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BRIEF COMMUNICATION

Direct somatic embryogenesis and plant regeneration from immature explants of chickpea

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Abstract

A protocol for plant regeneration *via* somatic embryogenesis was developed in two chickpea (*Cicer arietinum* L.) cultivars ICCV-10 and Annigeri. Somatic embryos were induced from immature cotyledons on Murashige and Skoog's (MS) medium supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), α-naphthaleneacetic acid (NAA) and picloram alone or in combination with 0.5 - 2.0 mg dm^{-3} N^6-benzylaminopurine (BA) or kinetin (KIN). NAA was better for somatic embryo induction compared to other auxins. The well formed, cotyledonary shaped embryos germinated into plantlets with 36.6 % frequency on MS medium supplemented with 2.0 mg dm^{-3} BA + 0.5 mg dm^{-3} abscisic acid (ABA). The frequency of embryogenesis and plantlet regeneration was higher in cv. ICCV-10 as compared to cv. Annigeri. Regenerated plants were transferred to soil (40 % survival) and grown to maturity. Histological studies of explants at various developmental stages of somatic embryogenesis reveled that somatic embryos developed directly from the cotyledon cells and they were single cell origin.

Additional key words: abscisic acid, auxins, cytokinins, germination, histology, mature somatic embryos.

Chickpea (*Cicer arietinum*. L.) is an important grain legume. There are a few reports on its somatic embryogenesis (Rao and Chopra 1991, Barna and Wakhulu 1993, Sagare *et al*. 1993, Kumar *et al*. 1994, 1995, Murthy *et al*. 1996). However, conversion of somatic embryos into plantlets remained inefficient and limited. Some of the serious limitations of these protocols are the low frequency, inconsistency, genotype specificity and occurrence of callus phase prior to embryogenesis. A variety of methods were used to increase the frequency of somatic embryos conversion including desiccation and plant growth regulator treatments (Distabanjong *et al*. 1997). Moreover, the entire cycle of plantlet regeneration in most of the reports is very long. To the best of our knowledge there are no reports on direct somatic embryogenesis and subsequent plant regeneration in the cultivars ICCV-10 and Annigeri using immature explants without the intervention of callus. The present study describes a reproducible protocol for plant recovery *via* somatic embryogenesis, from immature cotyledons in two important cultivars of chickpea.

Seeds of chickpea (*Cicer arietinum* L.) cvs. ICCV-10 and Annigeri were obtained from Agricultural Research Station, Gulbarga, Karnataka, India. Immature pods were harvested from plants 3 - 4 weeks after pollination, surface sterilized with 0.1 % (m/v) mercuric chloride for 4 - 5 min and rinsed with sterile distilled water. Immature seeds were aseptically isolated from the pods, the seed coat and embryonal axis were removed, and cotyledons were placed with abaxial side in contact with the Murashige and Skoog (1962; MS) medium supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), α-naphthaleneacetic acid (NAA) and picloram

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Abbreviations: ABA - abscisic acid; BA - N^6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acids; KIN - kinetin; MS - Murashige and Skoog; NAA - α-naphthaleneacetic acid; 2,4,5-T - 2,4,5-trichlorophenoxyacetic acids; TDZ - thidiazuron.
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alone or in combination with 0.5 - 2.0 mg dm^{-3} N^6-benzyl-aminopurine (BA) or kinetin (KIN). All the media contained 3 % (m/v) sucrose. The pH of the media was adjusted to 5.8 and solidified with 0.8 % agar, before autoclaving at a pressure of 1.06 kg cm^{-2} for 20 min. All the cultures were maintained at a temperature of 25 ± 2 °C and a 16-h photoperiod (irradiance of 50 - 100 µmol m^{-2} s^{-1} provided by cool white, fluorescent lamps). At the end of 4 weeks, percentage of explants with early and late stages of embryos visible to naked eye or stereo microscopically was recorded.

The somatic embryos obtained were later transferred to maturation medium supplemented with BA, KIN, abscisic acid (ABA), zeatin or thidiazuron (TDZ) alone or in combination with ABA. Plantlets regenerated from somatic embryos were transferred to half-strength MS liquid medium without hormones for two weeks for pre-hardening. Finally, plantlets were then transferred to pots containing sterilized soil and sand in the ratio 3:1.

