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Somatic Embryogenesis and Plant Regeneration from Petiole-Derived Callus of Spine Gourd (*Momordica dioica* Roxb. ex Willd)

M. Thiruvengadam* • K. T. Rekha • C. H. Yang

Graduate Institute of Biotechnology, National Chung-Hsing University, 250, Kuo-Kuang road, Taichung - 402, Taiwan, R.O.C.

Corresponding author: * **thiruv30@gmail.com**

ABSTRACT

An efficient protocol for plantlet regeneration from the cell suspension cultures of spine gourd (*Momordica dioica* Roxb. ex Willd.) through somatic embryogenesis is reported. Petiole-derived embryogenic calli were cultured on Murashige and Skoog medium augmented with 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 10% coconut milk (CM). The maximum frequency of somatic embryos (36.3%) was observed on MS medium supplemented with 2.2 µM 2,4-D for three weeks of culture. Ontogenic studies of somatic embryos revealed that the cells destined to become somatic embryos divided into spherical proembryos and then progressed to globular, heart and further differentiated properly into torpedo and cotyledonary stages within 5 weeks. Embryo development was asynchronous and strongly influenced by the 2,4-D concentration. The MS liquid medium augmented with 2.2 µM 2,4-D and 0.5 µM L-glutamine was effective to achieve high frequency of somatic embryo induction (44.5%). The cotyledonary-stage somatic embryos were transferred to MS liquid medium with no plant growth regulators to achieve complete maturation within 7 days. Lack of 2,4-D in suspensions increased somatic embryo maturation with decreased abnormalities. Sucrose was found to be the best carbon source for callus induction, embryo maturation and embryo germination. Relatively, only few numbers of embryos developed into root/shoot when transferred to 1/10 MS solid medium containing 0.5 µM abscisic acid (ABA), 2% (w/v) sucrose and 0.2% (w/v) Gelrite. About 11% of somatic embryos germinated into morphologically normal fertile plants within 2 weeks. Regenerated plantlets were successfully hardened, with a survival rate of approximately 60%, and established in the field. This regeneration protocol assured successful embryo induction, maturation and plantlet conversion.

Keywords: callus induction, germination, hardening, maturation, suspension culture

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **ABA**, (±)-*cis*, *trans*-abscisic acid; **BAP**, N^6-benzylaminopurine; **CM**, coconut milk; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **Kn**, kinetin; **NAA**, α-naphthaleneacetic acid; **PCV**, packed cell volume

INTRODUCTION

Spine gourd (*Momordica dioica* Roxb. ex Willd.) is a medicinally and economically important plant, cultivated for its fruit, which is used as vegetable. It originated from the Indo-Malayan region (Rashid 1976; Singh 1990) and has been highly cultivated in Asia especially in China, Taiwan, India, Bangladesh, Pakistan and Sri Lanka. The fruit is a rich source of protein, carbohydrates and vitamin C (Rashid 1976; Bhuiya *et al.* 1977). In addition to its nutritious value, the plant exhibits a wide range of anti-cancer properties (Li *et al.* 1999), due to the presence of terpenes and steroids in roots (Luo *et al.* 1998) and anti-microbial activity as it contains alkaloids and glycosides (Bhosle and Paratkar 2005). Besides, it is also used in the treatment of bleeding piles, urinary complaints, snakebite, scorpion stings and even as an antiseptic (Chopra *et al.* 1986).

Improvement of this crop has not been adequately attempted, because of its dioecious nature and its vegetative mode of propagation. At present, its propagation depends entirely on underground tuberous roots, which occupy the cultivable land for a long period, until the next planting season. Maintenance of tuber quality under field conditions and its storage are also difficult. Improvement through classical breeding has also been met with limited success due to the limited genetic variability in germplasm (Nabi *et al.* 2002). The establishment of an efficient regeneration system through somatic embryos will allow for crop improvement through a programme that includes somatic hybridization, *in vitro* selection and genetic transformation. Only few results on *M. charantia in vitro* studies such as direct shoot regeneration of different explants have been reported (Islam *et al.* 1994; Wang *et al.* 2001; Sultana and Miah 2003). In *M. dioica* results were reported only on direct organogenesis (Thiruvengadam and Jayabalan 2001; Nabi *et al.* 2002; Bhosle and Paratkar 2005; Thiruvengadam *et al.* 2006a). Although we have recently reported the development of somatic embryos in bitter melon (Thiruvengadam *et al.* 2006b), to our knowledge, no report on spine gourd regeneration via somatic embryogenesis from petiole has yet been described. This work described somatic embryogenesis, ontogeny and histology of embryo development and the effect of growth regulators and carbohydrates on high frequency induction of somatic embryogenesis in this nutritionally and medicinally important plant.

MATERIALS AND METHODS

Plant material

Tubers of *Momordica dioica* Roxb. ex Willd (one year old) were collected from Taichung mountains and the plants were raised in a greenhouse at the National Chung-Hsing University, Taichung, Taiwan. Petiole explants were collected and washed in running tap water for 5 min and surface sterilized in 70% (v/v) ethanol for 1 min. Further, explants were treated in 1.0% (v/v) sodium hypochlorite solution for 10 min with occasional agitation. Finally, the petioles were rinsed with sterile distilled water for seven times and sliced into explants of approximately 0.3-0.5 cm^2.

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Callus induction

Petiole explants were placed in a plastic Petri dish (90×15 mm; Alpha Plus Scientific Corp., Taiwan) with 25 ml medium, consisting of MS salts (Murashige and Skoog 1962), 3.0% (w/v) sucrose, 0.2% (w/v) Gelrite (Sigma, St. Louis, USA) with different concentrations (0.0-6.7 μM) of 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma, St. Louis, USA) alone and in combination with 10% coconut milk (CM; Coconut milk was prepared as described in Bhojwani and Razdan (1983) and added before autoclaving) for callus formation. The medium pH was adjusted to 5.8 by 1N NaOH or 1N HCl prior to sterilization. The medium was then solidified with 0.2% Gelrite and autoclaved at 121°C and 1.05 kg/cm^{-2} for 20 min. The incubation condition for culture, unless stated otherwise, was maintained at 25 ± 2°C and 16/8-h (light/dark) photoperiod of cool white fluorescent light (25 μmol m^{-2} s^{-1}).

