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INITIATION AND ORIGIN OF STIGMA-LIKE STRUCTURES (SLS) ON OVARY AND STYLE EXPLANTS OF SAFFRON IN TISSUE CULTURE

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Saffron, made from the dried stigmas of *Crocus sativus* L., contains pigments and valuable aromatic compounds, and can be used in medicine and as a spice. Nowadays its production is lower than demand. Tissue culture presents an alternative biochemical tool which can be used to produce stigma-like structure (SLS) *in vitro*. In this study, the origin and induction of SLS formation was investigated in ovary and style explants of floral buds on MS medium supplemented with 1-naphthalene acetic acid (NAA) and 6-benzlaminopurine (BAP). SLS were directly originated through meristematic cells or indirectly in the form of colorless globular structures from parenchyma tissue. The colorless globular structures initially were conical and pale yellow color at the sharp ends; subsequently they matured into trumpet-like red stigmas with or without finger-like papillae at the margins. Light and electron microscopic observations of ultra- and semithin sections of different developmental stages of SLS showed that these structures possess two kinds of cells: (1) small cells close to parenchyma tissues and (2) large cells oriented towards the peripheral area and apparently originated from the small ones. Our results suggest that the SLS originated from internal parenchyma tissues.

Key words: *Crocus sativus* L., meristematic cells, tissue culture, stigma-like structures, parenchyma tissues.

INTRODUCTION

Saffron is made from the dried stigmas of *Crocus sativus* L., a member of the Iridaceae. It is a monocotyledonous and sterile triploid plant, and is vegetatively propagated from corms. The corm produces 1 to 3 purple flowers each year. Its pistil is central, with an ovary from which the style originates. The top of the style is divided in three dark red trumpet-like stigmas. The major metabolites of saffron are water-soluble carotenoids, crocin, picrocrocin and safranal, which are responsible for its unique color, bitter taste and intense aroma. In order to ensure the future of saffron crop production, it is essential to improve plant materials, quality evaluation methods, and cultivation techniques such as tissue culture. Tissue culture methods provide a means of studying induction and formation of SLS in vitro and consequently have great potential in large scale propagation of saffron and in its genetic improvement.

There is an increasing demand for saffron production in the spice industry and in medicine, given the antimicrobial, anticarcinogenic and antioxidant properties of the water-soluble carotenoid derivatives of stigma. The demand is met by cultivating flowers with an increased number of stigmas (Estilai, 1978) or by developing stigmas with an increased amount of dye and aroma, or by expanding the area of pigment production to the style and even ovary to get more dye material. Grilli-Caiola and Canini (2004) demonstrated that the chromoplasts of stigma, style and parenchyma tissues of the ovary are the same and originate from amyloplasts, while differing from the plastids of leaf in vivo.

Sano and Himeno (1987) reported the formation of stigma-like structures (SLS) on young stigma, ovary and half-ovary explants in tissue culture. They noted two kinds of SLS, direct-compact and indirect-incompact, which originate from meristematic tissue of callus. Fakhrari and Evans (1989) used MS media sup-

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plemented with naphthalene acetic acid (NAA), benzylaminopurine (BAP) and 2,4-dichlorophenoxy acetic acid (2,4-D) to culture different parts of *C. chrysanthus* floral buds. Fakhrari and Evans (1990) reported SLS formation on different parts of floral bud of *C. sativus* on White media supplemented with different concentrations of zeatin, BAP and 2,4-D. Sarma et al. (1990) investigated the effect of age, explant type and exogenous hormones on the number of SLS formation on stigmas. Ebrahimzadeh et al. (1996) showed regenerated organs on different parts of floral buds using MS and LS media supplemented with NAA and BAP. Later they reported the formation of normal and abnormal flowers, in addition to SLS, on MS medium supplemented with kinetin and NAA (Ebrahimzadeh et al., 2000).

