BIOLOGIA PLANTARUM 44 (3): 333-337, 2001

# Shoot regeneration from immature cotyledons of *Cicer arietinum*

K. SRIVASTAVA*, K.N. TIWARI*, R. SINGH*, B.D. SINGH* and H.K. JAISWAL**

*School of Biotechnology, Faculty of Science, B.H.U. Varanasi-221005, U.P., India**
*Institute of Agricultural Sciences, B.H.U., Varanasi-221005, U.P., India***

## Abstract

Shoot regeneration was achieved from immature cotyledons of five chickpea (*Cicer arietinum* L.) genotypes: C235, ICC4971, ICC11531, ICC12257 and ICC12873. The cotyledons cultured on Murashige and Skoog (MS) medium supplemented with 3 or 5 mg $dm^{-3}$ zeatin with or without 0.04 mg $dm^{-3}$ indole acetic acid (IAA) showed formation of cotyledon like structures (CLS) at their proximal ends. Subsequently, shoot regeneration took place in some of the CLS forming explants. CLS were also formed in cotyledons cultured on MS + 0.2 - 1 mg $dm^{-3}$ thidiazuron (TDZ); direct shoot regeneration was observed in cotyledons cultured on 1 mg $dm^{-3}$ TDZ. The shoot buds elongated on media containing indole butyric acid (IBA), benzylaminopurine (BAP) and gibberellic acid ($GA_3$). Complete plantlets were obtained by rooting of shoots following pulse treatment with 200 mg $dm^{-3}$ IBA for 5 min and culture on growth regulator free half-strength MS medium.

*Additional key words*: chickpea, cotyledon like structures, regeneration *in vitro*, thidiazuron, zeatin.

## Introduction

Chickpea (*Cicer arietinum* L.) is a popular grain legume crop of Asian, Mediterranean and South American regions, favoured for high seed protein content. The improvement of chickpea is based primarily on intraspecific genetic variability as utilization of wild species is hampered by a lack of sexual compatibility. Non-conventional gene transfer methods appear to be a promising solution to this constraint, but these require a dependable high frequency whole plant regeneration protocol.

Several reports on somatic embryogenesis in chickpea are available, but the frequencies of somatic embryo development and conversion remain low (Barna and Wakhlu 1993, Sagare *et al.* 1993, Eapen and George 1994, Kumar *et al.* 1994, 1995, Suhasini *et al.* 1994). Some other studies have reported shoot regeneration either after callus formation (Surya Prakash *et al.* 1992) or directly from explants that contained meristems (Malik and Saxena 1992, Polisetty *et al.* 1997). Immature cotyledons of chickpea supported shoot regeneration *via* formation of cotyledon like structures (CLS) in ICC640 (Shri and Davis 1992).

The present report describes shoot regeneration from immature cotyledons of five chickpea genotypes.

## Materials and methods

Immature green pods of *Cicer arietinum* L. cv. C235 and the lines ICC4971, ICC11531, ICC12257 and ICC12873 were collected from the field and washed thoroughly under running tap water. The pods were surface sterilised by 1 % *Cetavlon* (*Cetrimide, ICI Ltd.*, Patiala, India) followed by a quick rinse in 70 % ethanol, a 15-min

Received 24 March 2000, accepted 11 September 2000.

*Abbreviations*: BAP - 6-benzylaminopurine; CLS - cotyledon like structures; $GA_3$ - gibberellic acid; GR - growth regulator; IAA - indoleacetic acid; IBA - indolebutyric acid; MS medium - Murashige and Skoog medium; TDZ - thidiazuron.

*Acknowledgements*: The award of senior research fellowships to KS from CSIR and to KNT and RS from UGC is gratefully acknowledged.

Present addresses: K. Srivastava, Indian Institute of Vegetable Research, 1 Gandhi Nagar, Naria, P.O. Box No. 5002, B.H.U., Varanasi - 221005, UP, India; K.N. Tiwari, Arvind Degree College, Kirandul, Bastar, MP, India.
Correspondence should be sent to B.D. Singh, fax: (+91) 0542 368693/368174, e-mail: brahmadsingh@khuljasim.sim.com

treatment with 0.2 % $HgCl_2$, a second rinse with 70 % ethanol and 4 - 5 rinses with sterilised double distilled water. The surface sterilised pods were opened with forceps and the green immature seeds were taken out. The seed coats were removed with the help of fine-tipped forceps avoiding injury to the cotyledons. The embryo axis was removed and the entire cotyledons were cultured with their abaxial surface in contact with the medium. In a separate experiment, the proximal ends of the cotyledons of cv. C235 were removed prior to culturing them on MS medium (Murashige and Skoog 1962) + 3 mg $dm^{-3}$ zeatin or 0.5 mg $dm^{-3}$ thidiazuron (TDZ) containing medium.