Suspension culture

For initiation of cell suspension cultures, two-week-old green-yellow, friable calli (>500 mg fresh weight each) were aseptically transferred to 125 ml Erlenmeyer flasks (Pyrex, USA) containing 25 ml of liquid MS medium supplemented with various concentrations (0.0- 4.5 μM) of 2,4-D and agitated on a gyratory shaker (95-110 rpm) in standard growth room conditions. Suspensions were subcultured every 7-10 days and supplemented by fresh MS medium with 2,4-D. At the end of the second subculture, the cells were filtered through 150 μM stainless steel sieves to separate possible embryogenic cells and small clumps of cells. Cells from the suspensions were observed under a microscope during the culture period. The growth rate of suspended cells was monitored for the first 12 days by determining the packed cell volume (PCV) of samples from 10 replicates. PCV was measured after centrifuging the suspension at 2000 rpm for 10 min in a graduated centrifuge tube (Kumar *et al.* 1988). MS basal medium lacking 2,4-D served as the control. Embryos at different stages of development were separated manually and subcultured in MS liquid medium with different 2,4-D concentrations. After two weeks of subculture, globular, heart, torpedo and cotyledonary shaped embryos formed.

Maturation and germination

Maturation of embryos did not progress in MS liquid medium with 2,4-D, hence cotyledonary-shaped embryos were transferred into 150 ml Erlenmeyer flasks containing 25 ml of hormone-free MS liquid medium for maturation and further development. Then the mature embryos were cultured on different MS media: MS + 3% sucrose, MS + 2% sucrose, 1/2 MS + 2% sucrose, 1/10 MS + 2% sucrose, MS + 3% sucrose + 0.5 μM ABA, 1/10 MS + 2% sucrose + 0.1 μM ABA, 1/10 MS + 2% sucrose + 0.5 μM ABA, and 1/10 MS + 2% sucrose + 1.0 μM abscisic acid (ABA; Sigma, St. Louis, USA), each with 0.2% Gelrite for plantlet conversion. The cultures were maintained in standard growth room conditions and plantlet conversion frequency was observed.

Effect of media, growth regulators, carbohydrates and amino acids

Two-week-old, green-yellow, friable embryogenic callus (750 mg fresh mass) derived from petiole explants were cultured in liquid on different basal media, including MS medium, B5 medium (Gamborg *et al.* 1968), LS medium (Linsmaier and Skoog 1965) and SH medium (Schenk and Hildebrandt 1972), were tested. Different concentrations (0.0-4.5 μM) of auxins (IAA, IBA, NAA and 2,4-D) individually or in combination with cytokinins (0.5 μM BAP and Kn) were tested for somatic embryo induction. The effects of different carbohydrates such as sucrose, fructose, glucose and maltose at various concentrations ranging from 0-5% and amino acids (L-alanine, L-proline, L-glutamine) at concentrations ranging from 0.0-0.6 μM were studied on the differentiation of somatic embryos. The frequency of embryo induction and stages of somatic embryos were recorded.

Study of ontogeny and frequency of somatic embryos

The cultures in liquid medium were examined every day during the experimental period to trace the ontogeny of somatic embryos. Samples from 10 replicate flasks per 2,4-D treatment were taken at random in order to determine the frequency of different stages of somatic embryos. The results do not represent the continuous monitoring of any particular single cell, but the observation of different cells at different stages of growth (Eapen and George 1990).

Soil transfer

After germinated plants were removed from the culture tubes and washed in running tap water, they were transferred to 6-cm plastic pots containing red soil, perlite and vermiculite mixture (3:1:1) maintained in a humidity chamber (Conviron, Winnipeg, Canada) under a 16 h photoperiod at 25 ± 2°C. After three weeks of hardening, the plants were transferred to the field.

Histology

The somatic embryos were fixed in a solution of formalin, alcohol and acetic acid (FAA) of 100 ml, which contains 5.4 ml formalin (37%), 65.6 ml ethanol (96%), 5 ml glacial acetic acid and 24 ml

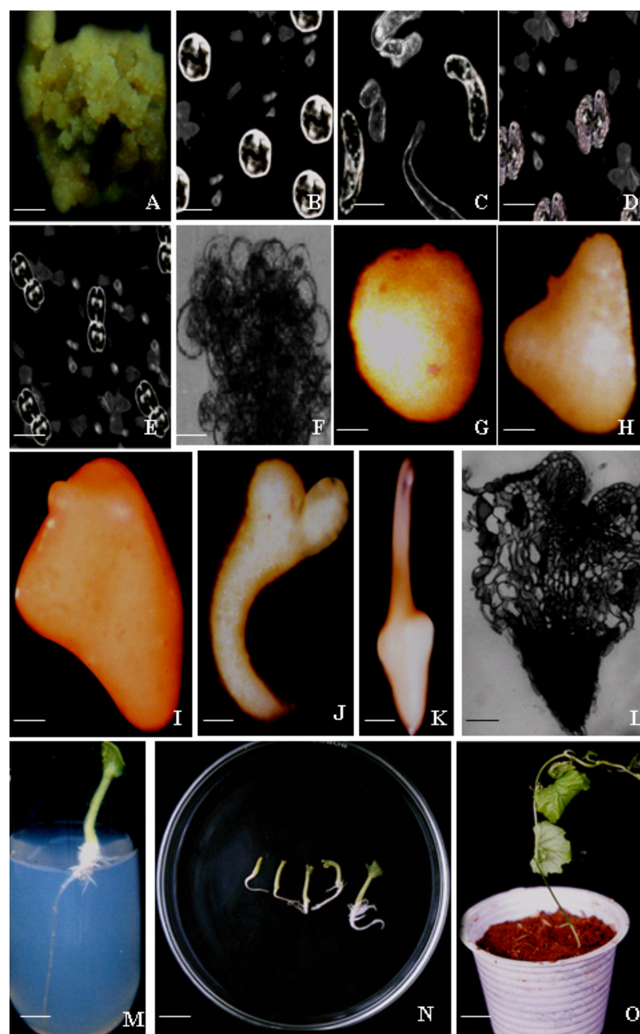

Fig. 1 Somatic embryogenesis from petiole explants through suspension cultures of spine gourd (*Momordica dioica*). (**A**) Petiole derived embryogenic callus; (**B**) Spherical embryogenic cells; (**C**) Elongated cells; (**D**) Two cell stages; (**E**) Four cell stages; (**F**) Proembryos; (**G**) Globular embryo; (**H**) Early heart-shaped embryo; (**I**) Heart-shaped embryo; (**J**) Torpedo shaped embryo; (**K**) Matured cotyledonary shaped embryo with shoot primordia and root primordia; (**L**) Longitudinal sections of heart-shaped embryo; (**M**) Germination of somatic embryos; (**N**) Serial arrangement in germination of somatic embryos; (**O**) Hardened plant. Bar represents 0.4 mm (**A-K**); 0.05 mm (**L**); 0.5 mm (**M-O**).

