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Effect of Primary and Secondary Somatic Embryogenesis in Safflower (*Carthamus tinctorius* L) at Morphological and Biochemical Levels

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Abstract: An efficient somatic embryogenesis system has been established in *Carthamus tinctorius* (L.) in which primary and secondary embryogenic calluses were developed from hypocotyls and primary cotyledonary somatic embryos (PCSEs). Two types of calluses were different in morphology and growth behaviour. Hypocotyl-derived embryogenic callus (HEC) was friable and fast-growing, while secondary callus derived from PCSE was compact and slow-growing. HEC differentiated into somatic embryos which proliferated quickly on medium supplemented with thidiazuron (TDZ) (1.0 mg l^{-1}) and spermidine (SPD) (1.5 mg l^{-1}). Although differentiation and proliferation of somatic embryos were faster in primary HEC, maturation and germination efficiency were better in somatic embryos developed from primary cotyledonary somatic embryo derived secondary embryogenic callus (PCSEC). At the biochemical level, two somatic embryogenesis systems were different. Both primary and secondary adventives somatic embryogenesis and the role of plant growth regulators in two modes of somatic embryo formation have been discussed.

Abbreviations: 2,4-D-2,4-dichlorophenoxyacetic acid, CPA-Chlorophenoxy acetic acid, HEC-Hypocotyl-derived embryogenic callus, HECSE-HEC-derived somatic embryo, IAA-Indole-3-acetic acid, NAA-a-naphthalene acetic acid, PCSE-Primary cotyledonary somatic embryo, PCSEC-PCSE-derived secondary embryogenic callus, SE-Somatic embryogenesis,(TDZ)-Thidiazuron, (SPD)-spermidine.

Key words: Amino acid · *Carthamus tinctorius* · *Compositae* · Spermidine · Somatic embryogenesis

INTRODUCTION

Safflower, *Carthamus tinctorius* L., is a member of the family *Compositae* cultivated mainly for its seed, which is used as edible oil and as birdseed. This crop was also grown for its flowers, used for colouring and flavouring foods and making dyes. Safflower is a highly branched, herbaceous, thistle-like annual, usually with many long sharp spines on the leaves. It has become an increasingly important crop in Turkey and the world due to the rich nutritional value of its edible oil. It is a rich source of oil (35-40%) and linoleic acid content (75-86%). It remains a minor crop with the world's seed production, around 8, 00, 000 tonnes per year [1].

Conventional breeding has helped in developing some elite cultivars, while *in vitro* technology could serve as an alternate means for further genetic upgrading, its successful application depends largely on a reliable plant regeneration system. Regeneration of whole plant transformants of safflower (*Carthamus tinctorius* L.) following *Agrobacterium tumefaciens*-mediated transformation has largely been unsuccessful [2-4]. Although several reports of *in vitro* regeneration of safflower have been published [5, 6] an efficient plant regeneration system applicable to a wide group of genotypes cultivars is still lacking. Sensitivity of regenerated shoots to media water content and high relative humidity in the culture vessels, differential rooting response among cultivars to the auxin source [7] and inhibition of culture growth by selection antibiotics [2] presently restrict genetic engineering of safflower to only those cultivars in which efficient whole plant regeneration *in vitro* has been demonstrated.

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When compared to other methods of *in vitro* propagation, somatic embryogenesis is favoured over other method of vegetative propagation because of the possibility to scale up, the propagation by using bioreactors [8].Often, somatic embryos or the embryogenic cultures can be cryopreserved [9, 10] which makes it possible to establish gene banks [11]. Furthermore *in vitro* somatic embryogenesis is an important prerequisite for the use many biotechnological tools for genetic improvement [12]. In this regard, somatic embryos play a key role in current genetic transformation methods. Spermidine (SPD), is a diamine obligate precursor, are small aliphatic polyamines that are ubiquitous in all living cells, extensive studies reported their role in a wide range of biological and physiological processes [13-15]. In recent years, research on polyamines has increased tremendously and has provided clues to improve plant developmental processes, including somatic embryogenesis, in a variety of economically important crops. Several reports have shown the involvement of polyamines, particularly in their free forms, in somatic embryogenesis. High concentrations of polyamines were commonly observed in tissues undergoing somatic embryogenesis [16-19].

