Physiol Mol Biol Plants (October–December 2010) 16(4):369–373
DOI 10.1007/s12298-010-0044-4

RESEARCH ARTICLE

# *In vitro* development of microcorms and stigma like structures in saffron (*Crocus sativus* L.)

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Published online: 8 January 2011


**Abstract** Saffron is an important spice derived from the stigmas of *Crocus sativus,* a species belonging to the family Iridaceae. Due to its triploid nature it is sterile and is not able to set seeds, so it is propagated only by corms. The natural propagation rate of most geophytes including saffron is relatively low. An *in vitro* multiplication technique like micropropagation has been used for the propagation of saffron. In the present study, various explants were cultured on different nutrient media supplemented with various concentrations of plant growth regulators to standardize the best media combination for obtaining optimum response with respect to corm production and development of Stigma Like Structures (SLS). Highest response (60%) was observed with half ovaries on G-5 media supplemented with 27 $\mu M$ NAA and 44.4 $\mu M$ BA followed by 55% on LS media with 27 $\mu M$ NAA and 44.4 $\mu M$ BA. Maximum size (1.3 g) of microcorms were obtained from apical buds on the LS media supplemented with 21.6 $\mu M$ NAA and 22.2 $\mu M$. Stigma Like Structures were developed from half ovary explants both directly and indirectly. Maximum number (120 indirectly and 20 directly) and size (5.2 cm) of SLS were obtained in G-5 medium supplemented with 27 $\mu M$ NAA and 44.4 $\mu M$ BA followed by 100 indirectly and 20 directly and 4.5 cm long on LS medium supplemented with 27 $\mu M$ NAA and 44.4 $\mu M$ BA.

**Keywords** Saffron · Stigma like structures · Growth regulators · Micropropagation · Half ovary · Microcorm

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## Introduction

The domesticated saffron (*C. sativus*), a fall-flowering perennial plant is a sterile triploid mutant of the eastern Mediterranean fall-flowering *Crocus cartwrightianus* (Deo 2003). The stigmas are dried for use in medicine, food seasoning and coloring since centuries and are characterized as the most expensive spice by weight. Saffron is propagated by corms as the flowers are sterile and fail to produce viable seeds. A corm survives for only one season, producing up to ten "cormlets" that eventually give rise to new plants (Deo 2003). Production of microcorm is important for plant production of saffron. Low microcorm production in saffron is due to lack of high yielding cultivars adopted to diverse growing conditions, large area under rainfed cultivation, biotic and abiotic stresses, poor plant stand, moisture stress at terminal growth stage, inadequate seed replacement rate, poor crop management, resource poor farmers, low risk bearing capacity, inadequate input and technical support, poor infrastructure and institutional support, inefficient technology delivery system, limited policy directives and incentives and crop damage due to menace of corm rot are the important production constraints which need to be taken care off (Nehvi et al. 2005). Conventional propagation methods are very slow and propagation by tissue culture represents an important potential to effectively propagate it. Micropropagation is very good alternative for quality planting material/seed production and large scale multiplication of disease free saffron (Ascough et al. 2009; Karaoglu et al. 2007). The successful tissue culture protocol was developed in saffron by several workers (Ding et al. 1981; Homes et al. 1987; Ilahi et al. 1987). Microcorm production under *in vitro* conditions shows promise with respect to rate of multiplication and number of microcorms produced in saffron (Bhagyalakshmi 1999; Raja et al. 2007; Jun et al. 2007; Sheibani et al. 2007; Darvishi et

al. 2007). Stigma-like structures were successfully induced by using different floral explants of *Crocus sativus* (Fakhrai and Evans 1990; Sarma et al. 1991). *In vitro* saffron production has been successfully induced from explants like stigma (Sarma et al. 1990), ovaries (Himeno and Sano 1987; Sano and Himeno 1987) style and perianth (Ebrahimzadeh and Karamian 2000). But stigma like structures produced through tissue culture contain lower amount of crocin which is responsible for color of stigma (Sarma et al. 1991). In the present study our objectives were to standardize the explants for microcorm production and development of stigma like structures under different cultural conditions.

