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Review

Saffron (*Crocus sativus* L.) in the light of biotechnological approaches: A review

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Saffron (*Crocus sativus* L.) is a sterile triploid plant belonging to the Iridaceae (Liliales, Monocots). Saffron is a spice derived from the flower and has for decades been the world's most expensive spice. 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. Therefore, reproduction is human dependent; the corms must be manually dug up, broken apart and replanted. The natural propagation rate of saffron is relatively low. Biotechnological approaches have increasingly become a valuable tool assisting breeders to release new species and cultivars into the market more rapidly. Biotechnological approaches offer the capability to produce large quantities of propagating material in short time as well as the production of commercially important chemical constituents like, crocin, picrocrocin, crocetin and safranal under *in vitro* conditions. However, the protocols available so far need further refinement for their commercial utilization. Here we review the progress made in genus *Crocus*, and highlight the potential for future expansion in this field through biotechnological interventions.

Key words: *Crocus sativus* L, biotechnological approaches, sterile, corms.

INTRODUCTION

Saffron (*Crocus sativus*) which belongs to Iris family *Iridaceae* is the most expensive spice in the world and is popularly known as the “Golden Condiment”. In India it is a legendary crop of Jammu and Kashmir, produced on well drained karewa soils where ideal climatic conditions are available for good shoot growth and flower production. Plants of this family are herbs with rhizomes, corms or bulbs. The family *Iridaceae* embraces about 60 genera and 1,500 species. The genus *Crocus* includes native species from Europe, North Africa and temperate Asia, and is especially well represented in arid countries of south-eastern Europe and Western and Central Asia. Among the 85 species belonging to the genus *Crocus*, *C. sativus* L. (Saffron) is the most fascinating and intriguing species (Fernández, 2004). Dried stigmas of saffron flowers compose the most expensive spice which has been valuable since ancient times for its odoriferous, coloring, and medicinal properties (Plessner et al., 1990). The name saffron is commonly used to refer both to the spice and the plant itself. Some archaeological and historical studies indicate that domestication of saffron dates back to 2,000 to 1,500 years BC (Grilli Caiola, 2004). The origin of saffron is obscure, but the plant is believed to have originated in the eastern Mediterranean, (Winterhalter and Straubinger, 2000). Most of the *Crocus* species grow naturally in fields between shrubs and grasses or in light woodlands. The species in the genus *Crocus* have underground fleshy corms and basal, grass-probably in Asia Minor and Persia. The name ‘saffron’ is derived from Arabic *zá-faran* which means ‘be yellow’

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like, dark green leaves with whitish median stripe. Their leaves appear with or after flowers. A plant may have one or several flowers. The fruit is a capsule and have numerous seeds with brownish or reddish color. Saffron is currently being cultivated more or less intensively in Iran, India, Greece, Spain, Italy, Turkey, France, Switzerland, Israel, Pakistan, Azerbaijan, China, Egypt, United Arab Emirates, Japan, Afghanistan, Iraq and recently Australia (Tasmania) (Nehvi et al*.,* 2006). Saffron has a life span of about 220 days. Rainfall in the autumn, warm summers and mild winters are the favorable climatic conditions for high yields of saffron. Water requirement of saffron is low. Garden soil (clay sand) is suitable for optimum growth of saffron. A relatively low water use, growth and development during fall and winter and a very low harvest index are some remarkable characteristics of saffron. Kashmir region in India produces between 5 to 6 t mostly dedicated to Indian's self consumption. Saffron export from India declined because sterility of saffron limits the application of conventional breeding approaches for its further improvement resulting in a low productivity (Ahmad et al., 2013). Traditional practices of saffron cultivation which ignores importance of water requirement, manual requirement, management of weeds, pests and disease and post harvest processing have been a matter of great concern particularly in Kashmir and have limited the benefits leading to a low I/O ratio. Non availability of saffron quality planting material has been another area which has affected the replantation and area expansion as the availability of quality planting material in India is less than 10%. Since more than 95,000 farm families are directly or indirectly associated with the crop in major saffron growing countries particularly in Iran and India, efforts have to be made to safeguard the interests of saffron growers by making the industry more profitable. Lack of high yielding cultivars adapted to diverse growing conditions, large area under rain fed 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 (Ahmad et al., 2013).

