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A microdroplet cell culture based high frequency somatic embryogenesis system for pigeonpea, *Cajanus cajan* (L.) Millsp.

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A protocol for high frequency production of somatic embryos was worked out in pigeonpea, *Cajanus cajan* (L.) Millsp. The protocol involved sequential employment of embryogenic callus cultures, low density cell suspension cultures and a novel microdroplet cell culture system. The microdroplet cell cultures involved culture of a single cell in 10 µl of Murashige and Skoog's medium supplemented with phytohormones, growth factors and phospholipid precursors. By employing the microdroplet cell cultures, single cells in isolation were grown into cell clones which developed somatic embryos. Further, 2,4-dichlorophenoxyacetic acid, kinetin, polyethylene glycol, putrescine, spermine, spermidine, choline chloride, ethanolamine and LiCl were supplemented to the low density cell suspension cultures and microdroplet cell cultures to screen for their cell division and somatic embryogenesis activity. Incubation of callus or the inoculum employed for low density cell suspension cultures and microdroplet cell cultures with polyethylene glycol was found critical for induction of somatic embryogenesis. Somatic embryogenesis at a frequency of 1.19, 3.16 and 6.51 per 10^6 cells was achieved in the callus, low density cell suspension cultures and microdroplet cell cultures, respectively. Advantages of employing microdroplet cell cultures for high frequency production of somatic embryos and its application in genetic transformation protocols are discussed.

Keywords: Callus culture, Choline chloride, Crop improvement, Kinetin, Lithium chloride, Phytohormones, Plantlet regeneration, Polyethylene glycol, Putrescine, Spermidine.

Pigeonpea is a large seeded protein rich grain legume cultivated in rain-fed areas of semi arid tropics and subtropics[1]. Despite its large area of cultivation, the total productivity is low because of several biotic and abiotic stress factors that affect the plant growth and development. Biotic factors that greatly affect crop productivity include sterility mosaic disease (SMD), *Fusarium* wilt, *Alternaria* blight, *Phytophthora* stem-blight (*Phytophthora* f.sp. *cajani*), pod-fly (*Melanagromyza obtuse*) and *Helicoverpa armigera* (legume pod-borer)[2]. Abiotic stress-factors affecting crop productivity include drought stress and photo- and thermo- sensitivity[1]. Agronomic traits such as tolerance to water logging and drought, grain quality involving higher protein content and amino acid composition with reference to essential amino acids of seed proteins, shortened vegetative phase and early maturity, compact and erect plant habit, identification and control of factors responsible for excessive flower shedding and synchronous fruiting have been identified to be the desirable traits for crop improvement[2]. Consequently, there is a dire need to improve crop productivity, nutritive value and stress tolerance of this species.

Pigeonpea *in vitro* is found to be recalcitrant, especially for high frequency plantlet regeneration[3,4]. Somatic embryogenesis (SE) is ideal for high frequency plantlet regeneration that could be used in genetic transformation protocols[3,5,6]. Plant cells either in callus or in a suspension culture are known to represent a heterogeneous population of cells[5,6]. Several *in vitro* protocols involving low density cell suspensions are employed to achieve homogeneity in the cell population[7,8]. Isolation and culture of single cells is attractive in developing cell clones comprising homogenous cell populations, and this system forms the basis for inducing desired *in vitro* responses. Extensive studies related to

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Abbreviations: ChCl, Choline chloride; 2,4-D, 2,4-Dichlorophenoxy acetic acid; DMRT, Duncan's Multiple Range Test; ETA, Ethanolamine; Kn, Kinetin; MS, Murashige and Skoog's nutrient medium; PEG, Polyethylene glycol; SE, Somatic embryogenesis; Spd, Spermidine.

induction of SE in suspension cultures have been carried out in *Dacus carota* and in *Medicago sativa*[9-11]. Single cell derived cell clones and plantlets have high application potential in transgenic biology in order to raise transgenic plants without the problem related to chimeric expression of the transgene[12,13]. In the present study, we explored a novel microdroplet cell culture system for pigeonpea and screened several potential embryogenic factors that induce high frequency SE.

Material and Methods

Chemicals—Choline chloride (ChCl), 2,4-dichlorophenoxyacetic acid (2,4-D), ETA, glutamine, kinetin (Kn), lithium chloride (LiCl), PEG-6000, putrescine, spermidine (Spd), spermine, sucrose and vitamins, were obtained from Sigma-Aldrich, USA. Onozuka Macerozyme-R10 was obtained from Yakult Pharmaceutical Industry Co. Ltd, Japan. Silane solution was obtained from BRL, USA. All other chemicals were procured locally.

Plant material—Seeds of pigeonpea [*Cajanus cajan* (L.) Millsp. cv ICPL-81179] were obtained from Tamil Nadu Agriculture University, Coimbatore, India. For initiating germination, seeds were washed 3 times with double distilled water, followed by treatment with 0.1% $HgCl_2$ for 4 min and subsequently rinsed 5 times with sterile double distilled water. Seeds were germinated aseptically on semisolid 0.7% agar-water medium.

Explants—Three days old sprouts were placed on a Petri dish lined with two layers of moist sterile Whatman No.1 filter paper and embryonal axis explants were dissected out. Four explants per culture tube were inoculated.

