Functional Plant Science and Biotechnology ©2010 Global Science Books

Saffron (*Crocus sativus* L.) Tissue Culture: Micropropagation and Secondary Metabolite Production

Kamal Dev Sharma[1*] • Abel Piqueras[2]**

[1] Department of Agricultural Biotechnology, CSK Himachal Pradesh Agricultural University, Palampur 176 062 (HP) India
[2] Department of Plant Breeding, CEBAS (CSIC), PO Box 164 30100 Espinardo, Murcia, Spain

Corresponding author: * **kml1967@rediffmail.com, kamaldev2002@yahoo.co.in** ** **piqueras@cebas.csic.es**

ABSTRACT

Saffron (*Crocus sativus* L.) is used as a spice, a dye and as a traditional medicine. It is a sterile geophyte and is propagated vegetatively through daughter corms. To meet the steady increase in worldwide demand of saffron, there is a need to expand area under its cultivation, however, limited availability of daughter corms is one of the major handicaps for the expansion of acreage under saffron. Alternatively, micropropagation of saffron using direct or indirect shoot induction or plantlet regeneration through somatic embryogenesis followed by microcorm production offers the capability to produce large quantities of propagating material in short duration of time, however, the protocols available so far need refinement for their commercial utilization. Alternatively, the spice saffron or its chemical constituents viz., crocin, picrocrocin, crocetin and safranal can be produced in tissue cultures. The structures similar to saffron stigmas called as stigma-like structures (SLS) have been generated *in vitro*. Tissue culture-derived SLS have a chemical composition and physical structure similar to natural stigmas; however, lack of their continuous production in tissue cultures is a major bottleneck to exploit this technology at commercial scale. The cell cultures of saffron also synthesize chemical constituents of stigma albeit at lower concentration. Among the four chemicals, production of crocin in cell cultures has been the main focus of research primarily because this chemical is implicated to have anticancer properties. Appropriate concentrations of growth regulators, media components, heavy metals, and two-stage culture system are some of the factors which offer potential to increase production of crocin in cell cultures.

Keywords: cell culture, microcorms, organogenesis, somatic embryogenesis, stigma-like structures, tissue-cultured stigmas

CONTENTS

INTRODUCTION

Saffron is the most expensive spice in the world. This spice is made from the dried stigmas of *Crocus sativus* L. which is a sterile triploid geophyte (2n = 3x = 24). *C. sativus* is propagated vegetatively by means of corms (Fernández 2004). The low rate of production of daughter corms limits the availability of propagating material in saffron. The daughter corms usually take three to four years to mature and give rise to the next progeny of daughter corms. Saffron plants worldwide are considered to be genetically uniform. Because of its narrow genetic base and autotriploid nature, saffron improvement by breeding is very difficult; however, large-sized corms produce more flowers and larger daughter corms (Agayev *et al*. 2009). Thus, there is a possibility of selection of superior clones for higher stigma yield. The multiplication of superior clones in adequate quantities for planting in larger areas is difficult as limited daughter corms are produced by mother corms. Alternatively, daughter corms or plantlets can be propagated rapidly through clonal propagation (Aguero and Tizio 1994; Sharma *et al*. 2008) or embryogenesis in callus cultures followed by generation of shoots/plantlets and daughter corms (Blazquez *et al*. 2004a).

Commercial value of saffron is due to its stigmas which are used as a spice. It is one of the most valuable spice and is recognized for its unique colour, aroma, taste and medicinal properties. The chemicals responsible for spicy properties of saffron are crocin, picrocrocin, crocetin and safranal. These chemicals, especially crocin, also have anticancer properties (Abdullaev 2004). Due to low productivity of saffron (4.6 kg/ha in Iran), limited cultivation in a few countries of the world and decrease in saffron production in all countries of the world in more recent years (Fernandez 2007), the possibilities of increasing saffron production seems to be remote. Another factor that contributes to high-

Received: 1 October, 2009. Accepted: 22 May, 2010.

Invited Review

er costs of saffron is the manual harvesting of stigmas. Tissue culture offers two alternatives to saffron production *in vivo* i.e. generation of saffron stigmas in cultures and production of chemical constituents of saffron in callus or cell cultures (Loskutov *et al.* 1999; Chen *et al.* 2003). Both the techniques have the capabilities for commercialization. In this review, the term metabolite refers to metabolites of saffron stigmas namely crocin, picrocrocin, crocetin and safranal, if not mentioned otherwise.

Research of saffron tissue culture has been reviewed by Plessner and Ziv (1999). A brief account of significant developments in saffron tissue culture has also been presented by Ascough *et al*. (2009) while reviewing micropropagation of Iridaceae. In this review, we have presented an updated account of the research developments on saffron micropropagation, generation of stigma-like structures (SLS) and biosynthesis of important metabolites of saffron stigmas in cell cultures.

PLANTLET REGENERATION FROM PROTOPLASTS

There is only one study on plantlet/shoot generation from isolated protoplasts of saffron (Isa *et al*. 1990). Saffron protoplasts were isolated from calli developed from apical buds or corms (Isa *et al.* 1990; Darvishi *et al*. 2007). A cocktail of two cellulases (1% Cellulase R-10 from *Trichoderma viride* and 1% Driselase from *Irpex lactes*) supplemented with low concentrations of a pectinase, pectlyase Y-23 and 0.3 M Mannitol at pH 5.7 was used as digestion mixture that released sufficiently higher number of protoplasts from callus cultures when treated at 25°C under dark for 1-3 h. Darvishi *et al*. (2007) also used 0.1% MES (2-*N*-morpholino ethane sulfonic acid) in the digestion mixture. Callus cultures treated for 3 h yielded 40×10^5 protoplasts per ml of the suspension with 98% viability (Darvishi *et al*. 2007). After isolation, subsequent growth and division was influenced by protoplast immobilization, nurse cultures and plating density (Isa *et al*. 1990). While protoplasts which were not immobilized in Ca-alginate beads did not divide, the ones embedded in Ca-alginate formed cell clusters with or without nurse cultures. The nurse cultures improved cell division from protoplasts as considerably more number (15%) of cell colonies were formed after two months than protoplasts without nurse cultures (3% cell colonies after two months) (Isa *et al*. 1990). The optimum density of protoplasts in medium for higher frequency of colony formation was 5×10^4 protoplasts per ml. The beads upon transfer to MS medium supplemented with benzyl adenine (BA) and 1-naphthaleneacetic acid (NAA) regenerated shoots and roots i.e., plantlets. About 80% of the calli regenerated on this medium.

The same strategy was used by Karamian and Ebrahimzadeh (2001) to regenerate plantlets from another species of *Crocus* i.e. *C. cancellatus*. Embryogenic callus of *C. cancellatus* initiated from shoot meristems at 17.8 μM kinetin and 4.4 μM 2,4 dichlorophenoxy acetic acid (2,4-D) was used for protoplast culture. Immobilization of protoplasts in Ca-alginate beads followed by 'nurse culture' with 4.4 μM 2,4-D, 8.9 μM kinetin and 0.57 mM ascorbic acid in the dark led to highest growth and cell division. Cells further divided to form cell colonies and embryogenic calli that regenerated plantlets (Karamian and Ebrahimzadeh 2001).

SOMATIC EMBRYOGENESIS

Micropropagation of several plant species has been achieved by callus induction followed by somatic embryogenesis or organogenesis. Among the two modes of plantlet regeneration, embryogenesis is preferred over organogenesis primarily because of the higher frequency of regeneration. The first report in saffron on successful induction of callus and regeneration of intact plantlets was from corm explants (Ding *et al.* 1979, 1981). This was achieved by using culture media supplemented with indole-3-acetic acid (IAA) and 2,4-D. Ilahi *et al.* (1987) also produced callus from corm explants on 2,4-D-containing media followed by bud and shoot differentiation. Different kinds of explants such as corms, corm pieces, apical and axillary buds, young ovaries, young leaves and shoot apices induce callus in saffron (Isa and Ogasawara 1988; Bhagyalakshmi 1999; Blazquez *et al*. 2004a; Sharma *et al*. 2005; Sharifi *et al*. 2010). These explants dedifferentiate to either embryogenic or non-embryogenic calli or mixture of embryogenic as well as non-embryogenic calli. Unlike many crop species where 2,4-D is essential for callus induction, callus in saffron can be induced in the absence of 2,4-D from wide variety of explants such as young ovaries, young leaves, shoot apices and vegetative buds (Igarashi and Yuasa 1994; Sharma *et al*. 2005). In the absence of 2,4-D, growth regulators NAA (auxin) and BA (cytokinin) together in an appropriate ratio induce calli with high efficiency.

