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BRIEF COMMUNICATION

Direct plant regeneration from cucumber embryonal axis

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Abstract

Embryonal axis explants from 2-d-old *in vitro* germinated seeds were used to induce multiple shoot production. The combination of 4.44 µM BA and 1.59 µM NAA in MS medium triggered the initiation of adventitious shoot buds. The explants with shoot buds produced maximum number of shoots (10.6 per explant) in MS medium supplemented with 4.44 µM BA and 0.065 mM L-glutamine in three successive transfers. The elongated shoots were rooted on MS medium with 4.92 µM IBA. Rooted plants were transferred to soil with a survival rate of 65 %.

Additional key words: adventitious shoot bud, benzyladenine, *Cucumis sativus* L., L-glutamine, naphthalene acetic acid.

Cucumber (*Cucumis sativus* L.) is an important vegetable crop. Cross-incompatibility and narrow genetic base in cucumber pose limitations to improvement by classical breeding (Ziv 1992). Tissue culture method for production of multiple shoots in cucumber using cotyledon explants was described by Gambley and Dodd (1990). A high frequency of variation in regenerated cucumber plants occurs at the callus or somatic embryo stage (Malepszy and Nadolska-Orezyk 1989). In contrast, in the shoots produced by direct regeneration from explants the morphological or physiological variation was much less (Burza and Malepszy 1995, Plader *et al.* 1998). Therefore, the present protocol was aimed to develop a technique which allows the production of multiple shoots directly from the embryonal axis of cucumber.

Seeds of cucumber (*Cucumis sativus* L.) cv. Poinsett 76 (Indo-American hybrid seeds, *Pvt Ltd*, Bangalore, India) were soaked in tap water for 15 min disinfected with 70 % alcohol for 1 min and 2.5 % (v/v) commercial bleach *Teepol* (5.25 % sodium hypochlorite; *Reckit & Benckiser of India Ltd,* Kolkatta, India) for 15 min followed by three rinses with sterile distilled water. Seeds were further disinfected by soaking in 0.5 % mercuric chloride (m/v) for 3 min and rinsed four times with sterile distilled water. The axenic seeds were inoculated in sterile culture tubes (25 × 150 mm) containing moist cotton and kept in the culture room. The embryonal axis was removed from germinating seeds at different growth periods under sterile conditions and was used as the explant. Intact embryonal axes without the radical region, embryonal axes without apical and radical regions, and embryonal axes in which a cut was made at the apical region without the radical were cultured on Murashige and Skoog's (1962) (MS) medium supplemented with N^6-benzyladenine (BA; 0 - 8.88 µM), kinetin (KIN; 0 - 9.28 µM) individually and in combination with α-naphthaleneacetic acid (NAA) at different concentrations (0.53 - 2.65 µM) (shoot induction medium). All the media contained 3 % sucrose and were gelled with 0.8 % *Phytagel* (m/v) (*Himedia Laboratories,* Mumbai, India). Plant growth regulators were added to the media under sterile conditions and the pH was adjusted to 5.8 before autoclaving at 1.06 kg cm^{-2} (121 ºC) for 20 min. MS medium without growth regulators served as control. Each culture tube containing 20 cm^3 of one of the above concentrations or combinations was inoculated with one explant and plugged with non-absorbent cotton wrapped in one layer of cheese cloth. The cultures were incubated at temperature of 27 ± 2 ºC and 16-h photoperiod with irradiance of 30 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps.

Explants which initiated the maximum number of

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Abbreviations: BA - benzyladenine; IBA - indolebutyric acid; KIN - kinetin; MS medium - Murashige and Skoog medium; NAA - α-naphthalene acetic acid.
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adventitious shoot buds at the plumular region on shoot induction medium were transferred to shoot proliferation medium (MS + 4.44 µM BA and 0.065 mM L-glutamine). Shoots of about 1.0 cm long were excised and elongated in the same medium. The shoots which attained approximately 6.0 cm long were transferred to rooting medium containing MS salts and different concentrations in indole butyric acid (IBA; 0 - 9.84 µM) for root induction.