## Materials and methods

### Explants

The present experiment has been carried out during 2008-09 at Biotechnology Laboratory of Central Institute of Temperate Horticulture, Rangreth, Srinagar. *Crocus sativus* corms were grown under controlled conditions in polyhouse of CITH, Srinagar Farm. The bulbs were harvested just before the onset of flowering and apical buds were removed and used for microcorm production experiment. For development of stigma, corms were harvested after sprouting and sprouts, containing floral buds, were separated from the bulbs, ovaries were separated from corm through dissection and carefully separated from the other floral parts. Explants were thoroughly washed under running tap water and sterilised by dipping in 70% ethanol for 3 min followed by sodium hypochlorite for 10 min and rinsed 4 times with sterile distilled water.

### Culture conditions

Basal media employed were (Murashige and Skoog 1962), LS (Linsmair and Skoog 1965) and G-5 Gamborg et al. 1968), each at pH 5.8 and with 0.9 or 1% agar and BA (2.22, 22.2, 4.44 and 44.4 μM) and NAA (10.8, 16.2, 21.6 and 27.0 μM) in combinations. After preparing the media explants like ovary, half ovary, style and apical bud cultured in glass tubes (90×25 mm) and flasks (150 ml).

### Microcorm formation and development of SLS

Cultures were maintained at 25±1°C under 16/8 h (light/darkness) photoperiod with a light intensity of approximately 4,000 lux. These cultures were sub-cultured every 4 weeks. Observations with respect to shoot development, microcorm formation, stigma formation etc were taken after every 6 weeks. This study was carried out as a factorial experiment based on completely randomized design (CRD) with 4 treatments, in 4 replications.

### Statistical analysis

Each treatment was replicated 4 times and observations in stages of development were recorded periodically. The data was analyzed by comparing means using one way ANOVA and the significance was determined by Duncan's Multiple Range Test using SPSS for windows (v. 15. SPSS Inc USA). Data given in percentages were subjected to arcsine ($\sqrt{X}$) transformation) [22] before statistical analysis.

## Results and discussion

### *In vitro* microcorm production

Differential response for microcorm production was observed on different explants/media combinations used in the study (Tables 1, 2 and 3). Explants like apical buds showed development of single shoots after 20 days of culture which further form well developed shoots with leggy appearance after 35 days of culture. Sub-culturing on the same medium showed regeneration of one or two cormlets from the main explants after 20 weeks of culture. Maximum size (1.3 g) of microcorms were obtained on LS media supplemented with 21.6 $\mu M$ NAA and 22.2 $\mu M$ BA followed by 1.2 g on MS media supplemented with 21.6 $\mu M$ NAA and 22.2 $\mu M$ BA. Our results showed further improvement over the protocols developed earlier (Ding et al. 1979; Ding et al. 1981), who first reported tissue culture of *C. sativus* using MS medium containing IAA and/or 2, 4-D. Development of microcorms on the surface of explants (1/8 corm fragments) (Homes et al. 1987) and differentiation of corms into buds (Ilahi et al. 1987) has been reported. *In vitro* microcorm production has been obtained by culturing leaf segments and apical buds of saffron (Raja et al. 2007; Sharma et al. 2008). Shoot formation was observed from ovaries and apical buds. *In vitro* development of microcorms from apical buds, corms, meristems and ovaries has been obtained by other researchers (Sharma et al. 2008; Mir et al. 2010). Microcorm formation from apical bud takes only 8 months under *in vitro* conditions as against 22 months under field conditions.

