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Plant regeneration through somatic embryogenesis from suspension cultures of gherkin (*Cucumis anguria* L.)

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Abstract

Gherkin (*Cucumis anguria* L.) is an important highly nutrient vegetable and traditional medicinal plant. An efficient protocol for plantlet regeneration from cell suspension cultures of gherkin (*Cucumis anguria* L.) through somatic embryogenesis is reported here. Leaf-derived embryogenic calli were cultured on Murashige and Skoog medium augmented with 4.0 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 µM kinetin (KIN). The maximum frequency of somatic embryos (33.5%) was observed on MS medium supplemented with 2.0 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 2.0 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 15% of somatic embryos germinated into morphologically normal fertile plants within 2 weeks. Regenerated plantlets were successfully hardened, with a survival rate of approximately 80%, 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: N6-benzylaminopurine; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; KIN: kinetin; NAA: α-naphthaleneacetic acid; PCV: packed cell volume.

Introduction

Gherkin (*Cucumis anguria* L.), also known as West Indian gherkin, burr gherkin and maxixe, belongs to the genus *Cucumis*, which is same as cucumber (*C. sativus* L.) (Kirkbride, 1993). It is mainly cultivated and consumed in Africa, Brazil, Cuba India, United States and Zimbabwe (Mangan et al., 2008, 2010). Gherkin fruits (100 g) contain high amounts of protein, calcium (27 mg), phosphorous (34 mg), iron (3.6 mg), vitamin A 325 IU, thiamin 0.15 mg, riboflavin 0.40 mg, niacin 0.5 mg and ascorbic acid 54 mg (Whitaker and Davis, 1962). Fruits and consumed as boiled, fried, stewed, or used fresh in salads, because of its valuable source of vitamins and minerals (Resende, 1998). In addition, this species is valued for several medicinal properties to treat against jaundice, kidney problems and hemorrhoids (Baird and Thieret, 1988). Phytochemists have isolated a number of potential medical components from *C. anguria*, such as cucurbitacin B, cucurbitacin D and cucurbitacins G (Sibanda and Chitate, 1990). *Cucumis anguria* consist of many useful compounds such as flavonoids, tannins alkaloids, saponins and steroids were used for high level of antioxidant activity (Dzomba and Mupa, 2012). Anthraquinones and saponins were used for antibacterial and antifungal activity of *C. anguria* fruits against clinical pathogens (Senthil Kumar and Kamaraj, 2010). Recently, it has been reported that gherkins are susceptible to the same diseases that affect some cucumbers or melons (*C. melo* L.). Some examples are mosaic virus (Srinivasulu et al., 2010), streak virus (Krishnareddy et al., 2003), powdery mildew (Lebeda, 1984; Alvarez et al., 2005), and fusarium wilt (Alvarez et al., 2005; Matsumoto et al., 2011). Although new cultivars have been developed by cross breeding (Modolo and Costa, 2004), yet no cultivar has developed with resistance to all these diseases. For suitable cultivation, it would be necessary to breed a cultivar that is resistant to these diseases. *In vitro* technology, especially plant tissue culture offers many unconventional techniques for crop improvement. It can be employed as an alternative means for genetic upgrading, and its application largely depends on the reliable plant regenera-

Table 1. Effect of MS solid medium augmented with 2,4-D and 1.0 µM KIN on the induction of embryogenic callus from leaf explants of *C. anguria*.

Concentration of growth regulators (µM)	Explants producing embryogenic callus (%)	Nature of callus	Adventitious rooting
2,4-D			
0.0	n.d.	n.d.	n.d.
1.0	32.5±1.5^{e}	GF	–
2.0	50.0±2.0^{c}	GF	–
3.0	55.3±1.0^{ab}	GC	+
4.0	60.0±1.5^{a}	GC	+
5.0	49.0±2.0^{cd}	BF	++
6.0	31.3±1.2^{ef}	BF	++
2,4-D + KIN (1.0 µM)			
0.0	n.d.	n.d.	n.d.
1.0	56.5±2.0^{e}	GF	–
2.0	68.8±1.0^{c}	GWF	–
3.0	84.5±2.^{0ab}	GF	–
4.0	89.1±1.0^{a}	GYF	–
5.0	65.5±1.5^{cd}	BF	+
6.0	45.2±2.0^{f}	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. n.d. = not determined due to nil response.