Nutrient media, experimental solutions and conditions—Murashige and Skoog's[14] (MS) medium was used as the basal medium along with specified phytohormones, growth factors and phospholipid precursors. The pH of the media was adjusted to 5.6 with 0.1 M KOH before autoclaving at 15 psi for 15 min. The different nutrient media employed in the present study are listed in Table 1.

Table 1—Different nutrient media employed for working out SE protocol in pigeonpea.

Medium composition	Experimental purpose and medium abbreviation
0.7% agar-water medium	Seed germination; germination medium (GM)
Semisolid MS+2,4-D-5 µM+glutamine-0.03 mM (solidified with 0.7% agar)	Developing embryogenic callus from embryonal axis explants; embryogenic callusing medium (ECM)
Liquid MS+2,4-D (10 µM)+Kn 15 µM) + pectinase 1.5%	Preparation of enzymatically dispersed cells; enzymatic cell dispersal medium (ECDM)
Liquid MS+2,4-D (10 µM)+Kn 15 µM)	Washing the enzymatically prepared cell suspension; preparation of non-enzymatically dispersed cells; growth of high density cell suspension cultures; high density cell suspension culture medium (HDCSCM)
Liquid MS+2,4-D (10 µM)+Kn (15 µM)+ CM (20% v/v)	Growth of low-density cell suspension cultures; low density cell suspension culture medium (LDCSCM-1)
Liquid MS+2,4-D (10 µM)+Kn (15 µM)+putrescine (5 mM)	Growth of low-density cell suspension cultures; LDCSCM-2
Liquid MS+2,4-D (10 µM)+Kn (15 µM)+spermine (5 mM)	Growth of low-density cell suspension cultures; LDCSCM-3
Liquid MS+2,4-D (10 µM)+Kn (15 µM)+Spd (5 mM)	Growth of low-density cell suspension cultures; LDCSCM-4; microdroplet cell culture medium (MDCCM-1)
Liquid MS+2,4-D (5 µM)+glutamine (0.03 mM)+ PEG 4% (w/v)	PEG incubation; stress factor incubation medium (SFIM-1)
Liquid MS+2,4-D (10 µM)+Kn (15 µM)+Spd (5 mM)+ PEG 4% (w/v)	PEG incubation; SFIM-2
Liquid MS+2,4-D (10 µM)+Kn 15 µM)+Spd (5 mM)+ ChCl (10 µM)	MDCCM-2; washing solution used subsequent to enzymatic dispersal of cells of the cell clones in microdroplet cell cultures
Liquid MS+2,4-D (10 µM)+Kn 15 µM)+Spd (5 mM)+ ETA (10 µM)	MDCCM-3; washing solution used subsequent to enzymatic dispersal of cells of the cell clones in microdroplet cell cultures
Liquid MS+2,4-D (10 µM)+Kn 15 µM)+Spd (5 mM)+ LiCl (5 mM)	MDCCM-4; washing solution used subsequent to enzymatic dispersal of cells of the cell clones in microdroplet cell cultures
Liquid MS+2,4-D (10 µM)+Kn 15 µM)+Spd (5 mM)+ ChCl (10 µM)+1.5% pectinase	Preparation of enzymatically dispersed cells of the cell clones in microdroplet cell cultures
Semisolid hormone free MS medium	Somatic embryo maturation; embryo maturation medium (EMM)
Semisolid half strength MS basal medium	Plantlet regeneration; regeneration medium (RM)

Media sterilization—MS medium along with 2,4-D, ChCl, ETA, Kn, PEG, putrescine, Spd, and spermine, as well as $CuSO_4$ and Ag_2SO_4 were sterilized by autoclaving. CM and pectinase containing media was filter sterilized.

Callus was initiated from 3 days old embryonal axis explants in MS + 2,4-D (5 μM) + glutamine (0.03 mM). Callus was routinely subcultured every 28 days. Callus cultures within two subculture ages (<84 days) were employed in the study.

Low density cell suspension cultures—Callus maintained on MS+2,4-D (5 μM)+glutamine (0.03 mM) was employed as the inoculum for initiating low density cell suspension cultures. Low density cell suspension cultures were initiated at an initial density of 10^3 cells ml^{-1} in contrast to the routinely used initial density of 10^6 cells ml^{-1} in high density cell suspension cultures. Inoculum was prepared by employing either enzymatic or non-enzymatic dispersal of the callus. Enzymatically dispersed cells were prepared by incubation of callus in 1.5% pectinase (Onozuka-Macerozyme R10) prepared in MS + 2,4-D (10 μM) + Kn 15 μM) at pH 5.6 in a shaker water bath at 25 ± 1°C for 2 h. Dispersed cells were washed 3 times in MS + 2,4-D (10 μM) + Kn (15 μM) by sedimentation of the cells at every stage of washing by centrifugation at 50 × g for 3 min. Subsequent to washing, the dispersed cells were suspended in the respective experimental media. Non-enzymatic dispersal of cells was carried out by incubating 1 gm of callus in 25 ml MS + 2,4-D (10 μM) + Kn 15 μM) on an orbital shaker at 60 rpm for 30 min. Subsequently, the cell suspension was passed through graded stainless steel sieves (40, 60, 100 and 200 mesh; cells sieving kit; Sigma Aldrich) to remove multicellular clumps. The resultant fine suspension of cells was centrifuged at 50 × g for 3 min and subsequently suspended in the experimental solution at desired cell density. In raising the low density cell suspension cultures, inoculum that contained single cells and few celled clusters (up to 3 cells) prepared by non-enzymatic dispersal of the callus were employed. Cell suspensions with desired constitution of cells were achieved by passing the cell suspensions through differently graded stainless steel meshes. Subsequently, the cells were pelleted at 50 × g for 3 min and suspended in MS + 2,4-D (10 μM) + Kn (15 μM) or in specified nutrient media depending on the experimental requirements. The conditioned medium (CM, 20% v/v) and polyamines such as putrescine, spermine and Spd were supplemented to the medium at specified concentrations as indicated in Table 1. Growth of the low density cell suspension cultures was monitored by evaluating the cell-growth parameters such as cell count, total soluble protein content and wet wt. Cell counting was done using a haemocytometer. Cell suspensions at desired cell density were made up by cell counting and making necessary dilutions. Total protein content was estimated according to the procedure of Lowry *et al.*[15] Wet weight of cells in low density cell suspension cultures was determined by collecting the cells on a 0.2 μm pore size filter paper disc in a vacuum filtration assembly. Collected cells were transferred to a butter paper and the wet wt determined.

