for cormlet induction as no cormlets were developed in the medium devoid of sucrose and only 0.29 per shoot in medium containing mannitol. *In vitro* derived cormlets sprouted from apical and axillary buds on MS medium containing 12 mg dm^{-3} BA, 3 mg dm^{-3} indolebutyric acid and 30 g dm^{-3} sucrose. Daughter cormlet formation from *in vitro* derived cormlets was also observed.

Additional key words: apical buds, growth regulators, micropropagation, multiple shoots, saffron, sprouting, sucrose.

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Crocus sativus L. (saffron crocus, *Iridaceae*) is cultivated in several countries of the world for its red stigmatic lobes that constitute the high value spice saffron. Saffron being triploid (2n=3x=24) is sterile and is propagated vegetatively through daughter corms. Rate of generation of daughter corms under natural conditions is low (Jirage *et al*. 1994, Chahota *et al*. 2003). Low rate of daughter corm production results in limited availability of propagating material for field cultivation. Micropropagation can be used to produce large quantities of the propagating material in saffron, however, efficient protocols to achieve this goal are not available (Plessner and Ziv 1999). Though, shoot regeneration has been achieved from callus cultures, terminal buds, lateral buds, small corms and ovaries, its frequency remains low (Isa and Ogasawara 1988, Plessner *et al*. 1990, Aguero and Tizio 1994, Milyaeva *et al*. 1995, Piqueras *et al*. 1999, Bhagyalakshami 1999, Karamian 2004, Blazquez *et al*. 2004). Other major problems in saffron micropropagation are the very poor establishment of plantlets in the field and low frequency of cormlet induction from tissue culture derived shoots (Milyaeva *et al*. 1995, Chauhan *et al*. 1999). Cormlets developed through tissue cultures usually remain smaller in size and rarely germinate. In the present paper, we report the efficiency of growth regulators and sugars in stimulating multiple shoot and cormlet differentiation in saffron.

Saffron corms of small size (about 3.0 g average fresh mass) were procured from CSK HPKV, Research Station, Sangla (H.P.), India. The corms were harvested immediately after saffron harvest and stored at 4 °C until used. Prior to culture, these were washed under tap water, rinsed for 10 s in 70 % ethanol, surface sterilized with 0.1 % mercuric chloride for 10 min and washed five times in sterile double distilled water. Apart from corms, corm tissues (5 × 5 × 4 mm^3) containing an apical bud (Fig. *1A*) were also used as explants for direct multiple shoot induction. The explants were cultured on Murashige and Skoog (1962; MS) medium supplemented with indole-3-butyric acid (IBA, 0 - 3 mg dm^{-3}), 6-benzyladenine (BA, 6 - 14 mg dm^{-3}) and sucrose (30 or 50 g dm^{-3}). Shoots emerging from explants were

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BA - 6-benzyladenine; IBA - indole-3-butyric acid; Kn - kinetin; MS - Murashige and Skoog (1962) medium; NAA - α-naphthaleneacetic acid.
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subcultured twice at an interval of 30 d and final data were recorded after 90 d. The multiple shoots obtained after 90 d were separated from each other and cultured individually on MS medium containing 0 - 3 mg dm^{-3} IBA, 6 - 14 mg dm^{-3} BA and 30 g dm^{-3} sucrose for further 90 d.

In vitro shoots were separated into bunches of two to three. The bunches as well as individual shoots were cultured on full or half strength MS medium containing sucrose (0 - 150 g dm^{-3}), mannitol (0 or 80 g dm^{-3}), IBA (2 mg dm^{-3}), BA (1 - 6 mg dm^{-3}) and naphthalene acetic acid (NAA, 1 mg dm^{-3}) for cormlet induction. Data on number of cormlets per shoot and their average fresh mass were recorded after 90 d of culture initiation. Cormlets of about 1.0 g fresh mass developed *in vitro* were pretreated at 4 °C in dark for 60 d and transferred thereafter on to MS medium supplemented with five combinations of BA (6 - 14 mg dm^{-3}), IBA (0 - 3 mg dm^{-3}), and sucrose (30 g dm^{-3}) for sprouting. Percentages of cormlets sprouted and number of sprouts per cormlet were scored after 30 d.

The pH of the media used was 5.8. The media were solidified with agar (8 g dm^{-3} *Sigma Chemical Company*, St. Louis, MO, US) and autoclaved at 121.6 °C for 15 min. The tissue cultures were incubated at 20 ± 1 °C and 16-h photoperiod (140 - 145 μmol m^{-2} s^{-1}) except mentioned otherwise. All experiments were conducted in a completely randomized design (Gomez and Gomez 1984). Each treatment had three replications and each replication consisted of 30 explants. The data were analyzed using analysis of variance (*ANOVA*) and are presented as the means of three replications ± SE.

