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BRIEF COMMUNICATION

In vitro organogenesis and plant formation in cucumber

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Abstract

In vitro organogenesis was achieved from callus derived from hypocotyl explants of *Cucumis sativus* L. cv. Poinsett 76. Calli were induced from hypocotyl explants excised from 7-d-old seedlings grown on Murashige and Skoog (MS) medium containing 87.64 μM sucrose, 0.8 % agar, 3.62 μM 2,4-dichlorophenoxy acetic acid and 2.22 μM 6-benzyladenine (BA). Regeneration of adventitious buds from callus (25 shoots explant^{-1}) was achieved on MS medium supplemented with 8.88 μM BA, 2.5 μM zeatin and 10 % coconut water after two subcultures in the same medium at 30-d interval. Gibberellic acid (1.75 μM) favoured shoot elongation and indole 3-butyric acid (7.36 μM) induced rooting. Rooted plants were hardened and successfully established in soil.

Additional key words: adventitious bud, callus, *Cucumis sativus* L., hypocotyl.

In vitro plant regeneration of cucumber is possible using different culture techniques (Malepszy 1988). It may be achieved by organogenesis and somatic embryogenesis using different explants: cotyledons (Jia *et al.* 1986, Trulson and Shahin 1986), seedling leaves (Orezyk and Malepszy 1985, Seo *et al.* 2000) cotyledons and leaves (Punja *et al.* 1990) and hypocotyls (Nishibayashi *et al.* 1996, Raharjo *et al.* 1996). Methods for plant regeneration from cucumber callus *via* organogenesis (Chee 1990, Punja *et al.* 1990, Nishibayashi *et al.* 1996, Seo *et al.* 2000), suspension culture (Chee and Tricoli 1988, Lou and Kako 1994) or by somatic embryogenesis on solid medium (Chee 1990) from the callus derived from cotyledon/hypocotyl explants have been also described. However, cucumber regeneration *via* organogenesis is still limited due to low regeneration frequency and the results found to be genotype dependent (Wehner and Locy 1981, Kim *et al.* 1988). The purpose of this study was to establish a protocol for regeneration *via* organogenesis of commercial cucumber cv. Poinsett 76.

Seeds of cucumber (*Cucumis sativus* L.) cv. Poinsett 76 (Indo-American Hybrid Seeds *Pvt Ltd*, Bangalore, India) were presoaked in distilled water for 15 min washed in 2 % *Teepol* solution (v/v) (commercial detergent; 5.25 % sodium hypochlorite) (*Reckitt & Benckiser Pvt. Ltd*., Kolkatta, India) for 10 - 15 min and disinfected with 0.1 % mercuric chloride (m/v) for 3 min followed by three rinses with distilled water. Disinfected seeds were germinated in darkness for 48 h in 25 × 150 mm test tubes (*Borosil*, Mumbai, India) containing sterile moist cotton and then cultured at an irradiance of 30 μmol m^{-2} s^{-1} with 16-h photoperiod and temperature of 25 ± 2 ºC. Hypocotyls from 7-d-old *in vitro* seedlings were excised and used as explants. Single hypocotyl explant (10 mm length) was inoculated in each culture tube containing 15 cm^3 MS medium (Murashige and Skoog 1962) supplemented with 87.64 μM sucrose (m/v) 0.8 % agar (m/v) and a range of concentrations and combinations of auxins, 2,4-dichlorophenoxyacetic acid (2,4-D: 0.90, 1.81, 2.72, 3.62, 4.52, 5.42 and 6.33 μM), naphthalene acetic acid (NAA: 1.07, 2.15, 3.22, 4.30, 5.37, 6.44 and 7.52 μM), indole-3-acetic acid (IAA: 1.14, 2.28, 3.42, 4.57, 5.71, 6.85, 7.99 and 11.42 μM) and cytokinins, 6-benzyladenine (BA: 2.22 and 4.44 μM) and kinetin (Kn: 2.32 and 4.65 μM). Four weeks after inoculation, well developed calli were produced from the cut ends of hypocotyls. These calli (1 g fresh mass) were transferred to MS medium with 87.64 μM sucrose (m/v),