Microdroplet cell cultures—Inoculum for microdroplet cell cultures was prepared from 1 day old low density cell suspension cultures. From the low density cell suspension cultures, fine suspension of cells was prepared by passing the cell suspensions through stainless steel sieves. The inoculum thus prepared contained predominantly single cells along with 2-celled and few-celled clusters.

Microdroplet cell cultures were established in 100 mm diameter × 15 mm high Borosil glass Petri dishes. Petri dishes for microdroplet cell cultures were rinsed with 20% Silane solution in order to ensure compactness of microdroplets and to avoid spreading. The inoculum of fine suspension of cells was inoculated as microdroplets by using a micropipette fitted with tips having cut ends at the tip side. Out of the tested volume of microdroplet cell cultures in the range of 2, 5, 10, 15 and 20 μl, 10 μl droplets were found to be ideal for precision and ease in microscopic counting in relation to spread area and depth of the microdroplets (data not shown). Inoculum density of fine cell suspension for the initiation of microdroplet cell cultures was *ca*. 2.1 × 10^4 cells ml^{-1}. Cell suspension was spotted as 10 microdroplets of 10 μl each in the Petri dish. Ethylene absorbants such as $CuSO_4$ (0.1%) and Ag_2SO_4 (0.1%) were included as separate 20 μl droplets in the microdroplet cell cultures. RH within the microdroplet cell cultures was optimally maintained and drying of microdroplets due to evaporation within the microdroplet cell cultures was prevented by incorporating a well with 1 ml of sterile double distilled water in a 35 mm diameter Costar Petri dish. The microdroplets were placed a little away from the edge of the Petri dish. In a cell count of microdroplet

cultures on 1st day, *ca*. 80 % of microdroplets contained one single cell alone. A minimum of 48 microdroplets were scored for cell count and frequency of somatic embryos in each experiment. Petri dishes were sealed with Parafilm strips and incubated. Media was replenished on 12th and 20th day in the microdroplet cell cultures with the addition of 10 µl medium for each droplet for sustained growth. Microscopic observations were made in 4 and 24 days old microdroplet cell cultures to count the number of cells and frequency of somatic embryos, respectively. Six Petri dishes were included in each of the experiments. Microdroplet cell cultures that had more than a single cell per microdroplet on 1st day were not included in the data. An idealized and actual microdroplet cell culture is shown in Fig. 1a & b.

Somatic embryogeneisis (*SE*) *frequency in microdroplet cell cultures*—SE frequency was determined in 24 days old microdroplet cell cultures on the basis of somatic embryos per microdroplet as well as per 10^6 cells. Number of cells in the cell clones was determined by enzymatic dispersion. Individual cell clones were incubated in 10 ml of 1.5% pectinase prepared in MS + 2,4-D (10 µM) + Kn 15 µM) + Spd (5 mM) + ChCl (10 µM) at pH 5.6 in a shaker water bath at 25 ± 1°C for 2 h. Dispersed cells were washed free of pectinase by 3 times washing in MS + 2,4-D (10 µM) + Kn 15 µM) + Spd (5 mM) + ChCl (10 µM). Subsequently, cells were pelleted down by centrifugation at 50 × g for 3 min. Cell count was made in the cell suspension and SE frequency was worked out. Since the somatic embryos in the cell clones were very soft and fragile, total dispersal of the cells including that of the somatic embryos in the cell clones was achieved. The Number of cells per cell clone indicates the total number of cells including that of the somatic embryos.

PEG incubation—Fourteen days old callus grown on MS + 2,4-D (5 µM) + glutamine (0.03 mM) or 7 days old low density cell suspension cultures maintained in MS + 2,4-D (10 µM) + Kn 15 µM) + Spd (5 mM) was incubated in respective media containing PEG at 4 % (w/v). One gram callus or 200 mg wet wt of low density cell suspension cultures was employed. Callus or cells were incubated in an orbital shaker at 60 rpm for 4 h. Subsequent to PEG incubation, callus or cell suspension was sedimented by centrifugation at 50 × g for 3 min. The pellets were washed 3 X in the medium employed in the subsequent stage for inducing SE.