### *In vitro* development of stigma-like structures

Different response was observed with respect to medium and explant used (Tables 1, 2 and 3, Fig. 1). Highest response (60%) was observed with half ovaries on G-5 media supplemented with 27 $\mu M$ NAA and 44.4 $\mu M$ BA followed by 55% on LS media with 27 $\mu M$ NAA and

**Table 1** Morphogenetic response of different explants cultured on MS-medium supplemented with 3% sucrose and NAA and BA in different combinations

| Medium: MS Hormone conc. ($\mu M$) | | Explant type | Response (%) | Nature of response |
|---|---|---|---|---|
| NAA | BA | | | |
| 27.0 | 44.4 | Ovary | 15 ($22.8^{c}\pm0.33$) | Profuse callus with occasional shoots. |
| | | Half ovary | 45($42.1^{e}\pm0.71$) | 10–12 SLS regenerated (3–3.5 cm long) directly and 90–100 indirectly. |
| | | Style | 3($9.9^{a}\pm0.70$) | Enlargement of explants |
| | | Apical bud | 18($25.1^{c}\pm0.61$) | Microcorm formation (<1 g in weight) and shoot formation |
| 21.6 | 22.2 | Ovary | 18($25.1^{c}\pm0.53$) | Shoot primordia with scanty callus |
| | | Half ovary | 44($41.5^{e}\pm1.25$) | 100 SLS regenerated indirectly and 6–9 directly |
| | | Style | 2($8.0^{a}\pm0.87$) | Enlargement of explants |
| | | Apical bud | 16($23.6^{c}\pm0.32$) | Microcorm formation (1–1.2 g in weight) and shoot formation |
| 16.2 | 4.4 | Ovary | 15($22.8^{c}\pm0.66$) | Cluster of shoots and elongated structures |
| | | Half ovary | 40($39.2^{d}\pm0.48$) | 5–7 SLS regenerated directly and 80–90 indirectly |
| | | Style | 2($8.0^{a}\pm0.87$) | Enlargement of explants |
| | | Apical bud | 10($18.4^{b}\pm0.78$) | Microcorm formation (0.8–1.0 g in weight) and shoot formation |
| 10.8 | 2.22 | Ovary | 16($23.5^{c}\pm0.96$) | Abnormal elongated structure with callus |
| | | Half ovary | 50($45.0^{f}\pm0.41$) | 2–4 SLS regenerated from the ovary and 40–50 indirectly |
| | | Style | 2($8.0^{a}\pm0.87$) | Enlargement of explants |
| | | Apical bud | 18($25.1^{c}\pm0.30$) | Profuse callus and shoot formation |

*Figures given in parenthesis are angular transformed values

Means followed by the same letter within the columns are not significantly different ($P=0.05$) using Duncan's multiple range test

**Table 2** Morphogenetic response of different explants cultured on LS-medium supplemented with 3% sucrose and NAA and BA in different combinations

| Medium: LS Hormone conc. ($\mu M$) | | Explant type | Response (%) | Nature of response |
|---|---|---|---|---|
| NAA | BA | | | |
| 27.0 | 44.4 | Ovary | 20($26.55^{e}\pm0.59$) | Shoot primordia (1.7±0.5 per ovary). |
| | | Half ovary | 55($47.87^{h}\pm0.71$) | 15–20 SLS regenerated (4–4.5 cm long) directly and 100 indirectly. |
| | | Style | 4($11.49^{b}\pm0.60$) | Enlargement of explants |
| | | Apical bud | 25($29.98^{f}\pm0.81$) | Microcorm formation (1 g in weight) and shoot formation |
| 21.6 | 22.2 | Ovary | 25($29.99^{f}\pm0.46$) | Shoot primordia (3.8±0.5 per ovary ) with scanty callus |
| | | Half ovary | 52($46.15^{h}\pm0.47$) | 12–15 SLS regenerated directly and 80–90 indirectly |
| | | Style | 3($9.90^{ab}\pm0.70$) | Enlargement of explants |
| | | Apical bud | 20($26.55^{e}\pm0.59$) | Microcorm formation (1–1.3 g in weight) and shoot formation |
| 16.2 | 4.4 | Ovary | 14($21.97^{d}\pm0.34$) | Cluster of shoots and elongated structures |
| | | Half ovary | 40($39.23^{g}\pm0.48$) | 10–12 SLS regenerated directly and 70–80 indirectly |
| | | Style | 2($7.99^{a}\pm0.87$) | Enlargement of explants |
| | | Apical bud | 10($18.42^{c}\pm0.39$) | Microcorm formation (<1 g in weight) and shoot formation |
| 10.8 | 2.22 | Ovary | 20($26.53^{e}\pm0.88$) | Profuse callus with occasional shoots |
| | | Half ovary | 53($46.72^{h}\pm1.07$) | 8–10 SLS regenerated directly and 50–60 indirectly. |
| | | Style | 2($7.99^{a}\pm0.87$) | Enlargement of explants |
| | | Apical bud | 18($25.09^{e}\pm0.53$) | Profuse callus and microcorm formation (0.5–0.8 g in weight) |