The cotyledons of different chickpea genotypes were cultured on MS + 3 or 5 mg $dm^{-3}$ zeatin with or without 0.04 mg $dm^{-3}$ indole acetic acid (IAA); cotyledons of cv. C235 were also cultured on MS + 0.2, 0.5 or 1.0 mg $dm^{-3}$ TDZ medium. In order to promote shoot regeneration, the cotyledon bearing CLS were transferred to MS medium containing combinations of indole butyric acid (IBA) (0.05, 0.1 and 0.2 mg $dm^{-3}$), gibberellic acid ($GA_3$) (0.05 and 0.1 mg $dm^{-3}$) and 6-benzylaminopurine (BAP) (0.1 and 0.2 mg $dm^{-3}$). Elongated shoots of approximately 2 cm were excised from the cotyledons and were rooted by giving them a pulse treatment of 200 mg $dm^{-3}$ IBA for 5 min followed by their culture on a GR-free medium having 1/2 strength MS salts – full strength MS vitamins + 15 g $dm^{-3}$ sucrose.

All the media were gelled using agar (*Sigma Chemical Co.*, St. Louis, USA). The pH of media was adjusted to 5.8 prior to autoclaving. The medium was autoclaved and dispensed in pre-sterilized culture tubes (100 × 25 mm). Zeatin, IAA, and $GA_3$ were filter sterilised and added to the autoclaved medium when it was cooled to approximately 40 °C. The culture tubes were arranged according to nested design, and incubated in a culture room under a 16-h photoperiod (irradiance of 20 µmol $m^{-2}$ $s^{-1}$ provided by cool fluorescent tubes) and constant temperature of 24 ± 2 °C. The data were subjected to analysis of variance (Steel and Torrie 1960) and the treatment means were compared by Duncan's Multiple Range Test (DMRT) (Gomez and Gomez 1984).

## Results and discussion

Immature cotyledons of chickpea genotypes, C235, ICC4971, ICC11531, ICC12257 and ICC12873, cultured on MS medium containing 3 or 5 mg $dm^{-3}$ zeatin with or without 0.04 mg $dm^{-3}$ IAA showed development of CLS within 15 d of culture initiation. The CLS developed exclusively at the proximal end of the cotyledons, close to, but never within the cotyledonary notch (Fig. 1). The CLS were 2 - 3 mm in size and their number per cotyledon varied greatly (1 - 50). The CLS were usually produced directly on the cotyledons, but occasionally they were borne on stalks (Fig. 2). By the end of 4 weeks, shoots developed at the base of some of the CLS (Fig. 3).

Table 1. CLS formation and shoot regeneration from immature cotyledons of 5 chickpea genotypes; the cotyledons were cultured on MS medium supplemented with 3 or 5 mg $dm^{-3}$ zeatin and 0 or 0.04 mg $dm^{-3}$ IAA for 4 weeks. Means having different letters as their superscripts are significantly different ($P < 0.05$); comparison by DMRT. Each mean is estimated from 4 replicates of 45 explants each.

| Genotype | Number of cotyledons forming CLS | CLS forming shoots [%] | Number of shoots [responding cotyledon$^{-1}$] |
|---|---|---|---|
| C235 | $22.0^{b}$ | 5.6 | $2.25^{b}$ |
| ICC11531 | $22.3^{b}$ | 14.6 | $2.08^{b}$ |
| ICC12873 | $10.8^{a}$ | 20.9 | $1.22^{ab}$ |
| ICC4971 | $11.0^{a}$ | 4.5 | $1.00^{a}$ |
| ICC12257 | $8.8^{a}$ | 17.1 | $1.83^{ab}$ |

Analysis of variance showed that the number of CLS forming explants and the number of shoots per responding explant were significantly affected by explant genotype. In C235 and ICC11531, significantly higher numbers of explants formed CLS as compared to the other genotypes; these two genotypes also showed the highest number of shoots per responding explant. However, the highest proportion of cotyledons formed shoots in the case of genotype ICC12873, followed by ICC12257 (Table 1).

Significant effect of GR concentration on number of CLS forming explants was detected through analysis of variance within ICC4971, ICC12257 and ICC12873, while the number of shoots per responding cotyledon differed significantly within all the genotypes, except ICC4971. In case of C235, ICC11531 and ICC4971, MS + 3 mg $dm^{-3}$ zeatin appeared to be the optimum, while in ICC12873 and ICC12257 MS + 5 mg $dm^{-3}$ zeatin seems to be optimal (Table 2).