Rooted plants were transferred to plastic cups containing a mixture of soil, sand and vermiculite in 1:1:1 (v/v/v). The plastic cups with rooted plants were covered with plastic wrap and grown for 15-d in the culture room at 25 ± 2 ºC with a 16-h photoperiod at 80 % relative humidity. The plantlets were acclimatized and hardened gradually by slowly removing the plastic wrap over a period of 5 d. Hardened plants were then transferred to the field.

All the experiments were repeated thrice with 20 explants for each treatment. Discrete data were analyzed by Duncan's Multiple Range Test (DMRT) (Gomez and Gomez 1975) and significance was determined at $P \leq 0.05$ level.

For histological examinations, the regenerating shoots from embryonic axis at different stages of growth period were fixed in formalin : acetic acid : ethyl alcohol (0.5:0.5:9.0, v/v/v) for 48-h, dehydrated through a graded series of ethyl alcohol and tertiary butyl alcohol and embedded in paraffin (55 - 60 ºC). Serial sections of 10 µm thickness were cut with a rotary microtome (*RM 2135*, *Leica*, Wetzlar, Germany) and stained with toluidine blue.

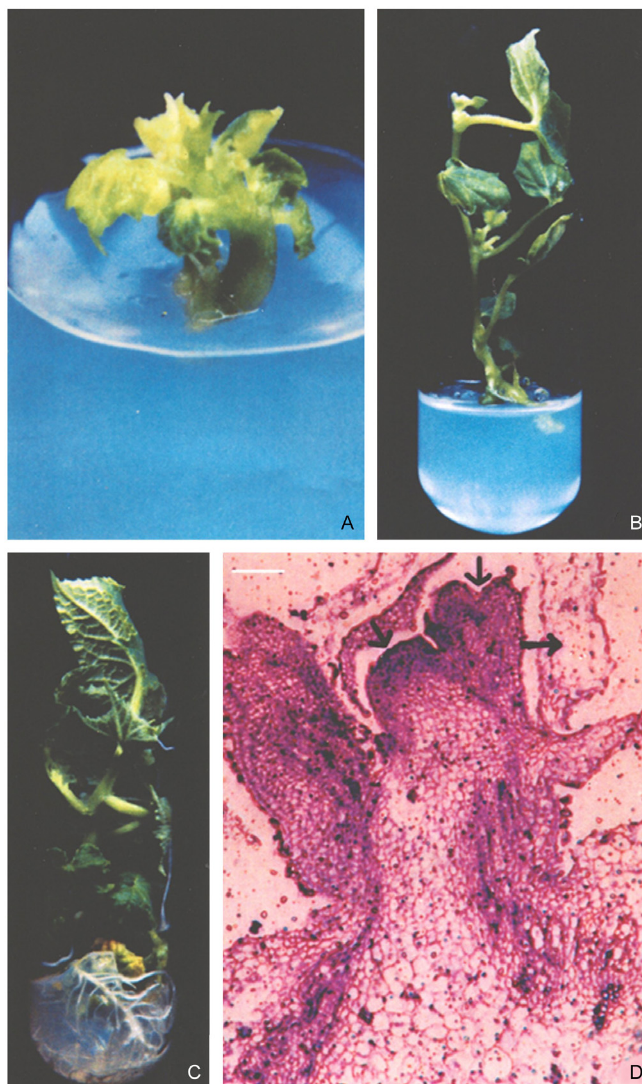

Fig. 1. Direct plant regeneration from cucumber embryonal axis. *A* - Multiple shoot formation from the apical end of the explant cultured on MS medium containing 4.44 µM BA and 0.065 mM L-glutamine after 4 weeks. *B* - Elongation of shoots cultured on MS medium containing 4.44 µM BA and 0.065 mM L-glutamine after 2 weeks. *C* - Elongated shoot showing well developed roots on MS medium supplemented with 4.92 µM IBA after 2 weeks. *D* - Longitudinal section of embryonal axis explants showing shoot primordial (*bar* = 10 µm).