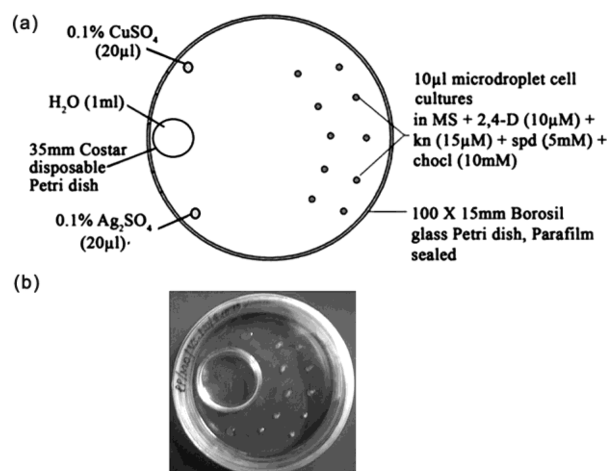

Fig. 1a & b—An idealized (a); and actual (b) microdroplet cell cultures indicating the different phytohormone/growth factor/ culture supplements employed in pigeonpea.

Conditioned medium (*CM*)—Cell free CM was prepared from 7 days old cell suspension cultures of the same species grown in MS + 2,4-D (10 µM) + Kn (15 µM). The cultures meant for preparing CM were raised at an initial density of 10^6 cells ml^{-1}. In raising the cell suspension cultures, inoculum that contained single cells and few celled clusters (up to 3 cells) were employed. Cell suspensions prepared by non-enzymatic dispersal of the callus was employed as the inoculum for raising the cell suspension cultures. Cell suspensions with desired constitution of cells were achieved by passing the cell suspensions through differently graded stainless steel sieves. Subsequently, the cells were pelleted at 50 × g for 3 min and the supernatant was subsequently filtered through 0.22 µm membrane filter and the filtrate obtained was employed as the CM. Whenever CM was supplemented to the nutrient medium, necessary adjustment was made in the constitution of the medium taking into consideration the concentration of nutrients in the CM.

Incubation conditions of cultures—Seedlings, callus and microdroplet cell cultures were incubated in a culture room at 25 ± 1°C with 80% RH, white light at 1200 µW cm^2 intensity and a photoperiod of 16 h L:8 h D. Low density cell suspension cultures were incubated in an orbital shaker (New Brunswick, USA) at 60 rpm at 25 ± 1°C.

Cell counting and photomicrography—Cell counting in the microdroplet cell cultures was done at 24 h intervals starting from 1st day up to 4th day by using an inverted microscope (Nikon, Japan) at 400X.

Photomicrography of other samples was done with Nikon fluorescence cum phase contrast microscope at 200X.

Data presentation—At least 120 explants were employed in each treatment. For callus samples, 30 tubes were inoculated with specified callus tissue either with or without PEG incubation. For suspension cultures, 10 flasks were employed for each experiment. Each experiment was repeated thrice. For microdroplet cell cultures six Petri dishes were included in each of the experiments. A complete randomized design was used in all the experiments and analysis of variance and mean separations were carried out using Duncan's Multiple Range Test (DMRT)[16]. Significance was determined at 5% level. Data presented are the mean of 3 replicates along with SD.

Results and Discussion

In order to establish a protocol for high frequency production of somatic embryos in cultures of pigeonpea, following experimental strategies were adopted in the present study. The experimental strategies involved the following stages: (i) establishment of embryogenic callus cultures initiated from 3 days old embryonal axis explants; (ii) establishment of low density cell suspension cultures initiated from embryogenic callus which served as the inoculum for the subsequent stage employing microdroplet cell cultures; and (iii) working out inductive conditions for cell division and SE in microdroplet cell cultures.

Establishment of embryogenic callus—Induction of SE in crop legumes in general and pigeonpea in particular is known to be difficult due to *in vitro* recalcitrance of this group of plants[3,17,18]. Developing a potential SE protocol requires initial establishment of embryogenic callus by optimizing factors such as choice of explants for the initiation of callus cultures, age of the seedlings from which explants were prepared, choice of nutrient media and phyto hormone/growth factor supplements[4,19,20]. In our laboratory, work related to the optimization of these parameters in pigeonpea cultures has already been accomplished[21]. Callus cultures from 3 days old embryonal axis explants were initiated with various concentrations of 2,4-D and glutamine. Observations showed that MS + 2,4-D (5 µM) + glutamine (0.03 mM) was optimal for developing proliferative mass of callus along with desired morphology of cells which marked the embryogenic callus (Fig. 2a). It is known that elongated cells with large vacuoles and scanty cytoplasm are non-embryogenic. In contrast, small globular cells with dense cytoplasm are known to be embryogenic[6,22] (Fig. 2b & c). Differentiation of single cells into somatic embryos in cell suspension cultures of carrot has been studied by many[5,9,10,23,24]. Girija *et al*.[6] demonstrated the relationship between the morphology of the cells and potency for embryogenesis in carrot suspension cells. Taras *et al*.[11] showed that the cell suspension cultures of carrot has two phases in SE including the early auxin dependent and the later auxin independent. Growth of embryogenic callus in 2,4-D supplemented medium for 7 days followed by transfer to 2,4-D lacking medium was shown to result in the development of somatic embryos[11]. However,

