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In Vitro Studies on Cormogenesis and Maximization of Corm Size in Saffron

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ABSTRACT

Saffron has long been recognized for its medicinal properties and is used principally as a flavoring and colouring agent in food preparations. The present study assesses the morphogenic response of various explants viz. corm slices, vegetative buds (dormant and active corms) in the formation of cormlets *in vitro*. Maximum cormlets formed on corm slices cultured on half-strength Murashige and Skoog (MS) medium + 6-benzyl amino purine (BAP) 20 µM + α-naphthalene acetic acid (NAA) 20 µM + 30 g/l sucrose. Increase in corm size from active vegetative buds were achieved on half-strength MS medium + indole-3-butyric acid (IBA) 8.8 µM + 40 g/l edible sugar from sugarcane whereas 40 g/l sucrose also favoured such an increase in size with a combination of 2 µM of both BAP + NAA. However, vegetative buds from dormant corms did not grow further or show other responses when cultured with different plant growth regulators or carbohydrate sources due to dormancy.

Keywords: *Crocus sativus*, carbon sources, micropropagation

INTRODUCTION

Saffron (*Crocus sativus*) cultivation in India is mostly confined to the table land of Pampore in Kashmir and Kishtwar in Jammu. The word "saffron" is derived from the Arabic word "zafran", which translates to "yellow" (Husaini *et al*. 2009). Saffron species belong to the family Iridaceae. Its stigmas are dried for use in medicine, food seasoning and colouring since centuries and have the distinction of being the most expensive spice by weight (Dauria *et al*. 2006). Its high cost is primarily due to low yield, labour intensive harvesting and processing of stigma (Nauriyal *et al*. 1977). Saffron contains more than 150 volatile and aroma yielding compounds, the major constituents of commercial importance being crocin (responsible for colour), picrocrocin (flavour) and safranal (smell). It also has many non-volatile active components, many of which are cartenoids including zeaxanthin, lycopene and various α- and β-carotenes. However, saffron's golden yellow-orange colour is primarily the result of α-crocin (Abdullaev 2002).

Major constraints in the production of saffron are non availability of corms (seed material), lack of efforts from farmers or State Government, lack of better cultivation and post harvest practices, rodents and diseases. Since conventional methods of saffron propagation are very slow therefore tissue culture represent an important potential to effectively propagate it (Wani and Mohiddin 2009). Micropropagation of saffron has been advocated as the best alternative for its propagation (George *et al*. 1992; Ahuja *et al*. 1994; Bagheri and Vesal 2006). Reports on *in vitro* corm production (Karaoglu *et al*. 2007; Quadri *et al*. 2008), plantlet regeneration (Majourhat *et al*. 2007), somatic embryogenesis (Raja *et al*. 2007) are indicative of recent attempts in saffron micro propagation. However these have a limited potential for employing in large scale production of corms and therefore an attempt was be made to evaluate morphogenetic response of various explants towards cormlet production and increase the size of mini corms, which is a major bottleneck in the *in vitro* technique.

MATERIALS AND METHODS

Plant material and surface sterilization of explants

Saffron corms (active and dormant) obtained from major saffron-growing sites within the Kashmir Valley served as a source for establishing the *in vitro* cultures. These corms were thoroughly washed with detergent Cedepol (0.5%) and Tween-20 (surfactant) under running tap water followed by final rinsing with double distilled water. Subsequently these were surface disinfected with 70% ethanol for 1 min followed by 0.1% $HgCl_2$ (w/v) for 10 min and washed five times with sterilized double distilled water.

Culture media

Half-strength MS basal medium (Murashige and Skoog 1962) supplemented with different concentrations of sucrose (Qualigens, India) viz. 3, 5, 6, 7, 8 and 10%, 0.8% Difcobacto agar and different concentrations of plant growth regulators (PGRs; Himedia, Mumbai, India) was prepared. All the medium constituents were added together and the pH was adjusted to 5.4 with 1 N NaOH or 1 N HCl and finally dispensed into 100-ml Erlenmyer flasks (borosilicate glass) plugged with non-absorbent cotton prior to autoclaving at 121°C and 15 psi for 20 min. All chemicals and reagents were purchased from Himedia, unless specified otherwise.