Induction of somatic embryogenic callus from non-embryogenic one (explant: meristematic regions of corm) was first reported by George *et al.* (1992). The non-embryogenic callus developed on 2,4-D was transferred to medium containing IAA, kinetin and ascorbic acid where somatic embryo development took place. This was followed by induction of embryogenic callus from bulblet explants in the presence of growth regulators BA and NAA (Ahuja *et al.* 1994). With the advent of somatic embryogenesis in saffron, role of 2,4-D and other growth regulators in embryogenesis became clear. 2,4-D is required for induction of embryogenic callus whereas its absence lead to the development of non-embryogenic calli (Karamian 2004; Sharma *et al*. 2005; Darvishi *et al*. 2007; Raja *et al*. 2007). 2,4-D alone, however, is not very effective in embryo induction and supplementation with kinetin or BA along with 2,4-D is essential for high frequency of induction. For example, embryogenic callus developed from shoot meristems in the presence of 4 mg L^{-1} kinetin and 1 mg L^{-1} 2,4-D (Karamian 2004) and that from shoot and leaf explants in the presence of 2,4-D and BA (Raja *et al.* 2007). In addition, jasmonic acid (Blazquez *et al.* 2004b) and thidiazuron (TDZ) (Sheibani *et al.* 2007) also improve efficiency of somatic embryogenesis. Compared to other hormones, low amounts of TDZ are required for embryo induction (0.5 mg L^{-1}) and proliferation (0.25 mg L^{-1}). Generally, whole of the callus does not convert uniformly to embryos and only some regions form embryos. These regions are called as embryo-rich regions and are selectively multiplied during subsequent subcultures to get enough embryos (Blazquez *et al.* 2009). Factors affecting maturation of embryos in saffron have not been studied in detail except that elevated levels of sucrose (6%) in hormone-free MS (Murashige and Skoog 1962) medium (Sheibani *et al.* 2007) or 1 mg L^{-1} abscisic acid lead to maturation of embryos (Karamian 2004). Mature embryos were germinated on 25 mg L^{-1} gibberellic acid, GA_3 (Karamian 2004). Upon germination, the basal parts of the embryos usually swell leading to the formation of microcorms after 3 months (Sheibani *et al.* 2007). Rapid increase in cell or callus mass is desirable to achieve sufficiently higher amounts of embryos. The use of a temporary immersion system increases fresh weight of embryogenic calli four times compared to those grown on solid media (Blazquez *et al.* 2004b).

The embryogenic calli look nodular. It takes about 6 weeks to get nodular embryogenic calli from corm tissue cultures (Blazquez *et al.* 2009). At the nodular stage, the calli contain proembryonic structures or proglobular embryos which develop to globular embryos after 3 weeks in culture, to monopolar (containing a meristem and cotyledon) after 7 weeks in culture and to bipolar embryo (consisting of an apical meristem with a cotyledon at one end and minicorm at other end) after 10 weeks in culture (**Fig. 1**, Blazquez *et al.* 2009). It takes about two weeks in culture for maturation of bipolar somatic embryos. The development of the globular structure of embryos is coupled to the concomitant development of protodermis which is the outermost layer of the developing embryo and has clinical

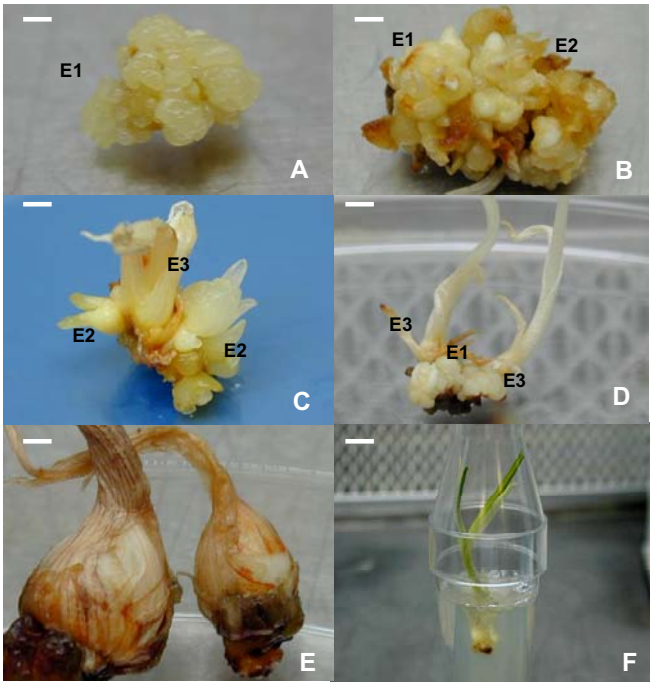

Fig. 1 Somatic embryogenesis in saffron. **A**, **B**, **C** and **D** show embryogenic calli with somatic embryos at different developmental stages (**E1**: globular, **E2**: monopolar, **E3**: dipolar). **E** presents a fully developed somatic embryo with a minicorm and **F** is a plantlet developed from an **E3** somatic embryo. The scale bar is 0.5 cm in **A**, **B**, **C** and **D** and 1 cm in **E** and **F**. (Blazquez 2004c).

divisions. The presence of this layer is considered as an evidence or characteristic feature of somatic embryo development (Von Arnold *et al.* 2002). The protodermis applies physical and cell divisional limitations and regulates further developments (Quiroz-Figueroa *et al.* 2002). The protodermis can be seen in histological sections during early stages of embryogenesis in saffron and embryogenesis follows the normal path (Blazquez *et al.* 2009) similar to other genera and species such as *Iris* (Wang *et al.* 1999), *Gladiolus* (Stefaniak 1994) and *Allium sativum* (Fereol *et al*. 2002). Apart from callus cultures, parthenocarpic fruits of saffron developed *in vitro* also develop embryos (Chichiricco and Grilli Caiola 1987), however, development stages of these embryos are not studied and hence, similarities or differences in the development of embryos in cells and parthenocarpic fruits are not known.

Like other plant species, embryogenesis in saffron is accompanied with cellular stresses (Blazquez *et al.* 2004a). Plant response to stress usually leads to accumulation of reactive oxygen species (ROS). In cell cultures, ROS such as hydrogen peroxide (H_2O_2) are generated during induction of embryogenesis and are suspected to play role in regenerative pathways in plant tissue cultures including embryo induction (Kairong *et al.* 1999; Tian *et al*. 2003). More specifically, H_2O_2 in the presence of ascorbate glutathione maintains cell wall plasticity and stimulate organized cell growth (De Gara *et al*. 1997). In several plant species, the role of ROS appears to be confined to early stages of somatic embryogenesis and these might not be required for subsequent development of the embryos. Saffron seems to be no exception. The activities of ROS, enzymes of the antioxidant system (ascorbate peroxidase, dehydroascorbic acid reductase and glutathione reductase) and catalase increase considerably during somatic embryogenesis in saffron (Blazquez *et al.* 2004a, 2009). Several members of the superoxide dismutase (SOD) family, the enzymes involved in stress tolerance in plants also play role in embryogenesis. Two Mn-SOD and four Cu, Zn-SODs are involved in embryogenesis while Mn-SODs increase during last two stages of embryogenesis and Cu, Zn-SODs decrease (Blazquez *et al*. 2009). In saffron, stress most probably the oxidative one followed by ROS appear to function as components of signal transduction chain required to reprogram gene expression and induce totipotency to gain embryogenic competence by the somatic cells (Blazquez *et al.* 2004a, 2009). The genes involved in embryogenesis in saffron are not known, however, based on small cDNA library prepared from corms, some genes with probable role in regulation of embryogenesis *in vitro* were identified (Alvarez-Orti *et al.* 2004). While constitutive genes showed similar expression patterns at all stages, developmentally regulated genes changed their expression. Expression of a xyloglucan endotransglycosylase [xyloglucan:xyloglucosyl transferase] (a cell wall loosening activity enzyme involved in cell growth), formaldehyde dehydrogenase and an abscisic stress ripening protein was strongly regulated.

The shoots/plantlets from callus cultures develop in the presence of cytokinins (BA, kinetin) either alone or in combination with auxins such as indole-3-butyric acid (IBA), NAA (Ahuja *et al.* 1994; Igarashi and Yuasa 1994; Sharma *et al.* 2005). As expected, 2,4-D inhibits shoot induction. Shoot/plantlet development was studied from both embryogenic and non-embryogenic calli and in both the cases growth regulators were used for shoot induction (from non-embryogenic calli) and plantlet development (embryogenic callus, George *et al*. 1992; Sharma *et al*. 2005). Type of nitrogen source also effects shoot regeneration from non-embryogenic calli. Nitrogen in the form of nitrate (NO_3^-) favours induction of shoots whereas the ammonical (NH_4^+) form of nitrogen has inhibitory effect (Igarashi and Yuasa 1994).

The cost of production of saffron shoots *in vitro* either through embryogenesis or organogenesis is high and alternatives are needed to lower the cost of their production. The major cost enhancing factors in shoot regeneration are labour, sucrose and agar. While the labour costs could not be reduced until the tissue culture operations are performed by using the robots, the amount of sucrose in media could be reduced by using photoautotrphic micropropagation under high light and CO_2 intensity especially after induction of shoots, and agar omitted from the media by using liquid cultures. By using cotton bed as a substitute of agar, the cost of shoot induction and development could be reduced by 33.5% (Sharma *et al.* 2005); however, the frequency of shoot regeneration was low in media devoid of agar. Probably agar altered nutrient availability and uptake by saffron cells as its type and concentration in medium is known to alter nutrient uptake and influence shoot development in tissue cultures (Beruto *et al.* 1999; Karim *et al.* 2003).

DIRECT SHOOT REGENERATION FROM DIFFERENT EXPLANTS

Somatic embryogenesis in saffron is not very efficient and plantlet generation from somatic embryos is low. These problems must be resolved before somatic embryogenesis can become a viable method for mass propagation of saffron. Direct shoot regeneration without an intervening callus pahse offers an alternative to somatic embryogenesis. The direct shoots have been generated from apical buds, lateral buds, small corms and ovaries (Plessner *et al.* 1990; Aguero and Tizio 1994; Bhagyalakshmi 1999; Sharma *et al.* 2008; see **Fig. 2** for direct shoots generated from apical buds). The direct organogenesis has the advantage of more genetic uniformity compared to adventitious regeneration from callus cultures or somatic embryogenesiss (Piqueras and Debergh 1999). Usually less time is required to generate direct shoots compared to indirect ones.