distilled water (Gerlach 1984). Samples were cut and embedded in hydroxyethylmethacrylate (Histo-Technique-Set Technovit 7100; Kulzer, Wehrhein, Germany). At the beginning of this process, the specimens were dehydrated in 2-h steps through a graded series of ethanol (70%, 90%, 96% and 100%). The samples were then pre-infiltrated overnight with a mixture of equal parts of 100% ethanol and Technovit 7100 base liquid. The somatic embryos were then transferred into an infiltration solution of 100 ml Technovit 7100 base liquid and 1 g hardener I for 1 day. A vaccum was established for 30 min at the start of the last two processes. The cultures were embedded in Teflon moulds with a mixture of 15 parts of infiltration solution and one part hardener II. The samples were polymerized for 1 h at room temperature and further for 6 h at 37°C. Specimens were mounted on block-holders with Technovit 3040. Slices (6 μM) were cut at room temperature using a Jung CM 1800 microtome equipped with type 818 disposable microtome blades (both from Leica Instruments, Nussloch, Germany). Slices were stretched on a bath of distilled water and mounted on slides. They were then stained with 0.05% toluidine blue O (Seva, Heidelberg, Germany) dissolved in 1% sodium tetraborate decahydrate buffer rinsed in distilled water, dried and covered with Entellan (Merck, Darmstadt, Germany) and a coverslip. This procedure stains the cytoplasm and unlignified cell walls blue (Gerlach 1984). Microscopic analysis was performed using a Leitz DMR microscope (Leica, Wetzlar, Germany) with a Wild MPS 48/52 camera (Leica, Heerbrugg, Switzerland).

Statistical analysis

For callus induction, at least 50 explants were used and each experiment was repeated three times. Samples of suspension cultures were taken randomly at the end of each subculture and the number of embryos was counted under a microscope. Counts were made from 10 different independent samples and percentage of embryos was calculated on the basis of the total number of pro-embryogenic cells present in the field. The analysis of variance (ANOVA), appropriate for the design was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Duncan's multiple range test at $P \leq 0.5$, % level by SPSS software version (SPSS 14 for windows, SPSS, Chicago, IL, USA).

RESULTS

Callus induction

Petiole explants from one year old greenhouse-grown plants of *M. dioica* were cultured on MS medium with various levels (0.0-6.7 μM) of 2,4-D alone and in combination with 10% CM for induction of callus. After 2 weeks of culture incubation indicated that MS solid medium containing 4.5 μM 2,4-D and 10% CM produced green-yellow friable calli (**Fig. 1A**). The callus showed dense cytoplasm, small vacuoles and large nuclei with deeply stained nucleoli when observed under a compound microscope. Such potentially embryogenic calli were suitable for suspension cultures. The maximum efficiency of callus and typical embryogenic cells was noted on MS medium containing 4.5 μM 2,4-D and 10% CM while at a 2,4-D concentration higher than 4.5 μM, the callus turned brown with numerous adventitious roots (**Table 1**).

Table 1 Effect of MS solid medium augmented with 2,4-D and 10% coconut milk (CM) on the induction of embryogenic callus from petiole explants of spine gourd.

Concentration of growth regulators (μM)	Explants producing embryogenic callus (%)	Nature of callus	Adventitious rooting
	2,4-D		
0.0	0.0	–	–
2.2	35.5 ± 1.0 i	GF	–
3.3	40.2 ± 3.0 h	GF	–
4.5	50.0 ± 2.5 g	GC	+
5.6	55.0 ± 3.0 f	BF	+
6.7	58.0 ± 1.0 d	BF	++
	2,4-D (μM) + 10% CM		
0.0	0.0	–	–
2.2	56.4 ± 2.0 de	GF	–
3.3	68.8 ± 3.0 c	GWF	–
4.5	84.0 ± 2.5 b	GYF	–
5.6	84.5 ± 3.0 ab	BF	+
6.7	86.0 ± 2.0 a	BF	++

Each value represents the mean ± SE of three replicates per treatment. The data were statistically analyzed using Duncan's Multiple Range Test (DMRT). In the same column, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different letters. BF = brown, friable; GC = green, compact; GF = green, friable; GYF = green-yellow, friable; GWF = green-white, friable. –, No adventitious rooting; +, less rooting; ++, profuse rooting.

Suspension culture, ontogeny and histology of somatic embryos

The green-yellow friable embryogenic calli were suspended in MS liquid medium containing various concentrations (0.0-4.5 μM) of 2,4-D to established the somatic embryogenesis. Active division and growth of cells were observed in 2.2 μM 2,4-D until the 7th and 8th day of culture. Initially, the calli exhibited highly vacuolated cells. After 6 days, the callus cells showed two morphologically distinct kinds of cells, namely, spherical (**Fig. 1B**) and elongated cells (**Fig. 1C**), both with visible cytoplasm and nucleus. Each sphere-cal embryogenic cell transversely divided into two cells, four cells and subsequently into a group of cells (**Fig. 1D, 1E**) that was considered to be the proembryo (**Fig. 1F**). The proembryo further divided and formed globular embryos (**Fig. 1G**), heart shaped embryos (**Fig. 1H, 1I**) within a period of two weeks, which later developed into torpedo and cotyledonary embryos (**Fig. 1J**) within a week. The optimal frequency of somatic embryo formation was achieved in 2.2 μM 2,4-D at shaking speed of 100 rpm were established in cell suspension culture (**Fig. 2**). On the same MS medium with 2,4-D, the torpedo and cotyledonary embryos did not develop but, some abnormal structures appeared such as stunted embryo polarity, poorly developed cotyledons, trumpet with leafy structure, which later turned to green calli (data not shown). Thus torpedo shaped embryos recallused on 2,4-D containing medium. The histological examinations revealed that the globular stage somatic embryos are

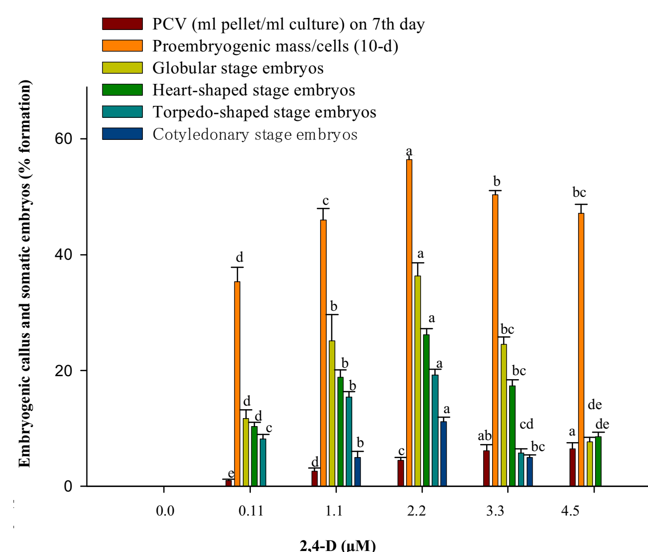

Fig. 2 Influence of MS liquid medium containing various concentrations of 2,4-D on differentiation of somatic embryos in spine gourd. The data were statistically analyzed using Duncan's multiple range test. In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different *letters*. Each value represents the mean of 10 independent replicates. The number of pro-embryogenic cells was compared with the total number of callus cells present in the microscopic field. The frequency of different stages of somatic embryos were determined by taking the samples from 10 replicate flasks per 2,4-D treatment. Vertical bars represented S.D.

new individuals and develop to bipolar structures showing clearly well developed shoot and root meristems, which consist of small cells with a dense cytoplasm (**Fig. 1L**).