It has been shown that synthesis of *Crocus* apocarotenoids occurs concomitantly with the amyloplast to chromoplast transition, and that it is regulated through development and enlargement of the stigmas (Bouvier et al., 2003; Grilli-Caiola and Canini, 2004)

Later, *CsZCD* and *CsCCD* genes, both coding for chromoplast enzymes, were identified. Castillo et al. (2005) investigated the genes, involved in the *C. sativus* apocarotenoid biosynthesis pathway, in vivo. New technologies such as in vitro tissue culture techniques are being developed for use to increase the amounts of crocin, picrocrocin and safranal, the main constituents of saffron. The pharmacological properties of saffron are another subject of recent extensive research (Schmidt, 2007; Chryssanthi, 2007). A review of the literature shows that all parts of the floral buds of saffron have the potential to produce SLS, especially on MS medium with different combinations of auxin and cytokinin.

This study used microscopy techniques to investigate the induction, development and origin of SLS from ovary and style explants.

MATERIALS AND METHODS

PLANT MATERIALS

Flowering corms of *Crocus sativus* from a saffron farm in Torbat-e-Haydarieh city (Khorasan Province) were collected in the early sprouting season (November-December 2007) and transferred to the lab. Flowering buds 7–12 cm long were removed from the corms and used for culture.

SAMPLE STERILIZATION

The crocus floral buds were rinsed under running tap water (30 min) and then sterilized by disinfection with 0.5% benzalkonium chloride (Tolidaru Co., Iran) solution for 15 min. Then the buds were treated with 70% ethanol for 2 min and then 5% sodium hypochlorite solution supplemented with a few drops of Tween 80 for 20 min. Finally the buds were washed three times with sterile distilled water. This is the first report of the use of disinfectant for part of the sterilization process in this procedure; it worked very well.

EXPLANT AND IN VITRO CULTURE CONDITION

Based on previous results on the frequency of calli and SLS production (Ebrahimzadeh et al., 1996, 2000), we set up a tissue culture experiment using MS medium (Murashige and Skoog, 1962) supplemented with 10 mg/L NAA and 10 mg/L BAP for induction of SLS. After removing the prophyll sheaths from the sterilized buds, we separated the ovary and style organs and used them for culture in Petri dishes.

Prior to culture, the ovary explants were divided into two groups: (1) explants vertically injured by scalpel, and (2) control explants with no injury. Then 4 to 6 explants were placed in each Petri dish in 10 replicates and the cultures were kept in a culture room in darkness at 22±2°C. The cultures were subcultured every 28 days.

LIGHT MICROSCOPY OBSERVATIONS

Prior to each subculture, the cultured ovary and style explants were observed with a stereomicroscope (SZH Olympus, Japan) and were divided into three developmental stages as follows: 1) stage I: formation of colorless globular calli; 2) stage II: formation of pale yellow conical calli on which SLS formation was initiated; 3) stage III: formation of matured trumpet-like red SLS.

The histological observations are from semithin sections (1–6 μm) prepared from samples (for method of preparation see below).

Photos of three different developmental stages of both ovary and style explants were taken through a stereomicroscope equipped with a camera (C-35AD-4) or with a G6 Cannon digital camera.

TRANSMISSION ELECTRON MICROSCOPY

Small portions of fresh callus from the ovary were used for preparation of ultra-thin sections. The procedure was carried out according to Bozzola's protocol (Bozolla and Russel, 1999) as follows: tissue specimens were separated and then fixed with two fixatives (glutaraldehyde, osmium tetroxide) for 1.5 h and 3 h, respectively, at 4°C. Then the fixed samples were washed three times with phosphate buffer (pH 7.2–7.4) for 5 min, after which all samples were dehydrated in a graded acetone/buffer series. Later the samples were infiltrated with an acetone-resin mixture, and the specimens were processed through increasing concentrations of resin and decreasing ratios of acetone to pure resin over 12–24 h. Finally the fixed specimens were molded and cured in an