For histological studies, tissues were fixed in formalin, acetic acid and alcohol, dehydrated in a tertiary butyl alcohol series, embedded in paraffin wax, sectioned at 10 µm, stained with saffranin, mounted on DPX.

The data pertaining to number of embryos, maturation and conversion were subjected to analysis of variance (*ANOVA*). Mean separation was done using Duncan's New Multiple Range test. Thirty cultures were raised for each treatment and all experiments were repeated thrice.

Somatic embryos were induced from immature cotyledons cultured on MS medium supplemented with different concentrations of auxins alone or in combination with cytokinins. The creamish embryos traversed all the known stages of ontogeny viz. globular, heart, torpedo and a bipolar cotyledonary shaped embryos. They subsequently developed into whole plant with shoot and root poles (Fig. 1*A*-*G*).

Highly organized, round, creamish coloured embryos differentiated from all over the surface of immature cotyledons when cultured on MS medium containing different concentrations of 2,4-D or 2,4,5-T or NAA or picloram (1.5 - 9.0 mg dm^{-3}). No embryogenesis was observed on MS basal medium without hormonal supplementation. Embryogenesis was better in presence of NAA followed by 2,4,5-T, 2,4-D and picloram. NAA at a concentration of 6.0 mg dm^{-3} produced the highest frequency (100 %) and maximum number of somatic embryos (40.2) in the cultivar ICCV-10 (Fig. 1*C*). However, when NAA concentration increased beyond 6.0 mg dm^{-3}, both frequency and number of embryos decreased. These findings are in agreement with the earlier reports of Lazzeri *et al*. (1990) in soybean, Eapen and George (1990) in *Vigna mungo*, Sehgal and Abbas (1994) in *Trachyspermum ammi,* Girija *et al.* (2000) in *Vigna radiata* and Ganesh Kumari *et al*. (2008) in *Ricinus communis*. Our results differ from those of Sagare *et al*. (1993). They reported maximum number of somatic embryos from immature cotyledon derived callus. Also Barna and Wakhulu (1993) and Kumar *et al*. (1994)

