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Establishment of Embryogenic Cell Suspension Culture and Plant Regeneration of Egyptian Cumin (*Cuminum cyminum* L.)

Kasem Z. Ahmed[1*] • Mohamed K. A. Aly[2] • Shadia K. Ahmed[3] • Jaime A. Teixeira da Silva[4] • Hossam M. Kamel[2]

[1] Department of Genetics and Minia Center for Genetic Engineering and Biotechnology, Faculty of Agriculture, Minia University, El-Minia, Et-61517 Egypt
[2] Department of Horticulture (Floriculture), Faculty of Agriculture, Minia University, El-Minia, Eg-61517 Egypt
[3] Department of Medicinal & Aromatic Plants, Horticulture Research Institute, Agriculture Research Center, Giza, Egypt
[4] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Ikenobe 2393, Kagawa-ken, 761-0795, Japan

Corresponding author: * **ahmed_kz@yahoo.com**

ABSTRACT

Embryogenic cell suspension (ECS) cultures were established from hypocotyl segments-derived embryogenic calli of Egyptian cumin (*Cuminum cyminum* L. cv. 'Balady'), the sole popular commercial variety of cumin in Egypt. After culture for 2-4 months on B5 solid callus induction medium supplemented with 0.88 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) plus 0.86 mg/l kinetin (Kin), meristematic globules and yellow, friable ECS were induced from the explants of seedling hypocotyls. ECS were initiated from these embryogenic calli in the same liquid B5 callus induction medium. After selection of small aggregates and single cell cultures at 15-day intervals for 2 months, homogeneous and yellow ECSs, composed of single cells, small cell aggregates were established. Based upon the growth dynamic of ECS, the entire old medium was replaced weekly by an equal volume of fresh medium. Plating of ECS (1-12 months old) on 3 different solid B5 media ($B5_Z$, $B5_K$ and $B5_{ZK}$) resulted in the induction and development of approximately six, two and six compact, organized calli/ml of ECS, respectively. Variation in callus induction ability was influenced by the time elapsed after subcultures and the medium used. Plated cells responded best 5 days after subculture; 11 calli/ml ECS were obtained while 8.2 shoots/ml ECS regenerated on $B5_{ZK}$ medium containing 0.065 mg/l Zeatin + 0.021 mg/l Kin. A total of 230 plants were obtained, ~75% of which were survived under *ex-vitro* conditions, flowered and produced normal seeds. Chromosome number of suspension cells ranged from 12-28 chromosomes, and the majority of cells (51%) had a normal (14) chromosome number, which was also observed in 63% of tested root tip cells of regenerated plants.

Keywords: chromosomes, embyrogenic calli, growth dynamic, hypocotyl, *in vitro* culture
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **B5**, hypocotyl Gamborg B5 medium; $\mathbf{B5_{DK}}$, B5 medium + 0.88 mg/l 2,4-D + 0.86 mg Kin/l; $\mathbf{B5_K}$, B5 medium + 0.021 mg Kin; $\mathbf{B5_Z}$, B5 medium + 0.065 mg/l Zea; $\mathbf{B5_{ZK}}$, B5 medium + 0.065 mg/l Zea + 0.021 mg/l Kin; **CSC**, cell suspension culture; **ECS**, embryogenic cell suspension; **IBA**, indole-3-butyric acid; **Kin**, kinetin; **MS**, Murashige and Skoog (1962) medium; $\mathbf{MS_R}$, ½MS basal medium + 0.5 mg/l IBA + 0.5 mg/l NAA + 20 g sucrose/l; **NAA**, 1-naphthaleneacetic acid; **Zea**, zeatin

INTRODUCTION

In the present paper we describe the *in vitro* protocol for initiation and maintenance of cell suspension cultures (CSCs) with morphogenic potential for Egyptian cumin cv. 'Balady', the sole popular commercial variety of cumin in Egypt. Plant regeneration form embryogenic cell suspension (ECS) as well as check the chromosomal number status of suspension cells and regenerated plants are reported.

Cumin (*Cuminum cyminum* L.), a member of the *Apiaceae* family, is a winter annual herb and an important medicinal, aromatic and spice plants. The plant grows native in Upper Egypt, and is cultivated in Egypt, Spain, France, India, Pakistan, Turkey and Iran (Jain *et al.* 1992; Lawless 1992; Lawrence 1995). In Egypt, cumin is one of the most widely cultivated medicinal and aromatic plants with a cultivated area 1740 ha (Egyptian Ministry of Agriculture and Land Reclamation 2009). Improvement of *Apiaceae* plants following classical breeding is generally slow, laborious and time consuming (Hunault *et al.* 1989). In addition, efforts to improve cumin have been constrained by the unavailability of genetic diversity for some desirable traits. It is highly susceptible to disease like wilt, blight and powdery mildew from seedlings to maturity (Deepak *et al.* 2008). Conventional breeding for biotic or abiotic stress resistance of Egyptian cumin remains a difficult endeavor, as the plant has very small flowers and narrow natural variability. Biotechnological techniques, e.g., genetic engineering, *in vitro* mutation breeding, or protoplast fusion, may overcome these difficulties and improve cumin germplasm. Establishment of a stable ECS culture is a prerequisite for many biotechnological breeding methods. Callus tissue is an essential material in plant cell culture systems, when it is introduced into a liquid medium and agitated, the cells disperse throughout the liquid to form a CSC. Such cells are, in theory, totipotent and should also have a potential to synthesize any of the compounds normally associated with the intact plant (Allan 1996). The direct use of *in vitro* selection at the cellular level is a powerful and valuable approach, especially when the improvement of one or two easily identifiable characters is desired in an important variety (Lestari 2006). The main advantage is that the basic genotype of the candidate variety is usually altered slightly as opposed to procedures involving hybridization of two distinct varieties. Desired characters can be induced in a variety, thus reducing the time required to breed and improve the variety with the same desired characters through conventional hybridization methods.