Immature cotyledons of cv. C235 cultured on 0.2 to 1.0 mg $dm^{-3}$ TDZ also showed development of CLS at their proximal ends (Table 3). The CLS elicited by TDZ were larger in size than those induced by zeatin. The differences between TDZ concentration for the frequencies of explants forming CLS and/or shoots were not significant. In addition to the CLS - mediated shoot regeneration, direct shoot regeneration was observed from the cotyledons cultured on 1 mg $dm^{-3}$ TDZ (Fig. 4).

CLS induction from immature cotyledons of chickpea seems to require a strong cytokinin activity since BAP

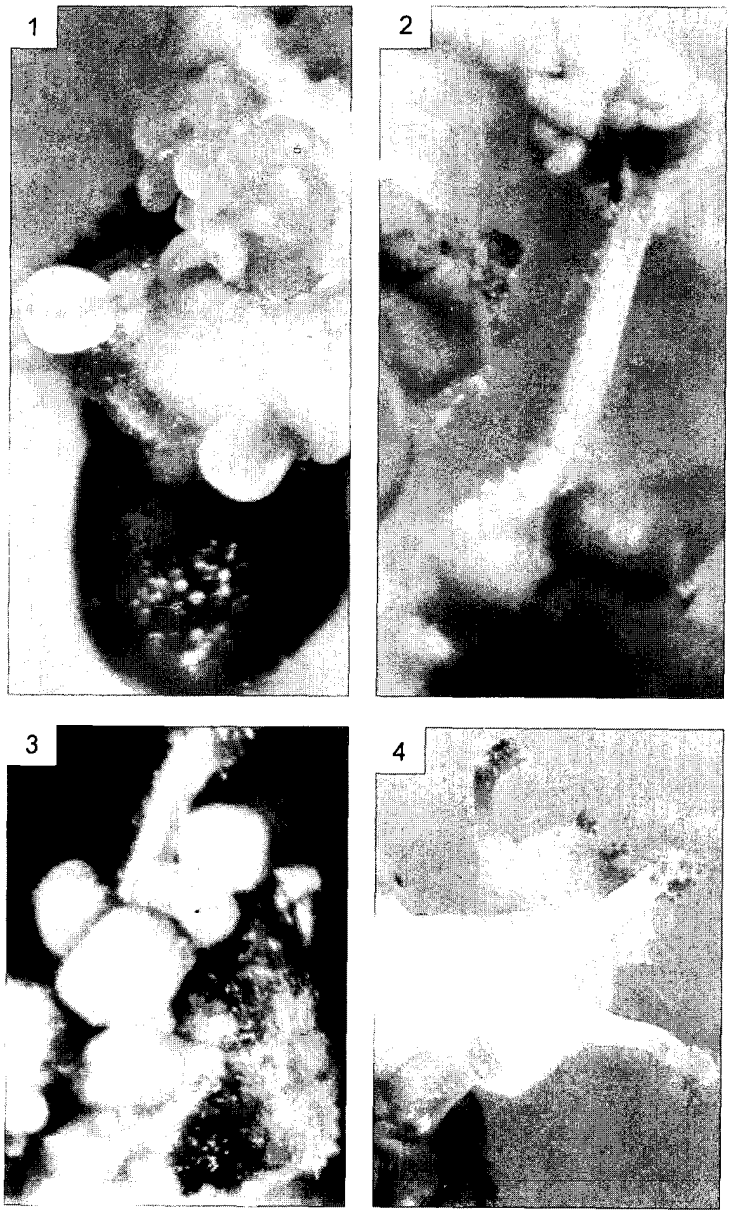


Fig. 1. CLS developed on an immature cotyledon of chickpea cv. C235 cultured on MS + 3 mg $dm^{-3}$ zeatin+ 0.04 mg $dm^{-3}$ IAA.
Fig. 2. CLS borne on a stalk.
Fig. 3. Shoot regeneration from the base of a CLS.
Fig. 4. Direct shoot regeneration from immature cotyledons of cv. C235 cultured on MS + 0.5 mg $dm^{-3}$ TDZ.

and kinetin were ineffective (Shri and Davis 1992), while zeatin and TDZ were effective in inducing CLS.

