Indian J. Plant Physiol., Vol. 11, No. 4, (N.S.) pp. 421-426 (Oct.-Dec., 2006)

SHORT COMMUNICATION

EFFICIENT *IN VITRO* REGENERATION PROTOCOL FROM DIFFERENT EX-PLANTS IN A DROUGHT TOLERANT VARIETY BGD72 OF *CICER ARIETINUM* L.

VEENA AGRAWAL* AND ANURADHA YADAV

Department of Botany, University of Delhi, Delhi-110007.

Received on 31 Jan., 2006, Revised on 10 Nov., 2006

Chickpea, commonly known as gram is extensively cultivated as one of the most important winter crop throughout India, especially in northern states. Incidentally, it suffers from both biotic and abiotic stresses causing low productivity. *In vitro* plantlet regeneration protocol has been developed employing seed and seedling explants for improving the crop through biotechnological manipulations. The three explant types - seed, embryonal axis and nodal segment (excised from 30-d-old seedling) were cultured on Murashige and Skoog's basal medium supplemented with various growth regulators, i.e. N^6-benzyladenine (BA), kinetin (Kn), α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) alone or in combination. Of the explants tried, the seed explants elicited best morphogenic response in terms of multiple shoots production. BA at 5 µM proved optimum for eliciting an average of 3.7 ± 1.2 shoots in 50% cultures in seeds whereas for embryonal axis 100% cultures induced an average of 3.1 ± 0.2 shoots on the same level in 30 d. However for nodal explants, 2.5 µM BA showed better response and an average of 3.25 ± 0.38 shoots per explants was induced in 91.7% cultures. For induction of roots, MS (½) + 5 µM IBA proved best where 72.5% shoots developed an average of 18.10 ± 1.37 roots within 20 d. The plantlets have been hardened and transferred to soil.

***Key words*: Benzyladenine, *Cicer arietinum*, indole-3-butyric acid, plant regeneration.**

Chickpea has been recognised as one of the important crops all over the world mainly because of its seeds which are used as rich source of dietary protein. Unfortunately, its productivity has been severely hampered due to several constraints like its cultivation on marginal lands, impacts of biotic and abiotic stresses, and low research and management efforts. Improvement of this crop through conventional breeding is not successful due to non-existence of the insect lepidopteran (*Heliothis* sp.) resistant germplasm (Varder Have 1979, Kar *et al*. 1996). Incidentally, improvement of the crop employing technique of genetic engineering too, poses difficulty due to non-availability of the efficient tissue culture protocols (Grover *et al*. 2003). Though, some *in vitro* regeneration reports have already appeared on various genotypes of *Cicer* through organogenesis using shoot meristem (Bajaj and Dhanju 1979 Sharma *et al*. 1979), immature cotyledons (Shri and Davis 1992, Shrivastava *et al*. 2001), leaflet callus (Barna and Wakhlu 1994), embryonal axis (Polisetty *et al*. 1997), seed (Murthy *et al*. 1996), cotyledonary node (Subhadra *et al*. 1998) or through somatic embryogenesis from immature cotyledons (Sagare *et al*. 1993) and leaflet callus (Barna and Waknlu 1993, Eapen and George 1994, Kumar *et al*. 1994), yet there is no report till date on regeneration in the drought tolerant variety of *Cicer*. Present communication reports the multiple shoot induction as well as successful plantlet regeneration from seed, embryonal axis, as well as seedling nodal explants of a drought tolerant *Cicer arietinum* variety BGD72.

* Corresponding author, E-mail : drveena_du@yahoo.co.in.

The seed material was procured from Pulse Seed Laboratory, Genetic Division, IARI. The explants - seeds, embryonal axis and nodal segments (derived from 30d old seedling) were cultured on Murashige and Skoog (1962) (MS) medium either alone or supplemented with the various growth regulators, like BA, Kn, IAA individually or in combination. The pH of the medium was adjusted to 5.8 and solidified with 0.8% agar. The medium was dispensed into 25 x 150 mm Borosil culture tubes and was autoclaved at 121°C temperature and 15 psi pressure. All cultures were incubated in a growth room under continuous light of 400-450 μW/cm^2 emitted by cool day light fluorescent incandescent tubes (40W Philips tubes). The temperature was maintained at 25 ± 2°C and 55±10% relative humidity.