A cell suspension offers advantages when rapid cell division or many cell generations are desired, or when a more uniform treatment application is required (Philips *et al.* 1995). Plant cell cultures represent a potential source of valuable secondary metabolites which can be used as food additives, nutraceuticals, and pharmaceuticals (Smetanska 2008). Regenerable ECS cultures are very suitable for mass propagation and for *in vitro* selection (Bisawas and Zapata

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1992). They have the potential for the production of crop varieties with new characteristics such as herbicide resistance (Saunders *et al.* 1992), salt tolerance (Freytag *et al.* 1990), cold tolerance, disease resistance and metal tolerance (Martínez-Estévez *et al.* 2001). Moreover, a CSC considers the best source for totipotent protoplasts (Ahmed and Sági 1994) and is a perfect target for gene transfer and for producing transgenic plants with new desirable traits (Andrade *et al*. 2009).

CSCs that resulted in efficient plant regeneration were achieved for different plant species: maize, barley, rice, wheat, sugar beet, ginger, *Pinus pinaster* (Ait.) and *Catharanthus roseus* (Mitchell and Petolino 1991, Jähne *et al.* 1991, Bisawas and Zapata 1992, Ahmed and Sági 1993, Gürel *et al.* 2002, Guo and Zhang 2005, Azevedo *et al.* 2008 and Fatima *et al.* 2009, respectively). However, only a single report exists on the establishment of ECS in cumin (Tawfik and Noga 2002). Plant growth regulators in particular play a central role in the establishment of ECS cultures. Gürel *et al.* (2002) examined the use different concentrations and combinations of benzylaminopurine and 2,4-dichlorophenoxyacetic acid (BAP and 2,4-D), the growth patterns of CSCs of sugar beet (*Beta vulgaris* L.) during a range of culture periods (0, 3, 5, 7, 9, 11, 13 and 15 days). They found that medium containing high BAP (0.25 mg/l) and 2,4-D (0.25 mg/l) induced higher rates of cell division than the medium containing low BAP (0.1 mg/l) and 2,4-D (0.1 mg/l) or the control. Many reports showed that a high concentration of 2,4-D could be regarded as a trigger for producing somatic embryogenesis in Apiaceae plants (Halperin 1964; Dudits *et al.* 1993), which inhibited certain specific stages of embryogenesis in this condition. The removal of 2,4-D facilitated the progression of pre-embryonic cells to the advanced stages of somatic embryos (Borkind *et al.* 1988). Masuda *et al.* (1995) reported that a high 2,4-D concentration (22 mg/l) induced alfalfa (*Medicago sativa* L.) cells proceeding from the G1 phase to the S phase in the cell cycle, which resulted in the formation of somatic embryos.

Azevedo *et al.* (2008) reported the establishment of a *Pinus pinaster* (Ait.) CSC in a modified MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l 6-benzyl adenine (BA). Fatima *et al.* (2009) were obtained calli from seedling root segments of *Catharanthus roseus*, while they successfully established isodiametric CSCs from friable embryogenic callus derived from hypocotyls.

Keeping of totipotency of ECS for longer with a higher growth dynamic rate is essential. Ahmed and Sági (1993) obtained highly embryogenic CSCs from immature embryo-derived embryogenic calli of winter wheat (*Triticum aestivum* L., cv. 'GK ságvári'). They obtained 22 compact, organized calli from each 1-ml suspension cell when plated on solid MS medium containing indole-3-acetic acid (IAA) and Zeatin (Zea) under a 16-h photoperiod, while only 9 calli were produced in the dark. On other hand, the majority of plants obtained had an abnormal chromosome number and low viability. This is in accordance with numerous earlier and subsequent reports describing cytological instability in CSCs and their regenerated plants, particular chromosome numbers e.g. wheat (*Triticum monococcum* L.), Kao *et al.* 1970; barley (*Hordeum vulgare* L.), Orton 1980; celery plants (*Apium graveolens* L.), Browers and Orton 1982; wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L), oats (*Avena sativa* L.), rye (*Secale cereale* L.) and triticale (× *Triticosecale*), Maddock 1985. The literature on chromosomal variation as the basis of somaclonal variation in plants was earlier reviewed by Gupta (1998). In the study of Guo and Zhang (2005), somatic embryogenic CSCs of four ginger (*Zingiber officinale* Rosc.) cultivars were established. The suspension cultures were placed on MSN agar medium (Guo and Zhang 2005) for callus proliferation, thereafter embryogenic callus that formed was transferred to solid medium (MS + 0.2 mg/l 2,4-D + 5.0 mg/l BA + 3% sucrose + 0.7% agar). Somatic embryos produced shoots and roots, and shoots developed into complete plantlets on solid MS medium supplemented with 3.0 mg/l BA and 0.1 mg/l 1-naphthaleneacetic acid (NAA). They observed that the suspension cultures maintained viability after subculture for 8 months.

Among the few published reports for cumin *in vitro* culture, Tawfik and Noga (2002) established and maintained the first CSCs from hypocotyl segments derived calli of cumin in liquid medium supplemented with 0.88 mg/l 2.4-D + 0.43 mg/l Kinetin (Kin). They found that somatic embryos differentiated 7 days after transferring the cell suspension into liquid media lacking plant growth regulators and a large number of somatic embryos germinated when either the cell suspension was directly plated or the differentiated embryos were cultured on gelled medium containing 0.02 mg/l Kin or 0.065 mg/l Zea.

