# SOMATIC EMBRYOGENESIS AND REGENERATION OF PLANTLET IN SAFFRON, *CROCUS SATIVUS* L.

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## Abstract

Somatic embryogenesis was initiated in *C. sativus* L. from shoot meristems on LS medium containing BAP ($2\times10^{-5}$ M)+NAA ($2\times10^{-5}$ M). Various stages of somatic embryogenesis were observed in the same medium and the development was asynchronous. Somatic embryo development proceeded through well recognized sequences (globular to embryoids) with clearly discernible bipolar regions. Matured embryos could be germinated on half-strength MS medium containing $GA_3$ (25 mg/l). Complete plantlets with well developed root system and corm formation were obtained on transferring germinated embryos to half strength MS medium supplemented with BAP ($5\times10^{-6}$ M)+NAA ($5\times10^{-6}$ M).

## Introduction

Saffron (*Crocus sativus* L.) is a monocotyledonous plant of the *Iridaceae* family that produces annual renewal corms which are indispensable for their propagation, since the plant is triploid and sterile. This plant is vegetatively propagated where breeding is generally difficult [4,5]. In recent years, there is increasing interest to exploit tissue culture and genetic engineering techniques for propagation and genetic improvement of saffron [1]. Plant regeneration via organogenesis [2,6-9], embryogenesis [1] and protoplast has been reported [10]. The present study reports induction of somatic embryogenesis from shoot meristem culture and subsequent plantlet regeneration in saffron at high frequency. Therefore, it may be used as an alternative method for propagation of saffron plants and development of artificial seed technology.

**Keywords:** *Crocus sativus* L.; Plant growth regulators; Plantlet regeneration; Somatic embryogenesis; Tissue culture

## Materials and Methods

Saffron plants grown in Karaj farm were utilized as source of explants. Microcorms with small portion of corm attached were separated from sprouted corms, collected during the following period. They were thoroughly washed with tap water and surface sterilized with 0.1% $HgCl_2$ for 10 min and rinsed three times in sterile distilled water. Shoot meristems were dissected and cultured on Linsmaier and Skoog (LS) basal medium [11] containing 3% sucrose, cytokinins [benzylaminopurine (BAP), kinetin (kn)] and auxins [indole acetic acid (IAA), naphthalene acetic acid

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**Table 1.** Morphogenetic response of bulblet examples (40 pieces) cultured on LS medium supplemented with growth regulators in different combinations (values are mean of two experiments)

| Growth regulators Aux+Cyt | [1]Concentration of Aux+Cyt | Number of responding explants | Callusing % | Embryogenic Callusing % | Callus growth | Morphogenetic nature |
|---|---|---|---|---|---|---|
| NAA+BAP | $A_1$ | 35 | $87.5^{a}$ | $40^{a}$ | ++++ | Compact, nodular embryogenic |
| | $B_1$ | 26 | $65^{ab}$ | $32.5^{ab}$ | +++ | |
| | $C_1$ | 24 | $60^{b}$ | $35^{ab}$ | +++ | |
| 2,4-D+BAP | $A_2$ | 20 | $50^{bc}$ | $32.5^{ab}$ | +++ | Yellowish soft friable and compact nodular |
| | $B_2$ | 13 | $32.5^{d}$ | $27.5^{b}$ | ++ | |
| | $C_2$ | 11 | $27.5^{de}$ | $30^{b}$ | ++ | |
| IAA+BAP | $A_3$ | 3 | 7.5e | $0^{d}$ | – | Very slow growing compact callus |
| | $B_3$ | 5 | $12.5^{e}$ | $0^{d}$ | – | with growing buds |
| | $C_3$ | 3 | $7.5^{e}$ | $0^{d}$ | – | |
| NAA+Kn | $A_4$ | 14 | $35^{d}$ | $20^{bc}$ | + | Compact hard callus with green buds |
| | $B_4$ | 24 | $60^{b}$ | $17.5^{c}$ | + | |
| | $C_4$ | 16 | $40^{cd}$ | $15^{c}$ | + | |
| 2,4-D+Kn | $A_5$ | 21 | $52.5^{bc}$ | $25^{bc}$ | +++ | Brownish soft friable and compact nodular |
| | $B_5$ | 19 | $47.5^{c}$ | $25^{bc}$ | +++ | |
| | $C_5$ | 8 | $20^{de}$ | $22.5^{bc}$ | +++ | |
| IAA+Kn | $A_6$ | 10 | $25^{de}$ | $15^{c}$ | + | Whitish hard callus with growing buds |
| | $B_6$ | 10 | $25^{de}$ | $12.5^{c}$ | + | |
| | $C_6$ | 6 | $15^{de}$ | $15^{c}$ | + | |