In seed and embryonal axis (with excised cotyledon only) explants, elongation of shoot and root apices started within 7 d of culture on MS basal, which developed into single shoot and root, respectively with normal growth in 100% cultures in next 20 days (Fig. 1A). Influence of two cytokinins such as BA or Kn alone or in combination with auxin IAA enhanced the morphogenic response in terms of multiple shoots production. Multiple shoot buds were induced on media containing BA within 7-10 days, which developed into shoots in next 20-25 days (Figs. 1 B,C). Though, the multiple shoots were obtained on all concentrations (2.5, 5, 7.5, 10, 12.5 & 25 μM) of BA tried, the differences were recorded in terms of percentage of explants forming multiple shoots as well as average shoot number (Table 1). However, the seed explants planted on MS medium fortified with 0, 1, 5, 10 and 20 μM Kn, elicited poorer response compared with those on BA supplemented media. Only single shoot per explant developed on basal as well as lower concentrations (1 or 5 mM) of Kn. On its higher levels (above 5 μM) multiple shoots were organized only in few cultures (Table 1). Since BA elicited best response in terms of multiple shoot production as well as average shoot number, its influence was studied in combination with auxins to further enhance their effect on morphogenesis. Explants were cultivated on MS medium supplemented with 1, 2 and 5 mM IAA in combination with 5 or 10 μM BA. Their response varied significantly on different combinations (Fig. 1D; Table 1).

The nodal explants, if reared on basal alone did not develop even a single shoot and the explants turned brown

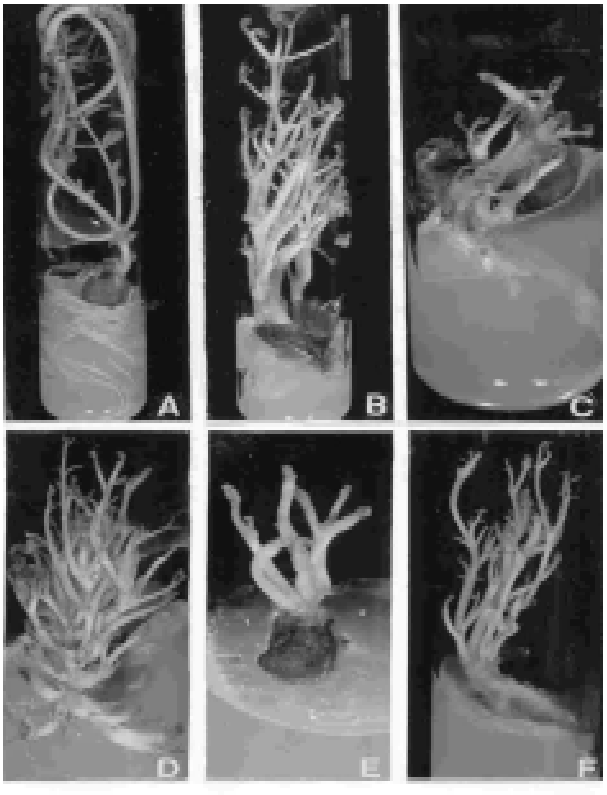

Fig. 1. A-F. Morphogenic response of different explants of a drought tolerant variety (BGD72) of *Cicer arietinum* cultured on MS medium alone or supplemented with BA, after 30 d of inoculation; A. Emergence of single shoot on MS basal medium from seed x 0.8; B,C. Multiple shoots developed from seed explant on 2.5 μM BA (B) and 25 μM BA (C) B x 1.2; C. x 1; D. Numerous multiple shoots with profuse branching from seed on 1 μM IAA + 10 μM BA x 1.6; E. A node bearing 4-5 direct axillary shoots on MS + 2.5 μM BA x 2.7; F. Multiple shoots developed in the axil of embryonal axis on 5 μM BA x 1.7.