*Figures given in parenthesis are angular transformed values

Means followed by the same letter within the columns are not significantly different ($P=0.05$) using Duncan's multiple range test

**Table 3** Morphogenetic response of different explants cultured on G-5-medium supplemented with 3% sucrose and NAA and BA in different combinations

| Medium: G-5 Hormone conc. ($\mu M$) | | Explant type | Response (%) | Nature of response |
|---|---|---|---|---|
| NAA | BA | | | |
| 27.0 | 44.4 | Ovary | 22 ($27.96^{d}\pm0.57$) | Shoot primordia (3.2±0.8 per ovary). |
| | | Half ovary | 60($50.78^{h}\pm0.96$) | 18–20 SLS regenerated (5–5.2 cm long) directly and 100–120 indirectly. |
| | | Style | 4($11.49^{a}\pm0.60$) | Enlargement of explants |
| | | Apical bud | 25($29.95^{d}\pm1.20$) | Microcorm formation (1–1.5 g in weight) and shoot formation |
| 21.6 | 22.2 | Ovary | 25($29.99^{d}\pm0.47$) | Shoot primordia (4.2±1.0 per ovary) |
| | | Half ovary | 50($45.00^{g}\pm0.47$) | 15–18 SLS regenerated directly and 100–110 indirectly |
| | | Style | 4($11.38^{a}\pm1.13$) | Enlargement of explants |
| | | Apical bud | 25($29.99^{d}\pm0.54$) | Microcorm formation (1–1.2 g in weight) |
| 16.2 | 4.4 | Ovary | 25($29.95^{d}\pm1.20$) | Shoots with callus |
| | | Half ovary | 35($36.27^{e}\pm0.43$) | 12–15 SLS regenerated directly and 90–100 indirectly |
| | | Style | 4($11.35^{a}\pm1.24$) | Enlargement of explants |
| | | Apical bud | 12($20.21^{c}\pm0.98$) | Microcorm formation (<1 g in weight) and shoot formation |
| 10.8 | 2.22 | Ovary | 15($22.76^{c}\pm0.66$) | Profuse callus with occasional shoots |
| | | Half ovary | 45($42.13^{f}\pm0.24$) | 6–8 SLS regenerated directly and 80–95 directly |
| | | Style | 3($9.65^{a}\pm1.48$) | Enlargement of explants |
| | | Apical bud | 8($16.31^{b}\pm1.20$) | Microcorm formation (0.2–0.6 g in weight) |

*Figures given in parenthesis are angular transformed values

Means followed by the same letter within the columns are not significantly different ($P$=0.05) using Duncan's multiple range test