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Abbreviations: BA - benzyladenine; 2,4-D - 2,4-dichlorophenolic acetic acid; GA_3 - gibberellic acid; IAA - indole acetic acid; IBA - indole-3-butyric acid; MS medium - Murashige and Skoog medium; NAA - naphthalene acetic acid.
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0.8 % agar (m/v) and 10 % coconut water (CW) with different concentrations and combinations of BA (4.44, 6.66, 8.88, 11.10 and 13.31 µM) and zeatin (1.0, 2.5 and 5.0 µM). Zeatin was filter sterilized (0.22 µm pore size filter, *Gelman*, Mumbai, India) and then added to the autoclaved media under aseptic conditions. Callus with regenerating adventitious buds was subcultured twice at an interval of 30 d in the same medium. The regenerated shoots were elongated in MS medium containing gibberellic acid (1.75 µM) and rooted in indole butyric acid (7.36 µM). All compounds and plant growth regulators from *Himedia Laboratories Pvt Ltd*, Mumbai, India. MS medium without growth regulators served as control. The medium was adjusted to pH 5.8 before autoclaving at 121 ºC for 15 min. Cultures were maintained as described above. Experiments were carried out with 20 replicates and each experiment was repeated three times. Rooted plants were washed thoroughly in tap water and transplanted into polyethylene pots containing autoclaved soil, sand and vermiculite mixture (1:2:1) (v/v/v). Potted plants were grown in growth chamber at 85 % relative humidity. Plants were covered with polyethylene covers for 2 weeks to maintain high humidity. Upon new leaf growth, the covers were removed and hardened plants were transferred to field.

For histological studies, callus cultures containing adventitious buds were fixed in formalin, acetic acid, ethyl alcohol (0.5:0.5:0.9 v/v/v) for 48 h, dehydrated through a graded series of ethyl alcohol and tertiary butyl alcohol and embedded in paraffin (58 - 60 ºC). Serial sections of 8 µm thickness were cut with a rotary microtome and stained with 1 % aqueous crystal violet solution.

Data were recorded at 30-d intervals and statistically analyzed using Duncan's Multiple Range Test (DMRT, Gomez and Gomez 1976).

Table 1. Effect of 2,4-D and cytokinins (BA and Kn) on callus induction from hypocotyl explants derived from 7-d-old *in vitro* seedlings of *Cucumis sativus* L. cv. Poinsett 76. Mean ± SE of three replicates with 20 explant per treatment. Means with different letters are significantly different according to DMRT at $P < 0.05$. B - brown, C - compact, D - dark, F - friable, G - green, L - light, N - nodular, Y - yellowish callus.

2,4-D [µM]	BA [µM]	Kn [µM]	Callus induction [%]	Nature of callus
1.81	2.22		65.7 ± 0.57de	GC
2.72	2.22		80.2 ± 0.90b	DGNC
3.62	2.22		90.3 ± 0.24a	NGC
4.52	2.22		60.4 ± 0.92f	GYC
5.42	2.22		50.2 ± 0.58k	BC
1.81	4.44		60.2 ± 0.24g	YGC
2.72	4.44		65.3 ± 0.24e	YG
3.62	4.44		70.5 ± 0.40c	GC
4.52	4.44		68.3 ± 0.95cd	LB
5.42	4.44		50.3 ± 0.96kl	DBC
1.81		2.32	50.4 ± 0.43k	YF
2.72		2.32	55.6 ± 0.48hi	GF
3.62		2.32	67.3 ± 0.24d	YF
4.52		2.32	50.5 ± 0.40jk	GF
5.42		2.32	40.3 ± 0.24n	LBF
1.81		4.65	40.7 ± 0.37m	GF
2.72		4.65	50.8 ± 0.28j	LBF
3.62		4.65	56.4 ± 0.16j	GF
4.52		4.65	30.2 ± 0.16o	DBF
5.42		4.65	26.7 ± 0.24p	DBF

Table 2. Effect of various concentrations and combinations of BA and zeatin along with 10 % coconut water (CW) on regeneration of shoots from organogenic callus of C*ucumis sativus* L. cv. Poinsett 76. Mean ± SE of three replicates with 20 explant per treatment. Means with different letters are significantly different according to DMRT at $P < 0.05$.