Efficacy of direct shoot regeneration in saffron depends upon the explant used, growth regulator composition and temperature of incubation. First report of direct shoot regeneration was from corms (Homes *et al*. 1987) that was followed by shoot regeneration from apical buds consisting of 2 mm sided cube of corm tissue (Plessner *et al.* 1990). Shoot induction from apical buds was promoted by cytokinins (kinetin or zeatin) and temperature of 15 or 20°C

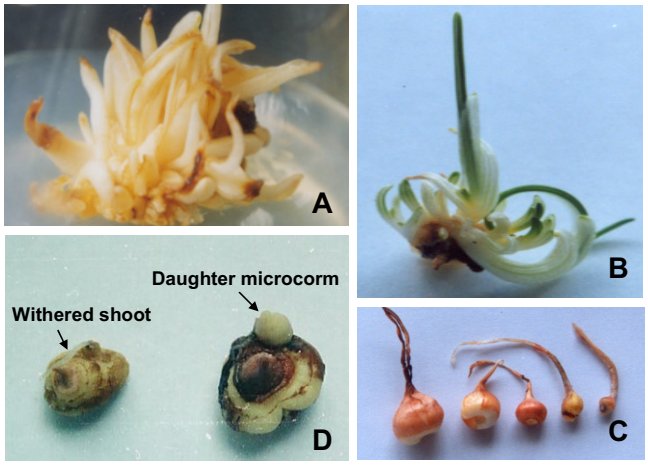

Fig. 2 Multiple shoot and microcorm generation from bud explants *in vitro* in *Crocus sativus*. (**A**) Direct multiple shoots induction; (**B**) green shoots; (**C**) mature microcorms; (**D**) microcorms bearing daughter microcorms. Microcorms first germinated (withered shoot is visible at the top of microcorm on the left hand side) followed by swelling at the base of shoots leading to daughter microcorm formation.

(Plessner *et al.* 1990). Among different cytokinins, BA is the most effective cytokinin in direct shoot induction from buds and small corms (Majourhat *et al.* 2007; Sharma *et al.* 2008). A recent study indicated that TDZ at concentrations below 10 µM is far more effective than BA in shoot induction from apical buds (Sharifi *et al.* 2010). Most characteristic feature of the shoot induction using TDZ is an intermediate nodular or embryo-like structures which lack root pole (Sharfi *et al.* 2010). Sucrose concentration is another factor that influences multiple shoot regeneration from corms as shoot induction is more at higher concentration (50 g L^{-1}) compared to lower one (30 g L^{-1}) (Sharma *et al.* 2008). The effect of sucrose is not unique in saffron as sugar concentrations are known to affect shoot and root regeneration in many plant species (Radhika *et al.* 2006; Soniya and Sujatha 2006; Novotna *et al.* 2007).

As for buds and corms, BA is also the best growth regulator for shoot induction from ovaries, however, shoot induction from ovaries is favoured by lower concentrations of BA compared to those required for direct shoot regeneration from corms or buds. At concentrations comparable to those used for shoot induction from buds or corms, it induced either calli or abnormal shoots from ovaries. The optimum concentration of BA for shoot induction from ovaries is 4.4 µM along with higher NAA (26.9 µM) concentration (Bhagyalakshmi 1999). Apart from growth regulators, growth stages of ovaries also influence shoot induction and ovaries with yellow stigma are most amenable (Bhagyalakshmi 1999).

Saffron ovaries have been used to generate calli, shoots and stigma-like structures (SLS) and ratio of BA to NAA seems to be the most critical factor leading to organogenesis or dedifferentiation. Absence or low levels of IBA or NAA along with BA lead to direct shoot induction from ovaries as well as other explants (see above; Darvishi *et al.* 2007; Sharma *et al.* 2008) whereas low concentrations of NAA (5.4 µM) and higher ones of BA (44. 4 µM) induce SLS from half ovaries (Loskutov *et al.* 1999, **Table 1**). A 1:2 ratio of these growth regulators (BA 5 mg L^{-1} and NAA 10 mg L^{-1}) supports callus induction (Castellar and Iborra 1997). Interactions among growth regulators, however, appear to be far more complex as equal ratio of both NAA (1 mg L^{-1}) and BA (1 mg L^{-1}) also differentiated ovaries into SLS (Castellar and Iborra 1997; Loskutov *et al*. 1999).

Temperature and light influence direct shoot induction from explants. Usually temperature around 20°C is best for shoot induction and development; however, 25°C also supported induction and development of shoots (Plessner *et al.* 1990; Bhagyalakshmi 1999). While the effect of light on shoot induction form buds/corms has not been studied, dark conditions enhance shoot bud induction from ovaries (Bhagyalakshmi 1999). Like embryogenesis, the changes in activities of antioxidative enzymes such as peroxidase, catalase, SOD, esterase and polyphenoloxidase were also noticed during shoot induction (Sharifi and Ebrahimzedah 2010). It appears that antioxidant enzymes play a major role in the process of organogenesis and embryogenesis in saffron.

Efficient micropropagation of saffron can not be achieved unless tissue culture derived shoots are multiplied *in vitro*. There is only one study (Sharma *et al.* 2008) on this aspect where individual *in vitro* derived shoots developed multiple shoots at low frequency. Maximum number of shoots (4.0) per cultured shoot induced at 14 mg L^{-1} BA + 3 mg L^{-1} IBA + 50 g L^{-1} sucrose was, however low.

IN VITRO MICROCORM PRODUCTION

Natural way of propagation of saffron is through cormels called as cormlets which develop on the mother corms. The cormlets grow in size while in soil and after attaining optimum size (three to four years) give rise to next generation of cormlets. During shoot/plantlet development experiments, it has been found that *in vitro* developed shoots have tendency to swell at the base followed by formation of the small cormlet also called as microcorm (**Fig. 2**, Gui *et al.* 1988; Plessner *et al.* 1990; Plessner and Ziv 1999; Sharma *et al.* 2008). The microcorms *in vitro* can be produced in large numbers in short duration of time and are considered ideal for saffron micropropagation. Being smaller in size, these are easy to transport and store under low temperature conditions and can be used for germplasm storage. However, generation of large number of microcorms for commercial use in saffron is elusive so far. The induction of microcorms from directly generated shoots was achieved for the first time by Plessner *et al.* (1990). The corms with adventitious shoots when rooted in medium without growth regulators also give rise to microcorms. Given appropriate culture condition, the tissue culture derived shoots form microcorms

Table 1 Effect of BA and NAA (mg l^{-1}) for callogenesis and organogenesis from ovaries, apical buds and corms of saffron.

Explant	BA	NAA	Organ induced	Reference
Ovary	5.0	10.0	callus	Castellar and Iborra 1997
Ovary	5.0	4.0	Shoots	Bhagyalakshmi 1999
Ovary	1.0	5.0	Shoot development	Bhagyalakshmi 1999
Ovary	1.0	10.0	SLS	Sarma *et al.* 1991
Ovary	1.0	1.0	SLS	Castellar and Iborra 1997
Half Ovary	10.0	1.0	SLS	Loskutov *et al*. 1999
Apical bud[a]	6.0	0	Shoots	Sharma *et al.* 2008
Corms	6.0	0	Shoots	Sharma *et al.* 2008
Apical bud	2.0	2.0	Non-embryogenic callus	Darvishi *et al.* 2007
Apical bud	1.0	[b]	Embryogenic callus	Darvishi *et al.* 2007
Apical bud	0	[c]	Callus and shoots	Sharifi *et al*. 2010

[a]Also called as apical meristem and consists of an apical bud along with about 2 mm or larger sided cube of corm tissue.
[b]1.0 mgl^{-1} 2,4-D
[c]1.0 mgl^{-1} TDZ

irrespective of the explant used to generate shoots. The shoots derived from ovaries, floral and corm segments as well as plantlets developed from somatic embryos from leaf explants form microcorms (Bhagyalakshmi *et al.* 1999; Karaoglu *et al.* 2007; Raja *et al.* 2007).

Ethylene treatment, microsurgery of the apical bud, concentration of media salts, growth regulators and growth retardants such as paclobutrazol and imazalil favour microcorm induction (Plessner *et al.* 1990; Piqueras *et al.* 1999; Sharma *et al.* 2008). The process of induction and development of microcorms is an energy requiring process and deposition of biomass at the base of the shoot is affected by sucrose concentration (Sharma *et al.* 2008). In saffron as well as in other crop species (Chow *et al.* 1992; Madubanya *et al.* 2006), sucrose/carbohydrate concentration is the most critical factor for cormogenesis and higher sucrose concentrations (6-9%) favoured microcorm induction and development (Aguero and Tizio 1994; Raja *et al.* 2007; Sharma *et al.* 2008). Sucrose, however, does not have the same affect on storage organ formation in all the crops. It affects number and size of storage organs differently in different crops, e.g. increase in sucrose concentration from 3-9% decreases microcorm induction frequency but increases corm mass in *Watsonia vanderspuyiae* (Ascough *et al.* 2008), in *Lachenalia* increase from 3 to 6% does not improve bulblet formation but does increase size (Slabbert and Niederwieser 1999). Saffron does not falls in both of these categories as higher concentrations of sucrose increase both the microcorm induction as well as their average mass. Sucrose is essential for microcorm induction as media devoid of sucrose form no microcorms whereas frequency of induction deceases considerably (0.29 per shoot) when mannitol (1.89 per shoot on medium containing comparable sucrose), a sugar alcohol that is not metabolized by plant tissue, is used as sole carbon source (Sharma *et al.* 2008). Sucrose is considered by many as an osmolyte that increases stress leading to storage organ induction, however, in saffron role of sucrose appears be more than osmolarity. No microcorm formation in the absence of sucrose, very little in the presence of mannitol, point that sucrose might be providing energy for corm induction and growth. Like saffron, addition of mannitol or sorbitol at concentrations comparable to sucrose in *Narcissus* 'St. Keverne and Hawera' does not stimulate bulblet formation (Staikidou *et al.* 2005). The type of carbohydrate source may also affect storage organ induction e.g. in *Hyacinth*, fructose is more effective than glucose or sucrose in bulblet induction (Bach *et al.* 1992). However, sucrose and mannitol are the only carbohydrates tested so far for microcorm induction in saffron and effect of other carbohydrate sources is not studied.