Saffron is a sterile triploid plant that is propagated by corms as the propagation through seed is impossible due to non setting of seeds. The natural propagation rate of most geophytes including saffron is relatively low. Besides conventional methods of propagation, biotechnological approaches such as *in vitro* cultural methods contribute importantly for the propagation of many important and economic plants. The application of contemporary biotechnological methods makes solving this problem more feasible. In view of this, a National mission on saffron was launched recently by the Government of India to ensure the revival of saffron production in Jammu and Kashmir. In 2010 the Central Government approved a plan to release Rs. 3.76 billion under this National Saffron Mission Programme for four years with the aim of improving overall saffron production, enhance its quality, build research and extension capability and develop an appropriate system for organized marketing. Premier research institutions like Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir and Central Institute of Temperate Horticulture are involved in the mission, while Development Departments of Jammu & Kashmir government are also key coordinators. Besides, many projects at these research institutions are funded under the Horticulture Technology Mission. The Indian Council of Agricultural Research is the nodal agency implementing these programs and the major thrust is on saffron corm multiplication, quality corm production, *in vitro* production of saffron/microcorms, corm rot management, saffron inter-culture, and demonstration of developed technologies to saffron growers. The Department of Biotechnology is also funding projects of individual researchers for development and refinement of saffron production and improvement technologies with a particular thrust on *in vitro* cormlet production and production of stigma-like structures. The technique, however, calls for development of convenient protocols and their standardization that will not only help in mass multiplication of elite-disease free clones but also open new vistas for application of recombinant DNA technologies for development of transgenics in this crop (Yasmin and Nehvi, 2013).

BIOTECHNOLOGICAL APPROACHES

Saffron is propagated solely by vegetative way using the annual renewal corms. Only four to five corms per mother corm are produced in one growing season through conventional methods. Hence, low multiplication rates and fungal infestation of corms are the bottlenecks for availability of sufficient quality planting material (Kiran et al., 2011). An enhancement in productivity per unit area that can lead to increase in net returns to farmers and encourage them to continue growing saffron is therefore necessary. Nevertheless, conventional breeding methodologies have not led to any improvement in saffron and alternative procedures like biotechnological, molecular biological interventions for enhancing yield and tolerance to biotic and abiotic stresses have to be explored. Tissue culture is a useful method for large scale production of disease free plants using different medium with different ratios of auxin and cytokinin (Ding et al., 1981; Chrungoo and Caiola, 1987; Ilahi et al., 1987; Plessner et al., 1990; Fakhrai and Evans, 1990;

Chen et al., 2003; Majourhay et al., 2007; Sheibani et al., 2007). However, smaller size of the tissue culture corms with least survival under actual field conditions is the limitation factor with the available tissue culture saffron protocols and further refinement for their commercial utilization is needed. Stigma-like structures were reported to be induced from almost every part of floral organs, including half ovaries (Himeno and Sano, 1987; Sano and Himeno, 1987; Loskutov et al., 1999), stigmas (Koyama et al., 1988; Sarma et al., 1990), petals (Lu et al., 1992; Jia et al., 1996), anthers (Fakhrai and Evans, 1990) and stamens (Zhao et al., 2001).