BA [µM]	Zeatin [µM]	Callus initiation [%]	Number of shoots [explant^{-1}]	Shoot length [cm]
4.44	0	15.2 ± 0.16o	1.3 ± 0.08kl	1.4 ± 0.32h
6.66	0	20.1 ± 0.08n	3.7 ± 0.08gh	1.3 ± 0.24i
8.88	0	40.3 ± 0.24gh	8.4 ± 0.08d	1.2 ± 0.43j
11.10	0	26.4 ± 0.24l	2.2 ± 0.08k	1.5 ± 0.24g
0	2.5	30.8 ± 0.43ij	5.3 ± 0.24ef	1.9 ± 0.08c
0	5.0	28.5 ± 0.14k	2.8 ± 0.08j	12.6 ± 0.16f
4.44	1.0	30.7 ± 0.40j	3.1 ± 0.08ij	1.7 ± 0.08e
4.44	2.5	41.5 ± 0.40fg	4.3 ± 0.35g	2.0 ± 0.16b
4.44	5.0	31.6 ± 0.48i	3.3 ± 0.24i	1.8 ± 0.16b
6.66	1.0	50.2 ± 0.32e	4.3 ± 0.08g	1.7 ± 0.08e
6.66	2.5	66.7 ± 0.42b	8.6 ± 0.16cd	1.9 ± 0.49c
6.66	5.0	56.8 ± 0.65c	7.2 ± 0.16e	1.8 ± 0.72d
8.88	1.0	66.7 ± 0.57b	13.4 ± 0.28b	2.0 ± 0.16b
8.88	2.5	80.3 ± 0.71a	25.3 ± 0.49a	2.3 ± 0.35a
8.88	5.0	55.2 ± 0.16cd	9.6 ± 0.16c	2.0 ± 0.65b
11.10	1.0	40.4 ± 0.32g	3.6 ± 0.76h	2.3 ± 0.08a
11.10	2.5	45.3 ± 0.24f	8.3 ± 0.14de	2.0 ± 0.16b
11.10	5.0	28.2 ± 0.16kl	3.4 ± 0.32hi	1.8 ± 0.16d
13.32	1.0	25.3 ± 0.24lm	2.6 ± 0.16jk	1.7 ± 0.57e
13.32	2.5	30.3 ± 0.24jk	4.4 ± 0.16fg	2.0 ± 0.16b

Callus was initiated from the cut ends of the hypocotyl in MS medium containing either auxin alone or its combination with cytokinin. After 4 weeks of culture callus rating was assessed. Among the three auxins tested individually, 2,4-D produced greenish friable callus, NAA produced yellowish green friable callus and IAA produced brownish friable callus and all of them were non organogenic (data not shown). The combination of 2,4-D at 3.62 µM with BA at 2.22 µM alone produced nodular greenish compact callus (Table 1) with a callusing response 90.3 %. The callus produced by 2,4-D and Kn was greenish yellow, friable and did not become compact or nodular. Both friable and compact nodular calli on hypocotyl explants were obtained in the presence of 2,4-D and BA after 12 - 14 d in cucumber culture (Ziv and Gadasi 1986). In the previous reports, calli from different explants and genotypes of cucumber were