Lower salt concentrations e.g. half strength of MS salts improves microcorm induction and development compared to full strength of salts (Raja *et al.* 2007; Sharma *et al.* 2008). Growth regulators IBA, BA, NAA and abscisic acid (ABA) affect microcorm induction with 3 mg L^{-1} BA in half-strength MS medium best for induction and development with formation of as many as 1.89 microcorms (1.18 g average weight) per shoot (Sharma *et al.* 2008). BA alone or in combination with other growth regulators is the most important growth regulator for cormogenesis (Piqueras *et al.* 1999; Sharma *et al.* 2008). BA induces storage organ formation in many crops such as *Gladiolus* (Steinitz *et al.* 1991; Ziv 1992), and *Crinum* (Slabbert *et al.* 1993). Fundamental differences appear to exist in different plant species for transmission of signals for induction of storage organs. In some crops, storage organ formation is induced by BA and anti-gibberellin compounds such as paclobutrazol whereas GA_3 inhibit their induction. In other crops like *Watsonia vanderspuyiae* (Ascough *et al.* 2008) and *Dierama luteoalbidum* (Madubanya *et al.* 2006), GA_3 increased cormogenesis. The role of GA_3 in microcorm inhibition or induction in saffron is not evaluated, and at the same time, role of growth retardants in corm induction is not established conclusively; though, there is an indication that paclobutrazol and imazalil increase growth of microcorms (Piqueras *et al.* 1999). Thus, saffron appears to fall in the first category where GA_3 inhibits cormogenesis. ABA which is associated with senescence inhibits corm induction and development as well as fructification leading to development of parthenocarpic fruits in saffron (Chichiricco and Caiola 1987; Sharma *et al*. 2008). The inhibitory effect of ABA on corm induction has also been reported in *Watsonia vanderspuyiae* (Ascough *et al.* 2008).

The type of explant also appears to affect cormogenesis. While individual shoots show poor cormogenic response, bunches of two to three shoots develop more number of microcorms having higher average weight (Sharma *et al.* 2008). The period of harvest of corms for explant isolation and season of culture can affect corm development as the seasonal developmental cycles typical for saffron in a natural environment do not change during *in vitro* culture (Milyaeva *et al.* 1995). The optimum temperature for corm development *in vivo* under phytotron conditions is 17°C day/ 12°C night with 27°C day/22°C night the highest for corm development (Plessner 1989). A wide variety of temperatures (10 to 25°C) were tested for microcorm induction *in vitro* (Plessner *et al.* 1990; Milyaeva *et al.* 1995; Bhagyalakshmi 1999; Sharma *et al.* 2008) and 20°C is the optimum for cormogenesis *in vitro*. Synergistic interactions between temperature and growth regulators for corm induction may also occur as has been observed in *Watsonia vanderspuyiae* (Ascough *et al.* 2008). The role of light in corm formation in saffron is also not clear as partial or continuous light has been used to induce corms from shoots (Plessner *et al.* 1990; Milyaeva *et al.* 1995; Sharma *et al.* 2008). Light affects storage organ formation differently in different crops. In some species, storage organ induction is inhibited by continuous dark e.g. *Lilium* (Lian *et al.* 2003), *Fritillaria* (Paek and Murthy 2002), *Watsonia vanderspuyiae* (Ascough *et al.* 2008), whereas in others it is either favoured by dark e.g. *Narcissus* (Steinitz and Yahel 1982) or not influenced by light (16 h photoperiod) or continuous dark e.g. *Hyacinthus* (Kim *et al.* 1981), *Lachenalia* (Slabbert and Niederwieser 1999). In saffron, information on the effect of both temperature and light on microcorm formation is limited and more research is needed to establish optimum temperature, light and interactions among temperature, light and growth regulators, if any for cormogenesis.

One of the recent protocols for microcorm induction from shoots (Sharma *et al.* 2008) is outlined in **Fig. 2**. Using this protocol, it took nine months to develop microcorms (1.89 microcorms per shoot, 1.18 g average weight) from culture of buds. The microcorms were comparable in shape and size to daughter corms obtained under field conditions (1.2 g average weight) after 22 months (Chahota *et al.* 2003). The majority (95.6%) of microcorms sprouted *in vitro* on MS medium supplemented with growth regulators and two of the cold pretreated (4°C for 4 months) microcorms (out of 10) also developed daughter microcorms. Sprouting and daughter microcorm differentiation was similar to daughter corm production under field conditions. The performance of these microcorms, however, was not evaluated under field conditions. It has been established that larger corms give rise to larger and more number of cormlets *in vivo* that bear more number of flowers (Gresta *et al.* 2008; Agayev *et al.* 2009). Larger corms also bear more flowers and yield more (Cavusoglu *et al*. 2009). Since microcorms vary considerably in size, it would be interesting to see if there exist differences in performance of larger and smaller microcorms under field conditions.

PRODUCTION OF CROCIN, CROCETIN, PICROCROCIN AND OTHER METABOLITES *IN VITRO* BY CALLUS/CELL CULTURES AND STIGMA-LIKE STRUCTURES

Saffron, the dried red stigmas of *C. sativus* L., being used as colouring and flavouring agent and as medicine are in huge demand, but low productivity and decline in area under crop limits availability. Being a labour intensive enterprise

and low average yield, the cost of natural stigmas is too high. Alternative economical ways to produce saffron include tissue culture methods (Loskutov *et al.* 1999). The approach being followed to accomplish this is the generation of saffron (stigmas) in tissue cultures (Loskutov *et al.* 1999; Zeng *et al.* 2003). Production of crocin, crocetin, picrocrocin and safranal, the important chemical constituents of spice saffron in calli or cell suspension cultures without induction of stigmas is another method that can be exploited by the industry. In other plants, colouring agents such as shikonin are being produced commercially in somatic cell cultures (Fujita *et al*. 1982). The tissue culture methods, if exploited, can reduce dependence on natural stigmas and may lower the cost of this spice.

Callus/cell cultures

Induction of secondary metabolites in callus or cell cultures of some plant species has been studied extensively; however, limited information on this aspect is available in saffron. Initial studies indicated that unorganized cells of saffron produced colouring agents like crocin or crocetin, albeit at very low concentrations compared to those produced in the natural or *in vitro* generated stigmas (Hori *et al*. 1988; Visvanath *et al.* 1990; Dufresne *et al.* 1997; Zeng *et al.* 2003). The crocin content in saffron callus was only 0.24% compared to 14.30% in the natural stigmas and up to 6.0% in SLS (Zeng *et al.* 2003). In addition to low amounts of secondary metabolites, saffron callus cultures also suffer from browning during culture (Visvanath *et al.* 1990). The brown parts of the callus are dead/dying cells with no capability to divide or produce secondary metabolites. Initial studies used one-stage culture system for metabolite production. In other plant taxa where cell cultures are used for secondary metabolite production, one-stage culture system is not very efficient because conditions for division and growth of cells usually differ from those for secondary metabolite production. The saffron is no exception as cell growth and crocin formation are separate events (Chen *et al.* 2003) and efficacy of crocin production was considerably more when two-stage culture system was used. In two-stage culture system for saffron, NAA (2.0 mg L^{-1}) and BAP (1.0 mg L^{-1}) in B5 medium (Gamborg *et al*. 1968) supplemented with casein hydrolysate (300 mg L^{-1}) yield maximum biomass whereas IAA (2.0 mg L^{-1}) and BAP (0.5 mg L^{-1}) produce maximum crocin. Using two-stage culture system, crocin production was 43 mg L^{-1} (6.32 mg g^{-1}) i.e. 295% of that of the one-stage culture system. As expected, one of the major factor contributing to higher crocin production in two-stage culture system is the high biomass yield (6.8 g L^{-1}). Unlike SLS, where light and 25°C enhance synthesis of secondary metabolites of saffron, darkness and 22°C is optimum for production of crocin from cell cultures.

Precursors enhance metabolite production in cell cultures considerably, however, impact of precursor feeding on production of secondary metabolites of saffron is not known. Two rare earth elements, La^{3+} and Ce^{3+}, enhance cell growth and crocin production in saffron (Chen *et al.* 2004). While La^{3+} promotes growth of cells with little impact on crocin production, the Ce^{3+} increases production of crocin with little effect on cell growth. Surprisingly, La^{3+} and Ce^{3+} together in the medium increase both the cell biomass (1.7-fold, 20.4 g l^{-1}) as well as crocin production (4.2-fold, 4.4 mg g^{-1}, 90 mg l^{-1}). Though, exact mechanism of action of rare earth elements is not clear, these are expected to react with certain enzymes in the cells and on the membrane and modify enzyme function and cell permeability. This can lead to increased uptake and utilization of nutrients (Guo 1999; Hong *et al.* 1999) and fast growth of saffron cells. The mode of action of these elements might be similar to that of heavy metal ions which are also known to induce production of secondary metabolites at lower concentrations.