Maturation and plantlet formation of somatic embryos

Cotyledonary-shaped embryos, when transferred to MS medium without 2,4-D, greatly improved maturation and further development within 7 days of culture (**Fig. 1K**), whereas in the same system, the presence of 2,4-D resulted in malformed embryo structures, which later developed into friable callus. Based on our experiment, after maturation, the embryos had the potential capacity to synthesize their own hormones for successive function during further development and conversion. Consequently, the matured somatic embryos produced shoots and roots from the poles, enhanced extension of hypocotyl and plantlet development on solidified 1/10 strength MS medium containing 2% sucrose, 0.5 μM ABA and 0.2% Gelrite within 14 days (**Fig. 1M, 1N**). Full strength MS medium consisting of 3% sucrose and 0.5 μM ABA considerably reduced embryo germination and recallusing (**Table 2**). However, 1/10 MS medium with 2% of sucrose and 0.5 μM ABA greatly enhanced embryo germination and effectively stimulated plantlet development from somatic embryos, in which the average frequency of germinating embryos into tiny plantlets (11%) was achieved (**Table 2**). These plantlets, when transplanted into 6-cm pots (**Fig. 1O**) containing red soil, perlite and verminculite (3:1:1), subsequently resulted in 60% survival and grew to maturity in the greenhouse. A total of 2 months was required for the production of plantlets that were ready to be potted.

Table 2 Conversion of mature cotyledonary-stage somatic embryos into plantlets of spine gourd.

Media composition	No. SE plated*	Regeneration (%)	Remarks
MS + 3% sucrose	25	0	Abnormal rooting
MS + 2% sucrose	25	1.5 ± 0.2 de	Browning and recallusing
1/2 MS + 2% sucrose	25	2.0 ± 0.5 d	Recallusing
1/10 MS + 2% sucrose	25	4.5 ± 0.8 c	Hypocotyl extension observed
MS + 3% sucrose + 0.5 μM ABA	25	2.0 ± 0.5 d	Shoot and root pole formation later recallusing
1/10 MS + 2% sucrose + 0.1 μM ABA	25	6.8 ± 0.6 b	Hypocotyl extension with greening at shoot primordia
1/10 MS + 2% sucrose + 0.5 μM ABA	25	11.0 ± 0.4 a	Enhanced extension of hypocotyl and plantlet development
1/10 MS + 2% sucrose +1.0 μM ABA	25	5.5 ± 0.8 bc	Hypocotyl extension with shoot primordial initiation

* Total No. of cotyledonary stage somatic embryos (SE). Each value represents the mean ± SE of three replicates per treatment. The data were statistically analyzed using Duncan's Multiple Range Test (DMRT). In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different *letters*.

Effect of growth regulators, media, carbohydrates and amino acids

Among the auxins (IAA, NAA, IBA, 2,4-D) and cytokinins (BAP, Kn) tested, 2,4-D was the most effective for inducing somatic embryogenesis. The highest frequency of embryo induction was observed on media with 2.2 μM 2,4-D (**Fig. 2**), while the lowest was on media with 1.1 μM. The other auxins (0.0-4.5 μM of IAA, NAA and IBA) could not induce embryogenesis (**Table 3**). The callus became green with the addition of cytokinins (BAP and Kn) and completely inhibited somatic embryogenesis (**Table 3**). The effect of carbohydrates (fructose, glucose, maltose, sucrose), basal medium (MS, B5, LS, SH) and amino acids (L-alanine, L-glutamine, L-proline) with 2.2 μM 2,4-D was assessed on the induction of somatic embryogenesis. The supply of 3% sucrose promoted effective somatic embryo induction (**Fig. 3**) but the same sucrose concentration inhibited embryo conversion (**Table 2**). A low frequency of somatic embryo induction was observed on MS with 3% fructose, glucose and maltose (**Table 4**). Somatic embryos were not obtained on B5, LS and SH medium (**Fig. 4**). The effective enhancement of embryo induction, growth and development was achieved with 0.5 μM glutamine (**Fig. 5**). Hence, the supply of proline and alanine was less efficient in embryogenesis than that of glutamine (**Table 5**).

Table 3 Influence of different growth hormones on differentiation of somatic embryos from petiole-derived callus of spine gourd.

Concentration of growth regulators (μM)	Proembryogenic mass/cells (10 d) (%)	Different stages of somatic embryos (%)		
		Globular stage	Heart stage	Torpedo stage
IAA				
2.2	2.2 ± 0.6 k	1.0 ± 0.2 e	–	–
4.5	3.0 ± 0.5 fg	2.5 ± 0.6 cd	–	–
IAA + BAP				
2.2 + 0.5	1.2 ± 0.2 o	–	–	–
4.5 + 0.5	2.0 ± 0.4 kl	–	–	–
IAA + Kn				
2.2 + 0.5	1.0 ± 0.4 op	–	–	–
4.5 + 0.5	1.5 ± 0.5 m	–	–	–
IBA				
2.2	2.0 ± 0.4 kl	3.0 ± 0.8 c	–	–
4.5	3.0 ± 0.8 fg	3.5 ± 0.6 a	1.0 ± 0.2 b	–
IBA + BAP				
2.2 + 0.5	1.0 ± 0.2 op	–	–	–
4.5 + 0.5	1.4 ± 0.2 mn	–	–	–
IBA + Kn				
2.2 + 0.5	0.5 ± 0.2 qr	–	–	–
4.5 + 0.5	1.0 ± 0.4 op	–	–	–
NAA				
2.2	3.2 ± 0.8 f	3.0 ± 0.5 c	1.0 ± 0.6 b	–
4.5	4.0 ± 1.0 de	3.4 ± 0.2 ab	1.6 ± 0.8 a	–
NAA + BAP				
2.2 + 0.5	1.2 ± 0.2 o	–	–	–
4.5 + 0.5	1.5 ± 0.4 m	–	–	–
NAA + Kn				
2.2 + 0.5	1.0 ± 0.4 op	–	–	–
4.5 + 0.5	0.8 ± 0.2 q	–	–	–
2,4-D + BAP				
2.2 + 0.5	7.2 ± 0.8 b	–	–	–
4.5 + 0.5	10.0 ± 1.0 a	–	–	–
2,4-D + Kn				
2.2 + 0.5	4.5 ± 0.6 d	–	–	–
4.5 + 0.5	6.4 ± 0.5 c	–	–	–

Each value represents the mean ± SE of three replicates per treatment. The data were statistically analyzed using Duncan's Multiple Range Test (DMRT). In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different *letters*. (– = Nil response).

