

Somatic embryogenesis, biochemical alterations and synthetic seed development in two varieties of coriander (*Coriandrum sativum* L.)

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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Abstract: Somatic embryogenesis (SE), biochemical alterations and synthetic seed formation were carried out in two *Coriandrum sativum* L. varieties (Rajendra Swathi 'RS' and Co-1). Callus was induced profusely in 1.0 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) added MS medium but Co-1 had more callus induction frequency (96.0%) compared to RS (89.3%). The callus turned into embryogenic tissue and variable embryogenic frequency (77.6% in RS and 72.8% in Co-1) was noted. Somatic embryos started to differentiate on the same 2,4-D added medium but the numbers of somatic embryos were more in RS (63.0 embryos per culture) compared to Co-1 (51.0 embryos per culture). These somatic embryos progressed well and showed maximum maturation in RS (78.7%) in 0.25 mg/l 6-benzyladenine (BA) + 0.5 mg/l α -naphthalene acetic acid (NAA) added medium. The biochemical analyses of non-embryogenic-, embryogenic-callus and different stages of embryos were conducted in order to know the changes of physiology in different tissues. Sugar and proline content were noted to be high at embryo induction stage while protein level was higher at embryo maturation stage. Biochemical analysis also revealed that the catalase (CAT) and superoxide dismutase (SOD) activities were higher at maturation stage of embryos compared to other embryogenic stages. Matured somatic embryos were germinated in MS added with 1.0 mg/l BA + 0.5 mg/l gibberellic acid (GA₃) in which 83.3% and 76.7% plantlet regeneration were noticed in RS and Co-1 respectively. Somatic embryos were encapsulated in various alginate and calcium chloride (CaCl₂) solutions and were kept in different temperature regimes for varied periods. On regeneration medium, the encapsulated embryos germinated into plantlets; in 3% sodium alginate + 100 mM CaCl₂, maximum plant regeneration (74.0% in RS and 70.6% in Co-1) was noted. The influence of low temperature on storage of synthetic seeds and their conversion into plantlets were also studied and we noted that the 4°C was the optimum temperature for synthetic seed conservation and plantlet regeneration compared to -20°C and 25°C temperature conditions.

1. Introduction

Coriandrum sativum L. is an annual herb and belongs to the family Apiaceae. The plant is used as a spice and flavouring compound (Burdock and Carabin, 2009). The plant has potential in pharmaceutical industry as it exhibits antimicrobial (Cao *et al.*, 2012), antioxidant (Hashim *et al.*, 2005), antidiabetic (Eidi *et al.*, 2009), hepatoprotective (Samojlik *et al.*, 2010) and antiarthritic properties (Rajeshwari *et al.*, 2012). Plant tissue culture technology is widely used for large scale plant propagation, beside being used as an *in vitro* experimental model for studying cellular processes like cell division, differentiation and morphogenesis, all play important key role in somatic embryogenesis (SE) and plant development (Zimmerman, 1993). SE is a process in which somatic cells undergo morphological and metabolic changes under *in vitro* cultural conditions, acquire embryogenic potency and later develop into somatic embryos (Feher *et al.*, 2003). Somatic embryos mimic zygotic embryos in various ways and hence it has proved to be a good model for studying morpho-physiological, biochemical and molecular events during the course of embryogenesis (Dodeman *et al.*, 1997). The fast identification of embryogenic tissue is very important as it plays a central role in marker-assisted selection (Dodeman *et al.*, 1997). Beside morphology, the embryogenic tissues are often differentiated from non-embryogenic callus by various biochemical markers and these markers based selection would be of immense value of SE based micro-propagation (Samar *et al.*, 2011). Embryogenic tissues have the ability of producing embryos for an extended period of time without any genetic alteration, which requires efficient conservation protocol for long-term preservation of coriander (Murthy *et al.*, 2008). Somatic embryos also have an important application in the formation of synthetic seeds and this artificial seed (an alternative to natural seed) technology may be exploited as a complementary method for *in vitro* production of plantlets (Reddy *et al.*, 2012). It also promotes fast plantlet regeneration, facilitates germplasm exchange and conservation (Palanyandy *et al.*, 2015). The synthetic seed technology with advantages like easy handling, conservation with germplasm exchange possibility is being widely used between national and international laboratories (Rai *et al.*, 2009). Beside bipolar somatic embryos, other explants like unipolar micro-bulbs, rhizomes, protocorms, nodal cuttings and shoot buds are used