In this study, an efficient somatic embryogenesis and rapid plant regeneration system have been established from hypocotyls and cotyledonary explants for primary and secondary somatic embryogenesis. Biochemical differences and the role of plant growth regulators in somatic embryo proliferation, maturation and germination into plantlets have also been discussed.

MATERIAL AND METHODS

Plants Material and *In vitro* Germination: Seeds of safflower (*Carthamus tinctorius* L.) cv. NARI-6 collected at the Nimbhkar Agricultural Research Institute, Maharashtra, India were used for establishing embryogenic cultures. Seeds were further sterilized as described by [20]. The surface sterilized seeds were aseptically germinated in Murashique and Skoog's germination (MSG) medium [21].

Induction of Callus and Primary Somatic Embryos: For induction of embryogenic callus, hypocotyls were cultured on MS medium supplemented with different auxins 2, 4-D (1.0 mg l^{-1}) + NAA (0.5 mg l^{-1}) +CPA (0.5 mg l^{-1}) and TDZ (1.0 mg l^{-1}). This type of embryogenic callus, referred to as hypocotyls derived embryogenic callus (HEC), was maintained with periodic sub culturing at an interval of three weeks. The HEC and somatic embryos derived from it (referred to as HECSEs) were used for maturation, germination and plant conversion studies. All cultures were incubated under a 16 h photo-period. The mature bipolar early cotyledonary stage embryos were developed directly from the torpedo stage embryos and were maintained on same medium with the same culture conditions for further development. Total number of primary somatic embryos and percentage of response was recorded at the end of 30 d of culture.

Proliferation of Secondary Somatic Embryogenesis: Somatic embryos were proliferated from friable calluses (20-30 mg) on MS medium supplemented with NAA (1.0 mg l^{-1}) +KN (0.5 mg l^{-1}) and different concentrations of BA (2.5 mg l^{-1}) +SPD (0.5 mg l^{-1}). Secondary calluses, induced on primary hypocotyls type somatic embryos (developed from HEC),were further used for proliferation in the same medium to compare the differences in their rate of proliferation, if any secondary somatic embryos were also obtained from primary hypocotyls somatic embryos on the same the per proliferation medium within 6-9 weeks. The number of secondary somatic embryos per primary somatic embryo was recorded.

Suspension Culture: For establishing suspension culture, secondary calluses were dissected from primary hypocotyls somatic embryos and cultured in liquid MS medium supplemented with BA (1.0 mg l^{-1}) $^{+}$SPD (0.5 mg l^{-1}) cultures were placed on a rotary shaker at 120 rpm at 25±2°C. After four days (10h day-1), the suspension was filtered in a laminar hood with sterile Whatman filter similarly cultured on proliferation medium supplemented with NAA(1.0 mg l^{-1}) +KN (0.5 mg l^{-1}) and BA (1.0 mg l^{-1}) + SPD (0.5 mgl^{-1}), where a heterogeneous masses of somatic embryos were produced. This type of callus derived from PCSE was referred to as PCSEC. The embryogenic competence and subsequent regeneration ability of PCSEC was monitored and compared with that of HEC. Before maturation and germination, percent somatic embryogenesis and number of somatic embryos at different stages, viz., globular, heat torpedo and cotyledonary were recorded.

Maturation of Somatic Embryos: For maturation, white opaque hypocotyls somatic embryos from both HEC and PCSEC were cultured on MS medium supplemented with GA_3 (1.0 mg l^{-1}) and 30 g l^{-1} from maltose since the carbon

sources were earlier found to be very effective in maturation of somatic embryos in *Catharanthus roseus* [22]. The percent maturation of somatic embryos and their growth were recorded from 4th week onwards. Matured somatic embryos (20 per culture) from both HEC and PCSEC were cultured on MS medium containing BA (0.5 mg l^{-1}) and 30 gl^{-1} maltose for ensuing quick germination, as reported earlier [23]. Growth features, like rooting, shoot development and conversion to plantlets were recorded at periodic intervals. Well-germinated mature hypocotyls embryos with roots were transferred into rooting medium MSG basal salts supplemented with NAA (1.5 mg l^{-1}) + KN(0.5 mg l^{-1}) and putrescine (0.5 mg l^{-1}). After 15 days, the rooting results were recorded. The experiment was repeated 4 times.