Experimental design

Corm slices and vegetative buds were subjected to different treatments as explained below.

Corm slices excised from active corms were cultured on: a) ½-MS medium + 3% sucrose supplemented with 6-benzyl amino purine (BAP) (2–25 µM) + α-naphthalene acetic acid (NAA) (2–20 µM); Kinetin (Kn) 2–25 µM + NAA (2-20 µM). b) ½-MS medium supplemented with 2 µM each of BAP and NAA and varying concentrations of different carbon sources viz. sucrose,

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Research Note

edible sugar (SLM Sugar, CL Products, India, Ltd.) ranging from 30 to 100 g/l. c) ½-MS medium + 0.5, 1.5 or 2.5 g/l KCl with 2 µM each of BAP and NAA + a carbon source (edible sugar 40 g/l + sucrose 30 g/l).

Vegetative buds from active and dormant corms were cultured separately on: a) ½-MS medium + 2 µM BAP + 2 µM NAA and varying concentrations of sucrose and edible sugar ranging from 30 to 100 g/l. b) ½-MS medium + 8.8 µM indole-3-butyric acid (IBA) + carbon source (edible sugar, sucrose 30-60 g/l each). c) ½-MS medium + KCl (0.5, 1.5 or 2.5 g/l) + BAP (2 µM) + NAA (2 µM) + carbon source (edible sugar 40 g/l + sucrose 30 g/l).

Cultures were maintained in an incubation room at a temp of 25 ± 3°C under a 16-hr photoperiod provided by cool white fluorescent tubes (3000 lux). The experiments were carried out in completely randomized block design (CRD), repeated three times; each treatment had 10 replicates. The size of corms were considered as 'less notable' (LN) when corms weighed < 1.0 g, 'notable' (N; 1.0-1.5 g), and 'prominent' (P; 1.5-2.0 g).

RESULTS AND DISCUSSION

The main aim of the present study was to assess the morphogenetic response of corm slices and explore the possibility for development and standardization of a protocol for multiple cormlet production *in vitro*. Half-strength MS medium supplemented with different concentrations of BAP, Kn and NAA were used to induce *in vitro* cormogenesis from corm slices of saffron (**Table 1**). Corm slices showed a good callus induction response with the combined use of BAP and NAA, with highest callus formation on BAP (20 µM), NAA (20 µM) and 30 g/l sucrose (**Fig. 1A**). However, replacement of BAP with Kn failed to elicit any morphogenetic response. The effect of PGRs is not specific and even different PGRs belonging to the same class may elicit different responses in a given tissue. Most PGRs are rapidly metabolized into physiologically inactive compounds (Bhan 1998) and same appears here with Kn. Callus formation with higher (83-93%) induction frequency was earlier recorded by Ding *et al*. (1981) from corms of saffron using MS medium containing NAA/IAA/2,4–dichloro phenoxyacetic acid (2,4-D) alone at 1 mg/l each. Callus induction has also been reported under the influence of different auxins and cytokinins using different explants saffron. Hori *et al*. (1988) obtained callus from pistils using BAP and NAA (1 mg/l each), while Karamian and Ebrahimzadeh (2001) induced callus from shoot meristems using Kn and 2,4-D. Raja *et al*. (2007) reported callus formation from basal leaf segments using 4.0 mg/l BAP and 0.5 mg/l NAA while Quadri *et al*. (2008) obtained callus from corm slices and needle segments using 13.2 µM BAP, 10 µM Kn, 15 µM 2,4-D and 80 g/l sucrose.