Though, limited studies have been conducted so far on crocin production in cell cultures, these might provide leads to the development of bioreactor based systems for commercial production of saffron metabolites in cell suspensions. Further experimentation is needed to standardize factors affecting cell growth and metabolite production in bioreactors. Some of these factors are, heavy metals, precursor chemicals, growth regulators, casein hydrolysae, light and temperature. The studies during the last a few years are only on crocin production, primarily because demand for crocin is expected to rise in near future as this compound has anticancer properties (Escribano *et al.* 1996). Moreover, crocin is synthesized from crocetin, another metabolite of saffron by enzyme glucosyltransferase, and bioconversion of crocetin to crocin is not successful (Dufresne *et al.* 1999).

Saffron corms also contain a proteoglycan that inhibits growth of human tumor cells. This glycoconjugate is cytotoxic against human cervical epithelioid carcinoma cells (IC_{50} = 7 mg mL^{-1}), and consists of approximately 90% carbohydrate and 10% protein. The proteoglycan was also synthesized in callus cultures developed from saffron corm (Escribano *et al.* 1999). Apart from human tumor cells, reversible cytostatic effect of the arabinogalactan protein from saffron on root growth and *in vitro* viability of plant cells has also been documented (Fernández *et al*. 2000). This seems to be the maiden report till date that describes cytotoxicity of proteins of plant origin on plant cells.

Stigma-like structures

The stigmas generated *in vitro* are called as stigma-like structures (SLS) or tissue culture stigmas (TCS). Followed by the first report on *in vitro* proliferation of SLS in *Nicotiana tabacum* (Matsuzaki *et al.* 1984), Sano and Himeno (1987) showed proliferation of young intact stigma plus ovaries, single stigmas and half ovaries of saffron into stigmas under *in vitro* conditions with half ovaries being the best explant (75 SLS per half ovary). The SLS contain crocin, crocetin, picrocrocin and safranal, all the pigments found in natural stigmas. The SLS can be generated from explants either directly or indirectly through meristematic tissue on media containing BA/kinetin and NAA. The generation of SLS in tissue cultures is common and can be achieved from wide variety of explants such as immature ovaries, half ovaries, stigmas, stigma plus ovaries, anthers, stamens and petals and on wide range of media (Himeno and Sano 1987; Sano and Himeno 1987; Namera *et al*. 1987; Fakhrai and Evans 1990; Sarma *et al.* 1990, 1991; Kohda *et al.* 1993; Loskutov *et al.* 1999; Zhao *et al.* 2001); however, frequency of generation of SLS and concentration of chemical constituents in SLS remained low (Fakhrai and Evans 1990; Sarma *et al.* 1990; Sarma *et al.* 1991; Kohda *et al.* 1993). The amount of crocin, and picrocrocin was lesser by 6 and 11 times in SLS compared to that in natural stigmas. In general, SLS were low in floral, spicy and fatty characteristics as compared to saffron obtained from flowers (Sarma *et al.* 1991). In addition to SLS, petal explants also generate petal-like structures (PLS) which develop SLS in frequencies higher than the SLS induced directly from petals (Wang *et al.* 2002).

Sustained proliferation of SLS is essential for commercialization of technology and needs attention for research. Major hurdles in sustained proliferation of SLS *in vitro* are low frequency of generation of SLS, browning of SLS, formation of non-SLS structures and single harvest. Besides sustained proliferation, low concentration of secondary metabolites, slow growth of callus tissues and short life of explants are the other problems. Browning and low rate of SLS production may be correlated as browning renders the cells dead which can otherwise generate SLS. Repeated subcultures at short intervals, use of B5 medium in place of MS medium and addition of activated charcoal in medium allowed three harvests of SLS within a period of 9-10 months (Loskutov *et al.* 1999). In addition to B5 medium and growth regulators, casein hydrolysate (CH) and L-alanine improved further the induction of SLS, and these along with rapid subculture, B5 medium and activated charcoal yielded SLS with content of crocin, crocetin,

picrocrocin and safranal comparable to or even higher than that produced by naturally grown stigmas. L-alanine is a precursor of saffron metabolites (Zeng *et al.* 2003); however, role of CH in enhancing metabolite content is not known. Though, Loskutov *et al.* (1999) improved considerably the SLS production, proliferation beyond three harvests requires further research efforts.

Understanding of the physiology of stigma induction and synthesis of chemical constituents of saffron is in infancy. A vast amount of literature indicates that yields of secondary metabolites increase with addition of the precursors. Compounds like crocin and crocetin are terpenoids (Dufresne *et al.* 1999). All terpenoids originate from acetyl CoA and compounds increasing production of acetyl CoA in cells can enhance production of saffron metabolites in SLS. Based on this assumption, L-alanine, sodium acetate, glycine and serine were used as precursors to enhance crocin production in SLS (Otsuka *et al.* 1992; Zeng *et al.* 2003). These compounds in plant cells are converted to acetyl CoA, albeit using different enzymes. Glycine first converts into serine and then to acetyl CoA. Of the four precursors used, L-alanine and sodium carbonate enhance not only production of SLS but also increase content of crocin, picrocrocin and safranal (Otsuka *et al.* 1992; Zeng *et al.* 2003). The concentration of crocin in SLS (6.0%) produced in the presence of sodium acetate was two fold more compared to the SLS generated on basal medium (2.21% crocin), however, the quantity was less by half compared to natural stigma (14.30% crocin, Zeng *et al.* 2003). The amount of crocin produced in SLS also depends upon light (4.91% on basal medium under light, 2.21% under dark) and polyvinyl pyrrolidone, PVP (5.22%). Despite the effectiveness of light and PVP in enhancing accumulation of crocin, these does not increase SLS induction, however, SLS formed in the presence of PVP were morphologically more like natural stigmas. It would be interesting to see how the combination of sodium acetate, light and PVP would affect SLS induction and accumulation of different metabolites of saffron. Loskutov *et al* (1999) have generated SLS with high concentration of crocin, picrocrocin, crocetin and safranal under dark. Since light enhances crocin accumulation by two times (Zeng *et al.* 2003), it might be possible to have SLS with an increased concentration of saffron metabolites compared to natural stigmas if the method of Loskutov *et al.* (1999) is combined with incubation under continuous light conditions.

Direct or indirect mode of induction of SLS depends upon hormonal composition of the media. While low concentrations of NAA and BA induce direct SLS, high concentrations of these growth regulators induce indirect types (Loskutov *et al.* 1999; Ebrahimzadeh *et al.* 2000, 2001). In general, the direct types are more similar to natural stigmas in shape, colour and size compared to the indirect ones. Similarly, accumulation of crocin, picrocrocin and safranal in direct types is more compared to indirect types (Ebrahimzadeh *et al.* 2001). The direct types also have proportion of different forms of crocetin i.e. monoglucoside esters, diglucoside esters crocetin ester types comparable to natural stigmas.

The systematic studies on affect of temperature on induction and proliferation of SLS are not available. Since, saffron is a crop of colder regions, induction and development of SLS might be thought to be better at lower temperatures. Initial experiments on SLS were also conducted at low (20°C) temperature (Sano and Himano 1987; Sarma *et al.* 1991). Flowering in saffron requires exposure of corms at higher temperatures (25°C) for as many as 55 days (Molina *et al.* 2005). The flowering could be accelerated (up to seven days) by increasing the temperature to 30°C prior to exposure at 25°C. However, elevated temperatures for longer durations might be deleterious for bud growth and flower formation. The optimum temperature for flower formation is 23 to 27°C, 23°C slightly better than 27°C and minimum exposure time of 50 days (Molina *et al.* 2004). Does that mean that the induction and development of SLS would be better at 25°C than that at 20°C? Though, there is no information available on this aspect, the incubation temperature used in later studies on SLS was 25°C or room temperature (Loskutov *et al.* 1999; Zeng *et al.* 2003).

CONCLUSIONS

Considerable progress has been made in saffron micropropagation and SLS formation whereas studies on synthesis of metabolites in cell cultures are limited only to crocin production (**Table 2**). Somatic embryogenesis, as a mode of propagation is still not very efficient because of low rate of induction of embryos in callus/cell cultures. The knowledge on possible role of oxidative stress (Kairong *et al.* 1999; Blazquez *et al*. 2009) and some chemicals (TDZ and NO_3^- form of nitrogen) on improvement of embryogenesis may be useful to enhance embryo induction *in vitro*.