Acclimatization: The plantlets with adventitious roots were carefully washed with tap water to remove medium and then transferred to plastic pots containing sand, garden soil and Vermiculite (1:2:1). Potted plants were incubated at 25 °C for 10 d under irradiance of 30 µmol m^{-2} s^{-1}. After emergence of new leaves, the acclimated plants were transplanted into greenhouse and ?nally planted in 100% soil under natural conditions.

Estimation of Protein: One gram fresh callus from the primary and secondary somatic embryos from *in vitro* plants were homogenized in 2 ml of 50 mM potassium phosphate buffer, pH 7.5, containing 2 mM EDTA and were centrifuged at 15,000g for 20 min at 4°C. Protein content in the supernatant was concentrated by ammonium sulfate precipitation and fltered through Whatman No.1 flter papers. The optical density was measured at 595 nm with a spectrophotometer. Protein content was determined by Bradford method [24].

Estimation of Free Amino Acids: Free amino acids were estimated by the method of [25]. In brief, 0.5 g callus tissue was incubated overnight in 70% methanol followed by washing with double distilled water. Then 1.5 ml of 55% glycerol and 0.5 ml ninhydrin solution were added, boiled at 100°C for 20 min and cooled down. The final volume was made up to 6 ml with double distilled water and the optical density was measured at 570 nm as described above.

Estimation of Total Sugars: Total sugars in developing somatic embryos and different parts of somatic embryo-derived plantlets were estimated according to [26]. All samples (0.5 g) were extracted twice with 90% ethanol and the extracts were pooled. The ?nal volume of the pooled extract was made up to 25 ml with double distilled water. To an aliquot of the extract, 1.0 ml of 5% phenol and 5.0 ml concentrated analytical-grade sulphuric acid were added and the ?nal volume was made up to 10 ml with double distilled water. The optical density was measured at 485 nm as described above. A solution containing 1.5 ml of 55% glycerol, 0.5 ml ninhydrin and 4.0 ml double distilled water was used as a calibration standard.

Statistical Analysis: The data on the effects of growth regulator on different stages of primary and secondary embryogenesis and other parameters were analyzed by one-way analyses of variance (ANOVAs). Values are means of ?ve replicates from two experiments and the presented mean value were separated using Duncan's Multiple Range Test (DMRT) both carried out using SPSS (version.12.0) software package, were used for expressing the statistical signi?cance obtained by comparing plant regeneration in primary and secondary somatic embryogenesis.

RESULTS

Callus Induction: The embryogenic callus of *Carthamus tinctorius* (Fig.1A and B) was initiated from hypocotyls on MS medium supplemented with 2, 4-D (1.0 mg l^{-1})+ CPA(0.5 mg l^{-1})+ NAA(0.5 mg l^{-1}) and TDZ (1.0 mgl^{-1}). A maximum of 96% callusing was recorded. Beside 2, 4-D, other synthetic auxins, such as NAA, TDZ and CPA were also effective in callus induction, but with low to moderate intensity. High embryogenic callus induction was achieved by continuous sub culturing on fresh nutrient medium for two weeks; the callus was friable, light yellow and fast-growing.

Somatic Embryo Initiation and Proliferation from Hypocotyls Callus: Various concentrations of BA (0.50-2.5 mg l^{-1}) were used to induce embryogenic callus on MS medium. However, somatic embryos developed rapidly on medium containing NAA (1.5 mg $l^{-1)}$ $^{+}$KN (0.5 mg l^{-1}). Somatic embryogenesis could be further improved when BA (1.0 mg l^{-1}) + SPD (0.5 mg l^{-1}) was added to the NAA+KN medium. Table 1 summarizes the response, which shows that NAA (1.0 mg l^{-1}) +KN (0.5 mg l^{-1}) +BA (1.5 mg l^{-1}) +SPD (0.5 mg l^{-1}) had a maximum favourable effect on somatic embryogenesis.