[1]Concentration of cytokinins and auxins in different combinations were A: cytokinin ($2\times10^{-5}$ M)+auxin ($2\times10^{-5}$ M); B: cytokinin ($2\times10^{-5}$ M)+auxin ($5\times10^{-6}$M) and C: cytokinin ($5\times10^{-6}$M)+auxin ($2\times10^{-5}$ M). Callus growth has been denoted as (–) no response; (+) 2 mm diameter; (++) 2-3 mm diameter; (+++) 4-5 mm diameter; (++++) >5 mm diameter. Values in each column marked by different letters are significantly different at 0.05 level.

(NAA), 2,4-dichlorophenoxy acetic acid (2,4-D)] (Table 1). All media were adjusted to pH 5.7-5.8 with NaOH or HCl 1 N prior to addition of agar (0.7%) and autoclaved at 120°C for 20 min. Cultures were incubated at 22±1°C in dark. The experiment was repeated twice using 40 explants per treatment. The data for callus initiation were scored after two transfers (8 weeks). Callus initiation frequency was calculated in percentage of cultured shoot tips producing callus. Data from total of two replications of experiments were statistically analyzed using a SAS program (1987). Embryogenic nature of cultures was maintained by visual identification and selection of embryogenic sectors and removal of nonembryogenic portions at the time of subculturing. Embryogenic calli (20 days old) were transferred to half-strength liquid MS basal medium [12] without growth regulators under constant agitation on rotary shaker (80 rpm) for maturation of somatic embryos. For germination, somatic embryos showing bipolarity were separated and transferred to half-strength solid MS medium supplemented with gibberellic acid ($GA_3$) at different concentrations (5, 10, 15, 20, 25 mg/l). Ten replicates were taken for each treatment. Data from total of replicates were statistically analyzed using a SAS program (1987) (Table 2). For plant regeneration, germinated somatic embryos were transferred to half-strength MS basal medium containing BAP ($5\times10^{-6}$ M)+NAA ($5\times10^{-6}$ M) and incubated at 22±1°C under 12 h (light/dark) photoperiod, provided by cool white fluorescent tubes.

## Results and Discussion

Callus initiation from cultured meristem was first observed 8 weeks after incubation. Frequency of callus

**Table 2.** Effect of different concentration of $GA_3$ on germination of saffron somatic embryos

| Concentration of $GA_3$ (mg/l) | % Production of root | % Production of root and shoot |
|---|---|---|
| 5 | $60^{abc}$ | $0^{c}$ |
| 10 | $70^{ab}$ | $10^{c}$ |
| 15 | $75^{ab}$ | $10^{c}$ |
| 20 | $85^{a}$ | $25^{bc}$ |
| 25 | $85^{a}$ | $40^{ab}$ |

Values are mean of ten replicates per each treatment. Values in each column marked by different letters are significantly different at 0.05 level.