for synthetic seed (Rihan *et al.*, 2011; Sharma and Shahzad, 2012). Among the various natural and synthetic polymers available for encapsulation, sodium alginate is used more frequently because of its easy gelling properties, non-toxicity and low cost (Saiprasad, 2001). Different concentrations of sodium alginate ranging from 1.5 to 6.0% are used in encapsulation in different plant species *Cacicus carota* (Latif *et al.*, 2007), *Spartina alterniflora* (Utomo *et al.*, 2008), *Catharanthus roseus* (Maqsood *et al.*, 2012), *Rinacanthus nasutus* (Meena *et al.*, 2013). In the present study, an efficient synthetic seed formation protocol was optimized in *C. sativum* and their regeneration potential was described after *in vitro* storage at different storage conditions. We also discussed SE and associated biochemical alterations in two varieties of *C. sativum*.

2. Materials and Methods

Seed germination and cultural conditions

Seeds of two varieties of *C. sativum*, Rajenda swathi (RS) and Co-1 were obtained from National Research Centre for Seed Spices (NRCSS) Ajmer, Rajasthan, India for this experimental study. The seeds were washed thoroughly under running tap water using cetrimide as detergent and surface disinfection was made with 0.1% (w/v) HgCl₂ for 2 min. The seeds were rinsed three times with sterilized double-distilled water before allowed to germinate on half strength MS medium (Murashige and Skoog, 1962) without plant growth regulators (PGRs). The basal medium was solidified by adding 8.0 g l⁻¹ agar. The pH of the medium was adjusted to 5.7 using 1.0 M NaOH and 0.1 N HCl and was sterilised in an autoclave for 15 min at 121°C. All the reagents were prepared using water supplied by a Milli-Q system (Billerica, Massachusetts, USA). The cultures were incubated at 25±2°C under 12-h photoperiod provided by cool white fluorescent lamps (100 µmol m⁻² s⁻¹ PFD).

Callus induction and somatic embryogenesis (SE)

Callus was induced from hypocotyl explants of 10-day old germinated seedlings. Different concentrations (0.5-2.0 mg/l) of 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.5-1.0 mg/l α-naphthalene acetic acid (NAA) were used for callus induction. After 4 weeks in induction medium, the callus was transferred in fresh medium for induction of somatic

embryos. Somatic embryos were induced on same 2,4-D added callus-induction medium. For embryo maturation, MS was supplemented with NAA (0.5-1.5 mg/l) and 6-benzyladenine, BA (0.25-0.5 mg/l). The germination of somatic embryos was made by transferring matured green embryos into MS, added with 0.5-2.0 mg/l BA and 0.2, 0.5 mg/l gibberellic acid (GA₃).

Biochemical analysis

Proline, protein and sugar assay. Proline estimation was made according to Bates *et al.* (1973). About 0.05 g of callus was homogenized in 2.0 ml of 3.0% aqueous sulphosalicylic acid under cold conditions; the homogenate was filtered with Whatman filter paper (No. 1). The filtrate (1.0 ml) was added with 1.0 ml ninhydrin and 1.0 ml glacial acetic acid; the reaction mixture was incubated for 1 h at 100°C. The reaction was terminated in ice bath and 2.0 ml toluene was added to it. Proline content was measured by spectrophotometric assay at 520 nm. The concentration of proline is expressed in mg per 1 g fresh weight.

Protein estimation was made following Bradford *et al.* (1976). The homogenate of 0.25 g callus in 1.5 ml phosphate buffer (0.1 M, pH 7.0) under pre-cold condition was centrifuged at 10⁴ rpm for 10 min and 1.0 ml supernatant was added with 0.5 ml trichloroacetic acid (10%). After centrifugation, the pellets were washed with acetone and dissolved in 1.0 ml of NaOH (0.1 N). To 1.0 ml of aliquot, 1.0 ml of Bradford reagent was added and optical density was measured at 595 nm. The protein concentration was expressed in mg per 1 g fresh weight.

Dey (1990) method was used for sugar estimation. Callus tissue 0.1 g was extracted twice with 90% alcohol at 60°C. Final volume of extract was made up to 10 ml by adding DDW. After mixing 0.5 ml of aliquot with 0.5 ml of 5% phenol, 1.0 ml of concentrated sulphuric acid was added and cooled in air. The optical density was measured at 485 nm. The sugar concentration was expressed mg per 1 g fresh weight.