At present, direct shoot induction followed by microcorm formation is an area that holds promise for commercialization especially if genetically improved saffron corms (Agayev *et al.* 2009) are to be multiplied with in a short span of time. There are three areas of concern in this field which are required to be addressed to make this technology viable for micropropagation, i) sustained multiplication of shoots from tissue culture derived shoots, ii) development of large sized microcorms and iii) field evaluation of microcorms for agronomic performance. There is only a report on *in vitro* multiplication of tissue culture derived shoots (Sharma *et al.* 2008) and no report on evaluation of microcorms under field conditions. In addition to this, there is also a need to study effect of light and temperature on shoot induction and microcorm formation because interactions between growth regulators and temperature determine the efficacy of hormonal treatments whereas light either inhibit storage organ formation, or enhance it or has no influence (Steinitz and Yahel 1982; Slabbert and Niederweiser 1999; Lian *et al*. 2003). Physiology of corm induction in saffron is also different from genera like *Watsonia vanderspuyiae* (Ascough *et al*. 2008) and *Dierama luteoalbidum* (Madubanya *et al.* 2006) where GA_3 promotes cormogenesis. In saffron, BA is a signaling molecule for cormogenesis and antigibberellic compounds promotes corm induction. Higher cost of micropropagation in many plants acts as a main hindrance for commercialization. Some of the major contributors of higher cost are sucrose and agar. One of the alternatives to reduce micropropagation cost in saffron is to switch to photoautotrophic micropropagation (not studied so far) and use of liquid medium.

SLS (the organized tissues) are the best source for the biosinthesis of crocin, picrocrocin, crocetin and safranal by tissue culture, however, continuous production of high quality SLS *in vitro* in sufficient quantity is a challenge for commercial upgradation of this technology. If the current status of research on this aspect is an indicative, there is a possibility of surpassing the contents of crocin, picrocrocin, crocetin and safranal in SLS compared to natural stigmas. Precursors improve crocin synthesis in SLS and a rare earth element, Ce^{3+} enhances crocin production in cell cultures (Otsuka *et al.* 1992; Zeng *et al.* 2003; Chen *et al.* 2004). Using the method of Loskutov *et al.* (1999) and combining it with incubation under continuous light (Zeng *et al.* 2003), precursors and rare earth elements might lead to higher concentration of metabolites in SLS. In comparison to SLS, cell culture system for production of crocin, crocetin, picrocrocin and safranal has the advantage of upscaling by use of bioreactors. Improvisation of the current cell culture protocols based on factors like media, casein hydrolysate, precursors, heavy metals/rare earth elements, temperature, light and a two-stage culture system, all of which improve either cell biomass or crocin biosynthesis, is required to develop bioreactor-based system for commercial production of important saffron metabolites in cell cultures. The crocin, crocetin, picrocrocin and safranal in saffron stigmas are synthesized via the terpenoid pathway. Studies on gene expression in saffron (Alvarez-Ortí *et al*. 2004; Castillo *et*

Table 2 Summary of *in vitro* research in *Crocus sativus*.

Explants used	Results	References
Callus/Somatic embryogenesis		
Corm	Callus, shoots	Ding *et al.* 1979
Corm	Callus, shoots	Ilahi *et al.* 1987
Corm	Callus	Isa *et al.* 1990
Apical buds	Callus, somatic embryogenesis	George *et al.* 1992
Corm	Somatic embryogenesis	Ahuja *et al.* 1994
Ovary	Callus, shoots	Igarashi *et al.* 1994
Corm, shoot, inflorescence, anthers	Callus, somatic embryogenesis	Milyaeva *et al.* 1995
Ovary	Callus	Castellar and Iborra 1997
Apical bud[a]	Callus, somatic embryogenesis	Piqueras *et al*. 1999
Shoots, corm	Somatic embryogenesis	Blázquez *et al.* 2004a, 2004b
Callus	Somatic embryogenesis	Blázquez *et al.* 2004a, 2004b
Shoot meristem	Somatic embryogenesis	Karamian 2004
Buds	Callus, shoots	Sharma *et al.* 2005
Apical bud	Callus, protoplasts, somatic embryogenesis	Darvishi *et al.* 2007
Corm, shoots, inflorescence, ovary, flower, stigma	Shoots, somatic embryogenesis	Karaoglu *et al.* 2007
Leaf	Callus, somatic embryogenesis, shoots	Raja *et al.* 2007
Corm	Callus, somatic embryogenesis, shoots, roots	Sheibani *et al.* 2007
Apical bud	Callus	Blázquez *et al.* 2009
Protoplast culture		
Calli from corms	Protoplasts, shoots	Isa *et al.* 1990
Direct shoot regeneration/ Microcrom formation		
Corm	Shoots	Homes *et al.* 1987
Apical bud	Shoots, microcorm	Plessner *et al*. 1990
In vitro shoots	Microcorm	Aguero and Tizio 1994
Corm, shoots	Shoots, microcorm, roots	Milyaeva *et al.* 1995
Ovary	Shoots, microcorm	Bhagyalakshmi 1999
Somatic embryos	Microcorm	Piqueras *et al*. 1999
Somatic embryos	Microcorm	Raja *et al.* 2007
Shoots	Shoots	Majourhat *et al.* 2007
Somatic embryos	Microcorm	Karaoglu *et al.* 2007
Somatic embryos	Microcorm	Sheibani *et al.* 2007
Corm, apical bud	Shoots, microcorm	Sharma *et al.* 2008
Apical bud	Shoots, plantlets	Sharifi *et al*. 2010
Stigma-like structure formation		
Ovary, stigma	SLS[b]	Sano and Himeno 1987
Ovary, stigma	SLS	Himeno and Sano 1987
Ovary	Parthenocarpic fruit	Chichiricco and Grilli Caiola 1987
Flower bud	SLS	Koyama *et al*. 1988
Anthers, petal, stigma, half ovary	SLS	Fakhrai and Evans 1990
Anthers, ovary	SLS	Sarma *et al.* 1990
Anthers, ovary	SLS	Sarma *et al.* 1991
Corolla, pistil	SLS	Ostuka *et al.* 1992
Stigma, style, ovary, ovule, petal	SLS	Kohda *et al*. 1993
Ovary	SLS	Castellar and Iborra 1997
Half ovary	SLS	Loskutov *et al*. 1999
Style, perianth	SLS	Ebrahimzadeh *et al*. 2000
Stamen	SLS	Zhao *et al.* 2001
Petal	Petal-like structures, SLS	Wang *et al*. 2002
Petal, stigma, style	SLS	Zeng *et al.* 2003
Style	Flower	Jun *et al.* 2007
Metabolite synthesis in cell cultures		
Pistil	Callus	Hori *et al.* 1988
Buds	Callus	Dufresne *et al.* 1997
Petal	Callus	Zeng *et al.* 2003[c]
Corm	Callus	Chen *et al.* 2003[c]
Corm	Callus	Chen *et al.* 2004[c]

[a]Also termed apical meristem and consists an apical bud along with about 2 mm or larger sided cube of corm tissue.
[b]SLS, stigma-like structures
[c]Only crocin production studied

al. 2005) are in infancy and none of the genes involved in biosynthesis of crocin, picrocrocin, crocetin and safranal has been isolated. The knowledge of these genes in the long run may open up the possibilities of improving yield of these metabolites in transgenic cells or plants.

Scale up of saffron tissue culture technologies for commercial exploitation in near future seems to be remote unless research efforts are intensified. Increasing industrial demand for crocetin esters, limited area under this crop and reduction in average yields during the recent years is expected strengthen research on production of crocin and other important metabolites of saffron stigmas in cell cultures or in SLS so that dependence on field grown saffron is minimized. In addition to this, there is a need to exploit tissue culture techniques to generate somaclonal variants so that genetic base of this genetically uniform crop (Agayev *et al.* 2009) may be widened and better yielding clones are generated.

ACKNOWLEDGEMENTS

KDS thanks the Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi for grant to work on saffron. AP thanks the Instituto de Desarrollo Regional (Universidad de Castilla La Mancha) at Albacete, Spain and the CEBAS (CSIC) at Murcia, Spain for supporting his research on saffron.

REFERENCES

Abdullaev FI (2004) Antitumor effect of saffron (*Crocus sativus* L.): Overview and perspectives. *Acta Horticulturae* **650**, 491-499

Agayev YM, Fernández JA, Zarifi E (2009) Clonal selection of saffron (*Crocus sativus* L.): the first optimistic experimental results. *Euphytica* **169**, 81-99

Aguero C, Tizio R (1994) *In vitro* mass bulbification as a preliminary contribution to saffron (*Crocus sativus* L.). *Biocell* **18**, 55-63

Ahuja A, Kaul S, Ram G, Kaul BL (1994) Somatic embryogenesis and regeneration of plantlets in saffron, *Crocus sativus* L. *Indian Journal of Experimental Biology* **32**, 135-140

Alvarez-Orti M, Schwarzacher T, Rubio A, Blazquez S, Piqueras A, Fernández JA, Heslop-Harrison P (2004) Studies on expression of genes involved in somatic embryogenesis and storage protein accumulation in saffron crocus (*Crocus sativus* L.). *Acta Horticulturae* **650**, 155-163

Alvarez-Orti M, Gómez LG, Rubio A, Escribano J, Pardo J, Jiménez F, Fernández JA (2004) Development and gene expression in saffron corms. *Acta Horticulturae* **650**, 141-153

Ascough GD, Erwin JE, Van Staden J (2008) Reduced temperature, elevated sucrose, continuous light and gibberellic acid promote corm formation in *Watsonia vanderspuyiae*. *Plant Cell, Tissue and Organ Culture* **95**, 275-283

Bach A, Pawłowska B, Pułczynska K (1992) Utilization of soluble carbohydrates in shoot and bulb regeneration of *Hyacinthus orientalis* L. *in vitro*. *Acta Horticulturae* **325**, 487-491