within 20 d of the culture. On BA supplemented medium, the inconspicuous axillary buds started growing within 7 d of incubation and developed into shoots in another 7 d on all the concentrations of BA tried. Though 100% explants responded on 10 μM BA but the average shoot number as well as average shoot length was optimum on 2.5 μM BA. At this level 91.7% shoots developed a maximum average of 3.3 ± 0.4 shoots having an average shoot length of 2.2 ± 0.3 cm. Higher level (25 μM) of BA proved inhibitory both for shoot growth as well as average shoot number (Fig. 1E; Table 1). However, shoots were seen to be elongated when transferred to MS basal from 25 μM BA. Explants reared on MS medium supplemented with 1, 5, 10 and 20 μM Kn showed comparatively less

Table 1. Morphogenic response of different explants of a drought tolerant variety of *Cicer arietinum* (BGD72) cultured on MS medium augmented with different growth regulators. Data recorded after 30 d.

Growth Regulators (µM)	Explants developing multiple shoots %			* Average number of shoots per culture			* Average length of shoot (cm)		
	Seed	**E. axis	Node	Seed	**E. axis	Node	Seed	**E. axis	Node
BA									
0	0	0	0	1.0±0.0	0.9±0.0	0	12.8±0.8	6.6±0.7	0
2.5	33.4	8.4	91.7	2.9+1.1	1.0 ±0.1	3.3 ± 0.4	1.9±0.5	7.0±0.9	2.2±0.3
5	50	100	25	3.7±1.2	3.1±0.2	0.8 ± 0.2	2.3±0.5	2.3±0.3	1.5±0.3
7.5	25	6.7	75	1.2±0.7	2.1±0.5	2.7± 0.5	1.8±0.2	1.7±0.4	1.3±0.2
10	8.4	83.4	91.7	0.6±0.3	2.9±0.4	2.8 ± 0.3	0.7±0.2	2.3±0.4	1.2±0.2
12.5	25	75	75	1.6±0.6	2.3±0.4	2.6 ± 0.4	1.7±0.5	1.5±0.3	0.9±0.2
25	25	100	58.4	2.5±1.4	3.8±0.3	1.5 ± 0.4	1.1±0.4	2.7±0.3	0.4±0.2
Kn									
0	0	0	0	1.0±0.0	0.9±0.0	0	12.8±0.8	6.6±0.6	0
1	0	0	-do-	0.7±0.0	0.7±0.0	0.7 ± 0.2	4.1±0.3	5.8±0.5	3.7±0.5
5	0	16.7	4.2	0.7±0.0	1.0±0.2	0.7 ± 0.0	3.8±0.4	4.2±0.4	3.5±0.3
10	6.7	59.1	16.7	0.1±0.2	1.7±0.2	0.8 ± 0.2	3.4±0.3	3.9±0.5	3.5±0.5
20	25	54.2	29.2	1.0±0.3	1.8±0.2	0.9 ± 0.1	2.2±0.4	2.9±0.3	1.2±0.4
BA+IAA									
0+0	0	0	0	1.0±0.0	0.9±0.0	–	12.8±0.8	6.5±0.7	–
5+1	8.4	75	33.4	0.9±0.4	2.0±0.3	1.9 ± 0.3	1.6±0.9	1.6±0.3	1.5±0.3
5+2	-	66.7	41.7	0	1.8±0.4	1.2 ± 0.2	0	2.0±0.3	1.1±0.5
5+5	25	75	16.6	0.5±0.0	2.3±0.3	0.5 ± 0.5	0.5±0.1	2.2±0.5	0.6±0.3
10+1	25	66.7	25	1.8±0.7	1.9±0.4	1.5 ± 0.3	0.8±0.4	1.3±0.2	1.5±0.5
10+2	50	41.7	50	1.9±0.4	1.7±0.4	1.9 ± 0.3	1.9±0.3	1.3±0.2	1.5±0.4
10+5	-	66.7	50	0	2.2±0.4	2.1± 0.5	0	1.6±0.4	1.1±0.2

*Mean value of 24 explants ± standard error.
** Embryonal axis.

morphogenic response in comparison to BA. Axillary buds developed after 7 d of inoculation which turned into shoots in next 7 d on all the concentrations of Kn tried. On 20 µM, a maximum of 29.2% explants formed multiple shoots after 30 d of inoculation (Table 1).