DISCUSSION

The regeneration of plants through embryogenic suspension culture is an ideal tool for the efficient *in vitro* selection and production of transgenic plants (Christou 1997). Embryogenic suspension cultures have been established in very few cucurbits, including melon (Oridate and Oosawa 1986; Kageyama *et al.* 1991), cucumber (Chee and Tricoli 1988; Bergervoet *et al.* 1989) and bitter melon Thiruvengadam *et al.* (2006b). The present study of embryogenic suspension cultures for regeneration of spine gourd have been established for the first time. The complete procedure from induction of somatic embryos to plantlet recovery could be completed within 60 days. This procedure is novel from earlier reports on somatic embryogenesis of melon (Oridate and Oosawa 1986), cucumber (Chee and Tricoli 1988), and bitter melon (Thiruvengadam *et al.* 2006b).

The choice of initial explant is a critical factor for embryogenic callus induction and initiation. In the majority of cucurbits seedling materials have been the most res-

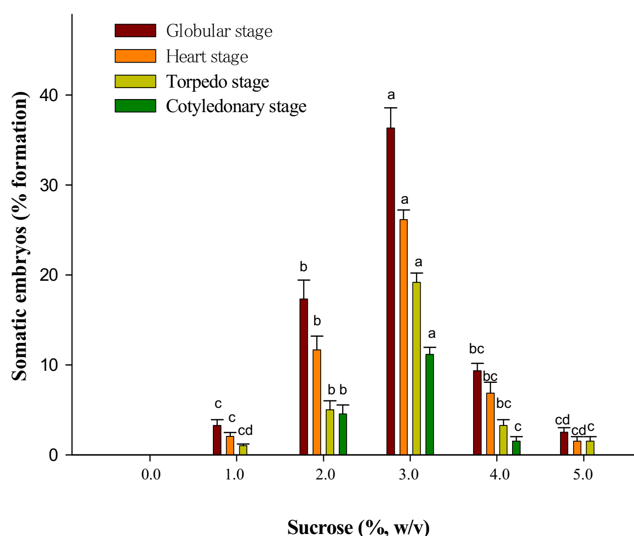

Fig. 3 Influence of varying concentration of sucrose on somatic embryo formation of petiole-derived callus in MS liquid medium with 2.2 µM 2,4-D. The data were statistically analyzed using Duncan's multiple range test. In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different *letters*. Each value represents the mean of 10 independent replicates. The number of pro-embryogenic cells was compared with the total number of callus cells present in the microscopic field. The frequency of different stages of somatic embryos were determined by taking the samples from 10 replicate flasks per 2,4-D treatment. Vertical bars represented S.D.

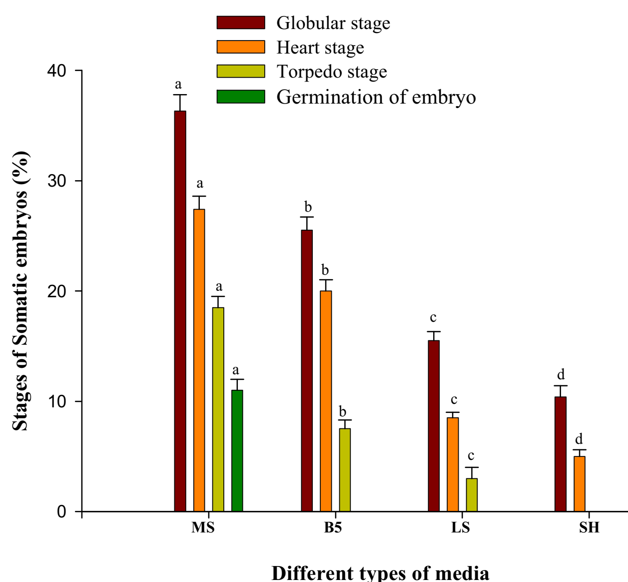

Fig. 4 Effect of different basal media with 2.2 µM 2,4-D on somatic embryogenesis of petiole-derived callus in spine gourd. The data were statistically analyzed using Duncan's multiple range test. In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different *letters*. Each value represents the mean of 10 independent replicates. The number of pro-embryogenic cells was compared with the total number of callus cells present in the microscopic field. The frequency of different stages of somatic embryos were determined by taking the samples from 10 replicate flasks per 2,4-D treatment. Vertical bars represented S.D.

ponsive explants for the induction of somatic embryogenesis. Debeaujon and Branchard (1993) demonstrated that most seedling material, such as cotyledon and hypocotyl tissues were successful for somatic embryogenesis in cucurbits. There are several reports that petiole explants are more suitable than other plant parts for *in vitro* plant regeneration. Petiole explants were used for plant regeneration via somatic embryogenesis in *Rosa hybrida* (Marchant *et*

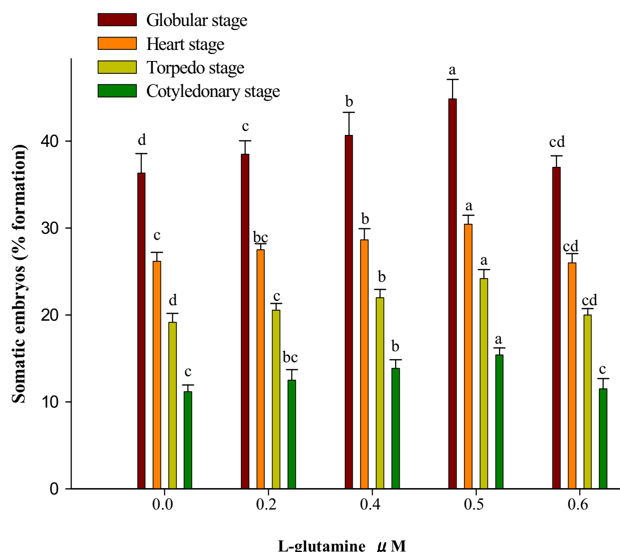

Fig. 5 Effect of L-glutamine on development of somatic embryos from petiole derived callus on MS liquid medium containing 2.2 µM 2,4-D. The data were statistically analyzed using Duncan's multiple range test. In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different *letters*. Each value represents the mean of 10 independent replicates. The number of pro-embryogenic cells was compared with the total number of callus cells present in the microscopic field. The frequency of different stages of somatic embryos were determined by taking the samples from 10 replicate flasks per 2,4-D treatment. Vertical bars represented S.D.