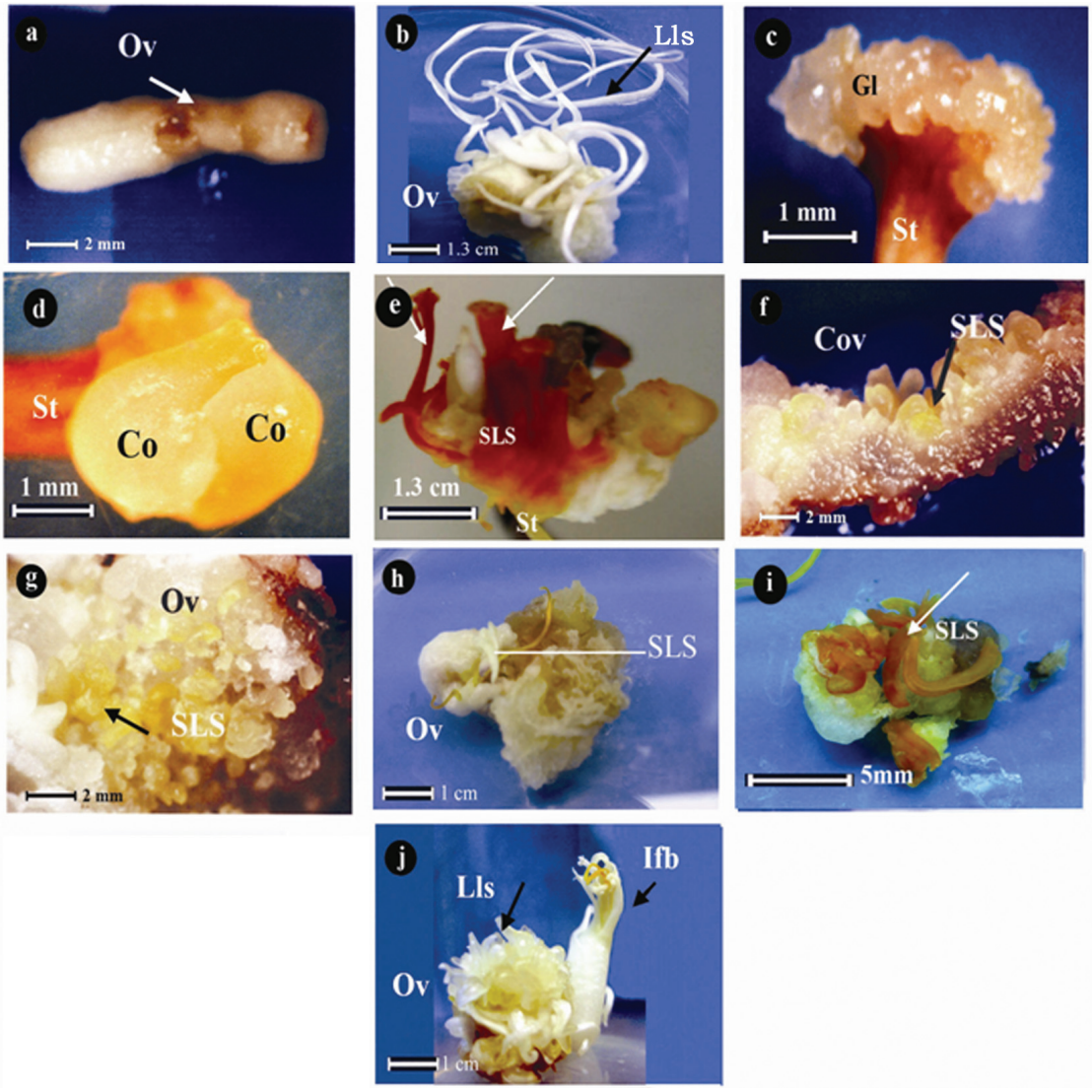

Fig. 1. (a) Injured ovary (Ov), **(b)** Leaf-like structures (Lls) on outer layer of callus from ovary (Ov), **(c)** Colorless globular (Gl) structures on cut edge of style (St), **(d)** Two pale yellow conical (Co) structures at cut edge of style, **(e)** Trumpet-like red stigma-like structures (SLS) on calli of style, **(f)** Cross section of injured ovary (Cov) with stigma-like structures (SLS), **(g)** Stigma-like structures (SLS) inside an injured ovary, **(h)** Callus from injured ovary with many stigma-like structures (SLS) inside it before and **(i)** after dissection, **(j)** Incomplete flower bud (Ifb) with leaf-like structures (Lls) from outer layer of ovary.

oven at 60°C for 2–3 days. Resin-embedded specimens were sectioned with glass or diamond knives (UCT, Leica, Switzerland). Ultra-thin sections (50-70 nm thick) were stained with uranyl acetate and lead citrate (Reynolds, 1963), mounted on copper grids and observed with a Zeiss-CEM 902 A-80kv TEM, and images were captured photographically on Kodak SO-163 film.