Callus cultures after 12 weeks induced mini-cormlets and most multiple cormlets (20 ± 0.4) were recorded on BAP (20 µM), NAA (20 µM) and 30 g/l sucrose (**Fig. 1B**). A similar response was noted by Quadri *et al*. (2008) with 26.4 µM BAP and 30 g/l sucrose from sub-cultured callus raised from corm slices. Auxin-cytokinin interactions change the course of morphogenetic processes in cultured explants which perhaps caused a shift in the endogenous levels of auxins and cytokinins (Bhan 1998), and in the present study BAP in combination with NAA resulted in reasonably good (20 ± 0.4) mini-cormlet production. *In*

Table 1 *In vitro* response of corm slices of *Crocus sativus* L. on ½-MS medium supplemented with BAP + NAA and Kn + NAA.

BAP (µM)	Kn (µM)	NAA (µM)	Callus*	Cormlet number** Mean ± S.D.
2	-	2	NR	NR
5	-	5	+	5 ± 0.2
10	-	5	+	10 ± 0.5
10	-	10	+	12 ± 0.3
15	-	10	++	13 ± 0.4
15	-	15	++	15 ± 0.2
20	-	15	++	15.5 ± 0.1
20	-	20	+++	20 ± 0.4
25	-	20	++	18 ± 0.3
-	2	2	NR	NR
-	5	5	NR	NR
-	10	5	NR	NR
-	10	10	NR	NR
-	15	10	NR	NR
-	15	15	NR	NR
-	20	15	NR	NR
-	20	20	NR	NR
-	25	20	NR	NR

*Data scored after 8 weeks of culture period; ** Data scored after 12 weeks of culture period; 10 replicates per treatment. S.D. Standard deviation; NR - no response; + low callus; ++ moderate callus; +++ intense callus.
BAP, 6-benzyl amino purine; Kn, kinetin; NAA, α-naphthalene acetic acid

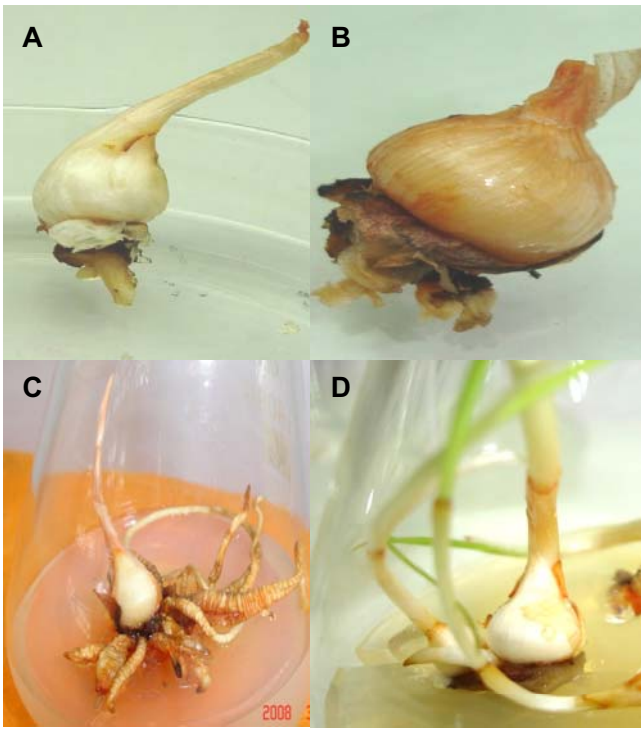

Fig. 2 ***In vitro*** **response of active vegetative buds of** ***Crocus sativus*** **L.** (**A**) Notable increase in corm size on BAP (2 µM) + NAA (2 µM) + 40 and 60 g/l edible sugar after 12 weeks. (**B**) Prominent increase in corm size on BAP (2 µM) + NAA (2 µM) + 40 g/l sucrose after 12 weeks. (**C**) Prominent increase in corm size and multiple thick root formation on IBA (8.8 µM) + 40 g/l edible sugar after 12 weeks. (**D**) Prominent increase in corm size and germination on BAP (2 µM) + NAA (2 µM) + KCl (2.5 g/l) + 40 g/l edible sugar + 30 g/l sucrose after 12 weeks.