Analysis of catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) activity. Calli at different stages such as non-embryogenic, embryogenic tissue and different embryo stages (induction, proliferation and maturation) were homogenized in 2.0 ml of 0.1 M extraction buffer (0.1 M K-phosphate, 0.5 mM EDTA, 1.0 mM ascorbic acid, pH 7.5). After centrifugation at 10⁴ rpm for 20

min, the supernatant was used for enzyme analysis.

CAT activity was determined according to Aebi (1984) method by measuring a decrease in the absorbance at 240 nm of reaction mixture containing 1.0 ml of 0.5 M reaction phosphate buffer (Na-phosphates, pH 7.5), 0.1 ml EDTA, 0.2 ml enzyme extract and 0.1 ml H₂O₂. The reaction was run for 3 min. One unit of enzyme determines the amount necessary to decompose 1.0 μM of H₂O₂ per min. CAT activity was calculated by using the co-efficient of absorbance at 0.036 mM⁻¹ cm⁻¹. The activity of CAT was expressed in EU mg⁻¹ protein.

The method developed by Nakano and Asada (1981) was used for APX activity. To the mixture of 1.0 ml sodium buffer (0.1 M, pH 7.2), 0.1 ml of EDTA and 0.1 ml of enzyme extract, 1.0 ml of 0.5 mM ascorbate were added and the reaction was run for 3 min at 25°C. The APX activity was estimated by monitoring the decrease in absorbance due to the breakdown of ascorbate by APX and was calculated by using the coefficient of absorbance 2.81 mM⁻¹ cm⁻¹. The activity of APX was expressed in EU mg⁻¹ protein.

The SOD activity was estimated following Dhindsa *et al.* (1981) method with slight modifications. Callus tissue (0.1 g) was homogenised in 2.0 ml of extraction mixture (0.5 M phosphate buffer (pH 7.3), 3.0 mM EDTA, 1.0% (w/v) polyvinylpyrrolidone (PVP), 1.0% (v/v) Triton X100) and centrifuged at 10⁴ for 10 min. The SOD activity in the supernatant was assayed by adding 0.1 ml enzyme extract with 1.5 ml reaction buffer, 0.2 ml methionine, 0.1 ml each of 1.0 M NaCO₃, 2.25 mM Nitro Blue Tetrazolium (NBT) solution, 3.0 mM EDTA, riboflavin and 1.0 ml of Millipore H₂O was taken in test tubes and was incubated under light for 10 min at 25°C. The absorbance at 560 nm, 50% reduction in colour is 1.0 unit and the enzyme activity was expressed in EU mg⁻¹ protein.

Synthetic seed preparation

Mature green somatic embryos at the cotyledonary stage were collected and suspended in a solution of MS, added with different concentrations of sodium alginate (2%, 3% and 4%) for a few seconds and then dropped into the sterile aqueous solution of calcium chloride (CaCl₂:75 mM, 10 mM and 125 mM) in order to encapsulate the embryo. Encapsulated embryos in CaCl₂ solution were shaken in an orbital shaker at 60 rpm. CaCl₂ solution was poured off and the beads were washed twice with sterilized water and placed on sterilized filter paper to remove excess water.

Storage and conversion of synthetic seeds

Encapsulated somatic embryos were stored at 4°C (in refrigerator), 25°C (incubation room temperature) and at -20°C for varied periods (weeks) in order to evaluate the viability and regeneration/conversion potential of stored synthetic seeds. The synthetic seeds were stored using airtight dark 100 ml conical flasks. The synthetic seeds were periodically removed from the respective storage temperatures and cultured in MS added with various plant growth regulators (PGRs). The conversion rate was recorded by observing the development of shoots in 1.0 mg/l BA and 0.5 mg/l GA₃ added MS after 2 and 4 weeks of culture.

Statistical analysis

For callus induction, hypocotyls explants were cultured in test tubes with a minimum of 30 explants per experiment. In case of somatic embryo, induction each experiment was replicated three times. Data on frequency of response and numbers of somatic embryos induced per 500 mg of callus were recorded after 4 weeks of culture. The data presented as mean and its standard deviation (mean ± SD). The significance of differences among means was carried out using Duncan's Multiple Range Test, DMRT (Duncan, 1995) at P= 0.05.