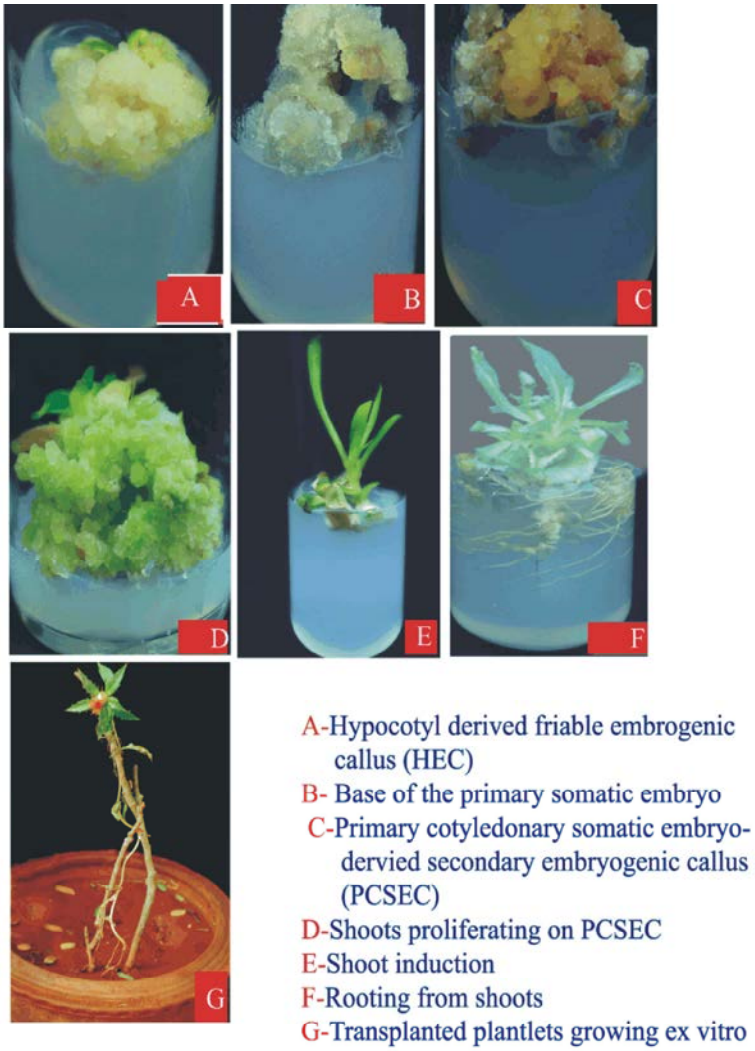

Fig. 1: Primary and secondary somatic embryogenesis in *Carthamus tinctorius* L.

Proliferation of Secondary Somatic Embryogenesis: Hypocotyl-derived somatic embryos were maintained on the same proliferation medium by repeated sub culturing. Three distinct responses were observed when somatic embryos, especially the hypocotyls derived type, were cultured on NAA(1.0 mg l^{-1})+KN (0.5 mg l^{-1}) and BA (1.0 mg l^{-1})+SPD (0.5 mgl^{-1})-containing proliferation media: (a) secondary somatic embryogenesis, (b) secondary callusing (Fig. 1C) and (c) secondary callusing plus secondary somatic embryogenesis (Table 2). Scanning electron microscopic observations showed that secondary somatic embryos were formed at the base of the primary somatic embryos. The induction of secondary callus and its embryogenic response were thoroughly evaluated and compared with that of primary callus induced from hypocotyls. Morphologically, secondary callus was compact, hard, yellowish and grew very slowly compared with hypocotyls-induced primary callus (HEC), which was friable, transparent to watery at initiation stage and relatively fast-growing. The hypocotyls responded quickly and within 7-10 days callusing occurred compared with PCSE-induced secondary callus, where a minimum of two to three weeks' incubation was necessary for callus induction. PCSE-derived secondary calluses were isolated (Fig. 1C, D) and transferred to liquid MS medium supplemented with 2, 4-D(1.0 mg l^{-1})+ NAA(0.5 mg l^{-1})+CPA (0.5 mg l^{-1}) and TDZ (1.0 mg l^{-1}) for faster embryogenic callus growth. Secondary callus masses (40-50 mg) were cultured on the same somatic embryo induction and proliferation media containing various concentrations of NAA (0.5 mg l^{-1}) and TDZ (1.0 mg l^{-1}). Within 2-3 weeks, secondary callus proliferated and