Beruto M, Curir P, Debergh P (1999) Influence of agar on *in vitro* cultures: II. Biological performance of *Ranunculus* on media solidified with three different agar brands. *In Vitro Cellular and Developmental Biology – Plant* **35**, 94-101

Bhagyalakshmi N (1999) Factors influencing direct shoot regeneration from ovary explants of saffron. *Plant Cell, Tissue and Organ Culture* **58**, 205-211

Blazquez S, Olmos E, Hernández JA, Hellın E, Fernández JA, Piqueras A (2004a) Somatic embryogenesis in saffron (*Crocus sativus* L.): morphological differentiation and the role of the antioxidant enzymatic system. *Acta Horticulturae* **650**, 261-267

Blazquez S, Piqueras A, Serna M, Casas JL, Fernández JA (2004b) Somatic embryogenesis in saffron: optimisation through temporary immersion and polyamine metabolism. *Acta Horticulturae* **650**, 269-276

Blazquez S (2004c) Physiological and structural characterization of somatic embryogenesis in saffron. PhD thesis, ETSIA Albacete, University of Castilla La Mancha, Spain, 295 pp

Blazquez S, Olmos E, Hernández JA, Fernández-Garcıa N, Fernández JA, Piqueras A (2009) Somatic embryogenesis in saffron (*Crocus sativus* L.). Histological differentiation and implication of some components of the antioxidant enzymatic system *Plant Cell, Tissue and Organ Culture* **97**, 49-57

Çavusoglu A, Erkel EI, Sülüsoglu M (2009) Saffron (*Crocus sativus* L.) studies with two mother corm dimensions on yield and harvest period under greenhouse condition. *American-Eurasian Journal of Sustainable Agriculture* **3**, 126-129

Castillo R, Fernández JA, Gómez-Gómez L (2005) Implications of carotenoid biosynthetic genes in apocarotenoid formation during the stigma development of *Crocus sativus* and its closer relatives. *Plant Physiology* **139**, 674-689

Castellar MR, Iborra JL (1997) Callus induction from explants of *Crocus sativus*. *Journal of Plant Biochemistry and Biotechnology* **6**, 97-100

Chahota RK, Dhiman C, Rana SS, Singh M (2003) Efficacy of different propagating methods for higher daughter corm production in saffron (*Crocus sativus* L.). *Indian Perfumer* **47**, 155-158

Chen S, Wang X, Zhao B, Yuan X, Wang Y (2003) Production of crocin using *Crocus sativus* callus by two-stage culture System. *Biotechnology Letters* **25**, 1235-1238

Chen S, Zhao B, Wang X, Yuan X, Wang Y (2004) Promotion of the growth of *Crocus sativus* cells and the production of crocin by rare earth elements. *Biotechnology Letters* **26**, 27-30

Chichiricco G, Grilli Caiola M (1987) *In vitro* development of parthenocarpic fruits of *Crocus sativus* L. *Plant Cell, Tissue and Organ Culture* **11**, 75-78

Chow YN, Selby C, Harvey BMR (1992) Stimulation by sucrose of *Narcissus bulbil* formation *in vitro*. *Journal of Horticulture Science* **67**, 289-293

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

De Bruyn MH, Ferreira DI (1992) *In vitro* corm production of *Gladiolus dalenii* and *G. tristis*. *Plant Cell, Tissue and Organ Culture* **31**, 123-128

De Gara L, de Pinto MC, Arigoni D (1997) Ascorbate synthesis and ascorbate peroxidase activity during the early stage of wheat germination. *Physiologia Plantarum* **100**, 894-900

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

Ding BZ, Bai SH, Wu Y, Fan XP (1981) Induction of callus and regeneration of plantlets from corm of *Crocus sativus* L. *Acta Botanica Sinica* **23**, 119-120

Dufresne C, Cormier F, Dorion S (1997) *In vitro* formation of crocetin glucosyl esters by *Crocus sativus* L. callus extract. *Planta Medica* **63**, 150-153

Dufresne C, Cormier F, Dorion S, Niggli UA (1999) Glycoslation of encapsulated crocetin by a *Crocus sativus* L. cell culture. *Enzyme and Microbial Technology* **24**, 453-462

Ebrahimzadeh H, Radjabian T, Karamian R (2000) *In vitro* production of floral buds in stigma-like structures on floral organs of *Crocus sativus* L. *Pakistan journal of Botany* **32**, 141-150

Ebrahimzadeh H, Karamian R, Nouri Delouei MR (2001) Comparison of pigments from *in vitro* stigma-like structures and authentic stigmata in saffron (*Crocus sativus* L.). *Pajouhesh-va-Sazandegi Winter* **13**, 52-55

Escribano J, Alonso GL, Coca-Prados M, Fernández JA (1996) Crocin, safranal and picroerocin from saffron (*Crocus sativus* L.) inhibit the growth of human cancer cells *in vitro*. *Cancer Letters* **100**, 23-30

Escribano J, Piqueras A, Medina J, Rubio A, Alvarez-Orti M, Fernández JA (1999) Production of a cytotoxic proteoglycan using callus culture of saffron corms (*Crocus sativus* L.). *Journal of Biotechnology* **73**, 53-59

Fakhrai F, Evans PK (1990) Morphogenetical potential of cultured floral explants of *Crocus sativus* L. for the *in vitro* production of saffron. *Journal of Experimental Botany* **41**, 47-52

Fereol L, Chovelon V, Causse S, Michaux-Ferriere N, Kahane R (2002) Evidence of a somatic embryogenesis process for plant regeneration in garlic (*Allium sativum* L.). *Plant Cell Reports* **21**, 197-203

Fernández JA, Escribano J, Piqueras A, Medina J (2000) A glycoconjugate from corma of saffron plan (*Crocus sativus* L.) inhibits root growth and affects *in vitro* cell viability. *Journal of Experimental Botany* **51**, 731-737

Fernández JA (2004) Biology, biotechnology and biomedicine of saffron. *Recent Research Development in Plant Science* **2**, 127-159

Fujita Y, Tabata M, Nishi A, Yamada Y (1982) New medium and production of secondary compounds with the two-staged culture method. In: Fujiwara A (Ed) *Plant Tissue Culture*, Japanese Association of Plant Tissue Culture, Tokyo, pp 399-400

George PS, Viswanath S, Ravishankar GA, Venkataraman LV (1992) Tissue culture of saffron, *Crocus sativus* L. Somatic embryogenesis and shoot regeneration. *Food Biotechnology* **6**, 217-223

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50**, 151-158

Gresta F, Lombardo GM, Siracusa L, Ruberto G (2008) Effect of mother corm dimension and sowing time on stigma yield, daughter corms and qualitative aspects of saffron (*Crocus sativus* L.) in a Mediterranean environment. *Journal of the Science of Food and Agriculture* **88**, 1144-1150

Gui YL, Xu TY, Gu SR, Liu SQ, Sun GD, Zhang Q (1988) Corm formation of saffron crocus *in vitro*. *Acta Botanica Sinica* **30**, 338-340

Guo BS (1999) Recent research advance of rare earth in the field of biology. *China Rare Earths* **20**, 64-68

Himeno H, Sano K (1987) Synthesis of crocin, picrocrocin and safranal by saffron stigma-like structure proliferated *in vitro*. *Agricultural and Biological Chemistry* **51**, 2395-2400

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

Hong FS, Fang N, Gu YH, Zhao GW (1999) Effect of cerium nitrate on seed vigor and activities of enzymes during germination of rice. *China Rare Earths* **20**, 45-47

Hori H, Enomoto K, Nakaya M (1988) Induction of callus from pistils of *Crocus sativus* L. and production of color compounds in the callus. *Plant Tissue Culture Letters* **5**, 72-77

Igarashi Y, Yuasa M (1994) Effects of NH^{4+} and total nitrogen content in culture medium on shoot regeneration from calli in saffron *(Crocus sativus* L.). *Plant Tissue Culture Letters* **11**, 61-64

Ilahi I, Jabeen M, Firdous N (1987) Morphogenesis with saffron tissue culture. *Journal of Plant Physiology* **128**, 227-232

Isa T, Ogasawara T (1988) Efficient regeneration from the callus of saffron (*Crocus sativus* L.). *Japanese Journal of Breeding* **38**, 371-374

Isa T, Ogasawara T, Kaneko H (1990) Regeneration from the protoplasts of saffron (*Crocus sativus* L.). *Japanese Journal of Breeding* **40**, 153-158

Jimenez VM (2005) Involvement of plant hormones and plant growth regulators on *in vitro* somatic embryogenesis. *Plant Growth Regulation* **47**, 91-110

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

Kairong C, Gengsheng X, Xinmin L, Gengmei X, Yafu W (1999) Effect of hydrogen peroxide on somatic embryogenesis of *Lycium barbarun* L. *Plant Science* **146**, 9-16

Karamian R, Ebrahimzadeh H (2001) Plantlet regeneration from protoplast-derived embryogenic calli of *Crocus cancellatus*. *Plant Cell, Tissue and Organ Culture* **65**, 115-121

Karamian R (2004) Plantlet regeneration via somatic embryogenesis in four

species of *Crocus*. *Acta Horticulturae (ISHS)* **650**, 253-259

Karaoğlu C, Çöcü S, İpek A, Parmaksız I, Uranbey S, Sarıhan E, Arslan N, Kaya, M D, Sancak C, Özcan S, Gürbüz B, Mirici S, Er C, Khawar KM (2007) *In vitro* micropropagation of saffron. *Acta Horticulturae (ISHS)* **739**, 223-227

