Indian Journal of Experimental Biology
Vol. 40, May 2002, pp. 600-602

Efficient protocol for *in vitro* direct plant regeneration in chickpea *Cicer arietinum* L.

Praveen Batra, Neelam R Yadav, Anil Sindhu, R C Yadav, V K Chowdhury* & J B Chowdhury**

Department of Biotechnology and Molecular Biology, CCS Haryana Agricultural University, Hisar 125 004, India

Received 30 March 2001; revised 16 January 2002

An efficient plant regeneration system was developed for two important Indian chickpea cultivars, C-235 and HC-1. Immature cotyledons (7-8 mm) directly formed shoots without an intervening callus phase on MS medium containing B_5 vitamins, BAP (2.0 mg/l), IBA (0.125 mg/l), $AgNO_3$ (1.69 mg/l) and phytagel (2.5 g/l). The regenerated shoots had normal morphology and were successfully rooted in half strength MS medium under partial dark conditions. Regenerated plants were transferred to potted soil. However, the survival rate of pot house transferred plants was 17.6 per cent.

Chickpea (*Cicer arietinum* L.), a pulse crop of South-East Asia, is an important source of dietary protein in third world countries including India. Chickpea yield is reduced due to various diseases (*Fusarium* wilt, *Ascochyta* blight) and insect pest (pod borer). Small seeded legumes in general and chickpea in particular have been found recalcitrant to *in vitro* regeneration. Somatic embryogenesis leading to plant formation from shoot tips[1], embryo axes[2] and leaflets[3] has been reported earlier but with low efficiency. Barna and Wakhlu[4] have observed plant regeneration in callus cultures with a mean of only 2.2 shoots per callus. Successful chickpea transformation for improving agronomic traits is limited due to low and inefficient whole plant regeneration[5,6]. In this communication, we have described direct shoot regeneration and complete plant formation from immature cotyledons. This procedure may find application for improving this important food legume through genetic transformation.

Seeds of two chickpea cultivars, HC-1 and C-235 were sown at the research experimental field of the institute. Green pods (20-25 days post-anthesis) were harvested, collected immature seeds and washed in-water containing a few drops of teepol. Seeds were sterilized with mercuric chloride (0.1%) for 6-8 min and washed several times with sterile distilled water. Seed coats were removed aseptically and the cotyledons were excised gently from the embryo axes. The abaxial part of the separated cotyledons was in contact with the culture medium. Varied number of cotyledons of size A (5-6 mm), and B (7-8 mm) were cultured on four different media in triplicate (Tables 1,2), incubated at 25°±2°C and 16/8 hr light and dark photoperiod using fluorescent tube lights at 38 µmol m^{-2} s^{-1}. Cotyledons were transferred to regeneration medium containing MS salts, vitamin B_5, benzyl amino purine (BAP 2.0 mg/l), indole butyric acid (IBA 0.125 mg/l), $AgNO_3$ (1.69 mg/l) and phytagel (2.5 g/l) after third subculture. The regenerated shoots (3-4 cm) were transferred to root induction medium having half strength of MS salts for regeneration of roots. Plantlets (5-6 cm long) were taken out from culture tubes, washed thoroughly with tap water to remove the medium and transferred to pre-sterilized potted soil and sand mixture (1:1) after hardening in water for 24 hr (Fig. 1D). A total of 65 regenerated plantlets were transferred to potted soil.

Cotyledons (7-8 mm) became swollen within 7-10 days of culture in both the cultivars (Fig. 1A). The colour of B sized (7-8 mm) cotyledons was green while A sized (5-6 mm) was light green. Cotyledons size, culture medium and cultivar affected shoot regeneration. After 15-20 days incubation of the cultures,

Table 1—Culture media used for shoot regeneration in cotyledons of *Cicer arietinum* L.

CR1	MS salts (Murashige and Skoog[8], 1962), B5 vitamins (Gamborg[9] *et al.*, 1968), IBA (0.125 mg/l), BAP (2.0 mg/l), $AgNO_3$ (1.69 mg/l^{-1}), Phytagel (2.5 g/l)
CR2	CR1 without $AgNO_3$
CR3	CR1, Agar (0.8% w/v replacing phytagel)
CR4	CR1, Mannitol (1.0% w/v)

*Correspondent author: Tel. +91-1662-33884,
Fax: +91-1662-33884
E-mail: vijayc@hau.nic.in
**Present address: Vice Chancellor, G.B. Pant Univ. of Agri. Sci. & Technology, Pantnagar (Uttaranchal)