3. Results

Callus induction and somatic embryogenesis

On 2,4-D (0.5-2.0 mg/l) supplemented MS medium, prolific callus was induced from hypocotyl explants. The optimum callus induction frequency of 96.0% and 89.3% was noticed in Co-1 and RS respectively with 1.0 mg/l 2,4-D added medium. The callus induction frequency decreased as the concentration of 2,4-D above 1.0 mg/l was used (Table 1). Callus induction was also observed on NAA at 0.5 mg/l

added MS but the induced callus was non-embryogenic in nature. The 2,4-D induced embryogenic callus was white and friable, which produced globular embryos on the same induction medium (Fig. 1 a). Of the different 2,4-D concentrations used, 1.0 mg/l exhibited higher embryogenic ability (77.6%) in RS with 63.3 somatic embryos; in Co-1, the embryogenic ability was also equally high (72.8%) with relatively lower numbers of embryos (51.0) (Fig. 1 b). Beside 2,4-D, other PGR combinations were also tested for embryo differentiation. In 0.5 mg/l NAA + 0.25 mg/l BA amended medium, maximum differentiation of embryos (78.7% in RS and 74.0% in Co-1) was noticed (Fig. 1 c-d) and in increasing NAA concentrations the embryo differentiation decreased. The somatic



Fig. 1 - Somatic embryo formation in *Coriandrum sativum* (RS). a) Globular embryos at induction stage; b) Somatic embryos at proliferation stage; c and d) Somatic embryos at maturation stage (scale bar, a=2 mm; b-c= 3 mm; d=1 cm).

Table 1 - Effect of different concentrations of 2,4-D on callus induction and somatic embryogenesis from hypocotyl explants of 'Rajendra Swathi' and 'Co-1' varieties of *Coriandrum sativum*

2,4-D (mg/l)	Rajendra Swathi			Co-1		
	Callus induction (%)	Embryogenic callus induction frequency	No. of embryos formed/culture (0.5 g)	Callus induction (%)	Embryogenic callus induction frequency	No. of embryos formed/culture (0.5 g)
0.5	86.0±4.0 a	55.3±2.5 b	39.3±2.1 b	89.3±3.0 b	52.0±2.64 b	36.3±2.08 c
1	89.3±4.2 a	77.6±3.2 a	63.0±4.5 a	96.0±2.3 a	72.8±3.0 a	51.0±2.64 d
1.5	74.0±4.0 b	58.0±2.6 b	44.5±2.5 b	80.7±4.1 c	54.2±2.51 b	41.5±2.0 b
2	47.3±1.1 c	45.2±2.3 c	31.0±3.0 c	64.0±2.0 d	42.0±3.0 c	27.0±3.0 d

Values are expressed as mean standard deviation, mean values within a column followed by different letters are significantly different (at p= 0.05) according to Duncan's multiple range test.

embryo germination was also influenced by PGRs combination; 1.0 mg/l BA + 0.5 mg/l GA₃ showed maximum germination of embryos in both varieties (83.3% in RS and 76.7% in Co-1). The increased levels of BA reduced plantlet conversion rate (Table 2).

Biochemical analysis

The embryogenic callus is often biochemically different from non-embryogenic tissues which could be used in marker assisted early selection. Protein, proline and sugar level were noted to be high in early embryogenic tissue compared to non-embryogenic callus (Fig. 2 a-c). The sugar content was also noted high at induction stage of embryogenesis (37.2 mg g m⁻¹ fresh weight in 'RS' and 33.4 mg g m⁻¹ fw in 'Co-1') as compared to other stages of embryo (matured) and non-embryogenic callus. The same was the case with proline where induction stage had more levels of proline in both the varieties (2.78 mg gm⁻¹ fw in RS and 2.54 mg g m⁻¹ fresh weight in Co-1). Protein content was higher at maturation stage of embryos showing 7.3 mg gm⁻¹ fw in 'RS' and 7.0 mg gm⁻¹fw in Co-1 compared to other stages of tissues.