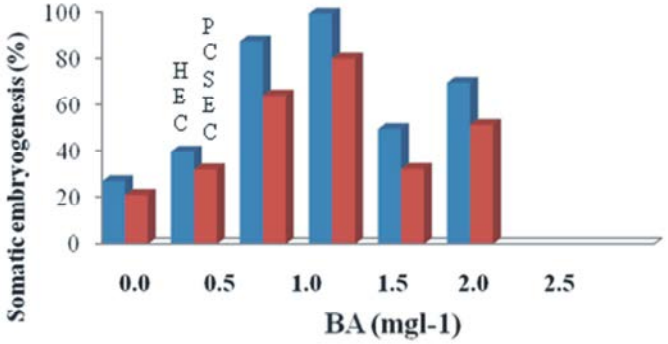

Fig. 2: Effect of BA on primary and secondary somatic embryogenesis in *Carthamus tinctorius* L. The basal medium is based on MS medium supplemented with 1.0 mg l^{-1} NAA. HEC = Hypocotyl-derived embryogenic callus and PCSEC = Primary cotyledonary somatic embryo-derived secondary embryogenic callus

Table 1: Effect of BA on somatic embryogenesis from two different callus lines after seven weeks culture on MS medium supplemented with 1.0 mg l^{-1} NAA, 0.5 mgL^{-1} Kin and 0.5 mgL^{-1} SPD

BA +	Total No. of Somatic embryos		Number of Somatic embryos Globular Heart Torpedo Cotyledonary							
(Mg l^{-1})	HEC[A]	PCSEC	HEC	PCSEC	HEC	PCSEC	HEC	PCSEC	HEC	PCSEC
0.0	27.0e[B]	21.2e	15.7	14.2e	9.4a	6.8e	4.9e	3.4e	2.1e	1.0d
0.5	39.9d	32.1de	23.9	21.3de	19.3d	12.1d	11.2d	9.3d	8.5c	1.0d
1.0	87.4b	64.3b	65.4	61.2b	32.5b	26.4b	24.3b	21.3b	18.3b	6.2b
1.5	99.4a	80.1a	81.5	79.4a	59.3a	43.2a	39.4a	32.3a	25.4a	8.3a
2.0	49.6d	32.3c	34.5	31.3d	19.1d	14.2d	11.3d	9.8d	7.8d	4.0c
2.5	69.7c	51.4c	50.4	48.9c	24.2c	20.1c	13.4c	10.5c	9.4c	3.1c

[A] HEC = Hypocotyl-derived embryogenic callus; PCSEC = Primary cotyledonary somatic embryo-derived secondary embryogenic callus.

[B] Means with common letters within a column are not signi?cantly different at P = 0.05, according to Duncan's Multiple Range Test (DMRT).

Table 2: Effect of BA on secondary callusing and somatic embryogenesis from primary cotyledonary somatic embryos (PCSEs) after six weeks' culture on MS medium supplemented with 1.0 mg l^{-1}NAA

BA(mg l^{-1}) Secondary	Secondary Callusing (%)	Secondary somatic embryogenesis (%)	Secondary callusing + secondary somatic embryogenesis (%)	Number of somatic embryos /PCSE
0.0	10.0e	18.0e	22.4e	2 1.9e
0.5	25.3 d	34.3d	44.5d	33.5d
1.0	37.8 c	55.4c	69.4c	52.2c
1.5	77.4 a	82.3a	86.5a	66.5b
2.0	55.3 b	66.4b	72.3b	69.5a
2.5	14.6e	46.5d	45.3d	51.1c

[A] Means with common letters within a column are not signi?cantly different at P = 0.05, according to Duncan's Multiple Range Test (DMRT).

produced somatic embryos of different morphological stages that could be easily isolated. A comparative analysis involving HEC and PCSEC systems revealed that somatic embryogenesis in terms of both percentage and number of somatic embryos proliferated was higher in HEC than in PCSEC.

Mature of Somatic Embryos: For maturation, morphologically advanced white opaque hypocotyls somatic embryos were individually isolated from each stock and were cultured on maturation medium, which primarily contained GA3 (1.0 mg l^{-1}) with maltose (30 g l^{-1}). In contrast to proliferation, PCSEC-derived somatic embryos matured faster (Fig. 1D) than HEC-derived ones. The maturation of somatic embryos was higher in maltose-medium. The somatic embryos developed from PCSEC were larger in size and their growth was found to be maintained even after nine weeks of growth period (Table 3).