MATERIALS AND METHODS

Callus induction and subculture

Seeds of Egyptian commercial cumin (*Cuminum cyminum* L. cv. 'Balady') were surface-sterilized with ethanol (70%) for 1 min and soaked for 15 min in a 20% solution of commercial bleach (Clorex®) containing 5.25% sodium hypochlorite and finally washed 3-5 times with sterile distilled water. Sterilized seeds (**Fig. 1A**) were placed on MS germination medium in vials (half-strength Murashige and Skoog 1962 salts supplemented with 0.8% (w/v) agar and 1% (w/v) sucrose, Sigma-Aldrich, St. Louis, MI, USA). Calli were induced from hypocotyl segments of cumin seedlings 2-3 days after emergence). Hypocotyls were cut into 3-5 mm long segments (**Figs. 1E, 1F**). These explants were cultured onto $B5_{DK}$ agar-gelled medium (Gamborg *et al.* 1968; B5 supplemented with 0.88 mg/l 2.4-D, 0.86 mg/l Kin, 30 g/l sucrose, 8 g/l plant agar, Sigma-Aldrich; pH 5.8) at 23°C in the dark. Calli were subcultured monthly on the same fresh solid B5 medium supplemented with 0.88 mg/l 2.4-D + 0.86 mg Kin; the first subculture was incubated at 23°C in the dark and the following two-three subcultures were kept in fluorescent light (16 h/day) (**Figs. 1G-I**).

Initiation and maintenance of suspension cultures

Emberyogenic calli (meristematic globules and yellow, friable embryogenic cultures) were selected visually for initiation of primary suspension cultures. One g fresh weight of friable embryogenic callus was placed into a 150-ml Erlenmeyer flask containing 50 ml of liquid B5 medium supplemented with 0.88 mg/l 2.4-D, 0.86 mg/l Kin, 30 g/l sucrose and pH 5.8. The flasks were placed on a rotary shaker at 25-27°C under a 16-h photoperiod and agitated at 130 rpm. Subcultures were performed biweekly for the first 2 months (later, subcultures were made weekly) by replacing the entire old medium by an equal volume of fresh medium. Only the small aggregates were decanted at every subculture. The ratio of cells to medium was 1:3-4 (v/v) in culture flasks.

For measuring the increase in ratio of cells, 1-ml samples of 4-, 8- and 12-month-old suspension cultures were cultured in 40 ml of liquid B5 medium (+ 0.88 mg/l 2.4-D, 0.86 mg/l Kin, 30 g/l sucrose; pH 5.8) in a 100-ml Erlenmeyer flask on a gyratory shaker at 130 rpm in fluorescent light (16 h/day) at 25-27°C; fresh and dry weights of the cells and pH of the culture medium were measured 0, 1, 2, 5, 7 and 9 days after culture initiation. Dry weight of the cells was measured after drying in an oven (60°C) for 5 days (Ahmed and Sági 1993). There were five replicates per treatment.

Plant regeneration from suspension cultures

For plant regeneration, 1-12 months old suspension cultures were used, one ml cell suspension at 5, 10, 15, 20, 25 days after subcultures, was pipetted onto solid $B5_Z$, $B5_{K1}$ or $B5_{ZK}$ medium (B5 medium containing 0.065 Zt or 0.021 mg/l Kin or 0.065 mg/l Zt plus 0.021 mg Kin, respectively (Tawfik and Noga 2002) and cultured at 25-27°C in a fluorescent light (16h/day) cycle for 4-5 weeks (**Fig. 2**). Organized compact calli developed from plated suspension cells were subcultured for 4 weeks on the same B5 medium used for calli induction. The developed shoots, embryo-

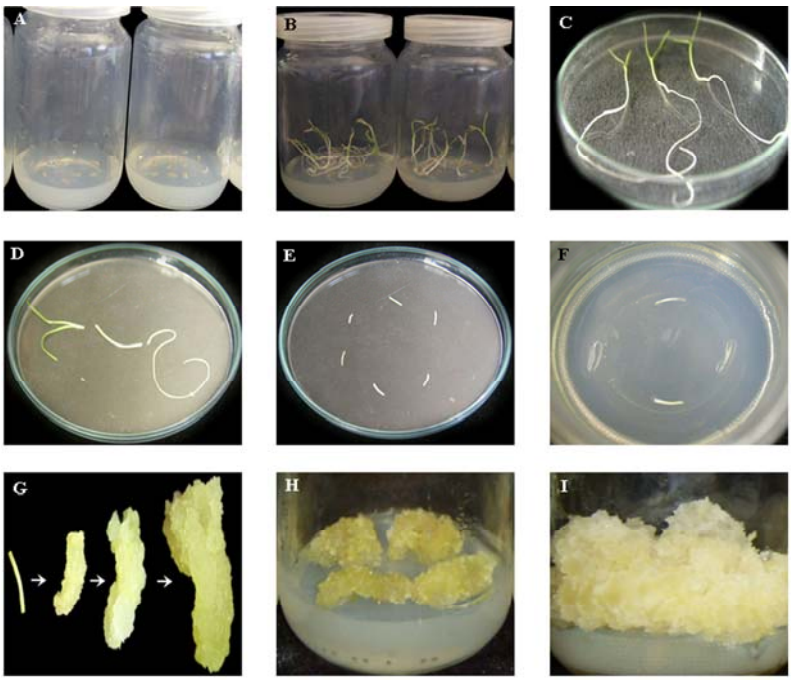

Fig. 1 (A-I) Explant preparation and callus induction. (**A**) Sterilized Egyptian cumin seeds placed on MS germination medium in vials. (**B, C, D**) Seedlings of cumin 2-3 days after emergence. (**E**) Hypocotyl was cut into 3-5 mm long segments. (**F**) Hypocotyl segments cultured onto calli induction medium. (**G**) Calli developed from hypocotyls. (**H**) Calli induced on $B5_{DK}$ medium after 5 weeks. (**I**) Friable, creamy, soft, smooth surface, highly embryogenic, aged calli (3 months old) used to initiate cell suspension culture.