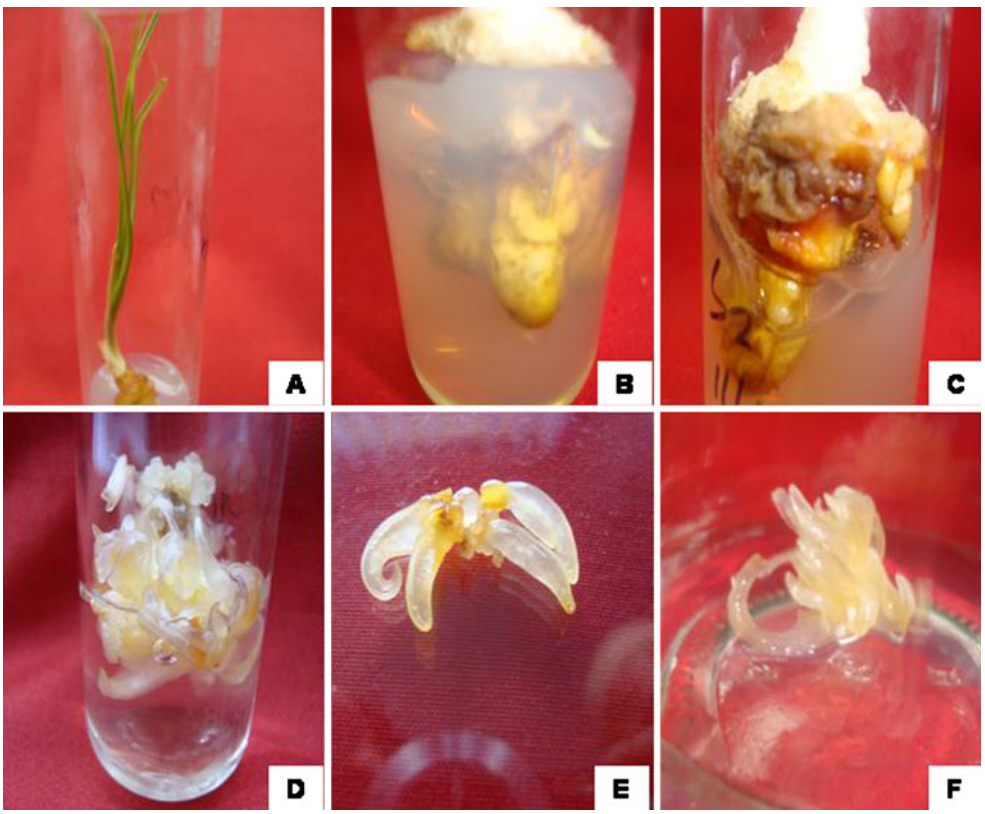


**Fig. 1** *In vitro* development of microcorm and SLS in saffron (*Crocus sativus* L.). **a** Shoot formation from apical bud. **b** & **c** Microcorm formation from apical bud, **d** Indirect SLS development from half ovary, **e** & **f**) Direct SLS development from half ovary

44.4 $\mu M$ BA (Table 2 and 3). Explants used in this study showed differential response on MS, LS and G-5 media with different concentrations of BA and NAA. Style when used as an explant form undifferentiated callus and showed undifferentiated enlargement of explants, *In vitro* flowering from styles of saffron have been reported (Jun et al. 2007), ovaries gave rise to shoot primordia and some times form shoots, direct shoot regeneration from ovary explants has also been reported (Bhagyalakshmi 1999), apical buds gave rise to shoots with leggy appearance and also produce micocorms. Half ovaries proliferated into direct-type SLS which had no intermediate callus phase and indirect-type SLS in which meristematic callus with somatic embryo like structure was an intermediate phase which later formed SLS. The new structures resembled each other in their shapes and tone of color, although there were two types, direct and indirect. The direct formation of stigma-like structures from the explant occurred early but the number of structures was few (10–20), while indirect ones were large in number (40–120) but showed late appearance. Maximum number (120 indirectly and 20 directly) with 5.2 cm long of SLS were obtained in G-5 medium supplemented with 27 $\mu M$ NAA and 44.4 $\mu M$ BA (Table 3) followed by 100 indirectly and 20 directly with 4.5 cm long SLS on LS medium supplemented with 27 $\mu M$ NAA and 44.4 $\mu M$ BA (Table 2). Development of stigma like structures from half ovaries on white medium supplemented with zeatin and NAA and half ovaries on different media using auxins has been reported (Fakhrai and Evans 1990; Himeno and Sano 1987).

During this study the explants for the microcorm production and SLS have been standardized. The frequency of *in vitro* stigma-like structure development was very low, but the observations reported here will be useful as the base to make a possible industrial production method of the spice "saffron".