Biotechnological approaches are presently mainly used as a tool to facilitate a better understanding of the bio-chemical synthesis of saffron secondary products. Regeneration/proliferation ability of the corms was dependent on genotype, type of explants, culture initiation time and composition of the culture medium. The plants were able to form shoots or corms within 5 to 30 weeks from the start culture (GulZaffar et al., 2004). Saffron is a monocotyledon member of the large family Iridaceae. Comparatively, bulbous and cormous monocotyledons are regarded as difficult *in vitro* material. Contamination is a serious problem during micropropagation of monocots especially if underground organs, such as corms, bulbs, rhizomes and tubers are used as an explants source. The size of geophytes, physical damage and dormancy are the other problems which make tissue culture studies difficult. Schenk and Hildebrandt (1972) reported the importance of medium composition and techniques for induction and growth of monocotyledonous and dicotyledonous plants in cell culture. They found that a high level of auxin-type growth regulating substances generally favored cell cultures of monocotyledonous plants, while low levels of cytokinin were essential for most dicotyledonous cell cultures. Within the last few decades, an increasing number of bulbous and cormous monocotyledons have been successfully cultured. Tissue culture technology was greatly influenced by the demand of rapid multiplication and clonal propagation of slow-growing monocots. Several economically important monocot species constituting nutritional, medicinal or ornamental groups of plants were used for *in vitro* clonal propagation (Sutter, 1986) and production of secondary metabolites (Aslanyants et al., 1988). Organogenesis and somatic embryogenesis from differentiated tissues of bulbous and cormous monocots, such as *C. sativus* L., are also reported in the literature. Ding et al. (1979, 1981) were the first to report the successful tissue culture of *Crocus*. They successfully regenerated callus and intact plantlets from corm explants when the culture media contained indole-3-acetic acid (IAA) and 2,4-D (2,4 Dichlorophenoxyacetic acid). Later, Homes et al. (1987) observed microcorms forming on 1/8th corm explants. These regenerated shoots when cultured on a medium with 9 µM 2, 4-D (Dichlorophenoxyacetic acid) using a similar medium (Ilahi et al., 1987), produced callus on corm explants that differentiated buds. Ilahi et al. (1986) described the morphogenesis in saffron tissue culture. Corms of saffron were cultured on half strength mass medium supplemented with different combinations of growth regulators; that is, auxin and cytokinins, and coconut milk. Callus was induced in a medium containing 0.5 mg/L each of 2,4-D (2,4 Dichlorophenoxyacetic_acid) and 6-Benzylaminopurine(BAP) and 2% coconut milk. The same culture was used for differentiation of callus into buds. They reported that an increase in 2,4-D also enhanced callus formation but suppressed shoot-bud formation. These shoots were induced to root when inoculated on a medium containing 2 mg/L NAA (Naphthaleneacetic acid) for 24 h. However, further growth of these roots was slow when reinoculated on half strength MS containing 0.1 mg/L each of 2,4-D and BAP. In another set of experiments when a piece of callus, growing in similar conditions, was transferred to MS medium containing 0.5 mg/L NAA, 0.1 of either BAP or kinetin and 2% coconut milk, the nodules gave rise to roots after four weeks of culture with subsequent suppression of the shoot development. Floral organs of four spring-flowering *Crocus* species were investigated for their competence to produce callus by Choob et al. (1994). Shoot development on corm explants was promoted by cytokinins (kinetin or zeatin, 14 - 56 lM) and 2, 4-D (4.5 lM), while corm formation and growth was promoted by ethylene exposure (Plessner et al., 1990). Ovary wall explants gave the best response, with stigma and style-type structures regenerating from the explants (Choob et al., 1994). Under continuous darkness, many shoot primordial were formed. These elongated when placed in the light, and formed normal plantlets with corms (Bhagyalakshmi, 1999). Loskutov et al. (1999) studied the optimization of *in vitro* conditions for stigma-like structure production from half-ovary explants of *C. sativus*. The optimum proliferation of stigma-like structure was observed on B5 basal medium (Gamborg B5 medium) containing NAA (5.4 µM), BA (44.4 µM), MS organics, casein hydrolysate (0.05%) and L-alanine (11.2 µM). They reported that the amounts of crocin, crocetin, picrocrocin and safranal in stigma-like structure, as determined by high performance liquid chromatography analysis, were similar to those found in natural saffron. Successful stigma and ovary formation has been attained when cultured on media containing BA (4.4 to 22.2 µM) and kinetin (4.7 to 23.3 µM) (Sano and Himeno, 1987). Style and perianth explants produced stigma-like structures that proliferated forming up to 100 structures per explant (Ebrahimzadeh Karamian, 2000). Zeng et al. (2003) recorded the increased crocin production and induction frequency of stigma-like structures from floral organs of *C. sativus* by precursor feeding. MS medium supplemented with 5 mg/L kinetin and 4 mg/L NAA was used as the basal medium. Almost all of the stigma-like structures formed directly from explants, instead of from

callus. The production of non-embryogenic and embryogenic callus for the purposes of protoplast formation was investigated by Darvishi et al. (2007).