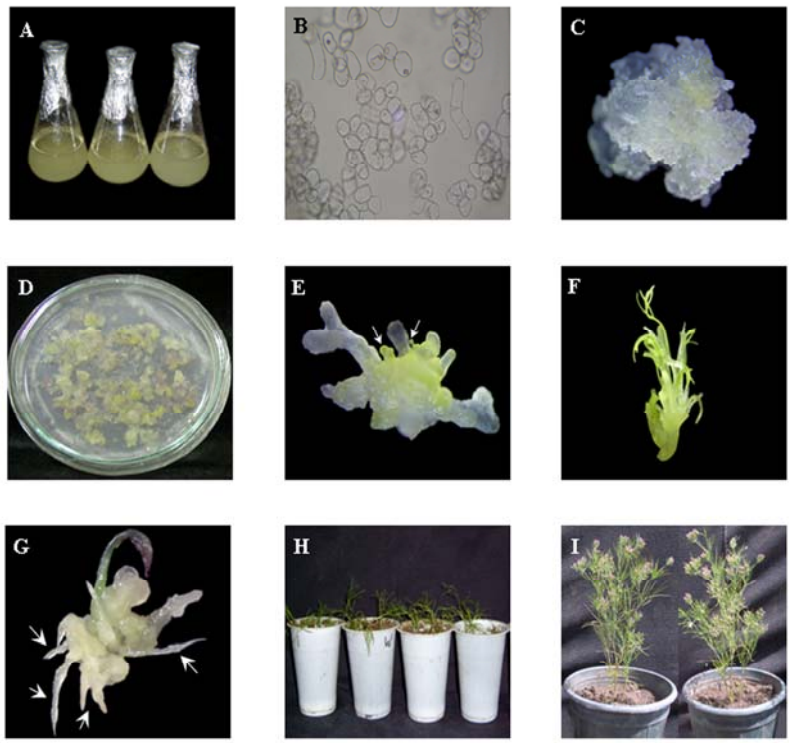

Fig. 2 (A-I) Establishment, maintenance of and plant regeneration from embryogenic Egyptian cumin cell suspension cultures (CSCs). (**A**) Somatic embryogenic suspension cultures (5 months old) growing in 100-ml Erlenmeyer flasks. (**B**) Cell aggregates of embryogenic CSCs pipetted onto callus induction medium (under photomicroscope, 400X). (**C**) Compact calli induced from pipetted CSCs (7-10 day-old, 16X). (**D**) Many of obtained compact and nodular microcalli growing on 9-cm Petri dish containing $B5_{ZK}$ medium (3-4 weeks old). (**E**) Differentiation of somatic embryos (40X). (**F**) Regenerated shoots (6.4X). (**G**) The appearance of a bunch of root primordia (arrowed) derived from a cumin shoot (16X). (**H**) A suspension cell-derived plants transplanted to *ex-vitro*. (**I**) Regenerated plants growing in clay pots under greenhouse conditions at flowering and seeds stages.

genic callus pieces were transferred to fresh regenerating medium. The pH of all media used in this study was adjusted to 5.8 before autoclaving at 121°C for 20 min. Zeatin was added to the cold autoclaved media after filter sterilization.

In vitro hardening and *ex-vitro* acclimatization

The regenerated shoots and plantlets with a poor or rootless system (about 1-3 cm long) were harvested and transferred to rooting medium (MS_R; ½ MS salts, 0.5 mg/l IBA, 0.5 mg/l NAA, and 20 g/l sucrose). Generally two shoots or plantlets were transferred into a vial (360 ml baby food jar) containing 50 ml rooting

medium (as one replicate). The cultures were incubated at 25-27°C under fluorescent light (16 h/day). Ten replicates were included for each treatment. After 2-4 weeks plantlets with well-developed roots were transplanted into plastic pots (5×12 cm) containing peat moss: vermiculite: clay (1: 1: 1, v/v/v), covered with transparent plastic lids and watered every two days. The plantlets were acclimatized and hardened gradually by slowly removing the lids over a period of 7-10 days. Hardened plants were then transferred to a greenhouse under natural light and temperature (Egyptian winter) conditions until seed seeding (~75-90 days).

Cytogenetic studies

Chromosome number of the suspension cells as well as of root tip cells of plants regenerated from cell suspension was counted as described next.

1. Preparation of chromosomes from cell suspensions

The CSCs (at 4-month old) were collected at the log phase (2 days after subculture) into fresh liquid medium; incubated with 0.1% (w/v) colchicine (Sigma-Aldrich) solution for 2 h at 26°C on a rotary shaker (130 rpm); the cells were centrifuged at 1000 rpm for 5 min and the medium and colchicine were decanted. The cells were washed once with sterile distilled water then centrifuged (1000 rpm), fixed in 95% ethanol and glacial acetic acid (3: 1) for 24 h. The fixative was then removed and the cells were washed with sterile distilled water using centrifugation (1000 rpm). The fixed cells were hydrolyzed in 0.2 N HCl for 5 min at 60°C, washed once with sterile distilled water followed by centrifugation (1000 rpm), resuspended in 2% pectinase (Sigma-Aldrich) for 10 min at 35°C. The enzyme was washed away thoroughly with distilled water and stored (~month) in 70% ethanol at 0°C until use (Kumar and Widholm 1984; Ahmed and Sági 1993). The hydrolyzed cells were dropped onto a clean slide and the alcohol was allowed to evaporate; cells were stained with 1% acetocarmine (Sigma-Aldrich). Metaphase chromosomes were counted in 100 well-spread cells.

2. Preparation of chromosomes from root tip cells of regenerated plants

Root tips of 4-6 regenerated plants from CSCs were collected, immersed in 0.1% (w/v) colchicine solution for 2 h at room temperature then washed with sterile distilled water, fixed overnight with glacial acetic acid: ethanol (1: 3), then placed in 70% ethanol as a storage solution. The root tips were rinsed with distilled water and hydrolysed for 6 min in 1N HCl at 60°C (Ahmed and Sági 1993). Tips were stained with 0.5% acetocarmine. One hundred well-spread metaphase cells were checked to score the variation in chromosome number. The root tips of normal germinated cumin seedlings were used as control.