Antioxidant enzymes activity behaved differently in different cultivating tissues (Fig. 2 d-f). The highest CAT activity was observed in RS with 5.4 EU mg⁻¹ protein min⁻¹ compared to Co-1 with 4.7 EU mg⁻¹ protein min⁻¹ at matured stage of somatic embryos; so was SOD activity (4.8 EUmg⁻¹protein min⁻¹ and 4.3 EU mg⁻¹ protein min⁻¹ in RS and Co-1 respectively). The APX activity was highest at induction stage of embryos with 2.1 EU mg⁻¹ protein min⁻¹ in RS and 1.8 EU mg⁻¹ protein min⁻¹ in Co-1 compared to other tissues.

Synthetic seed preparation and plantlet conversion

Green and matured somatic embryos were encapsulated in different gelling conditions. Good synthetic

seeds were produced in combination of sodium alginate and calcium chloride mix, which later responded differently in germination medium (Fig. 3 a-c). The study revealed that 3% sodium alginate + 100 mM CaCl₂ was the ideal mixture condition that had fairly good conversion rate (74.0% in RS and 70.6% in Co-1) compared to 2% (48.6% in RS and 43.3% in Co-1) and 4% (30.0% in RS and 26.6% in Co-1) sodium alginate solution (Table 3). The higher sodium alginate level (4%) reduced synthetic seed conversion ability. Similarly, the level of CaCl₂ was also equally important in making synthetic seeds and later in obtaining

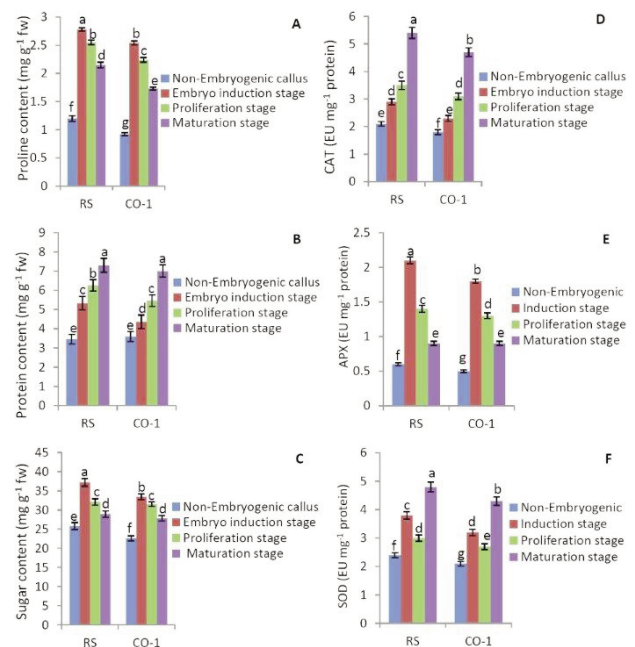


Fig. 2 - Biochemical and enzyme activities in two varieties of *Coriandrum sativum* (RS and CO-1). a) Proline content; b) Protein content; c) Sugar content; d) Catalase activity; e) Ascorbate peroxidase activity and f) Superoxide dismutase activity.

Table 2 - Somatic embryo differentiation and germination frequency in 'RS' and 'Co-1' on different concentrations of NAA, BA and GA₃ supplemented MS medium

NAA (mg/l)	BA (mg/l)	GA ₃ (mg/l)	Rajendra Swathi		Co-1	
			Embryo differentiation	Conversion rate	Embryo differentiation	Conversion rate
0	0.5	0.25	0	54.6±3.0 c	0	51.3±3.0 c
0	1	0.25	0	68.0±3.4 b	0	64.0±3.4 b
0	1	0.5	0	83.3±4.6 a	0	76.7±4.1 a
0	1.5	0.5	0	63.6±3.0 b	0	54.0±2.0 c
0.5	0.25	0	78.7±4.1 a	0	74.0±4.0 a	0
1	0.25	0	61.3±3.0 b	0	55.3±3.0 b	0
1	0.5	0	56.0±3.4 b	0	47.2±2.3 c	0
1.5	0.5	0	39.3±3.0 c	0	34.0±2.0 d	0

Values are expressed as mean standard deviation, mean values within a column followed by different letters are significantly different (at $p = 0.05$) according to Duncan's multiple range test.

plantlets; 100 mM CaCl₂ had a high plantlet conversion, followed by 75 mM and 125 mM. The synthetic seeds prepared at low (2%) sodium alginate +75 mM CaCl₂ were soft, fragile and showed poor conversion; while high (4%) level of sodium alginate and CaCl₂ (125 mM) produced hard beads, reducing conversion ability. The duration of CaCl₂ exposure also had an influence on conversion; 20 min exposure resulted in higher rate of germination compared to 10 and 30 min exposure (data not shown).