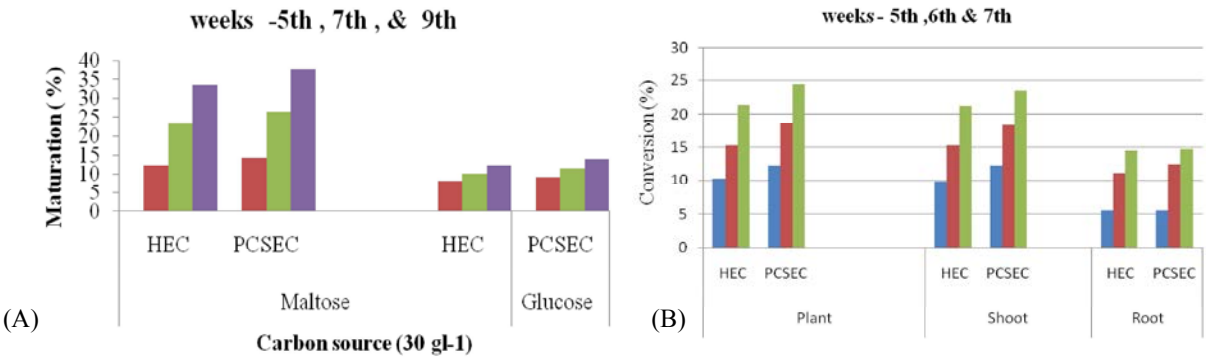

Fig. 3: Factors affecting the maturation and conversion of somatic embryos in *Carthamus tinctorius* L. (A) Effects of carbon sources on the maturation of somatic embryos developed from two different types of calluses; the maturation medium is based onMS medium supplemented with 1.0 mg l^{-1} GA3.(B) Conversion of somatic embryos, developed from two different types of calluses, into roots, shoots and plantlets; the conversion medium is based on MS medium supplemented with 0.5 mg l^{-1} BA plus 60 g l^{-1} maltose. HEC = Hypocotyl-derived embryogenic callus and PCSEC = Primary cotyledonary somatic embryo-derived secondary embryogenic callus.

Table 3: Effect of two different carbon sources on the maturation of somatic embryos (Length, mm) on MS medium supplemented with 1.0 mg l^{-1} GA_3

	Maltose (30 g l^{-1})		Glucose (30 g l^{-1})	
Growth period (weeks)	HEC[A]	PCSEC	HEC	PCSEC
5	12.3b[B]	14. 2a	8.3d	9.1c
7	23.4b	26.4a	10.3c	11.6b
9	33.4b	37.6a	12.4c	14.1b

[A] HEC = Hypocotyl-derived embryogenic callus;
PCSEC = Primary cotyledonary somatic embryo-derived secondary embryogenic callus.
[B] Means with common letters within a row are not signi?cantly different at P = 0.05,
according to Duncan's Multiple Range Test (DMRT)

Table 4. Effect of BA (0.5 mg l^{-1}) on the conversion of somatic embryos in liquid MS medium supplemented with 60 g l^{-1} maltose

	Plant-conversion (length, mm)				Shoot-conversion (length, mm)		Root-conversion (length, mm)	
Growth	Root	Shoot						
period (weeks)	HEC[A]	PCSEC	HEC	PCSEC	HEC	PCSEC	HEC	PCSEC
5	10.3	12.3	11.2	11.5	9.8	12.3	5.5	5.5
6	15.3	18.5	11.5	12.3	15.3	18.4	11.1	12.4
7	21.4	24.5	13.2	14.3	21.3	23.5	14.5	14.8

[A] HEC = Hypocotyl-derived embryogenic callus; PCSEC = Primary cotyledonary somatic embryo-derived secondary embryogenic callus.
[B] Means with common letters within a row are not signi?cantly different at P £ 0.05,
according to Duncan's Multiple Range Test (DMRT).

Table 5: Biochemical characterization of two different embryogenic callus lines and somatic embryos at various developmental stages

	Embryogenic callus		Proliferated somatic embryo		Matured somatic embryo		Germinated somatic embryo (mg g^{-1} FW)	
Parameters	HEC[A]	PCSEC	HEC	PCSEC	HEC	PCSEC	HEC	PCSEC
Protein	3.3e[B]	4.0d	4.5d	4.9c	5.5c	5.8b	6.7bc	7.2a
Amino acid	2.0d	2.1cd	3.2c	4.3bc	5.4b	6.5b	7.2b	7.7a
Sugar	29.2a	19.3ab	16.6b	14.3d	11.7c	10.9e	11.3e	9.7f