SCANNING ELECTRON MICROSCOPY

First, fresh specimens at stage I were dried in liquid nitrogen before performing PVD (physical vapor deposition) with a sputter coater (SCD00S, BAL-TEC, Switzerland). Images were prepared by SEM (XL 30, Philips, Netherlands) at accelerating voltage of 30 kv.

RESULTS

The results from tissue culture of injured ovaries (Fig. 1a) show that when the period of initial experiments continued to three months, both injured and non-injured (control) ovaries started to swell. This induction gradually increased within this period,

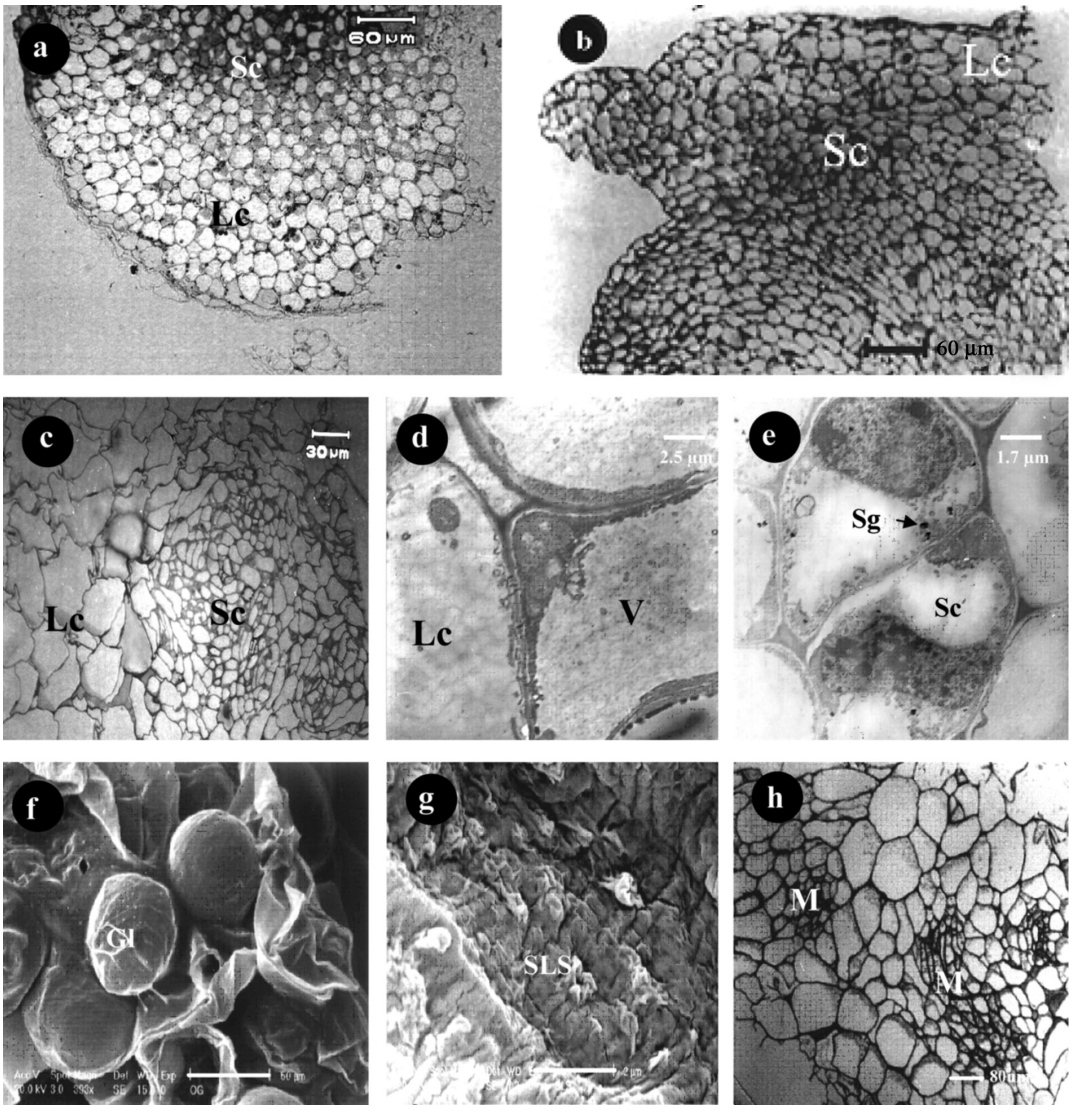

Fig. 2. (a,b) Semithin sections of globular and conical structures, with small cells (Sc) and large cells (Lc), **(c)** Section of trumpet-like red stigma structures with small cells (Sc) and large cells (Lc), **(d, e)** TEM images of large cells (Lc) with vacuole (V) and small cells (Sc) with starch grains (Sg), **(f, g)** SEM images of globular (Gl) structures and direct stigma-like structures (SLS) on calli of injured ovary, **(h)** Parenchyma tissue of calli from injured ovary, with meristematic areas (M).

and white leaf-like structures with many branches developed on the outer layers of some injured and non-injured ovary explants (Fig. 1b). Tissue and structural changes in style explants took from 1 to 2 months of culture. First the parenchyma tissue around the cut edge of the style explants started to swell, and then colorless globular structures formed at the swelled area (Fig. 1c). Then these colorless globular structures became pale yellow conical structures (Fig. 1d) and finally trumpet-like red SLS (Fig. 1e). Also, leaf-like structures emerged on the outer layer of calli from style explants.

Our observations at 3 to 5 months after culture mostly showed the formation of only white leaf-like structures on the outer layer of calli from non-injured ovary explants; but in addition to these structures, globular structures with many small SLS developed directly from inside the parenchyma tissue towards the cavity of calli from injured ovary explants (Fig. 1f–i). This was seen when the calli were dissected with a scalpel (Fig. 1f,i).