Thus, of all the aforesaid three explants tried, embryonal axis proved to be the best for inducing multiple shoots in 100% cultures at higher level of (25 µM) BA with an average of 3.8 ± 0.3 shoots per explant (Table 1; Fig. 1F). However, in terms of cytokinin requirement, nodal explant was best where 2.5 µM of BA proved optimum for eliciting morphogenic response in 91.7% cultures with an average of 3.3 ± 0.4 shoots per culture. For seed explants only 5 µM BA was needed to elicite multiple shoot production in 50% cultures with an average of 3.7 ± 1.2 shoots. Significantly low response has been seen in explants cultured on medium containing Kn. A maximum of 59.1% embryonal axis, 25% seed and 29.2% nodal explants developed an average of 1.8, 1 and 0.9 shoots per explants, respectively.

The requirement of high level of BA by the embryonal axis may be due to the reason that it might have to encounter the effect of apical dominance which subsequently trigger axillary shoot proliferation. BA in combination with IAA though supported multiple shoot induction but the % age responses were relatively low in

comparison to BA alone in all the explants (Table 1). Besides, the average shoot number seen was always less in comparison to those obtained on medium containing BA alone. A maximum of 75% embryonal axis explants induced an average of 2.3 ± 0.3 shoots per explant when a combination of 5 µM BA + 5 µM IAA was used (Table 1). For nodal explants a significant decline in response was seen when they were cultured on medium containing 10 µM BA with 5 µM IAA. Such explants developed an average of 2.1 ± 0.5 shoots per explant, whereas, seed explants did not give any response at this level. Thus, a differential response have been seen at similar levels of growth regulators (Table 1). The differential responses on various growth regulators may be due to differences in their endogenous levels.

In vitro raised shoots were excised from explants and transferred to MS 1/2 strength medium supplemented with 1, 5 or 10 mM of different auxins (IAA, IBA and NAA). Organisation of roots was seen on all the combinations, but 5 µM IBA proved to be the best for direct rhizogenesis in the excised shoots (Fig. 2B). An average of 18.10 ± 1.37 roots with an average length of 0.75± 0.83 cm were induced per shoot in response to this concentration.

The *in vitro* regenerated plantlets were washed thoroughly with tap water to remove all the adhering agar and then dipped in 0.2% (w/v) bavistin solution before transfer to the plastic cups containing autoclaved garden soil. The plants were maintained under culture room conditions, i.e. under high humidity and light conditions for initial 30 days. They were then transferred to soil where they are thriving (Fig. 2C).

The studies carried out so far on *Cicer arietinum* L. has revealed that the morphogenic response is mainly dependent on the genotype, type of explant and concentration of cytokinin used (Barna and Wakhlu, 1994, Polisetty *et al.* 1997, Subhadra *et al*. 1998, Sagare *et al*. 1993, Barna and Wakhlu 1993, Suhasini *et al.* 1994). During the present study, too, optimum response has been seen to be influenced by type of explant as well as concentration of BA. Maximum response has been given by embryonal axis, followed by nodal and seed explants in terms of percentage of morphogenic cultures producing multiple shoots.