The synthetic seeds containing somatic embryos, were kept in three different storage temperatures for varying periods in order to find the right temperature

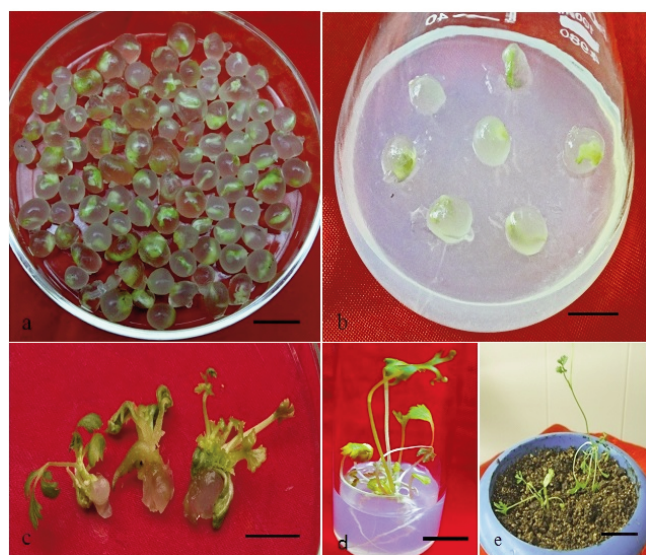


Fig. 3 - Synthetic seed development and plantlet formation in *Coriandrum sativum* (RS). a) Encapsulated somatic embryos; b) Synthetic seeds on the germination medium; c) Germinating synthetic seeds; d) Rooted plantlet and e) Synthetic seed derived plant, grown in outdoor condition (scale bar, a= 1.5 cm, b-d= 1 cm, e=2 cm).

for preservation. It was observed that the storage temperature and duration had a direct influence on synthetic seed viability and final conversion rate (Table 4). The encapsulated somatic embryos stored at 4°C for a week, showed higher regeneration ability (62.0% in RS and 58.6% in Co-1) compared to incubation at room temperature (25°C) and -20°C where the conversion rate was 42.6% and 13.3% in RS and 34.6% and 9.3% in Co-1 respectively. The conversion rate decreased as the storage duration increased as was observed in RS (44.0%) and Co-1 (37.3%) after 5 weeks of 4°C storage (Table 4). The synthetic seed's viability was lost in both the varieties when stored at ultralow temperature (-20°C) for extended period (3 weeks). The regeneration ability gradually declined with increasing storage time in all tested conditions.

The plantlets derived from synthetic seeds had a prominent shoot primordia without much root growth and these shoots were transferred to root induction medium, added with 0.5 mg/l IBA. Well rooted plants (Fig. 3 d) were taken out from the test tube, washed with water and transferred in paper cup (Fig. 3 e). After 2 weeks of hardening, the cups were transferred to the field. Out of 80 plants transferred to soil, 43 (55%) survived in the field. The survived plants in outdoor looked healthy and morphologically similar to mother plants.

4. Discussion and Conclusions

In these two varieties of coriander, callus was vigorously induced from hypocotyl in 1.0 mg/l 2,4-D added MS medium, consistent with previous studies in which 2,4-D played a crucial role in callusing in var-

Table 3 - Effect of different concentrations of sodium alginate and calcium chloride on the conversion rate of encapsulated somatic embryos on 1.0 mg/l BA and 0.5 mg/l GA₃ MS medium

Alginate (%)	Calcium chloride (mM)	Conversion rate (%)			
		Rajendra Swathi		Co-1	
		2 weeks	4 weeks	2 weeks	4 weeks
2	75	36.7±2.3 d	45.3±3.0 d	29.3±2.3 d	38.0±3.5 d
	100	42.6±3.0 c	48.6±2.3 cd	36.6±3.0 c	43.3±3.0 c
	125	30.0±2.0 e	37.3±3.0 e	24.0±2.0 e	32.6±2.3 e
3	75	46.0±3.4 c	52.0±3.4 c	41.3±3.0 e	51.3±3.0 b
	100	63.3±4.2 a	74.0±4.0 a	64.0±3.4 a	70.6±4.1 a
	125	52.0±3.4 b	57.3±2.3 b	45.3±2.3 b	54.7±3.0 b
4	75	21.3±2.3 f	24.6±3.0 g	18.0±2.0 f	24.0±2.0 f
	100	25.3±3.0 ef	30.0±2.0 f	21.3±2.3 e	26.6±2.5 f
	125	12.0±2.0 g	20.6±2.3 g	10.6±1.15 f	19.3±1.1 f