Finally, 5 to 8 months after the beginning of the tissue culture experiments, the formation of conical structures and then rather long SLS was observed mostly inside the calli in injured ovaries (Fig. 1i), and a few leaf-like structures and incomplete bud flowers were noted on the outer layer of the calli (Fig. 1j).

The microscopy data obtained from semithin sections of calli at three different developmental SLS stages (Fig. 2a-c) of injured ovary showed that they possess two kinds of cells: (1) small meristematic cells located within the parenchyma tissue, and (2) large cells which towards the peripheral areas were differentiated for formation of SLS.

TEM of stage III SLS showed a large, completely elongated cell possessing a large central vacuole with cytoplasm, with the nucleus squeezed to the corner of the cell (Fig. 2d), and small cells with starch grains located towards the parenchyma tissue (Fig. 2e). The observations from style explants were similar.

SEM showed the formation of globular structures (Fig. 2f) and small SLS which emerged directly from parenchyma tissue of injured ovary callus (Fig. 2g). Many meristematic areas were distributed on parenchyma tissue of injured ovary observed in semithin sections (Fig. 2h).

DISCUSSION

We studied the origin and different organogenesis stages of saffron stigma-like structures by culturing immature ovary and style explants from floral buds of *C. sativus* L., inducing them to produce these structures via callus formation in vitro, and using microscopy to clarify their mode of development. A model of organogenesis has been presented in which the process begins in calli with the formation of clusters of meristematic cells (meristemoids), which are able to react with many internal factors in order to produce primordial tissues, depending on the nature of those factors as well as the hormones (Torrey, 1966). Then the primordial tissues can give rise to shoots, roots, embryoids and other organs depending on different combinations of two auxin and cytokinin hormones.