Karim MZ, Amin MN, Azad MAK, Begum F, Rahman MM, Ahmad S, Alam R (2003) *In vitro* shoot multiplication of *Chrysanthimum morifolium* as affected by sucrose, agar and pH. *Biotechnology* **2**, 115-120

Kim YJ, Hasegawa PJ, Bressan RA (1981) *In vitro* propagation of hyacinth. *HortScience* **16**, 645-647

Kohda H, Yamasaki K, Koyama A, Miyagawa H, Fujioka N, Omori Y, Ohta Y, Ituh H, Hosono T (1993) Process for culturing saffron stigma tissues. *United States Patent N* 5,217,897

Koyama A, Ohmori Y, Fujioka N, Miyagawa H, Yamasaki K, Kohda H (1988) Formation of stigma-like structures and pigments in cultured tissues of *Crocus sativus*. *Planta Medica* **54**, 375-376

Lian ML, Chakrabarty D, Paek KY (2003) Bulblet formation from bulbscale segments of *Lilium* using bioreactor system. *Biologia Plantarum* **46**, 199-203

Loskutov AV, Benginger CW, Ball TM (1999) Optimization of *in vitro* conditions for stigma-like-structure production from half-ovary explants of *Crocus sativus* L. *In Vitro Cellular and Developmental Biology – Plant* **35**, 200-205

Madubanya LA, Makunga NP, Fennell CW (2006) *Dierama luteoalbidum*: liquid culture provides an efficient system for the *ex situ* conservation of an endangered and horticulturally valuable plant. *South African Journal of Botany* **72**, 584-588

Majourhat K, Martínez-Gómez P, Piqueras A, Fernández JA (2007) Enhanced plantlet regeneration from cultured meristems in sprouting buds of saffron corms. *Acta Horticulturae (ISHS)* **739**, 275-278

Matsuzaki T, Koiwai A, Iwai S, Yamada Y (1984) *In vitro* proliferation of stigma-like, style-like structures of *Nicotiana tabacum* and its fatty acid composition. *Plant and Cell Physiology* **25**, 197-203

Milyaeva EL, Azizbekova NS, Komarova EN, Akhundova DD (1995) *In vitro* formation of regenerant corms of saffron crocus (*Crocus sativus* L). *Russian Journal of Plant Physiology* **42**, 112-119

Molina RV, Valero M, Navarro Y, García-Luis A, Guardiola JL (2004) The effect of time of corm lifting and duration of incubation at inductive temperature on flowering in the saffron plant (*Crocus sativus* L.). *Scientia Horticulturae* **103**, 79-91

Molina RV, Valero M, Navarro Y, Guardiola JL, García-Luis A (2005) Temperature effects on flower formation in saffron (*Crocus sativus* L.). *Scientia Horticulturae* **103**, 361-379

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Namera A, Koyama A, Fujioka N, Yamasaki K, Kohda H (1987) Formation of stigma-like structure and pigments in cultured tissues of *Crocus sativus* 2. *The Japanese journal of Pharmacology* **41**, 260-262

Novotná WK, Vejsadová H, Kindlmann P (2007) Effects of sugars and growth regulators on *in vitro* growth of *Dactylorhiza* species. *Biologia Plantarum* **51**, 198-200

Otsuka M, Saimoto H, Murata Y, Kawashima M (1992) Method for producing saffron stigma-like tissue and method for producing useful components from saffron stigma-like tissue. *United States Patent N* 5, 085,995

Paek KY, Murthy HN (2002) High frequency of bulblet regeneration from bulb scale sections of *Fritillaria thunbergii*. *Plant Cell, Tissue and Organ Culture* **68**, 247-252

Piqueras A, Debergh PC (1999) Morphogenesis in micropropagation. In: Soh WY, Bhojwani SS (Eds) *Morphogenesis in Plant Tissue Cultures*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 443-462

Piqueras A, Han BH, Escribano J, Rubio C, Hellin E, Fernández JA (1999) Development of cormogenic nodules and microcorms by tissue culture, a new tool for the multiplication and genetic improvement of saffron. *Agronomie* **19**, 603-610

Plessner O, Ziv M, Negbi M (1990) *In vitro* corm production in the saffron crocus (*Crocus sativus* L.). *Plant Cell, Tissue and Organ Culture* **20**, 89-94

Plessner O, Ziv M (1999) *In vitro* propagation and secondary metabolite production in Crocus sativus L. In: Negbi M (Ed) *Saffron: Crocus sativus L.* - (*Medicinal and Aromatic Plants - Industrial Profiles* (Vol 8), Harwood Academic Publishers, Amsterdam, The Netherlands, pp 137-148

Quiroz-Figueroa F, Mendez-Zeel M, Sanchez-Teyer F, Rojas-Herrera R, Loyola-Vargas VM (2002) Differential gene expression in embryogenic and non embryogenic clusters form cell suspension cultures of *Coffea arabica*. *Journal of Plant Physiology* **159**, 1250-1267

Radhika K, Sujatha M, Rao NT (2006) Thidiazuron stimulates adventitious shoot regeneration in different safflower explants. *Biologia Plantarum* **50**, 174-179

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

Sano K, Himeno H (1987) *In vitro* proliferation of saffron (*Crocus sativus* L.) stigma. *Plant Cell, Tissue and Organ Culture* **I1**, 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. *Journal of Experimental Botany* **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, Tissue and Organ Culture* **26**, 11-16

Sharma KD, Singh BM, Sharma TR, Rathour R, Sharma R, Goel S (2005) Development of low-cost media for *in vitro* shoot regeneration in saffron (*Crocus sativus* L.). *Indian Perfumer* **49**, 333-337

Sharma KD, Singh BM, Sharma TR, Rathour R, Sharma R, Goel S (2008) *In vitro* cormlet development in *Crocus sativus*. *Biologia Plantarum* **52**, 709-712

Sharifi G, Ebrahimzadeh H (2010) Changes of antioxidant enzyme, activities and isoenzyme profiles during *in vitro* shoot formation in saffron (*Crocus sativus* L.). *Acta Biologica Hungarica* **61**, 73-89

Sharifi G, Ebrahimzadeh H, Ghareyazie B, Karimi M (2010) Globular embryo-like structures and highly efficient thidiazuron-induced multiple shoot formation in saffron (*Crocus sativus* L.). *In Vitro Cellular and Developmental Biology – Plant* **46**, 274-280

Sheibani M, Nemati SH, Davarinejad GH, Azghandi AV, Habashi AA (2007) Induction of somatic embryogenesis in saffron using thidiazuron (TDZ). *Acta Horticulturae (ISHS)* **739**, 259-267

Slabbert MM, de Bruyn MH, Ferreira DI, Pretorius J (1993) Regeneration of bulblets from twin scales of *Crinum macowanii in vitro*. *Plant Cell, Tissue and Organ Culture* **33**, 133-141

Slabbert MM, Niederwieser JG (1999) *In vitro* bulblet production of *Lachenalia*. *Plant Cell Reports* **18**, 620-624

Soniya EV, Sujitha M (2006) An efficient *in vitro* propagation of *Aristolochia indica*. *Biologia Plantarum* **50**, 272-274

Staikidou L, Watson S, Harvey BMR, Selby C (2005) Narcissus bulblet formation *in vitro*: effects of carbohydrate type and osmolarity of the culture medium. *Plant Cell, Tissue and Organ Culture* **80**, 313-320

Stefaniak B (1994) Somatic embryogenesis and plant regeneration of gladiolus (*Gladiolus* Hort). *Plant Cell Reports* **3**, 386-389

Steinitz B, Yahel H (1982) *In vitro* propagation of *Narcissus tazetta*. *HortScience* **17**, 333-334

Steinitz B, Cohen A, Goldberg Z, Kochba M (1991) Precocious gladiolus corm formation in liquid shake cultures. *Plant Cell, Tissue and Organ Culture* **26**, 63-70

Tian M, Gu Q, Zhu M (2003) The involvement of hydrogen peroxide and antioxidant enzymes in the process of shoot organogenesis of strawberry callus. *Plant Science* **165**, 701-707

Visvanath S, Ravishankar GA, Venkataraman LV (1990) Induction of crocin, crocetin, picrocrocin, and safranal synthesis in callus culture of saffron-*Crocus sativus* L. *Biotechnology and Applied Biochemistry* **12**, 336-340

Von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. *Plant Cell, Tissue and Organ Culture* **69**, 233-249

Wang Y, Jeknic Z, Ernst RC, Chen TH (1999) Efficient plant regeneration from suspension-cultured cells of tall bearded iris. *HortScience* **34**, 730-735

Wang L, Li Y, Dong XJ, Zhang BC (2002) Induction of petal-like structures from petals of *Crocus sativus* L. and the differentiation of style-stigma-like structures *in vitro*. *Sheng Wu Gong Cheng Xue Bao* **18**, 638-640

Zeng Y, Yan F, Tang L, Chen F (2003) Increased crocin production and induction frequency of stigma-like structure from floral organs of *Crocus sativus* by precursor feeding. *Plant Cell, Tissue and Organ Culture* **72**, 185-191

Zhao J, Chen F, Yan F, Tang L, Xu Y (2001) *In vitro* regeneration of style-stigma-like structure from stamens of *Crocus sativus*. *Acta Botanica Sinica* **43**, 475-479

Ziv M (1992) Morphogenic control of plants micropropagated in bioreactor cultures and its possible impact on acclimatization. *Acta Horticulturae (ISHS)* **319**, 119-124