Statistical analysis of data

A randomized complete block design with at least 4 replications was applied. Mean values for each treatment were subjected to ANOVA and compared by the least significant difference (LSD) test using MSTAT-C (1990).

RESULTS

Callus induction and subculture

A high percentage (97.5%) of callus was obtained when hypocotyl segments of cumin seedlings were cultured on $B5_{DK}$ agar-gelled medium (Gamborg *et al.* 1968; B5 supplemented with 0.88 mg/l 2.4-D, 0.86 mg/l Kin, 30 g/l sucrose, 8 g/l plant agar) at 23°C in the dark (**Fig. 1**, data not shown). This callus was subcultured monthly (4 times) onto the same fresh solid B5 medium: the first subculture was incubated at 23°C in the dark while the following three subcultures were kept in the light. The friable embryogenic callus obtained was used to initiate ECS (**Fig. 1I**).

Establishment of CSCs

From cumin friable embryogenic callus, true suspension cultures were established. A finely dispersed, embryogenic CSCs were obtained within 10 weeks from 2-3 months old embryogenic callus (**Fig. 2A, 2B**). Younger callus was a poor source for fine, embryogenic cell suspension. One g (fresh weight) of callus was placed into a 150-ml Erlenmeyer flask containing 50 ml of liquid B5 medium supplemented with 0.88 mg/l 2.4-D, 0.86 mg/l Kin, 30 g/l sucrose at pH 5.8. The flasks were placed on a rotary shaker at 25-27°C under a 16-h photoperiod and agitated at 130 rpm. Subcultures were made biweekly (initially at the 2nd month, then weekly subcultures throughout the life of CSCs) by replacing the entire old medium by an equal volume of fresh one.

Early in the establishment of a cumin CSC, large elongated and highly vacuolated cells with thick cell walls emerged. They were stepwise eliminated during the biweekly/weekly subcultures by systematic renewal of the medium and continuous selection for finer aggregates.

The growth dynamic of CSCs was determined at various cell ages (i.e. 4, 8 and 12 months old). Growth rate in the CSC was determined at 4 months. Cells grew and multiplied rapidly with a doubling time of 2.1 days. Fresh and dry weights of the cells increased nearly 4- and 3-fold, respectively, during a 9-day growth period during which time the pH of the culture medium dropped from 5.80 to 5.42 (**Table 1**).

The growth rate of the suspension culture was also determined at 8 months. These cells grew and multiplied rapidly with a doubling time of 2.2 days. Moreover, the FW and DW of cells increases nearly 5- and 4-fold, respectively during a 9-day growth period; the pH of the culture medium

Table 1 Chang in fresh weight, dry weight and medium-pH of Egyptian cumin cell suspension cultures (4-month old) during 9 days growth period. One ml settled cell was cultured in 40 ml liquid $B5_{DK}$ medium in 100-ml Erlenmeyer flasks. Values are means and standard error of 5 replicates.

Days	Fresh weight (g)	%	Dry weight (g)	%	pH of the medium
0	0.0884 ± 0.0065	100	0.0096 ± 0.0004	100	5.80
1	0.1299 ± 0.0083	147	0.0135 ± 0.0004	140	5.67
2	0.1703 ± 0.0114	192	0.0144 ± 0.0006	150	5.64
5	0.2596 ± 0.0324	294	0.0188 ± 0.0005	196	5.57
7	0.2836 ± 0.0266	321	0.0216 ± 0.0006	225	5.50
9	0.3207 ± 0.0346	363	0.0241 ± 0.0007	251	5.42

Table 2 Chang in fresh weight, dry weight and medium-pH of Egyptian cumin cell suspension culture (8-month old) during 9 days growth period. One ml settled cell was cultured in 40 ml liquid $B5_{DK}$ medium in 100-ml Erlenmeyer flasks. Values are means and standard error of 5 replicates.

Days	Fresh weight (g)	%	Dry weight (g)	%	pH of the medium
0	0.1245 ± 0.0020	100	0.0116 ± 0.0007	100	5.80
1	0.1676 ± 0.0029	134	0.0153 ± 0.0004	132	5.77
2	0.2241 ± 0.0031	180	0.0188 ± 0.0007	162	5.71
5	0.3768 ± 0.0063	302	0.0318 ± 0.0005	274	5.54
7	0.4671 ± 0.0130	375	0.0388 ± 0.0015	334	5.46
9	0.5842 ± 0.0113	469	0.0495 ± 0.0010	427	5.40

Table 3 Chang in fresh weight, dry weight and medium-pH of Egyptian cumin cell suspension culture (12-month old) during 9 days growth period. One ml settled cell was cultured in 40 ml liquid $B5_{DK}$ medium in 100-ml Erlenmeyer flasks. Values are means and standard error of 5 replicates.

Days	Fresh weight (g)	%	Dry weight (g)	%	pH of the medium
0	0.0211 ± 0.0005	100	0.0057 ± 0.0004	100	5.80
1	0.0274 ± 0.0009	130	0.0073 ± 0.0006	128	5.74
2	0.0420 ± 0.0032	199	0.0087 ± 0.0004	152	5.67
5	0.0631 ± 0.0038	299	0.0099 ± 0.0006	173	5.53
7	0.0865 ± 0.0038	410	0.0148 ± 0.0004	259	5.37
9	0.0912 ± 0.0017	432	0.0161 ± 0.0008	282	5.31

Table 4 Number of induced calli from cell suspension cultures of Egyptian cumin at various days after subculture on three different solid calli induction media ($B5_Z$, $B5_K$ and $B5_{ZK}$) at 25-27°C under a 16-h photoperiod.