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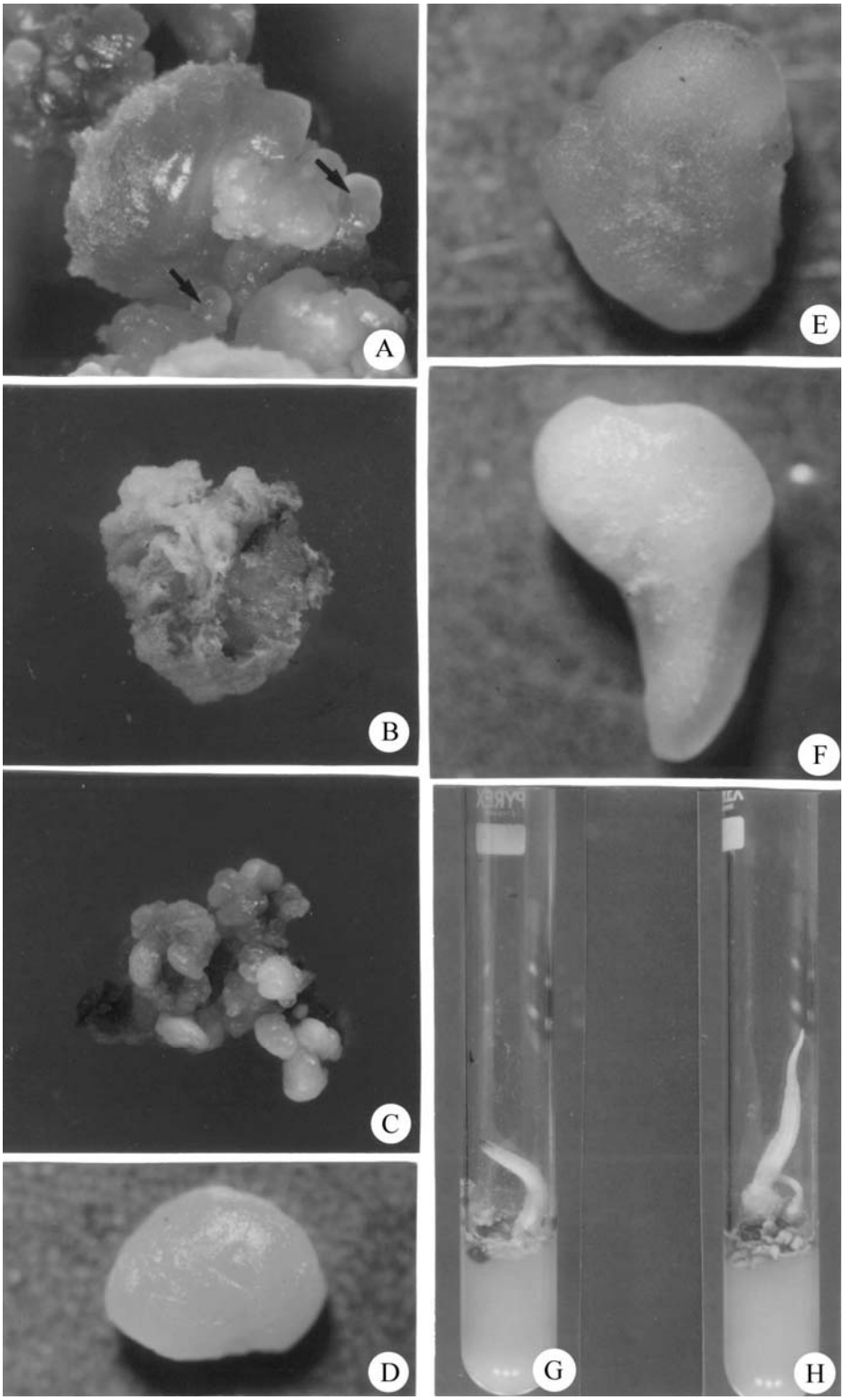


**Figure 1.** Plantlet regeneration via somatic embryogenesis in *C. sativus* L. A: appearance of nonembryogenic, embryogenic and clusters of embryoids; B: nonembryogenic, friable calli; C: embryogenic calli; D: globular embryoids ×20; E: heart-shaped somatic embryo ×20; F: torpedo somatic embryo showing bipolarity ×20; G: plantlet regeneration from somatic embryo; H: plantlet with corm.



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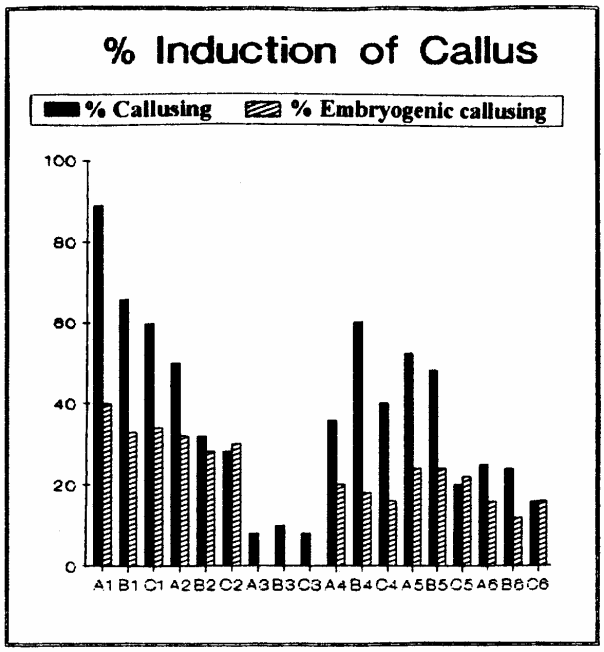