## References


Ascough GD, Erwin JE, Staden J (2009) Micropropagation of *iridaceae*-a review. Plant Cell Tissu Org Cult 97:1–19

Bhagyalakshmi N (1999) Factors influencing direct shoot regeneration from ovary explants of saffron. Plant Cell Tissu Org Cult 58:205–211

Darvishi E, Zarghami R, Mishani CA, Omidi M (2007) Effects of different hormone treatments on non-embryogenic and embryogenic callus induction and time-term enzyme treatments on number and viability of isolated protoplasts in saffron. (*Crocus sativus* L.). Acta Hortic 739:279–284

Deo B (2003) Growing Saffron—The World's Most Expensive Spice, Crop & Food Research (New Zealand Institute for Crop & Food Research), no. 20, http://www.crop.cri.nz/home/productsservices/publications/broadsheets/020Saffron.pdf

Ding B, Bai SH, Wu Y, Wang BK (1979) Preliminary report on tissue culture of corms of *Crocus sativus*. Acta Bot Sin 21:387

Ding B, Bai SH, Wu Y, Fang XP (1981) Induction of callus and regeneration of plantlets from corms of *Crocus sativus* L. Acta Bot Sin 23:419–420

Ebrahimzadeh RT, Karamian R (2000) *In vitro* production of floral buds in stigma-like structures on floral organs of *Crocus sativus* L. Pak J Bot 32:141–150

Fakhrai F, Evans PK (1990) Morphogenic potential of cultured floral explants of *crocus sativus* L. for the *in vitro* production of saffron. J Exp Bot 41:47–52

Gamborg O, Miller R, Ojima K (1968) Nutrient requirement suspensions cultures of soybean root cells. Exp Cell Res 50:151–158

Himeno H, Sano K (1987) Synthesis of crocin, picrocrocin and safranal by stigma-like structures proliferated *in vitro*. Agric Biol Chem 51:395–400

Homes J, Legros M, Jaziri M (1987) *In vitro* multiplication of *C. sativus* L. Acta Hortic 212:675–676

Ilahi I, Jabeen M, Firdous N (1987) Morphogenesis with saffron tissue cultures. J Plant Physiol 128:227–232

Jun Z, Xiaobin C, Fang C (2007) Factors influencing *in vitro* flowering from styles of saffron. Acta Hortic 739:313–320

Karaoglu C, Cocu S, Ipek A, Parmaksiz I, Sarihan E, Uranbey S, Arslan N, Kaya MD, Sancak C, Ozcan S, Gurbuz B, Mirici S, Khawar KM (2007) *In vitro* micropropagation of saffron. Acta Hortic 739:223–228

Linsmair EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–127

Mir JI, Ahmed N, Rashid R, Wani SH, Itoo H, Sheikh MA (2010) Corm multiplication and development of stigma like structures under *In vitro* conditions in saffron. In *: Proceedings of National Symposium on Botanical Researches*—Present Scenario, 18–19, Febrauary 2010. Department of Botany, Punjabi University, Patiala, pp. 143

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nehvi FA, Agarwal SG, Mir MA, Dar SA, Mir ZA, Nabi N (2005) Quality drying of Saffron. SKUAST J Res 7(2):343–346

Raja W, Zaffer G, Wani SA (2007) *In vitro* microcorm formation in saffron (*Crocus sativus* L.). Acta Hortic 739:291–296

Sano K, Himeno H (1987) *In vitro* proliferation of saffron *(Crocus sativus* L.) stigma. Plant Cell Tissu Org Cult 11:159–166

Sarma KS, Maesato K, Hara T, Sonoda Y (1990) *In vitro* production of stigma-like structures from stigma explants of *Crocus sativus* L. J Exp Bot 41:745–748

Sarma KS, Sharada K, Maesato K, Hara T, Sonoda Y (1991) Chemical and sensory analysis of saffron produced through tissue cultures of *Crocus sativus*. Plant Cell Tissu Org Cult 26:11–16

Sharma KD, Rathour R, Sharma R, Goel S, Sharma TR, Singh BM (2008) *In vitro* cormlet development in *Crocus sativus*. Biol Plant 52:709–712

Sheibani M, Nemati SH, Davarinejad GH, Azghandi AV, Habashi AA (2007) Induction of somatic embryogenesis in saffron using thidiazuron (TDZ). Acta Hortic 739:259–268