Chen et al. (2004) also examined the promotion of growth of *C. sativus* cells and the production of crocin by rare earth elements. They reported that La^{3+} and Ce^{3+}, either individually or as a mixture, promoted crocin production of *C. sativus* callus but Nd^{3+} (40 μM) had little effect and all metal ions were toxic above 100 μM. La^{3+} (60 μM) significantly promoted the growth of callus but only slightly increased crocin. Ce^{3+} (40 μM) significantly promoted crocin production but had little effect on cell growth. They showed that La^{3+} (60 μM) and Ce^{3+} (20 μM) together gave the highest dry weight biomass (20.4 g/L), crocin content (4.4 mg/g) and crocin production (90 mg/L).

Somatic embryogenesis in saffron was described also by Blázquez et al. (2004). They used MS culture medium supplemented with 0.5 mg/L BAP and 0.1 mg/L 2,4-D for induction of somatic embryogenesis. Embryogenic calli were subcultured in MS medium containing 1 mg/L BAP and 0.05 mg/L NAA for multiplication in solid medium. Temporary immersion systems (TIS) were used for this purpose. A four-fold increase in the production of embryogenic calli (fresh weight increase) was observed in TIS culture when compared to solid medium. They obtained the best result when 1 mg/L of paclobutrazol was added. They also improved the development of somatic embryos on solid medium supplemented with 0.5 mg/L jasmonic acid (JA) and obtained plant regeneration via somatic embryogenesis after eight weeks of treatment with JA in combination with sucrose. Leaf explants produced callus that regenerated somatic embryos and plantlets when cultured on 10 lM BA and 0.5 lM 2, 4-D (Raja et al., 2007). These were used for microcorm induction which was promoted by a half-strength MS medium plus 9% sucrose (Raja et al., 2007). Blázquez et al. (2004a) showed a link between type, occurrence and expression of antioxidant enzymes (superoxide dismutases and catalase) and the stage of somatic embryogenesis, suggesting these could act as markers of embryogenesis.

In vitro regenerated shoots from callus were cultured in Murashige and Skoog (MS) medium supplemented with (0.2 to 0.5 mg/L) 6-benzlaminopurine (BA), Paclobutrazol (PAC) 2 to 20 mg/L and sucrose (3 to 12%). Higher concentration of PAC (5 mg/L) along with BA (0.25 mg/L) and 9% sucrose resulted in formation of relatively large micro corms (Zaffar et al., 2012). 3C nuclear DNA of somatic embryos derived and directly formed shoots was 9.7±0.02 and 9.73±0.04 pg and 1C genome size was 4.81±0.01 x 10^9 bp and 4.80±0.01 x10^6 bp, respectively. 3C nuclear DNA and 1C genome size of mother plant was 9.77±0.02 and 4.82±0.01 x 10^9 bp, respectively indicating that genome size of tissue of raised plants remained stable (Devi et al., 2012). AP-3 gene expression was found maximum during late-preanthesis (bud) stage of flower development. Expression increases from pre-bud to bud stage and decreases from bud to flowering stage of flower development. Since AP-3 is the regulatory gene for floral development, its expression pattern determines the flowering fate in saffron (Wafi et al., 2012).