**Figure 2.** Induction of callus and embryogenesis in LS medium with different plant growth regulators.

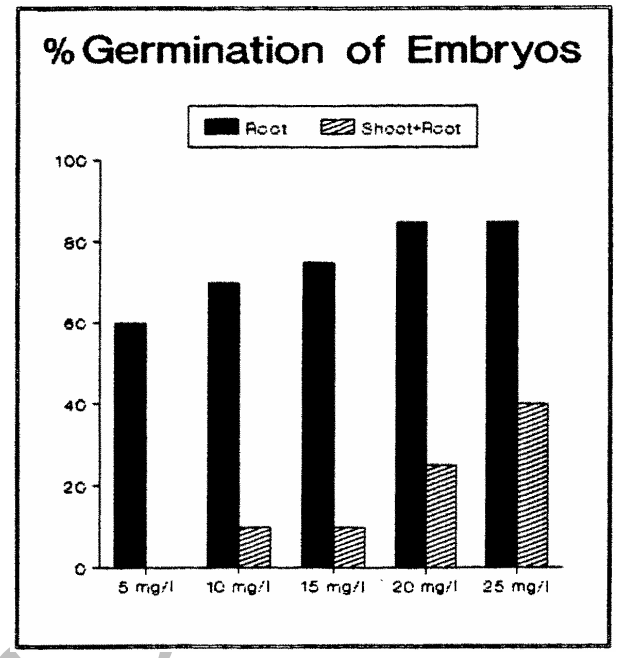


**Figure 3.** Germination of saffron somatic embryos in MS medium with gibberellic acid at different concentration.

initiation, production and morphogenetic nature of callus varied with the kind of media and growth regulator used (Tables 1, 2). Frequency of callus induction ranged from 7.5-87.5%. Highest frequency of callus induction (87.5%) and embryogenesis (40%) was recorded on LS medium supplemented with BAP ($5\times10^{-6}$ M) and NAA ($5\times10^{-6}$ M). This is suggestive of cytokinin and auxin requirement for induction of embryogenesis and growth of callus in saffron (Fig. 2). During initial stages of callus development, golden yellowish, soft, translucent calli appeared from cultured explants, which after subsequent transfers were visually distinguishable to be of two types: the embryogenic callus (EC) which was compact, opaque and whitish in appearance with shining globular embryogenic regions (Fig. 1A), and nonembryogenic callus (NEC) which was yellowish to brownish, soft, friable and translucent (Fig. 1B). Production of embryogenic callus was initially slow but careful selection of embryogenic regions and frequent subculturing resulted in vigorous proliferation of embryogenic callus (Fig. 1C). The asynchronous mode of development where the appearance of soft, translucent nonembryogenic calli, embryogenic calli and group of somatic embryos often loosely attached to the parent tissue as observed in the present case, has been reported to be a typical feature of a number of monocots [13]. Somatic embryo development in the present case proceeded through various stages of globular, heart-shaped and torpedo like embryos (Figs. 1D-F) which agrees with general development protocol of plant regeneration through somatic embryogenesis reported in a number of plants [14]. At each subculture, the embryogenic callus divided into clumps which consisted of several embryos when left on the same medium for eight or more weeks. 80% of the embryos geminated forming roots without any shoot. Transfer of embryogenic calli to half-strength MS basal medium without growth regulator, resulted in early maturation of somatic embryos. Somatic embryos showing bipolarity could be germinated on half-strength MS medium containing $GA_3$. Among the five concentrations of $GA_3$, the highest frequency of germination of somatic embryos was recorded at 25 mg/l of $GA_3$ (Fig. 3). In this medium radicle emerged from somatic embryos within two weeks. The highest frequency of somatic embryo germination with well developed root and shoot was 40% (fig. 3). Germinated somatic embryos transferred into half strength MS medium containing BAP ($5\times10^{-6}$ M)+NAA ($5\times10^{-6}$ M) resulted complete plantlet development after 8-10 weeks of inoculation at 22±1°C under light/dark cycle of 12 h (Fig. 1G). Plantlet thus developed, were left to grow in culture under the same conditions without further subculture produced corm (Fig. 1H). Then regenerated plantlets were kept on a medium with low nutrient levels to harden them before transfer to pots. Physiological immaturity or anatomical abnormalities, especially at the shoot apex may be one of the reasons for low frequency of embryos to form plantlets as

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reported earlier [1,15]. Tissue culture of *C. sativus* has been reported earlier [6] and bud development has been obtained from the cut surface of corms. Plantlets were also developed from callus culture of *C. sativus* using corm explants [2,7-9]. Regeneration of protoplast isolated from the cells of the suspension culture has been reported in saffron [3,10]. In the present study, callus cultures derived from meristems, facilitated the proliferation of plantlets from somatic embryos and proved to be an useful alternative method for micropropagation. It may be used for the isolation of totipotent protoplasts from embryogenic cell suspension [16] and development of artificial seed technology [17].

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