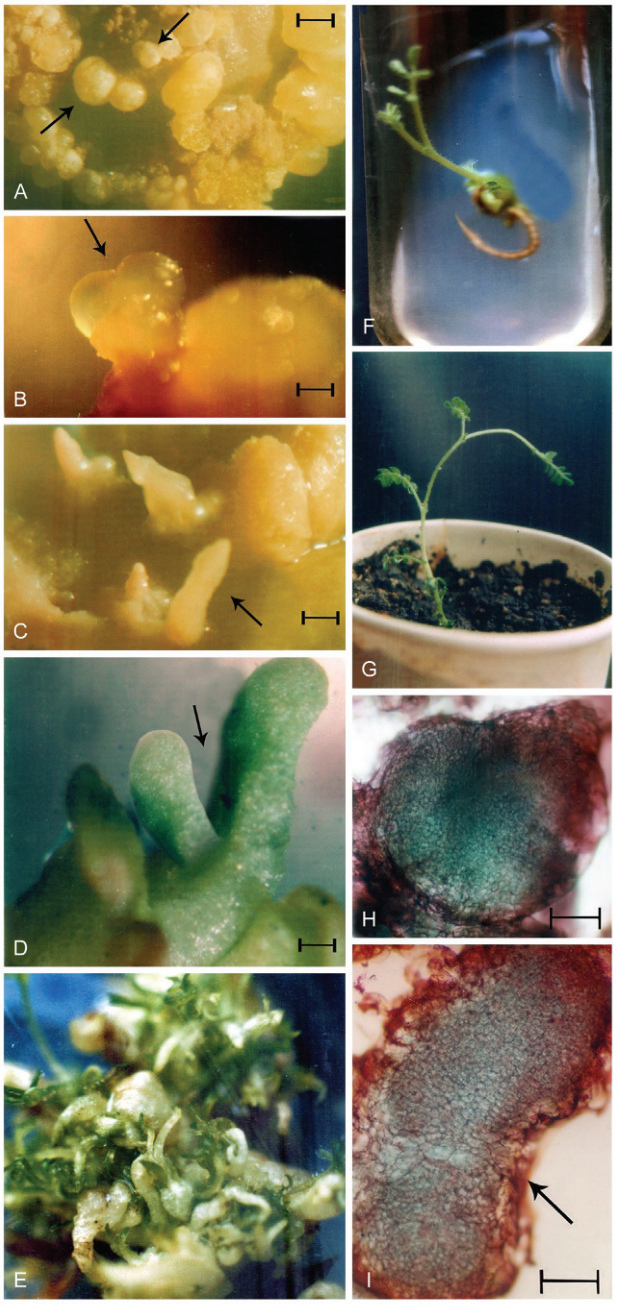

Fig. 1 Developments of plantlets through somatic embryogenesis in *Cicer arietinum*: *A* - globular stage of embryo (*arrow*) differentiated from immature cotyledons of *C. arietinum* on MS medium supplemented with 6.0 mg dm^{-3} NAA + 0.5 mg dm^{-3} KIN after 8 d of culture (10×); *B* - heart shaped embryo (*arrow*) differentiated on the same medium after 15-d of culture (25×); *C* - torpedo shaped embryo (*arrow*) differentiated after 20 d of culture (10×); *D* - cotyledonary shaped embryo (*arrow*) differentiated after 26 d of culture (25×); *E* - germination of somatic embryos (1×); *F* - germinating embryos showing shoot and root poles (1×); *G* - regenerated plant established in a plastic cup; *H* - transverse section showing actively dividing embryogenic mass of globular embryo (*bar* = 0.5 mm); *I* - transverse section through torpedo shaped embryo showing suspensor (*bar* = 0.5 mm).

obtained somatic embryos from leaf callus of chickpea. In the present study, somatic embryos were directly induced from immature cotyledon without an intervening callus and the number of somatic embryos per explants was high (40.2). Production of somatic embryos *via* callus phase can lead to somaclonal variation, while direct regeneration can avoid it. The cv. ICCV-10 produced more embryos as compared to cv. Annigeri (Fig. 2), suggesting genotype dependent embryogenesis as earlier reported in other legumes (Ozias *et al*. 1992, Sagare *et al*. 1993, Venu *et al*. 1999).

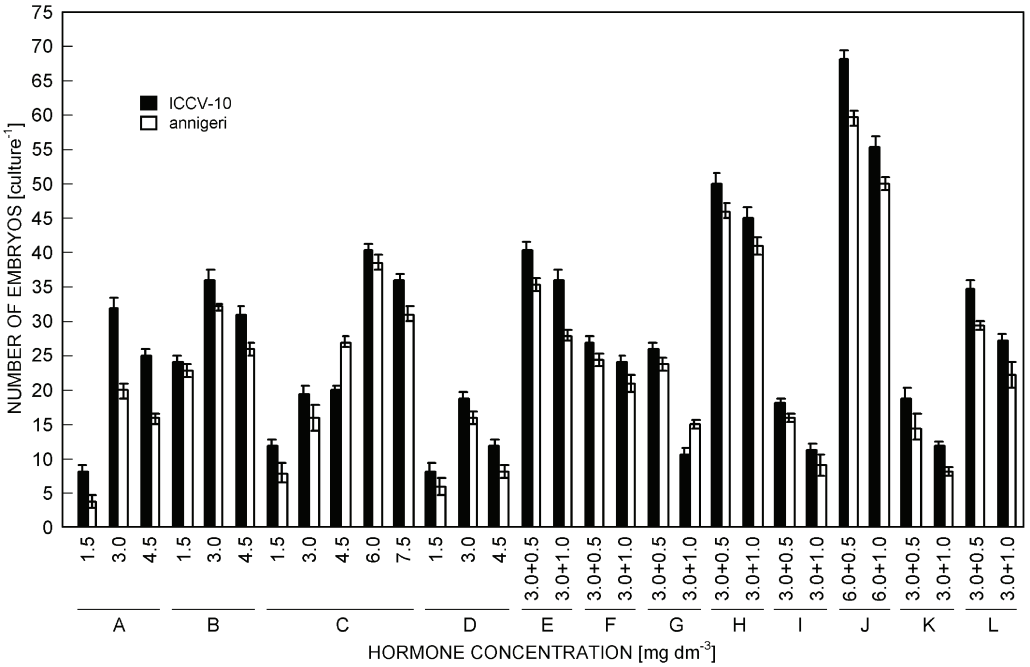

Fig. 2. Number of somatic embryos differentiated from immature cotyledon on MS medium supplemented with different concentrations of auxins alone or in combination with cytokinins: *A* - 2,4-D; *B* - 2,4,5-T; *C* - NAA; *D* - picloram; *E* - 2,4-D+ BA; *F* - 4-D+ KIN; *G* - 2,4,5-T + BA; *H* - 2,4,5-T + KIN; *I* - NAA + BA; *J* - NAA + KIN; *K* - picloram + BA; *L* - picloram + KIN. Data represent an average of 25 replicates. The experiment was repeated thrice and means ± SE are shown.