When the proximal ends of the immature cotyledons were trimmed, there was no CLS formation in response to 3 mg $dm^{-3}$ zeatin + 0.04 mg $dm^{-3}$ IAA or 0.5 mg $dm^{-3}$ TDZ. This indicates that regeneration competent cells in immature cotyledons were confined to their proximal ends; this seems to be the case in several grain legumes (Tetu *et al.* 1990, Genga and Allavena 1991, Ozcan *et al.* 1992, Shri and Davis 1992, Surya Prakash *et al.* 1992, Murthy *et al.* 1996).

CLS regeneration seems to occur even in those genotypes, which are known to be nonembryogenic. For example, in a separate study, ICC4971 was found to be nonembryogenic (Srivastava 1998) but it did show CLS regeneration. CLS appear to be modification of leaflets induced by strong cytokinin activity. This is supported by our observation that in some cases half of a leaflet was modified like CLS, while the other half appeared like a typical leaflet (Fig. 5).

Transferring the entire cotyledon explant bearing CLS onto media containing combinations of IBA, $GA_3$, and BAP could successfully elongate the shoot buds induced on zeatin containing media. A combination of 0.1 mg $dm^{-3}$ each of BAP and $GA_3$ and 0.2 mg $dm^{-3}$ IBA supported shoot elongation in upto 70 % of the explants (Table 4). But the shoots induced in the presence of TDZ were elongated either on 0.1 mg $dm^{-3}$ each of BAP, IBA and

Table 2. Shoot regeneration *via* CLS from immature cotyledons of different genotypes of chickpea cultured on MS medium supplemented with different concentrations of zeatin and IAA. Observations were recorded 4 weeks after culture initiation. Means having different letters as their superscripts are significantly different ($P < 0.05$); each mean is estimated from 3 replicates of 15 explants each.

| Genotype | Zeatin [mg $dm^{-3}$] | IAA [mg $dm^{-3}$] | Number of cotyledons forming CLS | Number of cotyledons forming shoots | Number of shoots per responding explant |
|---|---|---|---|---|---|
| C235 | 3.0 | 0.0 | 23 | 2 | $1.0^{a}$ |
| | 3.0 | 0.04 | 26 | 1 | $4.0^{b}$ |
| | 5.0 | 0.0 | 21 | 2 | $1.5^{ab}$ |
| | 5.0 | 0.04 | 18 | 0 | $0.0^{a}$ |
| ICC11531 | 3.0 | 0.0 | 22 | 4 | $3.75^{b}$ |
| | 3.0 | 0.04 | 26 | 4 | $1.25^{a}$ |
| | 5.0 | 0.0 | 20 | 2 | $1.50^{ab}$ |
| | 5.0 | 0.04 | 21 | 3 | $1.33^{ab}$ |
| ICC12873 | 3.0 | 0.0 | $6^{a}$ | 3 | $1.33^{b}$ |
| | 3.0 | 0.04 | $8^{a}$ | 0 | $0.0^{a}$ |
| | 5.0 | 0.0 | $19^{b}$ | 4 | $1.25^{b}$ |
| | 5.0 | 0.04 | $10^{ab}$ | 2 | $1.0^{ab}$ |
| ICC4971 | 3.0 | 0.0 | $4^{a}$ | 1 | 1.0 |
| | 3.0 | 0.04 | $16^{b}$ | 1 | 1.0 |
| | 5.0 | 0.0 | $11^{ab}$ | 0 | 0.0 |
| | 5.0 | 0.04 | $13^{ab}$ | 0 | 0.0 |
| ICC12257 | 3.0 | 0.0 | $4^{a}$ | 0 | $0.0^{a}$ |
| | 3.0 | 0.04 | $7^{a}$ | 0 | $0.0^{a}$ |
| | 5.0 | 0.0 | $20^{b}$ | 5 | $2.0^{b}$ |
| | 5.0 | 0.04 | $4^{a}$ | 1 | $1.0^{ab}$ |

Table 3. Shoot regeneration *via* CLS from immature cotyledons of chickpea cv. C235 cultured on MS medium supplemented with 0.2, 0.5 or 1.0 mg $dm^{-3}$ TDZ. Observations were recorded 4 weeks after culture initiation (* direct shoot regeneration).

| TDZ [mg $dm^{-3}$] | Number of cotyledons cultured | forming CLS | forming shoots from CLS | forming shoots directly | Number of shoots per explant |
|---|---|---|---|---|---|
| 0.2 | 60 | 21 | 8 | 0 | 2.6 |
| 0.5 | 60 | 16 | 9 | 0 | 2.4 |
| 1.0 | 60 | 19 | 14 | 5 | 3.57 (3.0)* |

Table 4. Shoot development from CLS of different genotypes transferred to MS medium having different combinations of BAP, $GA_3$ and IBA.