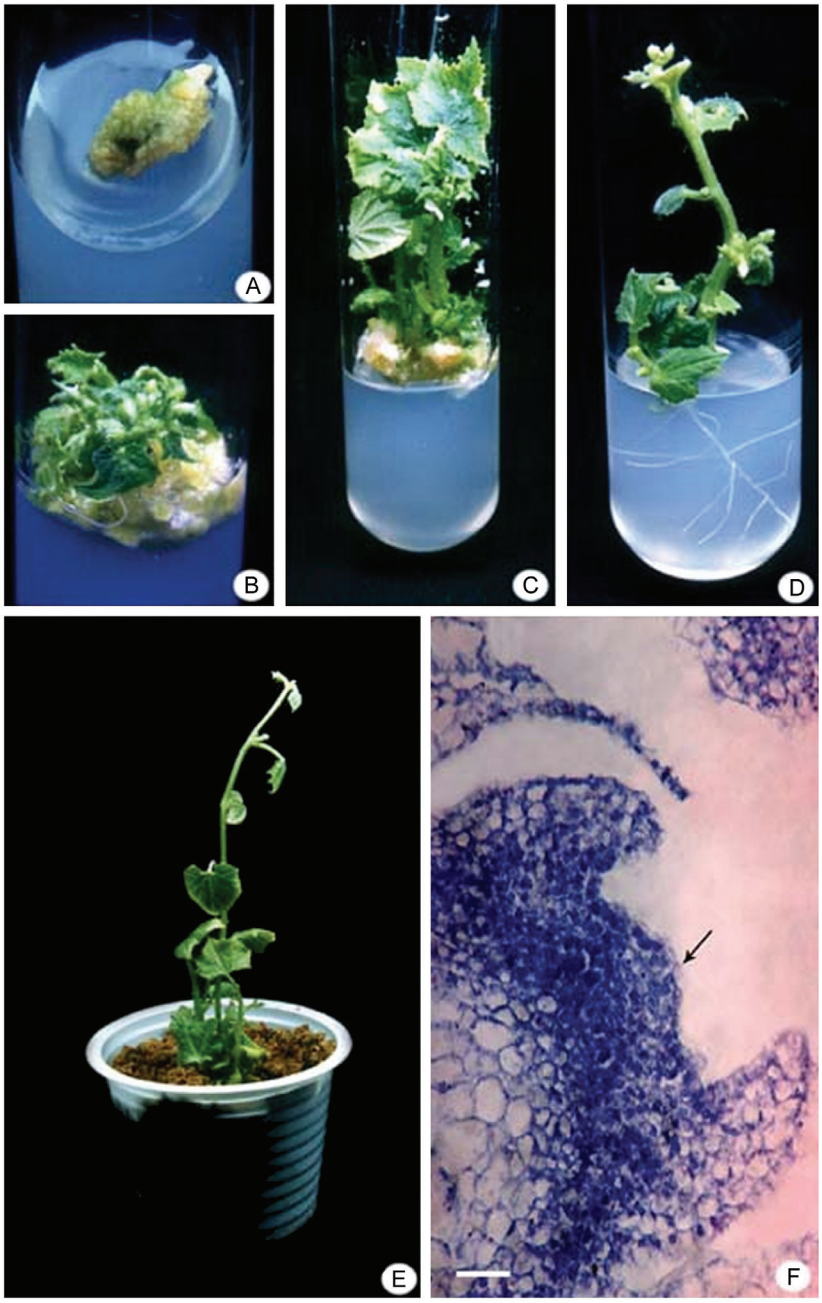

Fig. 1. Regeneration of plantlets from hypocotyl derived callus of *Cucumis sativus* L. *A* - Compact nodular callus initiating from hypocotyl explants (3.62 µM 2,4-D and 2.22 µM BA); *B* - shoot bud induction (8.88 µM BA and 2.5 µM zeatin); *C* - elongation of shoots (1.75 µM GA_3); *D* - rooting of *in vitro* raised shoots (7.36 µM IBA); *E* - hardened plant; *F* - longitudinal section of the organogenic callus showing adventitious bud. *Arrow* indicates bud primordium flanked by leaf primodia (*bar* = 40 µm).

obtained in different auxin and cytokinins combinations (Handley and Chambliss 1979, Punja *et al.* 1990, Nishibayashi *et al.* 1996, Seo *et al.* 2000). In the present study organogenic callus induction frequency was significantly higher with 2,4-D/BA combinations.

Four week old nodular green compact callus obtained from hypocotyl explants was transferred to MS medium containing various concentrations and combinations of BA and zeatin with 10 % coconut water (Table 2). Maximum number of shoot bud differentiation (20 - 25 shoots per explant) was obtained on MS medium containing BA (8.88 µM) and zeatin (2.5 µM) (Fig. 1*B*) after two subcultures of 30 d each with an average shoot length of 1.5 cm. Nadolska-Orezyk and Malepszy (1986) reported that during the first 20 d of culture on MS medium with 2,4-D, mostly meristematic tissues were

developed, proliferating around vascular bundles, forming centers of meristematic cells. Between 25 - 30 d of culture, simultaneously in different parts of the explant formed adventitious buds. The same developmental pattern has been observed in the present experiment in the combination of 2,4-D and BA.

Regeneration through organogenesis and shoot formation in cucumber using hypocotyl has been reported in IAA/Kn, NAA/BA and 2,4-D/BA combinations (Wehner and Locy 1981, Nishibayashi *et al.* 1996, Raharjo *et al.* 1996, Seo *et al.* 2000). When compared to these studies a higher regeneration frequency (25 shoots hypocotyl^{-1}) was achieved in the present study. Wehner and Locy (1981) found a great deal of variation in the percentage of shoots produced among different cucumber genotypes.

In the present study a high frequency of callus induction (90.3 %) and adventitious shoot regeneration (25 shoots explant^{-1}) for Poinsett 76 hypocotyl were achieved in combinations 2,4-D/BA and BA/zeatin/ coconut water.

Two subcultures of callus in the present study turn it to be more compact, nodular and organogenic. Malepszy and Nadolska-Oreszyk (1983) and Trulson and Shahin (1986) also suggested repeated subcultures for achieving cucumber regeneration. In our study, an average of two subcultures at an interval of 30 d each was required to induce optimum regeneration of adventitious shoots from organogenic callus.

The regenerated shoots (1.5 cm in long) when inoculated in medium containing GA_3 (1.75 µM) elongated (5.3 cm) within three weeks (Fig. 1*C*). The elongated shoots (5 - 6 cm) produced roots when transferred to MS medium supplemented with 7.36 µM IBA (Fig. 1*D*). The rooted plants were hardened (Selvaraj *et al.* 2002) and after four weeks of hardening, the plants (Fig. 1*E*) were transferred to field with a survival rate of 76 %. Histological examination of 4 week-old organogenic callus revealed the initiation of bud primordium beneath the epidermal region of the meristematic dome. The bud primordium is flanked by two leaf primordia (Fig. 1*F*). It reveals *de novo* origin of adventitious shoot buds. This protocol yielded about 25 plants per hypocotyl explant over a period of four months. This study could assist to introduce somaclonal variation and genetic manipulation of cucumber in general and cv. Poinsett 76 in particular.

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