Values are expressed as mean standard deviation, mean values within a column followed by different letters are significantly different (at p= 0.05) according Duncan's multiple range test.

ious investigated plants (Kim *et al.*, 2011). In the present study, embryogenic callus started to differentiate somatic embryos in 2,4-D added medium but the continuous presence of 2,4-D inhibited somatic embryo development beyond globular/heart shaped stage. The formation of somatic embryos on callus induction medium was earlier reported in several other species (Kim *et al.*, 2011). The hindrance of embryo differentiation and progression in continuous presence of 2,4-D has earlier been reported in a number studied cases and the transfer of embryogenic tissues or embryos into '2,4-D-free' or less PGR concentrated medium has been suggested for embryo development (Junaid *et al.*, 2006). The embryogenic tissues with developing somatic embryos were later cultured on NAA and BA added medium in which fast embryo development and maturation were observed. Somatic embryo differentiation and maturation in NAA and BA added medium was earlier reported in several investigated plants (Junaid *et al.*, 2006; Puhan and Rath, 2012). The use of sucrose, ABA and GA₃ has also been noted to be very efficient in promoting somatic embryo development and maturation (Yang *et al.*, 2013; Mujib *et al.*, 2014).

As the non-embryogenic callus transforms into embryogenic tissues, biochemical profiles also alter simultaneously. In this study, we noted differences in biochemical and enzyme activities in embryogenic and non-embryogenic tissues. Here, we noted increased protein and decreased soluble sugar levels in maturation stage of somatic embryo, which is in confirmatory with Kumar and Kumari (2010). Enhanced protein content was also observed here during maturation stage of embryo and it could be used as a good tissue- specific indicator. The increase

in biochemical attributes in embryogenic callus over non-embryogenic tissue was previously reported in several plants (Nieves *et al.*, 2003). The extra proline accumulation has been noted in response to stress, which is believed to act as osmo-regulator in protecting cells against osmotic perturbation (Elmaghrabi *et al.*, 2013). Similar changes in biochemical attributes were earlier observed during transition to differentiated embryogenic state from meristematic tissue under *in vitro* cultural conditions (Samar *et al.*, 2011). Kumar *et al.* (2010) observed alteration of enzyme activities during cellular differentiation, this and similar other findings are important in understanding the metabolic changes in developmental events (Santos *et al.*, 2011). Here, the enzyme activities were lower in non-embryogenic callus compared to embryogenic one, but it was higher at specific embryo stages. In *Hevea brasiliensis* similar increased peroxidase activity was noted during embryo organization (Blanc *et al.*, 2002). Increased APX and CAT activity in embryogenic tissue, designates its higher efficiency in scavenging H₂O₂ during SOD metabolism, prevents membrane lipids peroxidation process (Niknam *et al.*, 2006). Beside distinguishing morphogenic events, CAT facilitates in forming resistant cell walls and helps in defense mechanism against reactive oxygen species (Gaspar *et al.*, 2002).

The induced mature somatic embryos were encapsulated in gelling mix and the conversion of synthetic seeds into plantlets was monitored. Singh *et al.* (2010) observed that the quality bead formation and germination ability of synthetic seeds are partly dependent on concentrations of sodium alginate and calcium chloride solution and on duration of exposure. Our observation indicated that the somatic embryos, encapsulated in 3% sodium alginate + 100

Table 4 - Conversion rate at temperature conditions of somatic embryos encapsulated in 3% sodium alginate and 100 mm CaCl₂, after storage. MS was added with 1.0 mg/l BA and 0.5 mg/l GA₃