Table 1. Effect of BA, zeatin and TDZ alone or in combination with ABA on germination of somatic embryos. Means ± SE, $n = 25$. Means followed by same superscript in a column are not significantly different at $P = 0.05$ level.

Growth regulators	[mg dm^{-3}]	Number of embryos cultured		Number of embryos germinated		Conversion [%]	
		ICCV-10	Annigeri	ICCV-10	Annigeri	ICCV-10	Annigeri
BA	0.25	52	65	2.0 ± 0.00^{h}	2.0 ± 0.31^{f}	3.84^{h}	3.07^{h}
	1.0	62	60	15.0 ± 1.01^{c}	12.4 ± 0.60^{b}	24.11^{c}	20.66^{c}
	2.0	65	62	18.0 ± 1.01^{b}	14.4 ± 0.88^{b}	27.69^{b}	23.22^{b}
	3.0	55	55	6.1 ± 0.31^{f}	5.0 ± 0.45^{e}	10.34^{f}	09.09^{f}
Zeatin	0.5	60	55	0.1 ± 0.53^{i}	10.0 ± 0.68^{c}	13.50^{e}	18.00^{d}
	1.0	48	56	11.8 ± 0.55^{d}	9.3 ± 0.60^{c}	19.37^{d}	21.07^{bc}
	2.0	59	56	2.1 ± 0.31^{h}	3.8 ± 0.51^{e}	3.55^{h}	06.78^{g}
TDZ	0.1	60	65	10.1 ± 0.35^{d}	9.1 ± 0.45^{c}	16.83^{e}	14.00^{e}
	0.2	55	60	8.1 ± 0.45^{e}	7.1 ± 0.30^{d}	14.72^{e}	11.83^{f}
	0.5	52	58	4.5 ± 0.25^{fg}	4.1 ± 0.40^{e}	8.65^{g}	7.06^{g}
ABA	0.25	55	58	8.0 ± 0.88^{e}	6.0 ± 0.68^{d}	14.50^{e}	10.30^{f}
	0.5	60	56	11.0 ± 1.20^{d}	9.0 ± 0.45^{c}	18.30^{d}	16.30^{d}
ABA + BA	0.5 + 1.0	56	58	14.0 ± 0.00^{c}	13.0 ± 0.0^{b}	25.20^{c}	22.40^{b}
	0.5 + 2.0	60	57	22.0 ± 0.00^{a}	19.0 ± 0.6^{a}	36.60^{a}	33.30^{a}
ABA + zeatin	0.5 + 1.0	54	44	11.0 ± 0.70^{d}	8.0 ± 0.0^{cd}	20.30^{d}	18.80^{d}
ABA + TDZ	0.5 + 0.2	48	46	9.0 ± 0.50^{de}	8.0 ± 0.8^{c}	18.75^{c}	17.39^{d}
	0.5 + 0.5	55	51	8.0 ± 0.80^{e}	7.0 ± 0.8^{d}	14.50^{e}	13.70^{e}