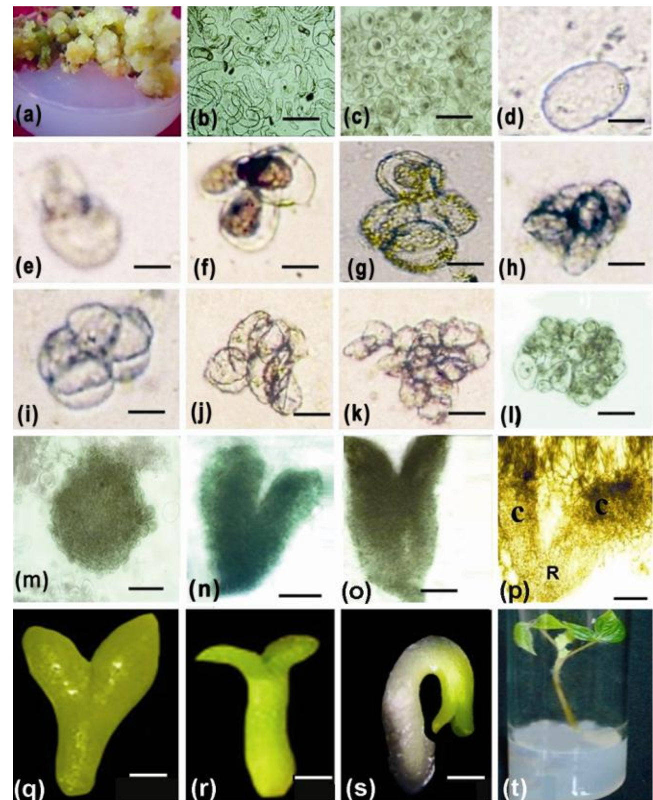

Fig. 2—Somatic embryogenesis of pigeonpea. (a) embryogenic callus; (b & c) non-embryogenic and embryogenic cells of the callus, respectively; (d-k) *In situ* microscopic observation of cell growth in microdroplet cell cultures showing different stages of growth in MS + 2,4-D (10µM) + Kn (15 µM) + Spd (5mM) + ChCl (10 µM); (l-p) SE in microdroplet cell cultures. [l, proembryogenic mass; and (m-p), somatic embryo stages such as, globular, heart, torpedo and cotyledonary, respectively]; (q & r) later stages of cotyledonary stage embryos isolated from microdroplet cell cultures; (s) germinating somatic embryo isolated from microdroplet cell cultures transferred to half-strength MS medium; (t) *in vitro* regenerated plantlet in half-strength MS medium. [bar in b, c and i to p: 50 µm; bar in d to k: 25 µm; bar in q to s: 1 mm. C, Cotyledon; R, root.]

in the present study we did not observe these distinct phases related to auxin requirement in the cultures of pigeonpea. Possibly, leguminous plants differ from the Solanaceous plants in their requirement of auxin during SE.

Establishment of low-density cell suspension cultures—Cells in callus are known to be heterogeneous in relation to their morphology and status of cytodifferentiation[17,22]. Differentiation responses manifested in callus are known to occur as a function of position of the potential differentiating cells in the callus[6,25]. This phenomenon termed as "position effect" is associated with differentiating cells of the callus cultures of different species. The concentration gradient of biomolecules established during differentiation responses in cultures are known to cause the cellular differentiation responses. Occurrence of cellular cross-inhibition in a heterogeneous mass of cells in callus results in the inhibition of differentiation responses by the cells which do not undergo differentiation[6,26]. In order to overcome the cellular cross-inhibition, it is necessary to establish homogeneity and synchrony of the cell population in cultures with the enrichment of differentiating cells[25]. Low density cell suspension cultures offer the advantage of establishing relatively high degree of homogeneity and synchrony of cells in cultures[10,25]. Studies employing cell suspension cultures of carrot highlighted the advantages of low density cell suspension cultures for inducing SE[9,10]. Establishing totally defined culture conditions, especially in low density cell suspension cultures, is relatively difficult[7]. However, systematic screening of the requirements for phytohormones, growth factors and metabolic precursors would yield positive results in establishing low density cell suspension cultures. Induction of cultured cells to acquire embryogenic competence or conferring embryogenic potential has been shown to be achieved in low density cell suspension cultures with greater ease in contrast to callus cultures[5,22].

In order to prepare inoculum for initiating low density cell suspension cultures, either enzymatic or non-enzymatic dispersal of the callus is employed. In the present study, inoculum prepared from non-enzymatic dispersion was found most suitable as the cell suspension was clean without any cell debris (data not shown). In order to establish low density cell suspension cultures, supplements such as CM and different polyamines were employed. There was no cell growth when the MS medium along with phytohormones was prepared at 1.2X concentration or when CM alone was supplemented to MS basal medium (Table 2). This showed the possibility that both the elevated concentration of hormone supplements and also component(s) of the CM stimulated the growth of cells in the low density cell suspension cultures.