al. 1996), *Begonia gracilis* (Castillo and Smith 1997) and *Heracleum candicans* (Wakhlu and Sharma 1998). Equally, in the present study, petiole explants were found to produce somatic embryos in *M. dioica*. Auxins were absolutely necessary for somatic embryo induction but were often omitted in the period of maturation and germination (Choi *et al.* 1991; Mohamed *et al.* 2004; Thiruvengadam *et al.* 2006b). The pretreatment of 2,4-D for somatic embryo induction in melon and squash were observed by Kintzios *et al.* (2002). Similarly, the present investigation also demonstrated the effect of 2,4-D as a vital role for inducing somatic embryogenesis. The induction of friable calli with embryogenic competence is a prerequisite in somatic embryogenesis for the regeneration of complete plantlets. In our experiments, green-yellow friable calli were induced on fortified MS medium containing 4.5 µM 2,4-D with CM (10%) for two weeks of culture. Similar results by Binzel *et al.* (1996) noted that MS medium containing 9 µM 2,4-D with 10% CM improved the potential of embryogeneic callus in *Capsicum annum*. The addition of BA or Kn to the culture medium had either a negative or no effect on somatic embryogenesis induction as demonstrated by Jelaska (1986) and Noel *et al.* (1992) in *Cucurbito pepo*. In contrast, 2,4-D with BAP or Kn was responsible for inducing somatic embryos in *Cucumis melo* (Oridate and Oosawa 1986), *Cucumis sativus* (Bergervoet *et al.* 1989) and *Cucurbito pepo* (Kintzios *et al.* 2002).

An attempt has been made to develop the ontogenic stages of somatic embryo differentiation from liquid cultures of cucurbits. Jelaska (1980) and Jelaska *et al.* (1985) reported that the presence of 2,4-D in the culture medium inhibited embryo development of squash beyond the globular stage. The complete removal of 2,4-D at later stages of culture stimulated further embryo development into the heart and torpedo stages in *Momordica charantia* (Thiruvengadam *et al.* 2006b). This problem was avoided in the present study, and induction and development of embryos up to the torpedo and cotyledonary stages were achieved on MS medium containing 2,4-D. Similar results were observed by Malepszy *et al.* (1982) and Malepszy and Nadolska-Orczyk (1983), in which MS medium supplemented with

Table 4 Influence of carbohydrate type and concentrations on somatic embryo formation of petiole-derived callus in MS liquid medium with 2.2 μM 2,4-D.

Carbohydrate	Concentration (%)	Different stages of somatic embryos (%)		
		Globular stage	Heart stage	Torpedo stage
Glucose	0	–	–	–
	1	1.0 ± 0.2 m	–	–
	2	3.0 ± 0.5 i	1.0 ± 0.2 fg	1.0 ± 0.2 cd
	3	6.4 ± 0.4 d	2.4 ± 0.3 cd	1.6 ± 0.4 b
	4	10.2 ± 0.5a	4.6 ± 0.4 a	2.0 ± 0.4 a
	5	9.4 ± 0.5 ab	3.8 ± 0.4 b	–
Fructose	0	–	–	–
	1	3.6 ± 0.8 g	1.0 ± 0.2 fg	–
	2	5.8 ± 0.4 de	2.6 ± 0.4 c	1.2 ± 0.5 c
	3	8.4 ± 0.2 c	3.8 ± 0.4 b	1.0 ± 0.2 cd
	4	2.8 ± 0.5 ij	2.2 ± 0.6 e	–
	5	1.2 ± 0.5 kl	–	–
Maltose	0	–	–	–
	1	3.4 ± 0.4 gh	1.2 ± 0.2 f	–
	2	4.8 ± 0.4 f	2.2 ± 0.2 e	–
	3	1.4 ± 0.5 k	–	–
	4	–	–	–
	5	–	–	–

Each value represents the mean ± SE of three replicates per treatment. The data were statistically analyzed using Duncan's Multiple Range Test (DMRT). In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different *letters*. (– = Nil response).

Table 5 Effect of amino acids on somatic embryogenesis of petiole-derived callus in MS liquid medium with 2.2 μM 2,4-D.

Amino acid Type	Concentration (μM)	Different stages of somatic embryos (%)		
		Globular stage	Heart stage	Torpedo stage
L-alanine	0	36.3 ± 1.0 a	25.4 ± 1.4 a	19.0 ± 0.8 a
	0.1	12.6 ± 1.2 c	5.2 ± 0.4 cd	1.0 ± 0.4 d
	0.2	4.0 ± 1.0 de	2.6 ± 0.2 ef	–
	0.3	1.2 ± 1.0 f	–	–
	0.4	–	–	–
	0.5	–	–	–
	0.6	–	–	–
L-proline	0	36.3 ± 1.0 a	25.4 ± 1.4 a	19.0 ± 0.8 a
	0.1	19.2 ± 1.2 b	11.4 ± 1.0 b	9.4 ± 0.5 b
	0.2	10.0 ± 1.0 cd	6.8 ± 0.8 c	4.2 ± 0.4 c
	0.3	4.2 ± 0.5 d	2.8 ± 0.5 e	1.0 ± 0.2 d
	0.4	–	–	–
	0.5	–	–	–
	0.6	–	–	–

Each value represents the mean ± SE of three replicates per treatment. The data were statistically analyzed using Duncan's Multiple Range Test (DMRT). In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different *letters*. (– = Nil response).

2,4-D (0.4 mg/l) was effective for the induction of somatic embryogenesis from leaf and stem explants of *C. sativus*.

Embryo maturation begins after the completion of histodifferentiation in somatic embryos. In *C. sativus*, hormone-free MS basal medium was found to be optimal for maturation and further development (Malepszy and Nadolska-Orczyk 1983; Rajasekaran *et al.* 1983; Orezyk and Malepszy 1985; Wyszogrodzka and Shahin 1985). Similar observations were made in our study, where the complete removal of 2,4-D from the culture medium improved embryo maturation. Our results contrast to the reports of Tabei *et al.* (1991) in which the maturation of somatic embryos from cotyledon, hypocotyls, stem and petiole explants of *C. melo* occurred on 1/2 MS medium fortified with GA_4 (0.2 mg/l). Later the matured embryos were transferred to 1/10 MS medium containing ABA 0.5 μM for successful germination. This result was coherent with that of Kim and Soh (1996) in *Allium* sp., Nakagawa *et al.* (2001) in *Cucumis melo*, and Mohamed *et al.* (2004) in *Macrotyloma uniflorum* for somatic embryo germination induced by ABA. Our histological report clearly confirmed the formation of bipolar structures in spine gourd. Globular embryos normally consist of small cells with dense cytoplasm and large nuclei and have been observed in regal geranium (Wilson *et al.* 1994). The results of this report are confirmed by the pre-sent study. The regenerated somatic embryos developed as new individuals that consisted in the globular stage of the typical small cells with a dense cytoplasm and developed to clear bipolar structures (**Fig. 1L**). In poppy (Kim *et al.* 2003), the optimum conditions for maintenance of cell suspension cultures were the shaking speed and packed cell volume (inoculums per 25 ml medium). Similar results were observed in the present study where agitation of the cultures at 100 rpm was found to be optimal.