small bud-like structures (Fig. 1A) appeared on the abaxial surface of the cotyledons on peripheral ends, which was in contact with the medium. These buds first appeared at the proximal end, where the embryo axis was attached. However, these buds did not resemble the cotyledon–like structures (CLS)[7]. Bud production took place earlier in cultivar C-235 than HC-1. Cotyledons (7-8 mm) responded earlier (15-20 days) and produced higher number of shoots (7-8) per cotyledon as compared to the cotyledons (5-6 mm), which responded after 30-35 days producing (0-1) shoots per cotyledon. The shoot buds developed into shoots in the fifth week of culture on the same medium (Fig. 1B). CR1 medium was found to be the most suitable medium, as it induced highest percentage of shoot formation in both the cultivars (Table 2). Cotyledons cultured on CR2, CR3 and CR4 media did not respond well. $AgNO_3$ is an ethylene inhibitor[10] and might have helped in initiation in shoots. In the present study when phytagel was replaced by agar regeneration frequency was reduced. Phytagel is more pure gelling agent than agar agar and reduction in shoot regeneration in agar supplemented medium may be attributed to impurities in agar. Addition of sugar alcohol in the form of mannitol did not have any effect on shoot regeneration (Table 2). Cotyledon age and size was observed to be an important factor influencing shoot formation. Larger explants (size B) regenerated at a higher rate than small sized cotyledons (Table 2). Cotyledons smaller than 5-6 mm did not regenerate shoots. Approximately 18 shoot buds were observed in 7-8 mm sized explants of HC-1 cultivar on CRI medium (Table 3). Cotyledons cultured abaxially were found to exhibit high regeneration frequency. Two components of the medium, $AgNO_3$ and phytagel, enhanced the frequency of plant regeneration (Table 3). Response of $AgNO_3$ and phytagel was found cumulative with respect to number of shoot buds, as well as number of shoots per cotyledon (Table 3). Also, the per cent cotyledons responding to shoot regeneration was more in the presence of $AgNO_3$ in both the cultivars. $AgNO_3$ has been reported to enhance plant regeneration in sunflower cotyledon cultures[11], cucumber immature cotyledons[12] and muskmelon leaf explant cultures[13]. Similar

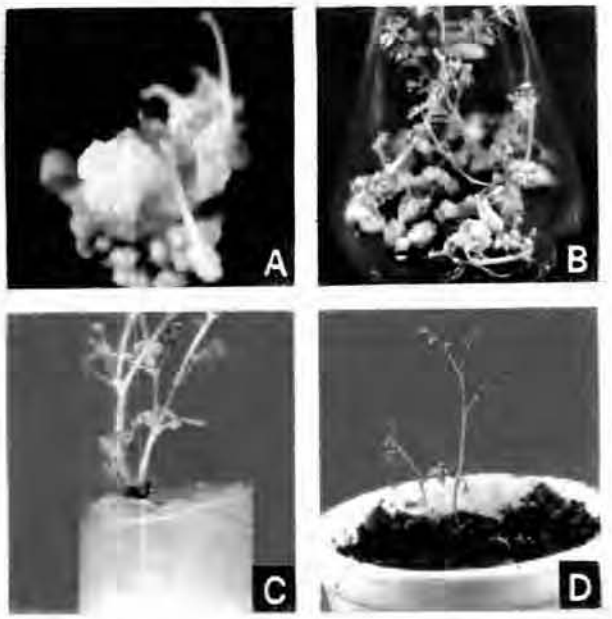

Fig. 1 (A)—Swollen cotyledon and formation of small bud like structures on the outer surface of cotyledon; (B)—Regenerated shoots with healthier leaves and normal morphology; (C)—Root formation in regenerated shoots; and (D)—Transfer of regenerated plantlets to soil.

Table 2—Per cent shoot regeneration response in culture of immature cotyledons of *Cicer arietinum* L. cultivars

Media	Cotyledon size	C-235		HC-1	
		Total explants	Per cent response	Total explants	Per cent response
CR1	A	180	57.16±6.17	239	47.10±4.36
	B	235	75.82±5.83	184	70.72±5.02
CR2	A	90	20.45±0.93	70	28.53±1.23
	B	110	26.85±2.38	70	27.66±2.92
CR3	A	40	5.20±1.47	30	6.48±2.35
	B	35	7.18±0.85	55	7.56±1.32
CR4	A	90	33.83±3.20	100	26.30±7.36
	B	60	47.27±8.88	90	32.66±3.12

A sized cotyledons (5-6 mm) light green and very soft in texture; and B sized cotyledons (7-8 mm) green and soft in texture.

Table 3—Effect of $AgNO_3$, phytagel and agar on shoot regeneration in culture of immature cotyledons of C-235 and HC-1 genotypes of *C. arietinum* L.