A set of experiments was performed to find out the efficacy of different polyamines, in the place of CM, for inducing the growth of the low density cell suspension cultures. Among the different polyamines, Spd supplementation alone effectively substituted the CM resulting in the growth of the low density cell suspension cultures at a rate comparable with the control. Supplementation of putrescine and spermine did not substitute the CM in supporting the growth of the low density cell suspension cultures. The comparable effects of CM and Spd indicated the possibility of Spd being the growth factor in the CM

Table 2—Effect of CM and polyamines on the growth of low density cell suspension cultures of pigeonpea

Culture conditions*	Growth parameters per flask		
	Cell countx 10000	Protein content (mg)	Wet wt (g)
Control (MS + 10 µM 2,4-D + 15 µM Kn)	2.2 ± 0.11^{c}	0.03 ± 0.001^{b}	0.004 ± 0.0002^{b}
MS + 10 µM 2,4-D + 15 µM Kn + CM (20%, v/v)	69.4±2.72^{a}	0.70 ± 0.032^{c}	0.141 ± 0.0068^{c}
MS-1.2X + 12 µM 2,4-D + 18 µM Kn (1.2 X)**	2.6 ± 0.11^{f}	0.03 ± 0.001^{b}	0.005 ± 0.0002^{b}
MS + CM (20%, v/v)	2.4 ± 0.10^{d}	0.03 ± 0.001^{b}	0.006 ± 0.0003^{b}
MS + 10 µM 2,4-D + 15 µM Kn + 5 mM putrescine	2.0 ± 0.07^{b}	0.02 ± 0.001^{b}	0.004 ± 0.0002^{b}
MS + 10 µM 2,4-D + 15 µM Kn + 5 mM spermine	2.5 ± 0.12^{e}	0.03 ± 0.001^{b}	0.005 ± 0.0002^{b}
MS + 10 µM 2,4-D + 15 µM Kn + 5 mM Spd	78.0± 2.44^{g}	0.95 ± 0.047^{a}	0.172 ± 0.0085^{a}

*cultures initiated at 1000 cells ml^{-1} density and their growth monitored; values were those observed after 18 days of culture in the indicated medium; **nutrient medium along with hormones was prepared at 1.2X concentration to correct for the supplementation of 20% (v/v) of CM; values in a column with the same letter are not significantly different according to DMRT at 5% level.

that induced the growth of the low density cell suspension cultures. Thus, in the present study, by working out conditions for the establishment of low density cell suspension cultures, a starter medium for the initiation of microdroplet cell cultures has been achieved. Table 3 provides the kinetic analysis of growth of the low density cell suspension cultures due to the supplementation of CM and Spd. Supplementation of the CM resulting in the induction of microspore embryogenesis has been reported in *Brassica napus*[27]. Change in the content of different polyamines and the activity profile of their biosynthetic enzymes have been shown to be related to somatic embryo development in cultures of red spruce[28]. Earlier, we have reported that Spd supplementation was effective in supporting the growth of low density cell suspension cultures in rice[7].

Embryogenesis in callus and low density cell suspension cultures—Incubation of shoot tip explants or cultured cells in medium containing abiotic stress factors, such as, D-mannitol and PEG prior to culture in 2,4-D containing embryogenesis induction medium induces SE in cultures of *Arabidopsis thaliana* and pigeonpea, respectively[21,29]. Similarly, incubation of explants, cultured tissues and cells in abiotic stress factors such as PEG, mannitol, sorbitol, NaCl, CdCl and Fe-EDTA induces SE in diverse plants, such as, *Arabidopsis thaliana*, *Daucus carota*, *Glycine max*, *Medicago sativa* and *Picea glauca*[21,30,31]. Dehydration and osmotic stress are known to mimic the conditions that exist in the embryo sac during zygotic embryogenesis[29,32].

Here, in order to induce SE, callus or inoculum for low density cell suspension cultures was subjected to PEG incubation. Results showed that callus or inoculum for low density cell suspension cultures without PEG incubation showed no observable stage of somatic embryos. In contrast, callus cultures and low density cell suspension cultures raised subsequent to PEG incubation of the inoculum resulted in the formation of cotyledonary stage somatic embryos (Table 4). Frequency of embryogenesis in callus

Table 3—Kinetics of growth due to supplementation of CM and Spd in low-density cell suspension cultures of pigeonpea*

Age of culture (d)	Culture supplements**	Growth parameters per flask		
		Cell count × 10000	Protein content (mg)	Wet wt (g)
0	+ CM	2.0 ± 0.1^{b}	0.02 ± 0.001^{b}	0.004 ± 0.0002^{b}
	+ Spd	2.4 ± 0.1^{b}	0.02 ± 0.001^{b}	0.004 ± 0.0002^{b}
6	+ CM	5.9 ± 0.3^{c}	0.05 ± 0.002^{b}	0.010 ± 0.0005^{b}
	+ Spd	6.2 ± 0.3^{c}	0.06 ± 0.003^{b}	0.014 ± 0.0006^{b}
12	+CM	33.4 ± 1.3^{d}	0.31 ± 0.012^{c}	0.069 ± 0.0034^{c}
	Spd	41.4 ± 2.0^{e}	0.38 ± 0.019^{d}	0.082 ± 0.0040^{d}
18	+CM	70.4 ± 3.4^{f}	0.62 ± 0.031^{e}	0.138 ± 0.0067^{e}
	+Spd	78.2 ± 3.1^{a}	0.87 ± 0.043^{a}	0.168 ± 0.0052^{a}

*cultures initiated at 1,000 cells ml^{-1} density; **supplemented with MS + 2,4-D (10 µM) + Kn (15 µM); values in a column with the same letter are not significantly different according to DMRT at 5% level.