tion system. The establishment of embryogenic suspension cultures has great potential to aid crop improvement and is suitable for *in vitro* selection of variants specially selection of salt tolerance, disease/toxin resistance, cold tolerance lines in crop plants (Dixon and Gonzales, 1994) and genetic transformation (Finer and McMullen, 1991). Somatic embryos have been induced from various cucumber explants such as cotyledons, hypocotyls, petioles and leaves (Jia et al., 1986; Ladyman and Girard, 1992; Raharjo and Punja, 1992; Lou and Kako, 1994; Burza and Malepszy, 1995; Lou et al., 1996; Nakagawa et al., 2001). Plant regeneration through direct and indirect embryogenesis from anther cultures of *C. sativus* (Ashok Kumar et al., 2003). Chee and Tricoli (1988) reported plant regeneration from cucumber embryogenic cell suspension cultures. Somatic embryogenesis in cucumber is generally limited by low efficiency or the formation of abnormal embryos (Vengadesan et al., 2006). The use of liquid medium has great promise in studies of embryogenesis, as it theoretically permits the isolation of embryogenic cells in a controlled environment. Additionally, liquid culture opens the possibility of mass production of somatic embryos in plant tissue culture (Levin et al., 1989). Cucumber embryogenesis in liquid culture has rarely been attempted. Chee and Tricoli (1988) found embryo conversion was problematic, and circumvented this problem by washing and reculturing in liquid medium. Ziv and Gadasi (1986) initially failed to produce normal embryos owing to problems with dedifferentiation and secondary embryogenesis. Cucumber somatic embryos with structural abnormalities were observed in liquid culture medium (Bergervoet et al., 1989). To our knowledge, there is no report on *C. anguria* regeneration via somatic embryogenesis from leaf explants. This work describes the somatic embryogenesis and the effect of growth regulators and carbohydrates on the high frequency induction of somatic embryogenesis in *C. anguria*.

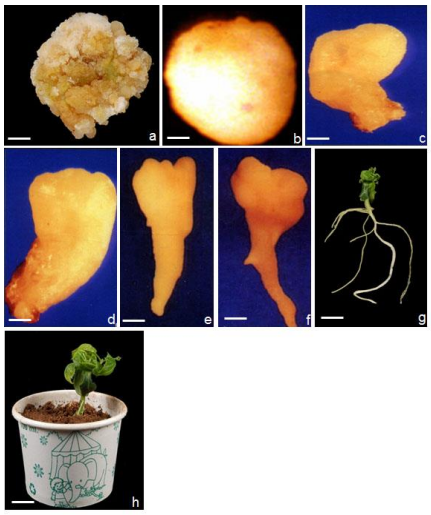

Fig 1. Somatic embryogenesis from leaf explants through suspension cultures of gherkin *C. anguria*. (a) Leaf derived embryogenic callus, (b) Globular embryo, (c) Early heart-shaped embryo, (d) Heart-shaped embryo, (e) Torpedo shaped embryo, (f) Matured cotyledonary shaped embryo with shoot primordia and root primordial, (g) Germination of somatic embryos, (h) Hardened plant. Bar represents 0.5 mm (a), 0.4 mm (b-g), and 3.0 cm (h).

Results and discussion

Callus induction

Leaf explants from one month old growth chamber-grown plants of *C. anguria* were cultured on MS medium with various levels (0.0 - 6.0 µM) of 2,4-D alone and in combination with KIN (1.0 µM) for induction of callus. After

Table 2. Conversion of mature cotyledonary-stage somatic embryos into plantlets of *C. anguria*.

Media composition	No. SE plated*	Regeneration (%)	Remarks
MS + 3% sucrose	20	0	Abnormal rooting
MS + 2% sucrose	20	1.6±0.5^{ef}	Browning and recallusing
1/2 MS + 2% sucrose	20	2.0±0.5^{e}	Recallusing
1/10 MS + 2% sucrose	20	4.5±0.8^{d}	Hypocotyl extension observed
MS + 3% sucrose + 0.5 µM ABA	20	2.0±0.5^{e}	Shoot and root pole formation later recallusing
1/10 MS + 2% sucrose + 0.1 µM ABA	20	8.8±0.6^{b}	Hypocotyl extension with greening at shoot primordial
1/10 MS + 2% sucrose + 0.5 µM ABA	20	15.0±0.4^{a}	Enhanced extension of hypocotyl and plantlet development
1/10 MS + 2% sucrose +1.0 µM ABA	20	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≤0.5 level are indicated by different letters.