Table. 1. Effect of BA, KIN and NAA on multiple shoot induction from the 2-d-old embryonal axis explants of cucumber cv. Poinsett 76. Data presented as means ± SE from 20 explants for each treatment and repeated three times. Means followed by same letters within a column are not significantly different according to Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$ level.

Growth regulators	Concentration [µM]	Explants with shoots [%]	Number of shoots [explant^{-1}] initial culture	after 2nd transfer	Shoot length [cm]
BA	0	21	0.4 ± 0.09ef	0.8 ± 0.9ef	0.4 ± 0.09f
	0.44	34	0.6 ± 0.09e	1.2 ± 0.18e	0.8 ± 0.18e
	0.88	42	1.0 ± 0.09d	1.8 ± 0.09d	1.0 ± 0.18d
	1.76	58	1.4 ± 0.09c	2.6 ± 0.24c	1.4 ± 0.37c
	2.22	66	2.0 ± 0.09ab	3.2 ± 0.33b	2.0 ± 0.24b
	4.44	72	2.2 ± 0.28a	4.1 ± 0.10a	2.6 ± 0.24a
	8.88	38	0.8 ± 0.18de	1.6 ± 0.24de	0.8 ± 0.28de
KIN	0	11	0.2 ± 0.08c	0.4 ± 0.09f	0.2 ± 0.01e
	0.92	16	0.4 ± 0.09de	0.8 ± 0.09c	0.4 ± 0.09de
	1.84	24	0.6 ± 0.18d	1.2 ± 0.09d	0.6 ± 0.09d
	2.76	36	1.0 ± 0.18c	1.8 ± 0.18c	1.0 ± 0.14c
	3.68	52	1.2 ± 0.28b	2.6 ± 0.33ab	1.4 ± 0.23ab
	4.64	64	1.8 ± 0.41a	3.4 ± 0.09a	2.0 ± 0.37a
	9.28	22	0.6 ± 0.09d	1.0 ± 0.18de	0.4 ± 0.28de
BA+NAA	4.44 + 0.53	64	2.2 ± 0.18bc	4.6 ± 0.18bc	2.8 ± 0.09c
	4.44 + 1.06	76	2.6 ± 0.37b	5.2 ± 0.24b	4.0 ± 0.32ab
	4.44 + 1.59	82	3.4 ± 0.37a	6.4 ± 0.41a	4.6 ± 0.16a
	4.44 + 2.12	56	1.6 ± 0.18d	3.8 ± 0.20c	2.6 ± 0.40cd
	4.44 + 2.65	48	1.4 ± 0.28de	3.0 ± 0.24cd	1.6 ± 0.09e
BA+KIN	4.44 + 0.53	46	1.2 ± 0.09c	2.8 ± 0.18c	2.0 ± 0.18c
	4.44 + 1.06	58	1.8 ± 0.18b	2.8 ± 0.24b	2.6 ± 0.16ab
	4.44 + 1.59	76	2.6 ± 0.58a	5.0 ± 0.41a	3.0 ± 0.41a
	4.44 + 2.12	38	1.2 ± 0.37c	2.2 ± 0.28cd	1.4 ± 0.09d
	4.44 + 2.65	26	0.8 ± 0.28cd	1.6 ± 0.37d	1.0 ± 0.16de