Table 4—Effect of PEG incubation of inoculum for callus and low density cell suspension cultures on SE of pigeonpea

Inoculum*	SE Frequency (per 10^6 cells)**	% distribution of SE stages			
		Globular	Heart	*Torpedo*	Cotyledonary
Callus without PEG incubation	0	-	-	-	-
Callus with PEG incubation***	1.19 ± 0.04^{b}	63 ± 3.1^{a}	24 ± 1.1^{b}	11 ± 0.5^{a}	2 ± 0.1^{b}
Cells from low density cell suspension cultures without PEG incubation	0	-	-	-	-
Cells from low density cell suspension cultures with PEG incubation***	3.16 ± 0.15^{a}	54 ± 1.9^{b}	34 ± 1.5^{a}	4 ± 0.2^{b}	8 ± 0.3^{a}

*callus inoculum subsequent to PEG incubation was cultured on semi-solid medium and that of cell suspension inoculated in liquid medium; medium employed for callus cultures was MS + 2,4-D (5 µM) + glutamine (0.03 mM); medium employed for low density cell suspension culture was MS + 2,4-D (10 µM) + Kn (15 µM) + Spd (5 mM); **SE frequency determined in callus and cell suspensions on 28th day and 18th day, respectively, subsequent to PEG incubation; ***incubated in 4% PEG (w/v) for 4 h; values in a column with the same letter are not significantly different according to DMRT at 5% level.

cultures and low density cell suspension cultures was to the tune of *ca*. 1.19 and 3.16, respectively per 10^6 cells. The frequency of somatic embryo formation was 2.7 times higher in low density cell suspension cultures as compared to that of callus cultures. In white spruce production of *ca.* 435 somatic embryos per gram fresh weight of callus has been reported as a high frequency SE system[31]. In soy bean and pigeonpea, only qualitative description of somatic embryo formation has been reported without indicating the frequency[33,34].

Cell division and SE in microdroplet cell cultures—The growth requirements of single isolated cells are known to be complex since these single cells lack organic contact with the neighboring cells[7]. In a multicellular system, all the cells in the multicellular cluster are able to exchange nutrients and biomolecules among the cell population so that all the cells are able to grow. Observations of the present study showed that a combination of effective growth factor supplements induced cell division in microdroplet cell cultures to the tune of *ca*. 30 cells per microdroplet culture on 4th day (Fig. 2d-k). Growth factors screened for cell division and SE activity included ChCl, ETA and LiCl (Table 5). Supplementation of membrane lipid precursors such as ChCl and ETA, specific precursors for phosphatidylcholine and phosphatidylethanolamine respectively, have been shown to be inducing various differentiation responses in cultures of *Datura innoxia*[35]. In the present study, cultures raised in the optimized low density cell suspension culture medium together with PEG incubation resulted in the production of somatic embryos although no maturation of somatic embryos was observed under those conditions. Results showed that supplementation of ChCl induced cell growth at a higher level by *ca.* 1.3 fold as compared to ETA supplementation (Table 5). It was observed that supplementation of ChCl/ETA to the microdroplet cell cultures together with PEG incubation resulted in the maturation of somatic embryos (Fig. 2l-r).

In an attempt to initiate SE in the microdroplet cell cultures, incubation of the inoculum of fine suspension of cells for initiating microdroplet cell cultures was carried out in PEG. Results showed that PEG incubation did not inhibit the growth of cells in the microdroplet cell cultures (Table 5). PEG incubation of the inoculum was found to be essential for the induction of SE. The SE frequency was found

Table 5—Establishment of conditions for growth of cells and SE in microdroplet cell cultures of pigeonpea

Medium for inoculum*	PEG incubation of inoculum**	Microdroplet cell culture medium***	Cell growth (number of cells per microdroplet)		SE frequency on 24th day					
			4th day	24th day (× 10^6 cells)	Per micro droplet	Per 10^6 cells	Somatic embryo stages			
							G	H	T	C
MS + 2,4-D + Kn+Spd	-	MS + 2,4-D + Kn+Spd	7.1± 0.32[c]	0.132± 0.004[c]	0	0	0	0	0	0
MS + 2,4-D + Kn+Spd	+	MS + 2,4-D + Kn+Spd	6.8± 0.33[c]	0.121± 0.006[c]	2.8± 0.10[b]	23.14± 1.1[a]	1.8± 0.06[b]	0.9± 0.03[b]	0	0
MS + 2,4-D + Kn+Spd	-	MS + 2,4-D + Kn+Spd+ChCl	30.4± 1.2[a]	4.03± 0.161[a]	0	0	0	0	0	0
MS + 2,4-D + Kn+Spd	+	MS + 2,4-D + Kn+Spd+ChCl	29.3± 1.18[e]	3.88± 0.147[e]	25.2± 1.23[a]	6.51± 0.3[b]	2.3± 0.08[a]	5.1± 0.17[a]	4.5± 0.21	13.3± 0.48[a]
MS + 2,4-D + Kn+Spd	-	MS + 2,4-D + Kn+Spd+ethanolamine	23.4± 1.16[d]	1.57± 0.071[d]	0	0	0	0	0	0
MS + 2,4-D + Kn+Spd	+	MS + 2,4-D + Kn+Spd+ethanolamine	21.9± 1.1[c]	1.38± 0.063[e]	9.4± 0.35[c]	6.76± 0.2[c]	1.7± 0.08[b]	1.8± 0.06[c]	0	5.9± 0.23[b]
MS + 2,4-D + Kn+Spd	-	MS + 2,4-D + Kn+Spd+ChCl+LiCl	4.2± 0.18[b]	0.002± 0.0001[b]	0	0	0	0	0	0
MS + 2,4-D + Kn+Spd	+	MS + 2,4-D + Kn+Spd+ChCl+LiCl	4.1± 0.18[b]	0.002± 0.0001[b]	0	0	0	0	0	0