Age of the suspension (days after subculture)		No. of compact calli/ml suspension cells			
		$B5_Z$	$B5_K$	$B5_{ZK}$	mean
5		9.6	3.6	11	8.0
10		6.9	1.9	6.8	5.2
15		6.5	1.3	5.2	4.3
20		5.4	0.9	3.6	3.3
25		2.8	0	1.8	1.5
mean		6.2	1.5	5.7	4.4
$LSD_{0.01\ Media}$	0.796	$LSD_{0.01\ Day}$	0.906	$LSD_{0.01\ Media \times days}$	1.569
$LSD_{0.05\ Media}$	0.581	$LSD_{0.05\ Day}$	0.685	$LSD_{0.05\ Media \times days}$	1.186

Table 5 Number of regenerated shoots & plants from Egyptian cumin cell suspension-derived calli at various days after subculture on three different solid regeneration media ($B5_Z$, $B5_K$ and $B5_{ZK}$) at 25-27°C under a 16-h photoperiod.

Age of the suspension (days after subculture)		No. of regenerated shoots and plants / 1 ml suspension cell-derived calli			
		$B5_Z$	$B5_K$	$B5_{ZK}$	mean
5		7.6	4	8.2	6.6
10		4.6	3.1	5.5	4.4
15		2.9	0.6	2.3	1.9
20		1.7	0.6	1.3	1.2
25		0.7	0	1.5	0.7
mean		3.5	1.6	3.7	3.0
$LSD_{0.01\ Media}$	1.721	$LSD_{0.01\ Day}$	1.44	$LSD_{0.01\ Media \times days}$	2.495
$LSD_{0.05\ Media}$	1.256	$LSD_{0.05\ Day}$	1.089	$LSD_{0.05\ Media \times days}$	1.886

dropped from 5.80 to 5.40 in this period (**Table 2**). After 12 months of growing and maintenance of CSCs, growth rate was also determined. CSCs cells grew and multiplied rapidly with a doubling time of only 2 days. FW and DW increased nearly 4- and-3 fold, respectively and the pH of the culture medium fell from 5.80 to 5.31 within a 9-day growth period (**Table 3**).

Regeneration from suspension-cultured cells and *ex-vitro* acclimatization

No organized calli formed when the CSCs were cultured in liquid B5 medium supplemented with 0.88 mg/l 2.4-D + 0.86 mg Kin/l. However, embryogenic, division-competent suspension cells plated onto solid $B5_Z$, $B5_K$, or $B5_{ZK}$ medium (B5 medium containing 0.065 mg/l Zt, 0.021 mg/l Kin or 0.065 mg/l Zt plus 0.021 mg Kin, respectively) developed white, compact, organized and nodular calli within 4-5 weeks, while most formed a soft, lawn-like nurse layer (**Figs. 2B, 2C, 3A, 3C, 3E**). Best callus induction occurred with $B5_Z$ and $B5_{ZK}$ media while the lowest induction rate was obtained with $B5_K$ medium (**Table 4**).

All cell cultures were kept under fluorescent light in a 16-h photoperiod; highly organized callus were obtained when cells, 5 days after subculture, were cultured on $B5_{ZK}$ medium (11 callus clumps per ml suspension), and a moderate number of callus clumps (9.6) were induced when CSCs were cultured on $B5_Z$ medium, while fewer (3.6) callus clumps per ml suspension were obtained on $B5_K$ medium (**Table 4**). In contrast, cultured cells 25 days after subculture induced the lowest number of callus clumps (2.8) on $B5_Z$ medium followed by $B5_{ZK}$ medium (1.8), while no callus clumps were obtained on $B5_K$ medium. However, significant differences were observed between most applied treatments (i.e. different types of callus induction media and days after subculture) in which ECS was induced to form callus clumps. 6.2, 5.7 and 1.5 callus clumps/ml suspension cells were obtained on $B5_Z$, $B5_{ZK}$ and $B5_K$ medium, respectively. 5-day cells produced 8 callus clumps/ml ECS overall for the 3 different tested media, while only 1.5 callus clumps were obtained at 25 days post subculture (**Table 4**).

After 10-15 days culturing on callus induction medium, green areas appeared on the compact calli (**Figs. 2D, 2E**). They were then transferred to the same fresh media ($B5_Z$, $B5_K$ or $B5_{ZK}$). Under the same temperature and light cycle, the majority of calli continued to grow and increased rapidly in size. After 4-5 weeks after transfer of the calli, the highest number of regenerated shoots/ml CSC-derived calli was 8.2, 7.6 and 4 for $B5_{ZK}$, $B5_Z$ and $B5_K$ medium, respectively, 5 days after subculture (**Table 5, Figs. 2F, 2G, 3A-F**). A total of 230 plants were obtained, ~75% of which survived *ex-vitro* conditions, flowered and produced normal seeds (**Fig 2H, 2I**).