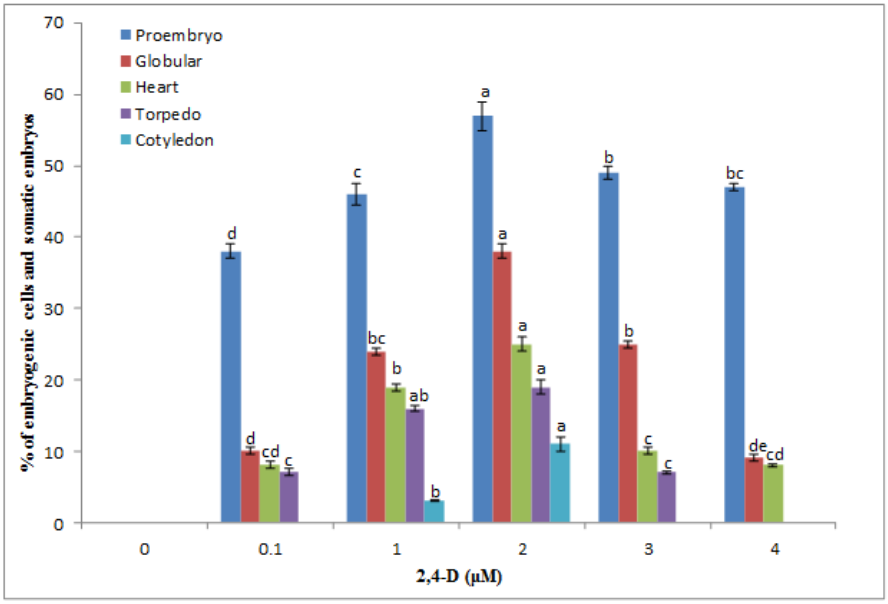

Fig 2. Influence of MS liquid medium containing various concentrations of 2,4-D on differentiation of somatic embryos in *C. anguria*. The data were statistically analyzed using Duncan's multiple range test. In the same co*lumn*, significant differences according to the least significant difference (LSD) at the P≤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.

2 weeks of culture incubation, MS solid medium containing 4.0 µM 2,4-D and 1.0 µM KIN produced green-yellow friable calli (Fig. 1a). Our results are in agreement with Cade et al., (1988), Kintzios et al., (2002), Raharjo and Punja (1992) and Ashok Kumar et al., (2003) who reported that embryogenic callus was produced in the presence of 2,4-D with KIN or BAP from explants of cucumber. 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 (89.1%) was noted on MS medium containing 4.0 µM 2,4-D and 1.0 µM KIN while at a 2,4-D concentration higher than 4.0 µM, the callus turned brown with numerous adventitious roots (Table 1). In our study, lower concentrations of 2,4-D (2.0 µM) produced 50% embryogenic calluses and higher concentrations of 2,4-D resulted in limited embryogenesis and browning of the callus. 2,4-D, one of the somatic embryo inducing agents with a strong mutagenic effect, at higher concentration would possibly be at a higher risk for inducing genetic variations and causing variations among the *in vitro* propagated plants (Venkov et al., 2000).

Suspension culture of somatic embryos

The green-yellow friable embryogenic calli were suspended in MS liquid medium containing various concentrations (0.0 - 4.0 µM) of 2,4-D to establish the somatic embryogenesis. Active division and growth of cells were observed in 2.0 µM 2,4-D until the 7th day of culture. Initially, the calli exhibited highly vacuolated cells. After 6 days, the callus exhibited two morphologically distinct cells, namely, spherical and elongated cells with visible cytoplasm and nucleus. Each spherical embryogenic cell transversely divided into two cells, four cells and subsequently into group of cells that was considered to be the proembryo. The proembryo further divided and formed globular embryos (Fig. 1b), heart shaped embryos (Fig. 1c, d) within a period of two weeks. Further developed into torpedo and cotyledonary embryos (Fig. 1e) in the same medium within a week. The optimal frequency of

Table 3. Influence of various carbohydrates and concentrations in MS liquid medium with 2.0 µM 2,4-D for somatic embryo formation of leaf-derived callus in *C. anguria*.