Storage temperature	Storage duration (weeks)	Regeneration (%)			
		Rajendra Swathi		Co-1	
		2 weeks	4 weeks	2 weeks	4 weeks
-20°C	1	12.6±2.3 f	13.3±1.1 f	6.6±1.1 f	9.3±1.15 e
	3	0	0	0	0
	1	57.3±4.1 a	62.0±4.0 a	54.0±3.5 a	58.6±3.0 a
4°C	3	38.0±2.0 b	44.0±3.4 b	31.3±3.0 b	37.3±3.0 b
	5	24.0±2.0 d	32.0±2.0 c	19.3±2.3 d	27.3±3.0 c
	7	10.6±1.1 f	18.6±1.1 e	09.3±1.1 f	16.0±2.0 d
25°C	1	33.3±3.0 c	42.6±3.0 b	27.3±2.3 c	34.6±2.3 b
	3	18.0±2.0 e	27.3±2.3 d	13.3±1.15 e	19.3±1.15 d

Values are expressed as mean standard deviation, mean values within a column followed by different letters are significantly different (at $p=0.05$) according Duncan's multiple range test.

mM CaCl₂ produced quality bead with more conversion as compared to the use of lower (2%) and higher level (4%) of sodium alginate. The earlier reports suggested that somatic embryos encapsulated with 3% sodium alginate and 100 mM CaCl₂ produced uniform and moderately hard beads with optimum regeneration potential (Sarmah *et al.*, 2010). The maximum plantlet generation frequency with 3% sodium alginate was found in *Paulownia elongata* (Gozukirmizi, 2003), *Coelogyne breviscapa* (Mohanraj *et al.*, 2009), *Stevia rebaudiana* (Ali *et al.*, 2012). In *Stevia rebaudiana*, hard beads were formed at 4% sodium alginate, which adversely affected germination rate (Andlib *et al.*, 2011). The 20 min exposure in CaCl₂ solution improved conversion of plantlets, the conversion rate however, declined with increasing exposure time and this observation corroborates with similar other previous studies (Malabadi and Van Staden, 2005). Over duration of exposure affects germination ability of the synthetic seeds and this decline of conversion may be due to growth inhibition caused by over absorption and penetration of calcium chloride (Malabadi and Van Staden, 2005). The use of liquid MS for preparing gel matrix was found to be beneficial in emerging shoot and this may be attributed to the presence of nutrients in gel matrix, which apparently serves as a nutrient bed around the propagules and facilitates synthetic seed survival and germination (Ara *et al.*, 1999). Matrix of synthetic seed mimics endosperm of natural seed and this nutrient rich artificial endosperm is essential for maintaining survival of germplasm (Antonietta *et al.*, 1999). The storage conditions also affect the germination potential of synthetic seeds. Our observation indicated that the viability and regeneration ability of synthetic seeds decreased with increasing storage duration. The same view i.e. the longer storage of synthetic seeds reduced conversion ability was reported in earlier few cases (Parveen and Shahzad, 2014). During long storage, carbohydrate reserves decrease gradually, which may be responsible for the reduced conversion ability of synthetic seeds (Ding *et al.*, 1998). The storage of *in vitro* grown tissues at -20°C showed a very poor regenerative ability and loss of tissue viability. It is believed that the short spell of low temperature exposure helps forming ice crystals in tissues, prevents food exchange, causes frost injury/stress and reduces tissue viability (Mittler, 2006).

The successful regeneration system requires germination of synthetic seeds/mature somatic embryos into plantlets at fast pace. The encapsulated somatic

embryos were germinated into plantlets on BA and GA₃ added MS. The combination of BA and GA₃ was found to be very responsive for somatic embryo germination in *Solanum lycopersicum* (Godishala *et al.*, 2011). The well-developed plantlets were transferred to IBA added half MS for promoting root growth. The positive effect of IBA on root induction was reported in several investigated plants like *Camellia nitidissima* (Lu *et al.*, 2013), *Talinum triangulare* (Swarna and Ravindhran, 2013). The synthetic seed derived plants were healthy and morphologically very similar to parent plants. The developed plant regeneration protocol from synthetic seeds will be very important in preserving coriander germplasm for short and medium term basis.

In summary, synthetic seed development and plant regeneration after storage may provide a good alternative strategy for coriander germplasm. The 'short and medium term' storage can open up and enable conservation possibility of elite important coriander. Observed biochemical and enzyme activity differences in various *in vitro* grown tissues may play an important role in marker based selection of tissues in cellular differentiation investigation. The developed synthetic seed preparation protocol can also be used as an alternative propagation method.

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