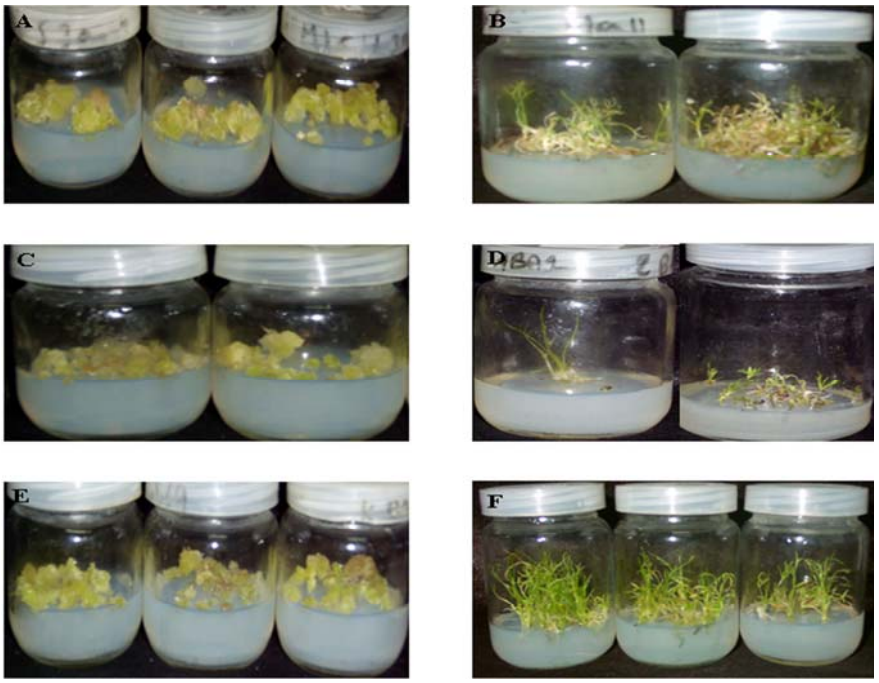

Fig. 3 (**A-F**) Plant regeneration from compact calli derived from suspension cultures of Egyptian cumin (4-month old). (**A**, **B**) Compact calli and their regenerated shoots on $B5_Z$ medium. (**C, D**) Obtained calli and regenerates on $B5_K$ medium. (**E, F**) Obtained calli and regenerates on $B5_{ZK}$ medium, respectively.

Table 6 Variation in chromosome number of the suspension cells, root tip cells of plants regenerated from cell suspension cultures and normal Egyptian cumin seedlings (control).

Chromosome number / Tested cells*	Number of cells having chromosome numbers													
	7	8	10	12	13	14	15	18	20	22	24	26	27	28
Root tip cells of normal cumin seedlings	-	-	-	-	-	100	-	-	-	-	-	-	-	-
Cumin cell suspension	-	-	-	29	4	51	-	-	-	-	-	-	1	15
Regenerated plants from suspension cells**	-	-	-	19	5	63	1	1	1	2	2	1	-	5

* Number of cells observed = 100 for each category. ** Root tip cells of more than 5 regenerated plants.

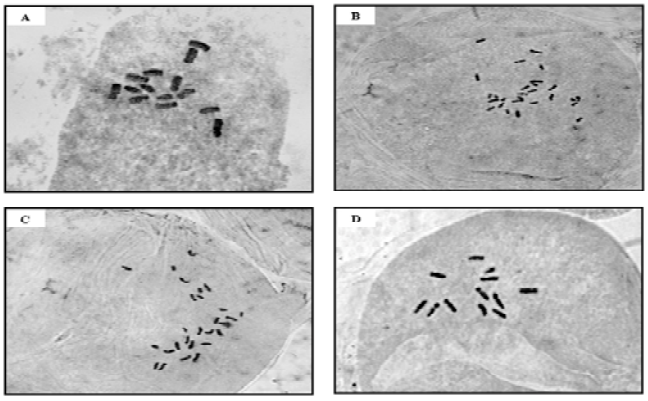

Fig. 4 (**A-D**) Chromosome number of Egyptian cumin suspension cells and their regenerated plants. (**A**) Normal diploid chromosome number (2n=14) of a cumin suspension cell. (**B**) Tetraploid chromosome number (2n=28) of a cumin suspension cell. (**C**) Aneuploid chromosome number (2n=27) of a cumin suspension cell. (**D**) Aneuploid chromosome number (2n=12) of root tip cell of regenerated cumin plant.

Cytogenetic studies

Mitotic chromosome number of the CSCs (4-months old) as well as plants regenerated from CSCs was tested. Cumin had 14 chromosomes (2n=14), observed for 100 cells of normal cumin seedling root tips (= control) (**Table 6**). Chromosomal aberrations were scored in CSCs and their regenerated plants.

Chromosome number of CSCs ranged from 12 to 28. The majority of cells (51%) had the normal chromosome number, i.e., 14 (**Fig. 4A**); 29% of cells had 12 chromosomes and 15% of cells had tetraploid chromosome number, i.e., 28 (**Fig. 4B**), 4% had 13 chromosomes while 1% had 27 chromosomes (**Table 6, Fig. 4C**).

In the root tip cells of regenerated plants, chromosome number also ranged between 12 and 28 chromosomes, tetraploid chromosomes (2n=28) and aneuploid chromosomes (13) were each observed in 5% of the cells while few cells (1-2%) had between 15 and 26 chromosomes. The two highest categories of root tip cells had 12 (**Fig. 4D**) and normal, i.e. 14, chromosomes, equaling 19 and 63%, respectively (**Table 6**).

DISCUSSION

The critical step for the initiation of ECS cultures is the selection of compact or friable, nodular, soft, creamy non-mucilaginous and rapidly growing, highly embryogenic callus as has been shown for a number of plant species e.g., maize (Mitchell and Petolino 1991), barley (Jähne *et al.* 1991), rice (Bisawas and Zapata 1992), wheat (Ahmed and Sági 1993), cumin (Tawfik and Noga 2002), sugar beet (Gürel *et al.* 2002), ginger (Guo and Zhang 2005), and *Catharanthus roseus* (Fatima *et al.* 2009).

In the present experiments CSCs derived from friable, embryogenic calli of cumin were also regenerable. The morphogenic anther callus used for suspension culture by Harris *et al.* (1988) was also a friable type. Müller *et al.* (1989) in barley and Tawfik and Noga (2002) in cumin found that only soft, compact and friable callus types were suitable for establishment of cell cultures.

It can be supposed that friable, embryogenic calli of cumin formed spontaneously, as also mentioned by Lowe *et al.* (1985) in maize and Ahmed and Sági (1993) in wheat. This change in callus type probably did not occur in the other tested plant species. Since our plated cells never developed roots and some of the regenerants had poor roots, it is also probable that the root-competent cells had been selected out automatically during the prolonged callus phase and the following suspension culture without any directed selection (Wang and Nguyen 1990). Restricted root development was also observed by Redway *et al.* (1990) on plantlets regenerated from suspension cells of long-term wheat embryogenic cell cultures.