KIN at 0.5 mg dm^{-3} in combination with 2,4,5-T or picloram or 2,4-D enhanced the number of somatic embryos per explant compared to 1.0 mg dm^{-3} KIN (Fig. 2*F*,*G*,*H*). Similar results were obtained in *Nicotiana tabacum* (Gill and Saxena 1993) and groundnut (Sabitha Rani and Reddy 1996). There was gradual reduction in the frequency and number of somatic embryos with an increase in the concentration of KIN. High frequency (100 %) and maximum number of globular and heart shaped embryos (68.0) per culture were observed on medium containing 6.0 mg dm^{-3} NAA plus 0.5 mg dm^{-3} KIN, followed by 6.0 mg dm^{-3} NAA and 1.0 mg dm^{-3} KIN. These results are in agreement with those of Chen *et al*. (1990) who reported an increase in somatic embryogenesis with the addition of KIN in *Vigna glabrescens*. Contrary to the above results, on the medium containing BA along with NAA or picloram or 2,4,5-T the number of somatic embryos and frequency in both the cultivars was rather low, with the exception of BA combined with 2,4-D. Thus BA induced less number of somatic embryos as compared to KIN. Similar responses were noticed by Kumar *et al*. (1994) in *Cicer arietinum* and also by Chaudhury and Rangda (2000) in *Cynodon dactylon* and also by Vila *et al*. (2009) in *Cedrela fissilis*. Occasionally, abnormal embryos (1 - 2 %) and cluster embryos were formed in the present study and they did not mature on any media. On the other hand, more abnormal embryos and cluster of embryos were reported when regenerated *via* callus in the case of groundnut (Patel *et al*. 1994) and niger (Sarvesh *et al*. 1994).

It was presumed that the failure of somatic embryos to form plantlets was either due to malformation of the apex or due to insufficient storage product in the less developed cotyledons. By manipulating the culture condition, the structure and behavior of these somatic embryos could be altered (Ammirato 1983). Therefore, these embryos were transferred to MS medium supplemented with different concentrations of BA, ABA, zeatin, or TDZ (Table 1). We noticed highest frequency of embryo germination on a medium with 2.0 mg dm^{-3} BA. Good embryo germination was also recorded in medium with 1.0 mg dm^{-3} zeatin, 0.5 mg dm^{-3} ABA and 0.1 mg dm^{-3} TDZ (Table 1), but KN did not support germination of somatic embryos (data not shown). The effect of a combination of ABA with BA or zeatin or TDZ on somatic embryo germination was also tested (Table 1) and 0.5 mg dm^{-3} ABA with 2 mg dm^{-3} BA gave the highest frequency of embryo germination. Thus, both ABA and BA seemed to be necessary for maturation and germination of somatic embryos. BA and zeatin affected many developmental stages of somatic embryos and their maturation in *Cajanus cajan* (Patel *et al*. 1994) and in *Arachis hypogea* L (Venkatachalam *et al*. 1997, Chengalrayan *et al*. 2001). Positive effect of ABA was reported for the maturation of somatic embryos in *Pennisetum americanum* (Vasil *et al*. 1981), soybean (Ackerson *et al*. 1984), alfalfa (Fuji *et al*. 1990) and niger (Sarvesh *et al*.1994). Ammirato (1983) and Chengalrayan *et al*. (2001) earlier described germination of embryos on ABA and BA containing media in groundnut. In the present study, it was found that BA with ABA was better for embryo conversion and germination than zeatin and TDZ (Table 1).

Histological sections of embryos revealed, a globular shaped embryos (Fig. 1*G*) and a torpedo shaped embryos with suspensor (Fig. 1*H*). The morphological and histological observation reported here provide evidence that embryogenesis were direct, *i,e* without an intermediate callus phase, which suggested that parental cells were pre-embryonically determined. These results clearly show that chickpea somatic embryos had not multi-cellular origin, but developed from a single cell. Embryos upon transfer to germination medium (2.0 mg dm^{-3} BA + 0.5 mg dm^{-3} ABA) turned green with folded cotyledons (Fig. 1*E*). Root and shoot poles were clearly distinct in such embryos (Fig. 1*F*). Germinated embryos developed into complete plantlets on ½ strength MS basal medium having a good root and shoot system with 36.6 % frequency. Regenerants were successfully transferred to poly cups containing a mixture of soil and sand in a ratio of 3:1 (Fig. 1*G*) and later to the field with 40 % survival rate. Tissue culture regenerated plants produced normal flowers and set the seeds and no morphological variations were observed.

This protocol has several distinct advantages over the earlier published protocols: *1*) about 95 - 100 % of the explants show embryo induction, the conversion of embryos into plantlets was as high as 36.6 %, and more than 40 complete plantlets could be obtained from 100 explants; *2*) since the protocol developed here results in direct somatic embryogenesis, time period is reduced by avoiding callus phase. Embryo induction, maturation and germination took approximately 8 - 9 weeks. To our knowledge, such a predictable rapid regeneration has not been reported earlier.

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