The components of media and various concentrations of carbohydrates and amino acids can exclusively play a vital role in determining the embryogenic competence of cell suspension and maturation of somatic embryo. This investigation on medium composition revealed that MS media were highly significant for the induction of somatic embryogenesis, due to high levels of nitrogen in the form of ammonium nitrate, which is responsible for both embryo initiation and maturation. Similar results were reported in the leaves of squash and melon (Kintzios *et al.* 2002), cucumber (Callebaut *et al.* 1987; Clusters and Bergervoet 1990). The occurrence of various carbohydrates has been found to affect the initiation of somatic embryos in spine gourd, with 3% sucrose resulting in highest frequency, followed by glucose and fructose with low frequency of embryo initiation. Maltose was completely ineffective for somatic embryogenesis in spine gourd. Similar results were also obtained in peanut and soybean (Eapen and George 1993; Samoylov *et al.* 1998). Guis *et al.* (1997) reported that glucose was the most important sugar for somatic embryogenesis in melon. However, our experiments confirmed sucrose plays an important role as an energy source, and when added to the medium might be essential for somatic embryogenesis. Our results support similar observations made in cucumber (Chee and Tricoli 1988), melon (Nakagawa *et al.* 2001) and bitter melon (Thiruvengadam *et al.* 2006b). Glutamine appears to be a suitable nitrogen source to support active protein synthesis associated with somatic embryo induction and maturation. In our study, the addition of glutamine at 0.5 μM resulted in the great improvement (44.5%) of embryogenic frequency and development. The similar results were demonstrated by Skokut *et al.* (1985) and Stuart and Strickland (1984) in *Medicago sativa*, Khlifi and Tremblay (1995) in *Picea mariana* and Vengatesan *et al.* (2005) in *Cucumis sativus*. This emphasized that the exogenous supply of these substances could foster the physiological maturity of embryos.

CONCLUDING REMARKS

The protocol developed could establish the potential to produce *Momordica dioica* plantlets from petiole explants through somatic embryogenesis. The entire procedure from induction of somatic embryos to plantlet recovery could be completed within 60 days. Such a novel protocol can emphasize the great potential of biotechnological approaches such as *in vitro* selection, production of synthetic seed and development of genetic transformation studies in spine gourd.

REFERENCES

Bergervoet JHW, Mark FV, Custers JBM (1989) Organogenesis versus embryogenesis from long term suspension cultures of cucumber (*Cucumis sativus* L.). *Plant Cell Reports* **8**, 116-119

Bhojwani SS, Razdan MK (1983) *Plant Tissue Culture*, Elsevier, Amsterdam, 502 pp

Bhosle DS, Paratkar GT (2005) Callus cultures from *Momordica dioica* (Roxb.). *Journal of Cell and Tissue Research* **5**, 431-434

Bhuiya MRH, Habib AKMA, Rashid MM (1977) Content and loss of vitamin C in vegetables during storage and cooking. *Bangladesh Horticulture* **5**, 1-6

Binzel LM, Sankhla N, Joshi S, Sankhla D (1996) Induction of direct somatic embryogenesis and plant regeneration in pepper (*Capsicum annuum* L.). *Plant Cell Reports* **15**, 536-540

Callebaut A, Motte JC, Cat DW (1987) Substrate utilization by embryogenic and non-embryogenic cell suspension cultures of *Cucumis sativus* L. *Journal of Plant Physiology* **127**, 271-280

Castillo B, Smith MAL (1997) Direct somatic embryogenesis from *Begonia gracilis* explants. *Plant Cell Reports* **16**, 385-388

Chee PP, Tricoli DM (1988) Somatic embryogenesis and plant regeneration from cell suspension cultures of *Cucumis sativus* L. *Plant Cell Reports* **7**, 274-277

Choi YE, Kim JW, Yoon ES (1999) High frequency of plant production via somatic embryogenesis from callus or cell suspension cultures in *Eleutherococcus senticosus*. *Annals of Botany* **83**, 309-314

Chopra RN, Nair SL, Chopra IC (1986) *Glossary of Indian Medicinal Plants*, Council of Scientific Industrial Research, New Delhi

Christou P (1997) Biotechnology applied in grain legumes. *Field Crops Research* **53**, 83-87

Clusters JBM, Bergervoet JHW (1990) Organogenesis and embryogenesis from cucumber suspension cultures. In: *Abstracts VIIth IAPTC Congress*, Amsterdam, Holland, **A1-35**, 12

Debeaujon I, Branchard M (1993) Somatic embryogenesis in Cucurbitaceae. *Plant Cell, Tissue and Organ Culture* **34**, 91-100

Eapen S, George L (1990) Ontogeny of somatic embryos of *Vigna aconitifolia*, *V. mungo* and *V. radiata*. *Annals of Botany* **66**, 219-226

Eapen S, George L (1993) Somatic embryogenesis in peanut: influence of growth regulators and sugars. *Plant Cell, Tissue and Organ Culture* **35**, 151-156

Gamborg OL, Miller RA, Ojima K (1968) Nutrient experiments of suspension culture of soybean root callus. *Experimental Cell Research* **80**, 150-158

Gerlach D (1984) *Botanische Mikrotechnik*, Georg Thieme, Stuttgart, Germany

Guis M, Latche A, Pech JC, Roustan JP (1997) An efficient method for production of diploid cantaloup charentais melon (*Cucumis melo* L. var. Cantalou Pensis) by somatic embryogenesis. *Scientia Horticulturae* **64**, 11-20

Islam R, Sarker KP, Naderuzzaman ATM, Joarder OI (1994) *In vitro* regeneration of plants from cotyledons of *Momordica charantia* L. *Plant Tissue Culture* **4**, 105-109

Jelaska S (1980) Growth and embryoid formation in *Cucurbita pepo* L. callus culture. In: Dore C INRA (Ed) *Application de la Culture In Vitro a L'amelioration des Plantes Potageres*, Reunion Eucarpia, Section Legumes, Versailles, France, pp 172-178

Jelaska S (1986) Cucurbits. In: Bajaj YPS (Ed) *Biotechnology in Agriculture and Forestry 2*, Crops 1, Springer-Verlag, Berlin, pp 371-386

Jelaska S, Magnus V, Seretin M, Lacan G (1985) Induction of embryogenic callus in *Cucurbita pepo* hypocotyl explants by indole-3-ethanol and its sugar conjugates. *Physiologia Plantarum* **64**, 237-242

Kageyama K, Yabe K, Miyajima S (1991) Somatic embryogenesis in suspension culture of mature seed of *Cucumis melo* L. *Japanese Journal of Breeding* **41**, 273-278

Khlifi S, Tremblay FM (1995) Maturation of black spruce somatic embryos. Part 1 Effect of L-glutamine on the germinability of somatic embryos. *Plant Cell, Tissue and Organ Culture* **41**, 23-32

Kim SW, In DS, Kim TJ, Liu JR (2003) High frequency somatic embryogenesis and plant regeneration in petiole and leaf explant cultures and petiole-derived embryogenic cell suspension cultures of *Hylomecon vernalis*. *Plant Cell, Tissue and Organ Culture* **74**, 163-167