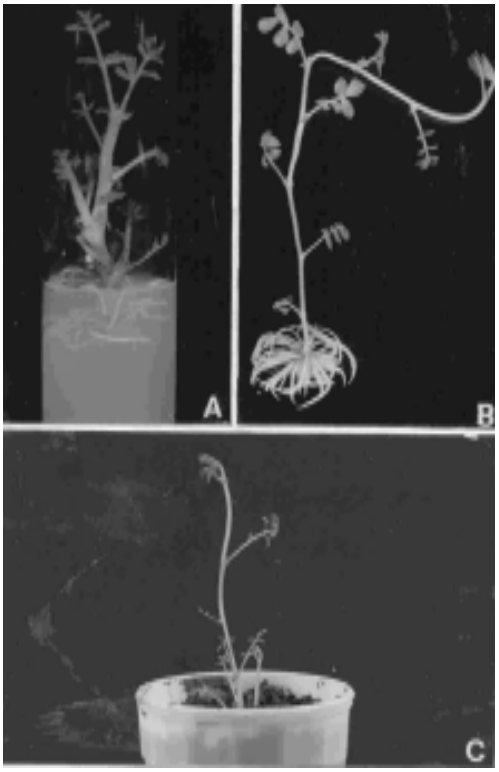

Fig. 2. A-C. Direct root induction at the base of *in vitro* raised shoots of a drought tolerant variety (BGD72) of *Cicer arietinum* on MS (½ strength) medium containing IAA, after 15 d of transfer; A. Developments of roots at the base of *in vitro* raised shoot on MS containing IAA 5µM A x 0.9; B. Tissue culture derived plantlets showing healthy shoot and roots; C. The tissue culture raised plantlet, after 30 d of transfer to soil.

Of the two cytokinins tried, maximum shoot regeneration has been achieved with BA, the Kn giving the poorer response. The levels of BA has been found to be yet another significant factor influencing morphogenesis. A very higher concentration of BA (beyond 75 and 150 µM) has been used in earlier paper (Polisetty *et al.* 1997) for inducing multiples shoots in different genotypes (BG-362, BG-329, BG-267, BG-256 and C-235), whereas relatively less concentration of BA was required for inducing multiple shoots in another cultivar of *Cicer* (Murthy *et al*. 1996). In contrast to the above reports, in our case a maximum of 5 µM BA was required for seed explants and 2.5 µM for nodal explants and 25 µM for embryonal axis (with excised cotyledon). The requirement of higher concentration of BA for the embryonal axis may be due to fact that it might encounter the effect of apical dominance shown by plumular axis

Table 2. Induction of roots in *in vitro* raised shoots of a drought tolerant variety of *Cicer arietinum* (BGD72) cultured on MS (1/2 strength) + auxins after 20 d.

Auxins (µM)		Shoot developing roots (%)		*Average number of root per culture	*Average root length (cm)
IBA	NAA	with callus	without callus		
0	0	17.4	52.2	5.66±0.99	1.54±0.78
-	1	17.4	43.5	4.47±1.06	1.77±0.44
-	5	40.0	25.0	3.75±0.94	0.42±0.06
-	10	29.2	37.5	4.59±1.21	0.32±1.09
1	-	0	68.2	6.78±1.41	1.10±0.84
5	-	05.8	66.7	18.10±1.37	0.85±0.55
10	-	12.5	62.5	15.54±1.77	0.75±0.83

*Mean value of 24 explants ± standard errors

which inhibits axillary proliferation. In this genotype, at higher concentration, the shoots turned stunted with concomitant callus formation at the basal ends. Incidentally when IAA was used along with BA, profused axillary branching of the shoots was noticed with abundance of callus.

Realising the effect of genotype versus BA level, it has, thus, become imperative to develop regeneration protocol for a recently developed genotype BGD72 for its further improvement. This is our first report of successful regeneration from seed, nodal as well as embryonal axis explants in this genotype which has already been selected as drought tolerant.

Such profuse rooting on different auxins levels has not been shown earlier. Significance of development of protocol for each genotype has been well documented in the light of the urgent need for the crop improvement all over the globe, employing techniques of genetic engineering. A remarkable influence of growth regulators has been exhibited on the *in vitro* propagation of chickpea. Thus, developed protocol can be effectively employed for further improvement of this crop using technique of genetic engineering.