Embryonal axis was used for shoot regeneration for the first time in cucumber. Previous studies reported the high regeneration potential of embryonic axis for shoot proliferation from different species (Mathews 1987, Mohamed *et al.* 1992, Kulothungan *et al.* 1993, Ananthakrishnan *et al.* 1999, Ignacimuthu and Franklin 1999). In the present study, maximum shoot bud induction (82 %) occurred in MS medium containing 4.44 µM BA and 1.59 µM NAA in 2-d-old embryonal axis explants cut at the apical region (Table 1). A low frequency of shoot induction occurred from embryonal axis of other age period (data not shown). Moderate shoot bud induction occurred in the individual treatment of either BA or KIN. Kulothungan *et al.* (1993) reported in cowpea that maximum number of shoot buds were regenerated from embryonal axis on the medium supplemented with 4.92 µM IBA and 4.64 µM KIN. Mathews (1987) and Mohamed *et al.* (1992) reported in *Vigna radiata* and *Phaseolus vulgaris* respectively that the de-apexed embryonic axis produced numerous buds on the medium containing 4.44 µM BA only. Yang *et al.* (1990) and Buising *et al.* (1994) reported that embryonic axis of soybean produced multiple buds after the suppression of normal growth of its plumule.

Among the different growth periods, embryonic axes isolated from 2-d-old germinating seeds were most effective and induced the highest frequency (74 %) of multiple shoot regeneration. When the growth period of isolated embryonic axis exceeded more than two days of seed germination, the multiple shoot induction efficiency decreased from 58 to 32 % (data not shown). The explants with emerging shoots (6.4 shoots per explant) in medium containing 4.44 µM BA and 1.59 µM NAA were transferred to shoot proliferation medium (BA + L-glutamine) after 2nd transfer. Three transfers were made at 15-d interval in the same medium for further shoot proliferation and development. At the end of third transfer, maximum number of shoots (average 10.6 per explant) (Fig. 1*A*) were produced from 20-d-old explants in MS medium fortified with 4.44 µM BA and 0.065 mM L-glutamine. Shoots that attained approximately 1.0 cm long were excised from explants during each transfer and were transferred to shoot elongation medium containing 4.44 µM BA and 0.065 mM L-glutamine. Compared to other cytokinins, BA was effective for adventitious bud induction and shoot proliferation from cotyledon explants of cucumbers (Aziz and McCown 1985, Misra and Bhatnagar 1995). Rather than the individual treatment of

either BA or KIN, the combination of NAA and BA promoted the production of maximum number of shoots as has been observed by Vasudevan *et al.* (2001) for shoot tip and Selvaraj (2002) for cotyledon explants of cucumber.

L-glutamine was successfully used to maintain a high growth rate for a larger period in soybean tissue culture as it provides a sustainable nitrogen source (Gamborg *et al.* 1968) but in much higher concentrations (Vasudevan *et al.* 2004). In earlier work, when embryonic axis devoid of cotyledon segments was used, only one shoot was obtained per explant (Ananthakrishnan *et al.* 1999). In the present study, we could get multiple shoots (10.6 shoots per explant) from embryonic axis without attached cotyledonary segments. Gambley and Dodd (1990), Misra and Bhatnagar (1995) reported maximum regeneration ability (95 to 100 %) and higher number of shoot production (14 to 30 shoots per explant) for various genotypes and explants of cucumber. However, in the present study, 88 % of embryonal axis explants regenerated producing only an average of 10.6 shoots per explant. Our results indicate that more than the genotype, it is the nature of the explant which played a vital role in terms of shoot production in *in vitro*. The regenerated shoots were elongated in MS medium containing BA and L-glutamine (Fig. 1*B*) and elongated shoots were rooted (Fig. 1*C*) in MS medium fortified with 4.92 µM IBA (data not shown). The rooted plants were successfully hardened with a survival rate of 65 %.

Histological examination of regenerated shoots revealed that the shoots have arisen from sub-epidermal region of the apex of the embryonal axis (Fig. 1*D*). Similar type of origin of shoot buds has also been reported for cucumber (Handley and Chambliss 1979, Gambley and Dodd 1990).

It is concluded that multiple shoots could also be obtained from the embryonal axis of cucumber like any other explants within 90 d of culture. As the protocol is simple, rapid and highly repeatable, it is suggested that this protocol may be adopted for genetic transformation of cucumber.

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