Plessner et al. (1990) showed that MS medium supplemented with auxins and cytokinins were needed for the development of bud explants. The areas occupied by meristemoids lie between the tissue and culture medium, so there are physiological flows from the culture medium towards the internal tissue which may play an important role in finding areas for meristemoids to form. These meristematic areas act as pores for transport of essential metabolites from surrounding cells (Street, 1977). In our experiments we found meristematic areas forming in two ways: both directly in parenchyma tissues, which were distributed throughout the parenchyma layer, with small compact cells from which the SLS differentiated; and also indirectly in the form of colorless globular structures which emerged from the parenchyma tissue.

The results from microscopy of semithin sections of colorless globular structures of both ovary and style calli revealed two kinds of cells: small compact cells (forming a meristematic area) in close proximity to the parenchyma tissue next to the medium; from these, large cells differentiated and then formed SLS. These observations confirm that organogenesis starts after the formation of meristemoids, leading to the production of primordial SLS. These results are in accord with SEM data reported by Himeno et al. (1988), who showed that SLS was initiated from various types of primordial tissues (organs) but did not examine the mechanism of their formation. Our TEM observations of SLS at stage III revealed that the small cells possessed starch grains and that the large cells had large vacuoles without any starch grains. In *C. sativus*, Castillo et al. (2005) showed that stigma development occurred concomitantly with the transition of amyloplasts to chromoplasts, and also with carotenoid accumulation.

Stereomicroscopy showed that when the colorless globular structures (stage I) differentiated to pale yellow conical structures (stage II) their color change was based on the transition of amyloplasts to chromoplasts. As conical structures began to form and continued to differentiate to trumpet-like red SLS (stage III), apocarotenoid accumulation were completed; within this transition the color changed from pale yellow to red. These results are comparable to those of Bouvier et al. (2003), who used electron microscopy to show that during stigma development in vivo the amyloplasts differentiated to chromoplasts; this was accompanied by the accumulation of apocarotenoids in the stigma of *C. sativus*, which then were sequestered in the central vacuoles of fully developed large cells of the stigma. They also showed that the many genes involved in the apocarotenoid biosynthesis pathway, which are regulated during stigma development in *C. sativus* L., include *CsZCD* and *CsCCD*, which code for chromoplast enzymes. Examining the transition of amyloplasts to chromoplasts in nectaries of tobacco, Horner et al. (2007) demonstrated that the color change in nectaries from green to orange is the result of active catabolism in amyloplasts, during which starch is broken down and replaced by carotenoid crystals to form chromoplasts.

Our stereomicroscopy observations verified that the maturation of SLS is accompanied by a change in color from colorless to pale yellow and then to dark yellow and finally to red. In this study we postulated that SLS formation is related to the formation of meristemoids. As part of our effort to understand and control factors that can increase the potential of parenchyma tissues to form meristemoids, we examined surgical injury of ovary tissue as a possible mechanism of SLS induction. It proved very successful, yielding more SLS (45%) than was achieved in previous work (Karamian, 2000). A proper combination of exogenous hormones, as well

as tissue culture conditions such as temperature and the age and physiological condition of explants (Plessner and Ziv, 1999), are also critical to the formation of meristemoids. By increasing the number of meristematic areas, directly throughout parenchyma tissues or indirectly in the form of globular structures, we were able to obtain more SLS from given explants. SLS formation is accompanied by crocin apocarotenoid biosynthesis (Bouvier et al., 2003).

Using sensory analysis, Sarma et al. (1991) found that production of color (crocin) in vitro was one tenth that of natural stigmas in *C. sativus*. Applying different chemical analyses to compare apocarotenoid constitution in SLS with that in natural stigmas, Ebrahimzadeh et al. 2000 found color production to be similar in vitro and in vivo. Loskutov et al. (1999), however, reported a considerably higher amount of crocin in SLS. Comprehensive biochemical analyses of the crocin (color), picrocrocin (bitter taste) and safranal (aroma) produced from SLS obtained in culture should help resolve questions about the yield from in vitro production of this invaluable commodity.

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