Apical buds started sprouting within 10 d of incubation on culture media, however, only those cultured at 6 mg dm^{-3} BA and 30 g dm^{-3} sucrose developed multiple shoots (Fig. 1*A*,*B*,*C*). Multiple shoot induction frequency (56.5 %) as well as number of shoots per bud (9.0) were high on this medium. This medium also elicited maximum shoot formation (9.0 per corm, 45.5 % corms forming shoots) from the corms

Fig. 1. Multiple shoot and cormlet generation *in vitro* in *Crocus sativus*: *A* - sprouting of apical bud on MS medium with 6 mg dm^{-3} BA and 30 mg dm^{-3} sucrose; *B* - multiple shoot emergence from the base of the cultured bud; *C* - green shoots; *D* - cormlet induction on half strength MS medium supplemented with 3 mg dm^{-3} BA and 80 g dm^{-3} sucrose; *E* - mature cormlets; *F* - a sprouted *in vitro* cormlet; *G* - mother cormlet bearing a daughter cormlet.

Table 1. Effect of growth regulators and sucrose concentration in MS medium on direct shoot regeneration from saffron corms. Means ± SE, n = 30. Means within each row followed by the same letter are not significantly different at 0.05 probability level.

BA [mg dm^{-3}]	IBA [mg dm^{-3}]	Sucrose [g dm^{-3}]	Corms forming shoots [%]	Number of shoots [corm^{-1}]
14	3	30	19.0 ± 2.2e	1.5 ± 0.1e
14	3	50	72.7 ± 2.1b	6.0 ± 0.5b
10	2	30	68.2 ± 2.4b	3.0 ± 0.3d
10	2	50	100.0	4.5 ± 0.4c
10	0	30	20.0 ± 2.4e	1.5 ± 0.2e
10	0	50	90.0 ± 6.2a	2.5 ± 0.2d
6	0	30	45.5 ± 1.5d	9.0 ± 0.6a
6	0	50	54.5 ± 4.1c	4.5 ± 0.4c
6	2	30	100.0	1.5 ± 0.1e
6	2	50	100.0	1.5 ± 0.2e

(Table 1). Multiple shoot formation from corms was also high (72.7 % corms forming shoots, 6.0 shoots per corm) at 14 mg dm^{-3} BA, 3 mg dm^{-3} IBA and 50 g dm^{-3} sucrose. In general, addition of IBA to BA containing medium increased number of corms forming shoots, however, IBA was not necessarily suitable for induction of more number of shoots per corm. For example, 100 % corms formed shoots at 6 mg dm^{-3} BA and 2 mg dm^{-3} IBA compared to that of 45.5 % at 6 mg dm^{-3} BA alone. However, the number of shoots per corm (1.5) was lowest in this medium. In addition to growth regulators, multiple shoot regeneration from corms was also influenced by sucrose concentration. The number of corms forming shoots and shoots per corm were more at 50 g dm^{-3} sucrose as compared to that at 30 g dm^{-3} (Table 1) with the exception of BA 6 mg dm^{-3} where number of shoots per corm (4.5) was significantly less at 50 g dm^{-3} than that (9.0 per corm) at 30 g dm^{-3}. Individual *in vitro* derived shoots also developed multiple shoots at low frequency. Maximum number of shoots (4.0) per cultured shoot was induced at 14 mg dm^{-3} BA + 3 mg dm^{-3} IBA + 50 g dm^{-3} sucrose. Frequency of shoot induction (3.9) obtained at 6 mg dm^{-3} BA was also comparable to that obtained at 14 mg dm^{-3} BA + 3 mg dm^{-3} IBA.

Table 2. Effect of sucrose concentration (g dm^{-3}) on cormlet formation from tissue culture derived shoots of saffron. The shoots were cultured on half strength MS medium supplemented with 6 mg dm^{-3} BA. Mean ± SE, n = 30. Means within each row followed by the same letter are not significantly different at 0.05 probability level.

Sucrose [g dm^{-3}]	Number of cormlets [shoot^{-1}]	Average cormlet fresh mass [g]	Fresh mass range [g]
0	0^{a}	-	-
30	0.75 ± 0.02^{b}	0.91 ± 0.05^{a}	0.20 - 1.30
80	1.84 ± 0.10^{c}	1.10 ± 0.08^{a}	0.05 - 1.17
150	0.69 ± 0.03^{d}	0.50 ± 0.02^{b}	0.41 - 0.77