Carbohydrate	Concentration (%)	Different stages of somatic embryos (%)		
		Globular stage	Heart stage	Torpedo stage
Glucose	0	n.d.	n.d.	n.d.
	1	1.0±0.2^{k}	n.d.	n.d.
	2	3.0±0.5^{i}	1.0±0.2^{h}	1.0±0.2^{ef}
	3	6.4±0.4^{fg}	2.4±0.3^{fg}	1.6±0.4^{e}
	4	10.2±0.5^{d}	4.6±0.4^{de}	2.0±0.4^{de}
	5	9.4±0.5^{e}	3.8±0.4^{ef}	n.d.
Sucrose	0	n.d.	n.d.	n.d.
	1	9.5±0.5^{de}	5.4±0.6^{d}	3.0±0.2^{d}
	2	19.4±1.0^{b}	14.8±1.0^{b}	11.0±1.0^{b}
	3	33.5±2.0^{a}	27.0±1.0^{a}	20.4±1.5^{a}
	4	16.8±1.0^{c}	11.0±0.5^{c}	8.5±0.5^{c}
	5	7.0±0.5^{f}	4.5±0.5^{e}	2.0±0.1^{de}
Fructose	0	n.d.	n.d.	n.d.
	1	3.6±0.8^{h}	1.0±0.2^{h}	n.d.
	2	5.8±0.4^{g}	2.6±0.4^{f}	n.d.
	3	8.4±0.2^{ef}	3.8±0.4^{ef}	1.0±0.1^{ef}
	4	2.8±0.5^{ij}	2.2±0.6^{g}	n.d.
	5	1.2±0.5^{jk}	n.d.	n.d.
Maltose	0	n.d.	n.d.	n.d.
	1	3.4±0.4^{hi}	1.2±0.2^{gh}	n.d.
	2	4.8±0.4^{gh}	2.2±0.2^{g}	n.d.
	3	1.4±0.5^{j}	n.d.	n.d.
	4	n.d.	n.d.	n.d.
	5	n.d.	n.d.	n.d.

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≤0.5 level are indicated by different letters. n.d. = not determined due to nil response.

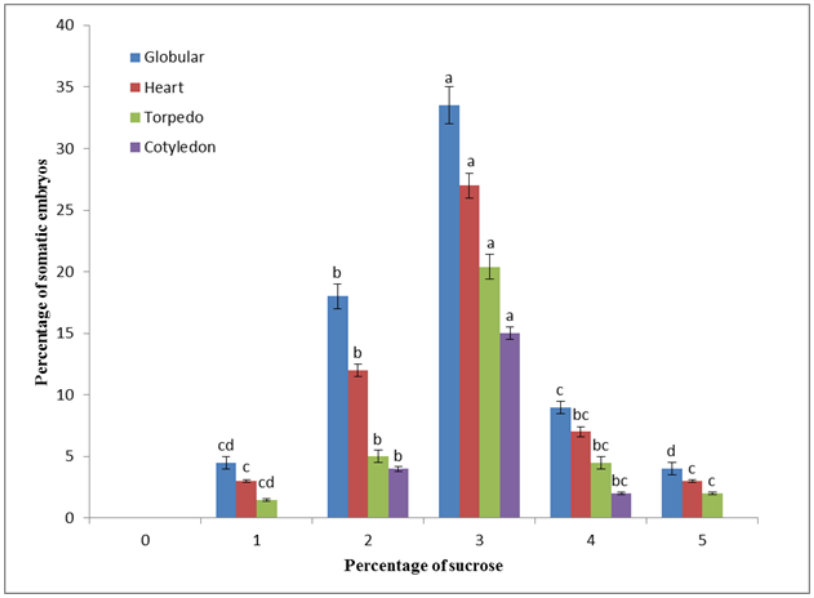

Fig 3. Influence of various concentration of sucrose in MS liquid medium with 2.0 µM 2,4-D for somatic embryo formation of leaf-derived callus in *C. anguria*. The data were statistically analyzed using Duncan's multiple range test. Values are mean ± SE. In the same *column*, significant differences according to the least significant difference (LSD) at the P≤0.5 level are indicated by different letters.