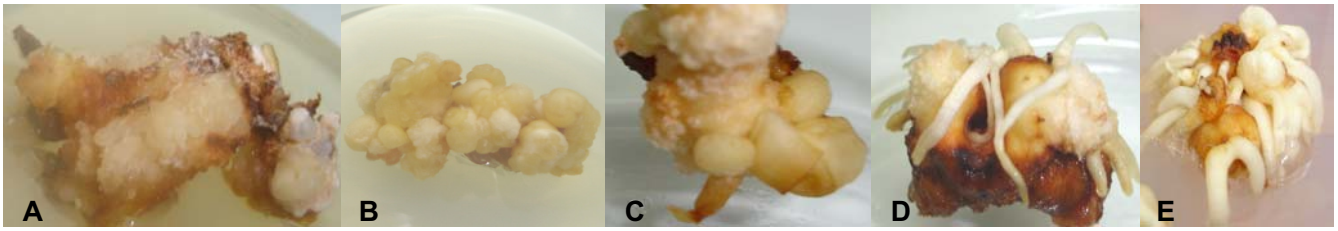

Fig. 1 ***In vitro*** **response of corm slices of** ***Crocus sativus*** **L.** (**A**) Callus formation on corm slices after 8 weeks. (**B**) Multiple cormlet production after 12 weeks. (**C**) Cormlet size (less notable) and callus formation after 12 weeks. (**D**) Cormlet (notable size) accompanied with multiple root regeneration and callus formation after 12 weeks. (**E**) Multiple root formation and increase in cormlet size.

Table 2 Effect of ½-MS medium supplemented with BAP + NAA and different concentrations of carbon sources on size of *in vitro* mini-cormlets of *Crocus sativus* L*.

BAP (µM)	NAA (µM)	Sucrose (g/l)	Edible sugar (g/l)	Corm size	Other responses
2	2	30	-	NR	NR
2	2	-	30	NR	Callus formation
2	2	40	-	NR	Callus formation
2	2	-	40	NR	Callus formation
2	2	60	-	N	Rhizogenesis and callus formation
2	2	-	60	LN	Callus formation
2	2	80	-	NR	Callus formation
2	2	-	80	NR	Callus formation
2	2	100	-	NR	Callus formation
2	2	-	100	NR	Callus formation

*Data scored after 12 weeks of culture period; 10 replicates per treatment. NR - no response; LN - less notable; N - notable.
BAP, 6-benzyl amino purine; NAA, α-naphthalene acetic acid

Table 3 Effect of ½-MS medium supplemented with BAP + NAA and different concentrations of KCl and carbon sources on size of *in vitro* mini-cormlets of *Crocus sativus* L*.

BAP (µM)	NAA (µM)	KCl (g/l)	Sucrose (g/l)	Edible sugar (g/l)	Corm size	Other responses
2	2	0.5	30	40	LN	Rhizogenesis
2	2	1.5	30	40	LN	Rhizogenesis
2	2	2.5	30	40	N	Rhizogenesis

*Data scored after 12 weeks of culture period; 10 replicates per treatment.
BAP, 6-benzyl amino purine; NAA, α-naphthalene acetic acid; KCl, potassium chloride

Table 4 Effect of ½-MS medium supplemented with BAP + NAA and different concentrations of carbon sources on active vegetative buds of *Crocus sativus* L*.

BAP (µM)	NAA (µM)	IBA (µM)	Sucrose (g/l)	Edible sugar (g/l)	Corm size	Other responses
2	2		30	-	LN	-
2	2		-	30	LN	-
2	2		40	-	P	-
2	2		-	40	N	-
2	2		60	-	LN	-
2	2		-	60	N	-
2	2		80	-	NR	-
2	2		-	80	NR	-
2	2		100	-	NR	-
2	2		-	100	NR	-
-	-	8.8	30	-	LN	Rhizogenesis
-	-	8.8	-	30	LN	Rhizogenesis
-	-	8.8	40	-	LN	Rhizogenesis
-	-	8.8	-	40	P	Rhizogenesis
-	-	8.8	60	-	LN	Rhizogenesis
-	-	8.8	-	60	LN	Rhizogenesis