*inoculum was prepared from 1-day old low density cell suspension cultures initiated at 1000 cells ml^{-1}; concentration of media supplements: 2,4-D, 10 µM; Kn, 15 µM; Spd, 5 mM; ChCl, 10 mM; ethanolamine, 10 mM; and LiCl, 5 mM.

**incubated in 4% PEG (w/v) for 4 h.

***media replenished on 12th and 20th day. Observations made on 24th d. G, H, T and C indicate the somatic embryo stages such as globular, heart, torpedo and cotyledonary, respectively. Values in a column with the same letter are not significantly different according to DMRT at 5% level.

to be high in ChCl supplemented microdroplet cell cultures. The cultures exhibited both higher cell growth as well as SE frequency on 24th day in the ChCl supplemented microdroplet cell cultures. It is pertinent to mention that there was an ambiguity with regard to the expression of SE frequency in relation to cell number of the cell clones. When the cell growth was low, SE frequency was also found to be low on the basis of per microdroplet count of somatic embryos. However, for the same sample SE frequency on the basis of per 10^6 cells was found to be very high. Ongoing work in our laboratory focuses on separating the somatic embryos from the mass of cells in microdroplets to precisely quantify the SE frequency.

Supplementation of LiCl was found to inhibit cell division as well as SE in the microdroplet cell cultures (Table 5). Lithium salts are known to inhibit *myo*-inositol-1-phosphatase which is involved in the biogenesis and turn-over of phosphoinositides which constitutes the plasmamembrane based signal transduction cascade[36]. Inhibition of cell division and SE in cultures of *V. radiata* by LiCl indicated the possibility of the involvement of phosphoinositides based signal transduction during growth and development.

It was observed that no embryo stage was detected in the cell clones that were developed in the microdroplet cell cultures up to a cell number of *ca*. 30 cells. Possibly, embryo formation could not be ascertained by microscopic observation at this stage of development in the microdroplet cell cultures. It has been shown in carrot cell suspension cultures that commitment to SE happened in few-celled stage in cell suspension cultures and there was establishment of polarity in few-celled clusters[10]. Establishment of cellular polarity during SE has been demonstrated on the basis of spatial pattern of DNA synthesis in proembryogenic masses of carrot cultures[37]. In the present study, SE frequency in microdroplet cell cultures was found to be *ca*. 6.51 per 10^6 cells, which was 2.1 fold higher than that of the low density cell suspension cultures.

In contrast to the low density cell suspension cultures, the microdroplet cell cultures employed in the present study offers the advantage of eliminating the cellular cross-inhibition totally. The microdroplet cell cultures are found to be novel since it supports the growth of single cells in isolation that are cultured in microdroplets. Accordingly, the microdroplet cell cultures offer for the screening of potential, as yet elusive, morphogenetic and embryogenic factors for the induction of desired *in vitro* responses. The culture of pollen grains as a hanging drop in microscope slides is found to be a forerunner for the microdroplet cell cultures of the present study[38]. It is pertinent to mention that the microdroplet cell cultures offer the advantage of *in situ* microscopic visualization of cell divisions up to *ca.* 30 cells in glass Petri dishes in which they are grown (Fig. 2d-k).

Development of somatic embryo derived plantlets and their establishment—The somatic embryos that were developed in the microdroplet cell cultures were subsequently transferred to semi-solid hormone free MS medium for their germination (Fig. 2s). This, as well as the subsequent stages, leading to the development of *in vitro* plantlets and hardening of the *in vitro* developed plantlets was essentially according to the protocols worked out in our laboratory[21]. To mention briefly, the development of *in vitro* plantlets was carried out in half strength MS basal semi-solid medium (Fig. 2t). Those plantlets were subsequently established in the garden soil. The establishment rate of plantlets in the garden soil was found to be *ca*. 100%. Thus, the present study contributes to induction of high frequency SE by employing microdroplet cell culture system and development of plantlets and their establishment in the garden soil.

Culture of single isolated cells and development of somatic embryos from the single cell derived cell clones would have applications in transgenic biology[3,12]. When callus cultures were transformed, especially by employing *Agrobacterium tumefaciens* based transformation systems, cells dispersed from the transformed callus could be cultured and developed into somatic embryos by following the protocols established in the present study. Thus, the present study focused on establishing conditions for cell growth and induction of SE in microdroplet cell cultures with emphasis on its application in genetic transformation protocols. This study has clearly demonstrated that metabolic conditioning of single isolated cells in microdroplets by supplementing phospholipid precursors contributes to induction of SE in pigeonpea cultures.

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