somatic embryo formation (33.5%) was achieved with 2.0 µM 2,4-D at shaking speed of 100 rpm for cell suspension culture (Fig. 2). Similarly, 2,4-D was efficient for inducing somatic embryos in cucumber (Malepszy et al., 1982; Malepszy and Nadolska-Orczyk 1983; Cade et al., 1990; Chee, 1990; Punja et al., 1990). The presence of 2,4-D in the culture medium inhibited embryo development beyond the globular stage in *Cucurbita pepo* (Jelaska, 1980; Jelaska et al., 1985) and *Momordica charantia* (Thiruvengadam et al., 2006). Auxins were absolutely necessary for somatic embryo induction but were often omitted in the period of maturation and germination (Choi et al., 1999; Mohamed et al., 2004; Thiruvengadam et al., 2006). On the same MS medium with 2,4-D, the torpedo and cotyledonary embryos did not develop but, some abnormal structures appeared 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. Similar results were reported in cucumber (Vengadesan et al., 2006).

Table 4. Effect of different concentration of various amino acids in MS liquid medium with 2.0 µM 2,4-D for somatic embryogenesis of leaf derived callus in *C. anguria*.

Type of Amino acid	Concentration (µM)	Different stages of somatic embryos (%)		
		Globular stage	Heart stage	Torpedo stage
L-alanine	0	33.5±2.0^{a}	27.0±1.0^{a}	20.4±1.5^{a}
	0.1	10.6±1.2^{c}	5.2 ±0.4^{c}	1.0 ±0.4^{c}
	0.2	4.0 ±1.0^{ef}	2.6±0.2^{d}	n.d.
	0.3	1.2 ±1.0^{f}	n.d.	n.d.
	0.4	n.d.	n.d.	n.d.
	0.5	n.d.	n.d.	n.d.
	0.7	n.d.	n.d.	n.d.
L-proline	0	33.5±2.0^{a}	27.0±1.0^{a}	20.4±1.5^{a}
	0.1	17.2 ±1.2^{b}	10.4 ± 1.0^{b}	7.4 ± 0.5^{b}
	0.2	10.0±1.0^{c}	6.8 ± 0.8^{bc}	4.2 ± 0.4^{bc}
	0.3	4.2±0.5^{e}	2.8 ± 0.5^{cd}	1.0 ± 0.2^{c}
	0.4	n.d.	n.d.	n.d.
	0.5	n.d.	n.d.	n.d.
	0.7	n.d.	n.d.	n.d.

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≤0.5 level are indicated by different letters. n.d. = not determined due to nil response.

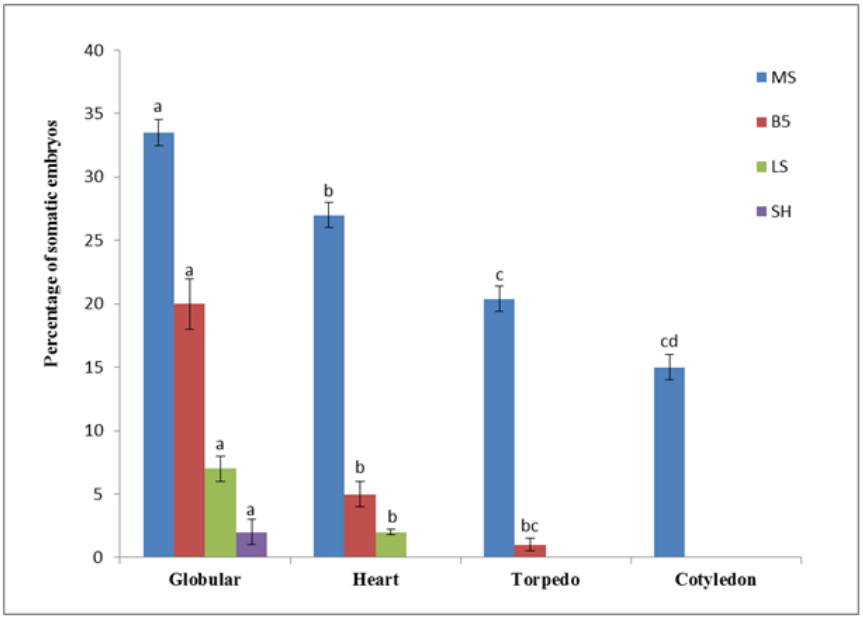

Fig 4. Effect of different basal media with 2.0 µM 2,4-D on somatic embryogenesis of leaf-derived callus in *C. anguria*. The data were statistically analyzed using Duncan's multiple range test. Values are mean ± SE. In the same column, significant differences according to the least significant difference (LSD) at the P≤0.5 level are indicated by different letters.