Kim JW, Soh WY (1996) Plant regeneration through somatic embryogenesis from suspension cultures of *Allium fistulosum* L. *Plant Science* **114**, 215-220

Kintzios S, Sereti E, Bluchos P, Drossopoulos JB (2002) Growth regulator pretreatment improves somatic embryos from leaves of squash (*Cucurbita pepo* L.) and melon (*Cucumis melo* L). *Plant Cell Reports* **21**, 1-8

Kumar AS, Gamborg OL, Nabors MW (1988) Plant regeneration from cell suspension cultures of *Vigna aconitifolia*. *Plant Cell Reports* **7**, 138-141

Linsmaier FM, Skoog F (1965) Organic growth factor requirements of tobacco tissue monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany* **50**, 199-204

Li Z, Luo L, Ling M (1999) Studies on the anti-cancer active constituents of Yunnan *Momordica* (*Momordica dioica*). *Chinese Traditional and Herbal Drugs* **30**, 6

Luo L, Li Z, Zhang Y, Huang R (1998) Triterpenes and steroidal compounds from *Momordica dioica*. *Yao Xue Xue Bao* **33**, 839-842

Malepszy S, Niemirowicz-Szczytt K, Wiszniewska J (1982) Cucumber (*Cucumis sativus* L.) somatic embryogenesis *in vitro*. *Acta Biologia* **10**, 218-220

Malepszy S, Nadolska-Orczyk A (1983) *In vitro* culture of C*ucumis sativus* L. regeneration of plantlets from callus formed by leaf explants. *Zeitschrift für Pflanzenphysiologie* **111 S**, 273-276

Marchant R, Davey MR, Lucas JA, Power JB (1996) Somatic embryogenesis and plant regeneration in floribunda rose (*Rosa hybrida* L.) cvs. Trumpeter and Glad Tidings. *Plant Science* **120**, 95-105

Mohamed SV, Wang CS, Thiruvengadam M, Jayabalan N (2004) *In vitro* plant regeneration via somatic embryogenesis through cell suspension cultures of horsegram [*Macrotyloma uniflorum* (Lam.) Verdc.]. *In Vitro Cellular and Developmental Biology – Plant* **40**, 284-289

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Nabi SA, Rashid MM, Al-Amin M, Rasul MG (2002) Organogenesis in teasle gourd (*Momordica dioica* Roxb). *Plant Tissue Culture* **12**, 173-180

Nakagawa H, Saijyo T, Yamauchi N, Shigyo M, Kako S, Ito A (2001) Effects of sugars and abscisic acid on somatic embryogenesis from melon (*Cucumis melo* L.) expanded cotyledon. *Scientia Horticulturae* **90**, 85-92

Noel M, Comeau D, Lenee P (1992) Embryogenese somatique de la courgette (*Cucurbita pepo* L) a partir de cotyledons. In: IAPTC (Ed) *Comptes Rendus du ler Forum Jeunes Chercheurs du Gene a l'Entreprise*, Amiens, France, pp 14-18

Oridate T, Oosawa K (1986) Somatic embryogenesis and plant regeneration from suspension callus culture in melon (*Cucumis melo* L.). *Japanese Journal of Breeding* **36**, 424-428

Orezyk AN, Malepszy S (1985) *In vitro* culture of *Cucumis sativus* L. V. Stabilizing effect of glycine on leaf protoplasts. *Plant Cell Reports* **4**, 269-273

Rajasekaran K, Mullins MG, Nair Y (1983) Flower formation *in vitro* by hypocotyl explants of cucumber (*Cucumis sativus* L.). *Annals of Botany* **52**, 417-420

Rashid MM (1976) *Bangladeshi Shabjee* (1st Edn) Dhaka, Bangladesh, Bangla Academy, 494 pp

Samoylov VM, Tucker DM, Parrott WA (1998) A liquid medium based protocol for rapid regeneration from embryogenic soybean cultures. *Plant Cell Reports* **18**, 49-54

Schenk RV, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany* **50**, 199-204

Singh AK (1990) Cytogenetics and evalution in the cucrbitaceae. In: Bates DW, Robinson RW, Jaffy C (Eds) *Biology and Utilization of Cucurbitaceae*, Cornell University Press, Ithaca, Comstock Publishing Associates, NY, pp 10-28

Skokut TA, Manchester J, Schaefer J (1985) Regeneration of alfalfa tissue culture: stimulation of somatic embryo production by amino acids and N-15 NMR determination of nitrogen utilization. *Plant Physiology* **79**, 579-583

Stuart DA, Strickland SG (1984) Somatic embryogenesis from cell cultures of *Medicago sativa* L. I. The role of amino acid additions to the regeneration medium. *Plant Science Letters* **34**, 165-174

Sultana RS, Miah MAB (2003) *In vitro* propagation of Karalla (*Momordica charantia* L.) from nodal explant and shoot tip. *Journal of Biological Science* **3**, 1134-1139

Tabei Y, Kanno T, Nishio T (1991) Regulation of organogenesis and somatic embryogenesis by auxin in melon *Cucumis melo* L. *Plant Cell Reports* **10**, 225-229

Thiruvengadam M, Jayabalan N (2001) *In vitro* shoot multiplication and field establishment of kakrol (*Momordica dioica* Roxb.). *Journal of the Indian Botanical Society* **80**, 31-33

Thiruvengadam M, Rekha KT, Jayabalan N (2006a) An efficient *in vitro* propagation of *Momordica dioica* Roxb. *ex*. Willd. *Philippine Agricultural Scientist* **89**, 165-171

Thiruvengadam M, Mohamed SV, Yang CH, Jayabalan N (2006b) Development of an embryogenic suspension culture of bitter melon (*Momordica charantia* L.). *Scientia Horticulturae* **109**, 123-129

Vengadesan G, Selvaraj N, Anand RP, Gaba V, Ganapathi A (2005) Ontogeny of somatic embryos in cucumber (*Cucumis sativus* L.). *In Vitro Cellular and Developmental Biology – Plant* **41**, 789-793

Wakhlu AK, Sharma RK (1998) Somatic embryogenesis and plant regeneration in *Heracleum candicans* Wall. *Plant Cell Reports* **17**, 866-869

Wang S, Tang L, Chen F (2001) *In vitro* flowering of bitter melon. *Plant Cell Reports* **20**, 393-397

Wilson DPM, Sullivan JA, Marsolais AA, Tsujita MJ (1994) Histology of somatic embryogenesis in regal geranium. *Journal of the American Society for Horticultural Science* **119**, 648-651

Wyszogrodzka AJ, Shahin EA (1985) Regeneration of cucumber plants (*Cucumis sativus* L.) from callus and protoplasts. *HortScience* **20**, 357-571