Single shoots did not develop cormlets on semi-solid media, however, shoot bunches did so on semi-solid MS medium supplemented with 6 mg dm^{-3} BA and 30 g dm^{-3} sucrose (Table 2). Sucrose appeared to be essential for cormlet differentiation as no cormlet was formed in medium devoid of sucrose and only 0.29 per shoot (0.18 g average fresh mass) in medium containing mannitol, a sugar alcohol that is not metabolized by plant tissues. The optimum sucrose concentration for cormlet develop-ment was 80 g dm^{-3} at which 100 % shoots developed cormlets of high fresh mass (1.10 g) with an average of 1.84 cormlets per shoot bunch. Lowering the strength of the MS salts and vitamin to half did not increase cormlet induction significantly over full strength medium, however, five growth regulator combinations (3 mg dm^{-3} BA, 6 mg dm^{-3} BA, 4 mg dm^{-3} BA + 2 mg dm^{-3} IBA, 3 mg dm^{-3} BA + 2 mg dm^{-3} IBA and 2 mg dm^{-3} IBA) in ½ MS medium induced cormlets in all the cultured shoots (Table 3). The best growth regulator concentration for cormlet development was 3 mg dm^{-3} BA in ½ MS medium (1.89 cormlets per shoot, 1.18 g average fresh mass) (Fig. 1*D*,*E*).

Table 3. Effect of growth regulators in half strength MS medium on cormlet induction from tissue culture derived shoots of saffron. Only those media have been listed on which more than one cormlet per shoot were formed. Sucrose concentration in the media was 80 g dm^{-3}. Mean ± SE, n = 30. Means within each row followed by the same letter are not significantly different at 0.05 probability level.

BA [mg dm^{-3}]	IBA [mg dm^{-3}]	Number of cormlets [shoot^{-1}]	Average cormlet fresh mass [g]	Fresh mass range [g]
3.0	0	1.89 ± 0.15a	1.18 ± 0.03a	0.22 - 1.84
4.0	0	1.00 ± 0.18c	0.48 ± 0.03c	0.23 - 0.88
6.0	0	1.85 ± 0.22a	1.08 ± 0.02a	0.05 - 1.14
0	2.0	1.25 ± 0.18bc	0.29 ± 0.04d	0.04 - 1.42
3.0	2.0	1.50 ± 0.16b	0.66 ± 0.06b	0.17 - 1.26
4.0	2.0	1.71 ± 0.20a	0.37 ± 0.04cd	0.05 - 0.80
6.0	2.0	1.08 ± 0.16c	0.62 ± 0.06b	0.40 - 1.03

Apical and axillary buds of cormlets sprouted at five growth regulator combinations in MS medium (Fig. 1*F*). More than one shoots per cormlet were obtained on all the media. While the percentage of cormlets sprouted (95.6 %) was high at 12 mg dm^{-3} BA + 3 mg dm^{-3} IBA, the number of buds sprouted per cormlet (3.7) was maximum at 6 mg dm^{-3} BA. Sprouting (84.2 %) as well as number of buds sprouted per cormlet (3.2) were also high at 14 mg dm^{-3} BA and 3 mg dm^{-3} IBA. Two out of the ten cold pretreated (4 °C for 4 months) cormlets formed at MS + 3 mg dm^{-3} BA developed one daughter cormlets each (Fig. *1G*), however, those formed at other growth regulator combinations failed to do so.

The percentage (56.5 %) of apical buds inducing multiple shoots and number of shoots per apical bud (9.0) obtained in our studies were higher than that reported earlier from corms (Milyaeva *et al*. 1995, Chauhan *et al*. 1999), isolated buds (Huang 1987, Plessner *et al*. 1990) or ovaries (Bhagyalakshami 1999). Moreover, the multiple shoot induction from *in vitro* derived shoots has been observed for the first time in tissue cultures. Effects of sugars and growth regulators on shoot and root regeneration as observed by us has also been well documented for other plant species (Radhika *et al*. 2006, Soniya and Sujitha 2006, Hiregoudar *et al*. 2006, Rao and Purohit 2006, Novotná *et al*. 2007).

Our findings on high sucrose requirement for cormlet differentiation from shoots support the findings of Aguero and Tizio (1994) who reported 6.0 % sucrose to be optimum for cormlet formation. Shoot bunches forming cormlets (100 %) and cormlet/shoot (1.89) was more than that reported earlier using single shoots (Ahuja *et al*. 1993, Chauhan *et al*. 1999). In contrast to the earlier reports, the average fresh mass of cormlets (1.18 g) developed in the present study was comparable to that of 1.2 g obtained under field conditions (Chahota *et al*. 2003). Moreover, in comparison to 22 months under field conditions, it took nine months *in vitro*. Sprouting and daughter cormlets differentiation from buds of cold pretreated *in vitro* derived cormlets was similar to daughter corm production under field conditions. Apart from saffron, cold-pretreatment has also been shown to enhance growth and development of segments of the bulblets of *Hyacinthus orientalis* L. (Chung *et al*. 2006).

As per our knowledge, this is the first report of daughter cormlet formation from *in vitro* developed cormlets. The present observations are expected to lead to elaboration of efficient protocols of saffron *in vitro* multiplication. Improved *in vitro* cormlet production might also find use in elucidation of physiology of cormlet differentiation and genes involved in this process.

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