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. 1f), whereas in the same system, the presence of 2,4-D resulted in malformed embryo structures, which later developed into friable callus. Similarly, Cade et al., (1990) and Raharjo and Punja, (1992) obtained somatic embryo development and maturation was obtained on MS basal liquid medium. 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 producing shoots and roots from the poles, enhanced the 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. 1g). 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 (15%) was achieved (Table 2).) The efficient production of matured somatic embryos from the culturing of embryogenic calluses in MS medium fortified with sucrose and ABA were reported in *Carica papaya* (Ascencio-Cabral et al., 2008; Anandan et al., 2012). These plantlets, when transplanted into a plastic cup (Fig. 1h) containing soil, perlite and vermiculite (3:1:1), subsequently resulted in 80% 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.

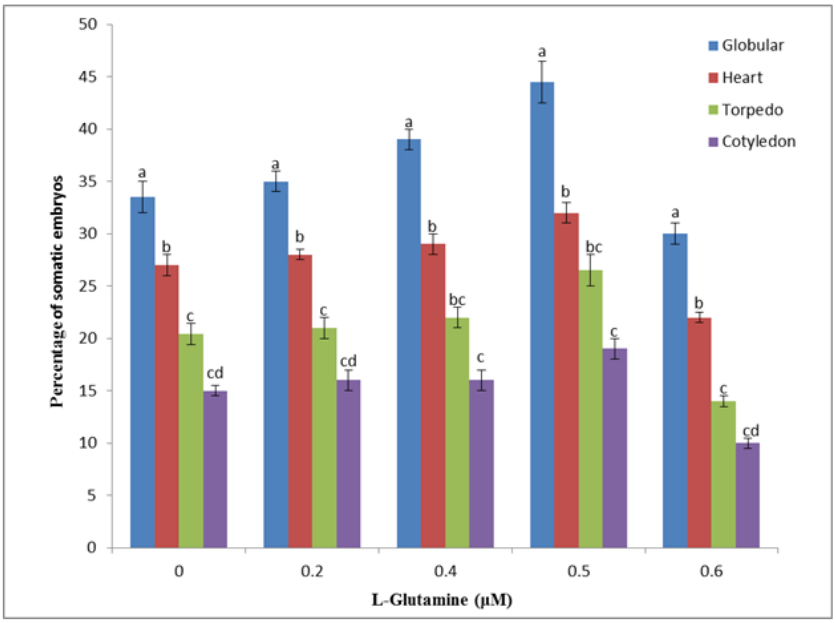

Fig 5. Effect of various concentrations of L-glutamine in MS liquid medium containing 2.0 µM 2,4-D on development of somatic embryos from leaf derived callus in *C. anguria*. The data were statistically analyzed using Duncan's multiple range test. Values are mean ± SE. In the same column, significant differences according to the least significant difference (LSD) at the P≤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, KIN) tested, 2,4-D was the most effective for inducing somatic embryogenesis. The highest frequency of embryo induction was observed on media with 2.0 µM 2,4-D (Fig. 2), while the lowest was on media with 0.1 µM. The other auxins (0.0 - 4.0 µM of IAA, NAA and IBA) could not induce embryogenesis (data not shown). The callus became green with the addition of cytokinins (BAP and KIN) and completely inhibited somatic embryogenesis (data not shown). The effect of carbohydrates (fructose, glucose, maltose, sucrose), basal medium (MS, B5, LS, SH) and amino acids (L-alanine, L-glutamine, L-proline) with 2.0 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). 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 are responsible for both embryo initiation and maturation (Fig. 4). Similar results were reported in the leaves of squash and melon (Kintzios et al., 2002) and cucumber (Callebaut et al., 1987; Clusters and Bergervoet, 1990). A low frequency of somatic embryo induction was observed on MS with 3% glucose, fructose and maltose (Table 3). B5 media induced globular, heart and torpedo stage somatic embryos, LS media developed globular and heart-shaped somatic embryos and SH media only formed globular stage somatic embryos but no germination was developed with B5, LS and SH medium (Fig. 4). The occurrence of various carbohydrates has been found to affect the initiation of somatic embryos in *C. anguria*, with 3% sucrose resulting in the highest frequency, followed by glucose and fructose with low frequency of embryo initiation. Maltose was completely ineffective for somatic embryogenesis in *C. anguria*. Similar results were also observed in cucumber (Ladyman and Girard, 1992; Vengatesan et al., (2006). Guis et al., (1997) reported that glucose was the most important sugar for somatic embryogenesis in melon. However, our experiments confirmed that 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., 2006). The effective enhancement of embryo induction, growth and development was achieved with 0.5 µM L-glutamine (Fig. 5). Hence, the supply of proline and alanine was less efficient in embryogenesis than that of glutamine (Table 4). 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. 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., (2006) in *C. sativus*. This emphasized that the exogenous supply of these substances could foster the physiological maturity of embryos.