*Data scored after 12 weeks of culture period; 10 replicates per treatment. NR - no response; LN - less notable; N - notable; P - prominent.
BAP, 6-benzyl amino purine; IBA, indole-3-butyric acid; NAA, α-naphthalene acetic acid

vitro mini-corm production has also been obtained by culturing basal leaf segments of saffron on MS medium containing BA (4.0 mg/l) and NAA (0.50 mg/l) (Raja *et al*. 2007). Thidiazuron (TDZ) (0.1 mg/l), however, is much more efficient in producing micro-corms, 60% of regenerants fully developing a leaf primordium compared to only 20% of regenerants with BAP (2 mg/l) (Blazquez *et al*. 2001). This shows that PGRs have an important role to play in cormlet induction.

In another experimental trial *in vitro* raised mini-cormlets and vegetative buds (dormant and active) were assessed for their response to different PGR combinations and carbon sources to enhance their size and regeneration. *In vitro* raised mini-cormlets subcultured on constant BAP + NAA (2 µM each) in ½-MS medium with different concentrations of carbon sources showed a limited response (**Table 2**). A less notable increase in size accompanied with callus formation was registered on edible sugar (60 g/l), while 60 g/l of sucrose resulted in a notable increase in size accompanied with multiple root regeneration and callus formation (**Fig 1C, 1D**). Sucrose plays a vital role for cormlet production in saffron (Sharma *et al.* 2008) but in the present study corm size, rather than cormlet production, could be increased by the use of sucrose and edible sugar as carbon sources (**Table 2**). The addition of KCl (used as an additional potash supplement) resulted in an increase in size followed by rhizogenesis and maximum increase in size and multiple thick root formation on ½-MS, BAP (2 µM), NAA (2 µM), KCl (2.5 g/l), 40 g/l edible sugar and 30 g/l sucrose (**Table 3; Fig. 1E**).

Apical and lateral vegetative buds from actively growing corms were cultured on ½-MS medium with a range of PGRs and carbon sources. Apical vegetative buds showed an increase in corm size which was significantly affected by the carbon source used (**Table 4**). An increase in size was noticed after 12 weeks of culture ranging from LN to P. However, lateral vegetative buds (upper and lower) showed no response at all, which is not consistent with the results of Rajabpoor *et al*. (2007), who reported that the lower portion of corm segments were more responsive than the upper portion. Different responses observed from apical and lateral vegetative buds of the corms can be possibly attributed to polar transportation of PGRs and carbon sources and the existence of a gradient (Cook *et al*. 1993). An increase in corm size was registered when edible sugar (40 and 60 g/l) was added to ½-MS medium supplemented with 2 µM each of BAP and NAA (**Fig. 2A**), while an increase in size was recorded on 40 g/l sucrose (**Fig. 2B; Table 4**). An increase

Table 5 Effect of ½-MS medium supplemented with BAP + NAA and different concentrations of KCl and carbon sources on size of active vegetative buds of *Crocus sativus* L*.

BAP (µM)	NAA (µM)	KCl (g/l)	Sucrose (g/l)	Edible sugar (g/l)	Corm size	Other responses
2	2	0.5	30	40	LN	Germination
2	2	1.5	30	40	LN	Germination
2	2	2.5	30	40	P	Germination

*Data scored after 12 weeks of culture period; 10 replicates per treatment.
BAP, 6-benzyl amino purine; NAA, α-naphthalene acetic acid; KCl, potassium chloride

in corm size accompanied by maximum multiple thick root formation was recorded on 40 g/l edible sugar and 8.8 µM IBA (**Fig. 2C; Table 4**). The addition of 2.5 g/l KCl in medium containing ½-MS, 2 µM BAP, 2 µM NAA, edible sugar (40 g/l) and sucrose (30 g/l), resulting in an increase in the size of corm followed by germination (**Table 5; Fig. 2D**).

Vegetative buds from dormant corms did not express their potential for growth because of deep dormancy. It is not only the concentration of a particular PGR that determines a developmental response, but the cell sensitivity towards it also seems to be important (Trewavas 1998). The variation in responses may therefore be due to the cell sensitivity towards the carbon sources used.

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