| Growth regulator [mg $dm^{-3}$] | | | Number of cotyledons with CLS | | Number of shoots per explant |
|---|---|---|---|---|---|
| BAP | $GA_3$ | IBA | cultured | showing shoot regeneration | |
| 0.1 | 0.05 | 0.10 | 10 | 1 | 2.0 |
| 0.1 | 0.10 | 0.10 | 10 | 2 | 1.0 |
| 0.1 | 0.10 | 0.20 | 10 | 7 | 1.0 |
| 0.2 | 0.05 | 0.05 | 10 | 3 | 2.7 |
| 0.2 | 0.10 | 0.10 | 10 | 5 | 1.6 |

Fig. 5. A leaflet on a shoot regenerated in presence of 3 mg $dm^{-3}$ zeatin + 0.04 mg $dm^{-3}$ IAA; part of it is CLS - like in appearance (*arrow*).

$GA_3$ or on 0.2 mg $dm^{-3}$ BAP with 0.1 mg $dm^{-3}$ each of GA3 and IBA. As the shoots were stunted, these were further elongated on GR - free MS, MS + 0.1 mg $dm^{-3}$ $GA_3$ + 0.1 mg $dm^{-3}$ IBA, of which GR-free MS appeared optimal as it supported elongation of 20 % of the cultured shoots as compared to only 10 % in the case of other two media.

In a separate study carried out to develop an optimal medium for rooting, it was found that IAA or IBA (0.1 - 1 mg $dm^{-3}$) included in the medium did not support normal rooting, whereas a pulse treatment of the shoots with 200 mg $dm^{-3}$ IBA for 5 min supported healthy rooting (Srivastava 1998). Therefore, a total of 10 elongated shoots were excised, given a pulse treatment of 200 mg $dm^{-3}$ IBA and cultured on GR-free MS medium with only half-strength salts and 15 g $dm^{-3}$ sucrose and 6 g $dm^{-3}$ agar. A total of 6 rooted plantlets so obtained were transferred to plastic cups containing *Soilrite* (*Kel Perlite*, India) and covered with clear plastic bags. However, the plantlets were lost within 14 d mainly because of fungal growth on *Soilrite*. In other reports of regeneration in chickpea, the transfer of plantlets to soil is reported without indicating the frequency of survival. In a detailed study, Polisetty *et al.* (1997) reported the survival frequency to be less than 10 %. Thus, there is an urgent need to refine the hardening protocol for regenerated chickpea plantlets so that the full potential of biotechnological methods can be realized for chickpea improvement.

## References


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

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

Genga, A., Allavena, A.: Factors affecting morphogenesis from immature cotyledons of *Phaseolus coccineus* L. - Plant Cell Tissue Organ Cult. **27**: 189-196, 1991.

Gomez, K.A., Gomez, A.A.: Statistical Procedures for Agricultural Research. - John Wiley & Sons, New York 1984.

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

Kumar, V.D., Kirti, P.B., Sachan, J.K.S. Chopra, V.L.: Picloram induced somatic embryogenesis in chickpea (*Cicer arietinum* L.). - Plant Sci. **109**: 207-213, 1995.

Malik, K.A., Saxena, P.K.: Thidiazuron induces high-frequency regeneration in intact seedlings of pea (*Pisum sativum*), chickpea (*Cicer arietinum*) and lentil (*Lens culinaris*). - Aust. J. Plant Physiol. **19**: 731-740, 1992.

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

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

Ozcan, S., Barghchi, M., Firek, S., Draper, J.: High frequency adventitious shoot regeneration from immature cotyledons of pea (*Pisum sativum* L.). - Plant Cell Rep. **11**: 44-47, 1992.

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

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

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

Srivastava, K.: Studies on Somatic Embryogenesis in and Selection for *Ascochyta* Culture Filtrate Resistant Lines of Chickpea. - Ph.D. Thesis, Banaras Hindu University, Varanasi 1998.

Steel, R.G.D., Torrie, J.H.: Principles and Procedures of Statistics. - McGraw Hill Book Co., New York 1960.

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

Surya Prakash, Chowdhury, J.B., Jain, R.K., Chowdhury, V.K.: Factors affecting plant regeneration in chickpea, *Cicer arietinum*.- Indian J. exp. Biol. **30**: 1149-1153, 1992.

Tetu, T., Sangwan, R.S., Sangwan-Noreel, B.S.: Direct somatic embryogenesis and organogenesis in cultured immature zygotic embryos of *Pisum sativum* L. - J. Plant Physiol. **137**: 107-109, 1990.