Materials and methods

Collection of seeds and germination

Seeds of gherkin (*Cucumis anguria* L.) were obtained from IAP Farms (P) Ltd., Nilakkottai, India. Seeds were potted in a mixture of peat : vermiculite : soil (1:2:1) and maintained in a growth chamber (MLR-350H, Sanyo, Tokyo, Japan) at 27°C day/22°C night under 16 h light and 8 h dark photoperiod. The plants were fertilized and watered at weekly intervals. Leaf explants were excised from highly proliferating (30 day old) plants in a growth chamber and

rinsed thoroughly in running tap water for 2 h. The leaf explants were then surface sterilized by agitating in 1% laboline and 0.1% $HgCl_2$ for 10 min and rinsed five to seven times with sterile distilled water. The leaf explants were sliced into approximately 0.1 mm width and 0.7 mm length.

Callus induction

Leaf explants were placed in a plastic Petri dish with 25 ml medium, consisting of MS salts (Murashige and Skoog, 1962), 3.0% sucrose, 0.2% Gelrite (Sigma, St. Louis, USA) with different concentrations (0.0 - 6.0 µM) of 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma, St. Louis, USA) alone and in combination with 1.0 µM kinetin (KIN; Sigma, St. Louis, USA) for callus formation. The pH of the medium 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 (45 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 100 ml Erlenmeyer flasks (Pyrex, USA) containing 25 ml of liquid MS medium supplemented with various concentrations (0.0 - 4.0 µM) of 2,4-D. Then agitated on a gyratory shaker (100 rpm) maintained at 25 ± 2°C for 16/8-h (light/ dark) photoperiod of cool white fluorescent light (45 µmol m^{-2} s^{-1}). 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. 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 were developed.

Maturation and germination

Maturation of embryos did not progress in MS liquid medium with 2,4-D. Hence cotyledonary-shaped embryos were transferred into 100 ml Erlenmeyer flasks containing 25 ml of hormone-free MS liquid medium for maturation and further development. Then the matured 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 at 25 ± 2°C and 16/8-h (light/ dark) photoperiod of cool white fluorescent light (45 µmol m^{-2} s^{-1}) and plantlet conversion frequency was observed.

Effect of media, growth regulators, carbohydrates and amino acids

Two-weeks-old, green-yellow, friable embryogenic callus (750 mg fresh mass) derived from leaf 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 KIN) 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 - 0.7 µM were studied on the differentiation of somatic embryos. The frequency of embryo induction and stages of somatic embryos were recorded.

Soil transfer

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

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 the percentage of embryos was calculated on the basis of the total number of proembryogenic cells present in the field. All the experiments were carried out in completely randomized design. Mean ± SE values of results are presented. 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).

Conclusion

The present study of embryogenic suspension cultures for regeneration of *C. anguria* 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., 2006). The protocol developed could establish the potential to produce *C. anguria* plantlets from leaf explants through somatic embryogenesis. 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 gherkin.

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