Recently, T and B-cell epitopes of Iranian *C. sativus* were mapped using bioinformatics tools and the predicted peptides were found useful for vaccine development. Expression pattern of CsLYC, CsZCD, CsBCH and CSgt-2 was studied in different flower parts and highest expression was found in stigma followed by style and petal (Mir et al., 2012). The Reverse transcriptase-PCR analysis revealed that CsZCD gene expression followed different patterns during stigma development. Highest levels of CsZCD gene expression was observed in fully developed scarlet stage of stigma. Real time PCR analysis showed that there is a sharp increase in gene expression from yellow to orange and orange to scarlet stages of stigma development. Increase in CsZCD gene expression with the development of stigma suggests its regulatory role for stigma development in saffron (Mir et al., 2012). According to (Karamian, 2004), maturated embryos could be germinated on half strength MS medium supplemented with 25 mg/L GA3. Finally, complete plantlets were obtained by transferring germinated embryos into half strength MS medium supplemented with 1 mg/L NAA and 1 mg/L BA at 20ºC under 16/8 h (light/dark) cycle. Among different types of explants, intact ovaries are more suitable for production of direct SLS than others, while the induction of indirect SLS was higher on styles than on intact and half ovaries. The best hormonal combination for induction of direct and indirect SLS was 2 mg/L kin, 8 mg/L NAA and 20 mg/LNAA, 1 mg/L BA, respectively. The HPLC comparison of natural stigma and SLS showed that all three saffron constituents are present in SLS derived, but at lower levels compared with natural ones (Ziaratina et al., 2012). Lateral and terminal meristem of plants were collected and inoculated in MS media supplemented with plus (1,2 and 4 mg/L) 2, 4-D and kinetin (0.5, 1,4 and 8 mg/L) in different combinations with 3% sucrose for callus induction after thorough surface sterilization. The first callus was induced after 35 days of inoculation from terminal meristem explants. However, lateral meristem was observed to be less responsive. The highest frequency of callus induction was achieved on MS medium supplemented with 2 mg/L 2, 4-D plus 0.5 mg/L Kin (Vahedi et al., 2012). 3C nuclear DNA of somatic embryos derived and directly formed shoots was 9.7±0.02 and 9.73±0.04 pg and 1C genome size was 4.81±0.01 x 109 bp and 4.80±0.01 x106 bp, respectively. 3C nuclear DNA and 1C genome size of mother plant was 9.77±0.02 and 4.82±0.01 x 109 bp, respectively indicating that genome size of tissue of raised plants remained stable (Devi et al., 2012). CsSERK expression is associated with induction of shoot

organogenisis and could be a potential marker for cells competent to form shoot in saffron tissue cultured *in vitro*. Also SERK gene may have a broader role in morphogenesis in cultured tissue rather than being specific to somatic embryogenesis (Vatankhan et al., 2012). Maturated embryos could be germinated on half strength MS medium containing 20 mg/L gibberellic acid (GA3). Complete plantlets with well developed root system and corm formation were obtained on transferring germinated embryos to half strength MS (Murashige and Skoog basal medium) supplemented with $5x10^{-6}$ M BA, $5x10^{-6}$ M NAA and 2% activated charcoal (Ahuja et al., 1994). MS (Murashige and Skoog basal medium Media supplemented) with 0.5 mg/L naphthalene acetic acid (NAA) and 1.5 mg/L 6-benzyl amino purine (BAP) ensured maximum bud sprouting in September with direct multiple shoot primordia initiation on 6.5 mg/L BAP in November. 6.5 mg/L BAP + 0.2 mg/L NAA resulted in maximum shoot proliferation (24); however, at higher concentration, the PGRs were detrimental in arresting the growth. Viable shoot clumps established maximum *in vitro* corms in April after sub culturing on growth retardant (CCC) at 0.25% supplemented with 9% sucrose. Sub culturing of non-flowering *in vitro* corms on growth retardant with sucrose eliminated season dependence of *in vitro* protocols in the 2nd cycle of protocol. Primary and secondary hardening before field transfer ensured 100% corm viability (Yasmin et al., 2013).

Conclusion

There is a need of expanding the area of cultivation of saffron to meet the steady increase of its demand worldwide. However, limited availability of daughter corms is one of the major hindrances for the expansion of acreage under saffron. Biotechnological approaches such as micropropagation of saffron using direct or indirect shoot induction or plantlet regeneration through somatic embryogenesis followed by microcorm production offer the capability to produce large quantities of propagating material free of disease 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 through biotechnological approaches. Among the four chemicals, production of crocin in cell cultures has been the main focus of research because of the anticancer properties of this chemical. Biotechnological approaches are the alternative to meet the worldwide demand and to preserve this “Golden Condiment”.

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