[A] HEC = Hypocotyl-derived embryogenic callus; PCSEC = Primary cotyledonary somatic embryo-derived secondary embryogenic callus
[B] Means with common letters within a row are not signi?cantly different at p £ 0.05,
according to Duncan's Multiple Range Test (DMRT)

Germination and Conversion of Somatic Embryos: Dark-green matured somatic embryos from both HEC and PCSEC were similarly cultured on MS medium supplemented with 0.5 mg l^{-1} BA for germination and conversion to plantlets (Table 4) shows three types of responses and their growth: (a) plantlets with both shoots and roots, (b) juvenile shoots without any roots and (c) only roots without any shoots. Plantlet-conversion, in terms of not only conversion rate but also shoot and root growth, was much higher for somatic embryos developed from PCSEC (Fig.1E and Fig.1F). When the plantlets with well-developed roots and shoots were finally transferred to the field, about 100% survival occurred and they flowered normally (Fig.1G).

Biochemical Analysis at Different Stages of Somatic Embryogenesis: As the morphology and embryogenic response of HEC and PCSEC were different, biochemical analyses were carried out in both the types of somatic embryogenesis systems. In PCSEC, there was increase in protein and amino acids contents than in HEC. The amino acid and protein contents gradually increased with advancing stages of somatic embryogenesis (Table 5). In contrast, there was a decline in sugar content with increasing complexities in embryogenic process. Plant-lets regenerated from two callus lines (HEC and PCSEC) via somatic embryogenesis were also characterized for biochemical changes. We observed that there were no major quantitative changes in protein and amino acid contents HEC and PCSEC derived somatic embryogenesis.

DISCUSSION

Amino acids have been found critical to induce s omatic embryogenesis in plant tissue culture medium. In orchard grass, embryos formed on amino acid containing medium showed high percentage of conversion and considerably less incidence of precocious germination [27]. The yield of alfalfa embryos was also considerably improved when amino acids were added to callus maintenance medium. We report here a rapid plant regeneration system via primary and secondary somatic embryogenesis in *Carthamus tinctorius L.* Two different embryogenic callus, viz., primary hypocotyl-callus and secondary embryogenic callus derived from primary cotyledonary somatic embryos, produced repetitive somatic embryos in culture.

These two processes collectively produced a large number of somatic embryos within a limited time and space. In this system, secondary somatic embryogenesis was also operative where adventives somatic embryos regenerated directly from primary cotyledonary somatic embryos. The development of secondary somatic embryos (on primary somatic embryos) is, however, not uncommon in tissue cultures [28-32]. We found that a range of combinations of BA and NAA was useful for secondary somatic embryogenesis. The same combinations of growth regulators were earlier reported to be very effective in primary somatic embryogenesis in *Catharanthus roseus* [22]. In the present study, the proliferation, maturation and germination of somatic embryos were different in two systems involving separate tissue sources. The proliferation of somatic embryos was much higher in callus derived from primary cotyledonary somatic embryos. Although two callus-types were different biochemically, their differential responses to somatic embryogenesis (process) might be due to variation in the level of endogenous plant growth regulators. The difference in callus morphology and subsequent embryogenic competence was similarly observed in other plant systems [33,34]. Related studies with wild carrot have shown that SPD alone can restore embryogenesis in cultures treated with polyamine biosynthesis inhibitors, indicating a direct role of SPD in somatic embryogenesis [35, 36]. SPD was also the most abundant polyamine in conditions that allow cell cultures of *Papaver somniferum* to form embryo-like structures [37]. The morphology of somatic embryos and simultaneous accumulation of storage reserves have been shown to be a good indicator of their maturity and development [38]. It has also been demonstrated that this process is positively in?uenced by various compounds, like carbohydrates, sugar alcohol, PEG, etc. [39, 40, 23]. As a carbon source, the use of maltose and glucose for somatic embryo maturation has been reported in a number of studies [41, 42].

Somatic embryogenesis has several applications including mass propagation of plants. In *Carthamus tinctorius L*, we demonstrate a secondary somatic embryogenesis system, operative along with primary somatic embryogenesis originated from hypocotyls. Using both the methods, a large number of somatic embryos and plantlets were obtained which could be used as medicinal raw material for producing alkaloids. Moreover, the present protocol may offer an efficient and ideal system for large-scale genetic transformation in *Carthamus tinctorius.*

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