Cultivars	Medium	No. of shoot buds	No. of shoots
C-235	1	15.00±1.67	7.10±1.41
	2	7.20±1.41	1.01±0.70
	3	3.10±1.87	1.01±0.00
HC-1	1	18.00±2.30	10.00±2.60
	2	8.25±2.27	2.00±0.70
	3	8.75±1.47	1.01±0.00

Medium (1) – CR1; (2) – CR2; and (3) – CR3 as indicated in Table 1.

enhancement was also noticed with $AgNO_3$ supplemented medium in leaf cultures of JG-62 and P114 chickpea cultivars, however in C-235 cultivar, shoot regeneration did not increase significantly[3]. Exact mechanism for enhancement of shoot formation by $AgNO_3$ is not clear, however, it may be possible that this is related to polyamine biosynthesis which also promotes shoot formation[14]. Per cent shoot regeneration in each cultivar was higher on medium containing phytagel instead of agar as gelling agent. Approximately multi-fold increase in shoot regeneration was noticed on medium containing phytagel (Table 3) and number of shoots per explant also increased (Table 3). Yadav *et al.*[13] have also reported that plant regeneration enhances by using phytagel (2.6 g/l) in muskmelon leaf explants. The regenerated chickpea shoots readily formed roots (Fig. 1C), on root induction medium in 15-20 days as reported earlier[4,5]. The complete plantlets had normal morphology in both culture tubes as well as in pot house conditions (Fig. 1C,D) in contrast to earlier reports[1,4]. In our study, the survival percentage of regenerated plantlets to potted soil was low (17.6%). Establishment of regenerated chickpea plants in soil has been found extremely difficult. Barna and Wakhlu[4] have found 20.4% success in transferring regenerated plants to soil. Krishnamurthy *et al.*[6] have obtained mature plants by grafting regenerated shoots on five day old etiolated chickpea seedlings. Further progress is needed to increase the efficiency of transferring plants to soil, since it will be a major limitation in using transformation strategy for chickpea improvement[4].

In the present protocol, regeneration was not preceded by callus formation and the shoots were well formed with normal morphology. Direct shoot bud formation without callus phase leads to uniformity in regenerated plants[15,16], which could be effectively utilized for transformation studies in this important pulse crop.

The authors are thankful to Dr. Ram Kumar, chickpea breeder for providing materials and technical advice. The financial assistance by the Department of Biotechnology, India is gratefully acknowledged.

References

1 Surya Prakash, Chowdhury J B, Jain R K & Chowdhury V K, Factors affecting plant regeneration in chickpea, *Cicer arietinum* L., *Indian J Exp Biol*, 30(1992) 1149.

2 Sagare A P, Suhasini K & Krishnamurthy K V, Histology of somatic embryo initiation and development in chickpea (*Cicer arietinum* L.), *Plant Cell Rep*, 12(1995) 652.

3 Kumar P A, Bisaria S, Pai R A & Sharma R P, Comparative shoot regeneration in different genotypes of chickpea, *Cicer arietinum* L., *Indian J Exp Biol*, 33 (1995) 77.

4 Barna K S & Wakhlu A K, Whole plant regeneration of *Cicer arietinum* from callus cultures *via* organogenesis, *Plant Cell Rep*, 13(1994) 510.

5 Subhadra Vashishat R K, Chowdhury J B, Singh M & Sareen P K, Multiple shoots from cotyledonary node explants of non nodulating genotype (ICC435m) of chickpea *Cicer arietinum* L, *Indian J Exp Biol*, 36 (1998) 1276.

6 Krishnamurthy K V, Suhasini K, Sagare A P, Meixner M, Dekathen A, Pickardt T & Schieder O, *Agrobacterium* mediated transformation of chickpea (*Cicer arietinum* L.) embryo axes, *Plant Cell Rep*, 19(2000) 235.

7 Shri P V & Davis T M, Zeatin induced shoot regeneration from immature chickpea (*Cicer arietinum* L.) cotyledons, *Plant Cell Tissue Organ Cult*, 28(1992) 45.

8 Murashige T & Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol Plant*, 15(1962) 473.

9 Gamborg O L, Miller R & Ojima K, Nutrient requirements of suspension cultures of soyabean root cells, *Exp Cell Res*, 50(1968) 151.

10 Beyer E M, Silver ion: A potent anti-ethylene agent in cucumber and tomato, *Hort Sci*, 11(1976) 195.

11 Chraibi K M B, Cstelle J C, Latche, A Roustan, J P & Falot J, A genotype independent system of regeneration from cotyledons of sunflower (*Helianthus annus* L.), *Plant Sci*, 86(1992) 215.

12 Roustan J P, Latche A & Fallot J, Enhancement of shoot regeneration from cotyledons of *Cucumis melo* by $AgNO_3$ an inhibitor of ethylene action, *J Plant Physiol*, 140(1992) 485.

13 Yadav R C, Saleh M T & Grumet R, High frequency shoot regeneration from leaf explants of muskmelon, *Plant Cell Tissue Organ Cult*, 45(1996) 207.

14 Eapen S & George L, Enhancement in shoot regeneration from leaf discs of *Brassica juncea* L. Czern and Coss by silver nitrate and silver thiosulphate, *Physiol Mol Biol Plants*, 2(1996) 83.

15 Gill R & Saxena P K, Direct somatic embryogenesis and regeneration of plants from seedling explants of peanut (*Arachis hypogaea*) : Promotive role of thidiazuron, *Can J Bot*, 70(1992) 1186.

16 Suhasini K, Sagare A P & Krishnamurthy K V, Direct somatic embryogenesis from mature embryo axes in chickpea (*Cicer arietinum* L.), *Plant Sci*, 102(1994) 189.