ACKNOWLEDGEMENTS

Authors are grateful to the University Grants Commission, New Delhi for providing financial support. Thanks are also due to Dr. S.S. Yadav, PS, IARI, New Delhi for providing seed material.

REFERENCES

Bajaj, Y.P.S. and Dhanju, M.S. (1979). Regeneration of plants from apical meristem tips of some legumes. *Curr. Sci.* **48:** 906-907.

Barna, K.S. and Wakhlu, A.K. (1993). Somatic embryogenesis and plant regeneration from callus cultures of chickpea (*Cicer arietinum.*). *Plant Cell Rep.* **12:** 521-524.

Barna, K.S. and Wakhlu, A.K. (1994). Whole plant regeneration of *Cicer arietinum* from callus cultures *via* organogenesis. *Plant Cell Rep.* **13:** 510-513.

Eapen, S. and George, L. (1994). Somatic embryogenesis in *Cicer arietinum* L.: influence of genotype and auxins. *Biol. Plant.* **36:** 343-349.

Grover, A. and Pental, D. (2003). Breeding objectives and requirements for producing transgenics for major field crops of India. *Curr. Sci.* **84:** 310-320.

Kar, S., Johnson, T.M., Nayak, P. and Sen, S.K. (1996). Efficient transgenic plant regeneration through *Agrobacterium*-mediated transformation of chickpea. *Plant Cell Rep.* **16:** 32-37.

Kumar, V.D., Kirti, P.B., Sachan, J.K.S. and Chopra, V.L. (1994). Plant regeneration *via* somatic embryogenesis in chickpea (*Cicer arietinum* L.). *Plant Cell Rep.* **13:** 468-472.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. **15:** 473-497.

Murthy, B.N.S., Victor, J., Singh, R.P., Fletcher, R.A. and Saxena, P.K. (1996). *In vitro* regeneration of chickpea (*Cicer arietinum* L.): Stimulation of direct organogenesis and somatic embryogenesis by thidiazuron. *Plant Growth Regul*. **19:** 233-240.

Polisetty, R., Paul, V., Deveshwar, J.J., Khetarpal, S., Suresh, K. and Chandra, R. (1997). Multiple shoots induction by benzyladenine and complete plant regeneration from seed explant of chickpea (*Cicer arietinum* L.). *Plant Cell Rep*. **16:** 565-571.

Sagare, A.P., Suhasini, K. and Krishnamurthy, K.V. (1993). Plant regeneration *via* somatic embryogenesis in chickpea (*Cicer arietinum* L.). *Plant Cell Rep*. **12:** 652-655.

Sharma, D.R., Kumari, R. and Chowdhury, J.B. (1979). Plant regeneration in *Cicer* species through tissue culture. *Indian J. Exp. Biol*. **27:** 607-609.

Shri, P.V. and Davis, T.M. (1992). Zeatin induced shoot regeneration from immature chickpea (*Cicer arietinum* L.) cotyledons. *Plant Cell Tiss. Org. Cult*. **28:** 45-51.

Shrivastava, K., Tiwari, K.N., Singh, R., Singh, B.D. and Jaiswal, H.K. (2001). Shoot regeneration from immature cotyledons of *Cicer arietinum. Biol. Plant*. **44 :** 333-337.

Subhadra, R.K., Vashishat, J.B., Chowdhury, M., Singh, P.K. and Sareen, P.K. (1998). Multiple shoots from cotyledonary node explants of non-nodulating genotype (ICC435M) of chickpea, *Cicer arietinum* L. *Indian J. Exp. Biol*. **36:** 1276-1279.

Suhasini, K., Sagare, A.P. and Krishnamurthy, K.V. (1994). Direct somatic embryogenesis from mature embryo axes in chickpea (*Cicer arietinum* L.). *Plant Sci*. **102:** 189-194.

Varder Have D.J. (1979). : Plant Breeding Perspectives, Pudco, Centre for Agricultural Publishing and Documentation, Wageningen.