In our study, cumin suspension cells had a doubling time of 2.1, 2.2 and 2 when cells were 4, 8 and 12 months old, respectively. This is within the 2-2.5 days observed by Redway *et al.* (1990) but is considerably shorter than the doubling time (4.7 days) noted by Ahmed and Sági (1993), or the 3-6 days observed by Qiao *et al.* (1992) in wheat. The increase in FW and DW of the cells during a 9-day growth period was nearly 4- and 3-fold, respectively in 4- and 12-months old callus, or 5- and 4-fold, respectively in 8-months old CSCs. During this 9-day active cell growth period of cumin, pH of the nutrient medium changed from 5.80 to 5.42, 5.40 and 5.31 when cells were 4, 8 and 12 months old, respectively. A pH shift was also mentioned by Prioli and Söndahl (1989) in maize, Ahmed and Sági (1993) in wheat, Guo and Zhang (2005) in ginger and Azevedo *et al.* (2008) in *Pinus pinaster* CSCs. Moreover, a direct relationship between the growth of CSCs and pH changes in medium was reported by Guo and Zhang (2005).

After plating, the cumin CSCs on $B5_Z$, $B5_K$ or $B5_{ZK}$ solid callus induction and regeneration medium, compact and organized calli developed. This observation is in agreement with that of Jähne *et al.* (1991); Ahmed and Sági, (1993); Gürel *et al.* (2002); Guo and Zhang (2005) made in barley, wheat, sugar beet and ginger CSCs, respectively; but its in contrast with the statement of Bisawas and Zapata (1992) and Tawfik and Noga (2002), who reported the development of somatic embryos from rice and cumin CSCs in liquid medium. Use of solid callus induction medium similar to the CSC medium at the beginning of regeneration is probably advantageous for cellular adaptation (Jähne *et al.* 1991; Ahmed and Sagi 1993; Guo and Zhang 2005), although some of the calli formed after transferring the cell suspension to solid regeneration medium stopped growing and died, as also found by Chang *et el.* (1991) in wheat.

Our results indicate that $B5_Z$, $B5_K$, and $B5_{ZK}$ solid media supplemented with 0.065 mg/l Zt, 0.021 mg/l Kin, or 0.065 mg/l Zt + 0.021 mg Kin, respectively, could efficiently regenerate shoots or plants from suspension-derived calli of cumin as also pointed out by Tawfik and Noga (2002).

In the experiments of Ahmed and Sági (1993) in wheat, one ml of CSCs resulted in 9.3 and 22.2 organized calli in darkness and a 16-h photoperiod, respectively, which is comparably higher than the present results, i.e. 4.4 callus clumps per ml cumin CSCs under fluorescent light and a 16-h photoperiod. However, a light/dark regime is more efficient than darkness in inducing organized callus from CSCs plated onto solid media (data not shown).

It is well known that CSCs are cytologically unstable and chromosomal aberrations frequently occur in them (Kao *et al.* 1970; Orton 1980; Browers and Orton 1982; Evans *et al.* 1984; Kumar and Widholm 1984; Maddock

1985; Gupta 1998). In the present study, mitotic chromosome number of CSCs (4-months old) regenerated plants ranged from 12 to 28 chromosomes, however, chromosome number of more than 50% of cumin CSCs and more than 60% of their regenerated plants were normal (2n = 14). Low rates of chromosome number are distributed between tetraploid chromosomes (2n=28) or aneuploid chromosomes (>14 or >28) and were observed in 1 to 29% of the cells. Such variation that arises through *in vitro* culture is thought to be due either to physiological modification (epigenetic) or to genetic changes (somaclonal variation) such as chromosomal alteration, DNA amplification, translocation, point mutation, or transposable elements (Lee and Phillips 1988; Karp 1989). The explants with high ploidy and high chromosome numbers show more variability than species with low ploidy and low chromosome number (Creissen and Karp 1985). Polyploidy in tissue culture-derived plants generally results from endopolyploidization or nuclear fusion (Sunderland 1977; Bayliss 1980). The altered karyotypes in somaclones include chromosomal rearrangements as well as aneuploidy and euploidy. Aneuploidy may be caused by non-disjunction, aberrant spindles, lagging chromosomes, chromosome breakage that produces dicentric and acentric chromosomes (Sunderland 1977). Normal cell cycle controls, which prevent cell division before the completion of DNA replication, are presumed to be disrupted by tissue culture, resulting in chromosomal breakage (Phillips *et al.* 1994). More detailed studies on polyploidy are discussed in a special issue of *New Phytologist* in 2010 (e.g., Cifuentes *et al*. 2010).

Briefly, the suggestion is that normally late-replicating regions of the chromosome replicate even later than usual in the tissue culture environment. The separating chromatids are held together at this point of late replication, producing a stress that could result in breakage. The nature, extent, and possible origin of somaclonal variation (Larkin and Scowcroft 1981) have been reviewed on numerous occasions from such various viewpoints as chromosomal variation in culture cells (Bayliss 1980) and regenerated plants (D'Amato 1977), the biological basis and possible role in plant improvement programs (Evans *et al.* 1984; Karp and Bright 1985) and the epigenetic basis (Meins 1983). According to the best knowledge, this study of *in vitro* chromosomal variation for cumin plant is the first one in this field.

CONCLUSIONS

This study confirms that cells and tissues of Egyptian cumin (cv. 'Balady') respond to tissue culture under specific *in vitro* conditions. Several protocols were successfully applied to induce callus and maintain long-term (4-6 months) cultures. Also, from these calli, regenerable embryogenic CSCs could be initiated; these have a high potential for growth more than one year at 25-27°C under a light/dark illumination regime and the growth rate was estimated at different ages (4, 8, 12 months). We successfully regenerated intact plants from CSCs that were acclimatized in the soil and continued normal growth, flowering and produced normal seeds. Mitotic chromosomes were monitored in suspension cells as well as in root tip cells of regenerated plants; most had the normal diploid chromosome number for cumin (i.e., 14 chromosomes) but many cells had abnormal chromosome numbers.

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