Rapid *in vitro* micropropagation of *Cicer arietinum* L.

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ABSTRACT: A rapid, simple and efficient protocol for *in vitro* multiple shoot induction and plantlet regeneration was achieved from three different explants of *Cicer arietinum*. The explants viz shoot tip, cotyledonary node and node were cultured on MS medium fortified with Benzyl Adenine (BA) (0.44–8.88μM) for multiple shoot induction. Multiple shoots proliferation was best observed at 4.44μM BA from all the three explants within two weeks of culture. Of the three different explants tested, cotyledonary nodes produced the maximum number of shoots. Shoot number per explant ranged between 7 and 15. Individual shoots were aseptically excised and subcultured in the same media for shoot elongation. The elongated shoots were transferred to Indole Butyric Acid (IBA) (2.46–12.30μM) for root induction. Rooting was observed within two weeks of culture. Rooted plantlets were successfully hardened under culture conditions and subsequently established in the field conditions. The recorded survival rate of the plants was 76.3%. Plants looked healthy with no visually detectable phenotypic variations.

Keywords: shoot tip; cotyledonary node; node; multiple shoots; rooting; hardening

Pulse crops, also known as grain legumes, belong to the family Fabaceae, the second largest natural order of flowering plants. Generally, legumes are of a great economic importance as a source of food, fodder as well as for the significant role they play in biological fixation of atmospheric nitrogen. India is the largest producer of pulses in the world and more than a dozen pulse crops are grown on an estimated area of 22–23 million hectares. Of the grain legumes produced in the world, chickpeas stand second as for occupied area (10 million ha) and third in production (7 million t).

Chickpea (*Cicer arietinum*) is an important grain legume of the Indian subcontinent, West Asia, Mediterranean region, North and East Africa, Southern Europe and Central America and Australia. Various attributes of chickpea made it the most cultivated pulse crop and the most appreciated protein source among vegetarians all over the world. Chickpea straw has forage value comparable to other straws commonly used for livestock feed. It is able to drive more than 70% of nitrogen from symbiotic dinitrogen fixation, which makes it a promising crop for "alternative agriculture" that is now attracting a considerable attention in the industrialized world. The heavy demand created by the pressure of increasing population in the developing world requires a tremendous scientific effort to meet the requirements of food, fibre, fuel and other necessities of life. Since the conventional techniques employed in crop improvement may not keep pace with the demands of the increasing population (3 person/s) and decreasing land resources, the importance of *in vitro* technologies in crop improvement has great relevance. Recent advances made in the field of tissue culture have brought about new emerging technologies for crop improvement. Micropropagation offers the potential to produce thousands or even millions of plants per annum. Application of tissue culture techniques for genetic upgradation of economically important plants has been reported (Scowcraft, Ryan 1985). Plant tissue culture offers new ways for the improvement of this crop after many years of recalcitrance. Several researchers have reported on the regeneration of *Cicer arietinum* via direct organogenesis (Kartha et al. 1981; Islam et al. 1995; Barna, Wakhlu 1995; Anju, Chawla 2005). Thus the objective of the present study was to induce maximum number of shoots and regenerate whole plants from shoot tips, cotyledonary nodes and node explants of *Cicer arietinum*.

MATERIALS AND METHODS

Explant source

Seeds of *Cicer arietinum* cv. CO-3 were collected from Tamil Nadu Agricultural University, Coimba-

tore, Tamil Nadu, India. *In vitro* raised 8-d old seedlings were used as the source of explants. To raise the seedlings, the seeds were washed thoroughly in tap water 3–5 times and placed in 1% (v/v) Teepol solution (Reckitt Benckiser, India) which was kept under running tap water for 15 min. Then the seeds were disinfected with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 min. Finally the seeds were rinsed 3–4 times in sterile distilled water and inoculated on moist cotton in sterile test tubes. To assure uniform and rapid germination of seeds, test tubes were placed in dark at 28°C for 24–48 h. Then the germinated seeds were transferred to light intensity (15 μmol/s^2/s), 16 h light per day photoperiod for another 4–7 d and maintained at 25 ± 2°C and 55–60% relative humidity.

Selection of explants

Shoot tips with one or two leaf primordia, node and cotyledonary nodal segments of 8-d old *in vitro* raised seedlings were selected as explants for direct shoot multiplication. The shoot tips, cotyledonary node and nodal segments of 5–8 mm in length were excised aseptically.

Culture medium

Single disinfected explants viz. shoot tip, cotyledonary node and node were cultured on MS basal medium (Murashige, Skoog 1962) supplemented with 3% (w/v) sucrose (Himedia, India) for multiple shoot initiation. The pH of the medium was adjusted to 5.7 with 0.1N NaOH or 0.1N HCl before solidifying with 0.7% (w/v) agar (Himedia, India). The solidified medium was sterilized by autoclaving at 121°C for 15 min with 1.06 kg/cm pressure.

Multiple shoot induction

Shoot tips, cotyledonary nodes and nodal segments were excised and inoculated by vertical orientation on the culture medium containing different concentrations of BA (0.44–8.88μM). Hundred single explants were assigned randomly to each treatment and cultures were kept under 16 h light/day photoperiod at 25 ± 2°C. Shoot multiplication was assessed after 2 weeks of culture by counting the proliferated shoots. Individual shoots were excised and subcultured in the same media composition for further elongation.

Rooting and hardening

The elongated shoots were transferred to MS medium augmented with IBA (2.46–12.30μM) for root induction. Rooting was observed within two weeks of culture. Well-rooted plantlets were isolated and washed in running tap water. Later they were transplanted into plastic cups containing sterile sand and soil mixture (3:1) for hardening purposes. The well-grown plants were transferred to larger pots containing soil mixture and maintained in the field conditions. Plants grown in the field were further observed for growth and survival (Kulothungan 1997).

Statistical analysis

Hundred single explants were assigned randomly to each treatment and the data recorded were subjected to statistical analysis according to the New Duncan's Multiple Range Test (Gomez, Gomez 1976).

RESULTS AND DISCUSSION

Explant initiation and establishment of aseptic culture

The important part of the present study was the preparation of contamination free explants. This was achieved by using *in vitro* germinated seedlings as an explant source. Sterilization of seeds required

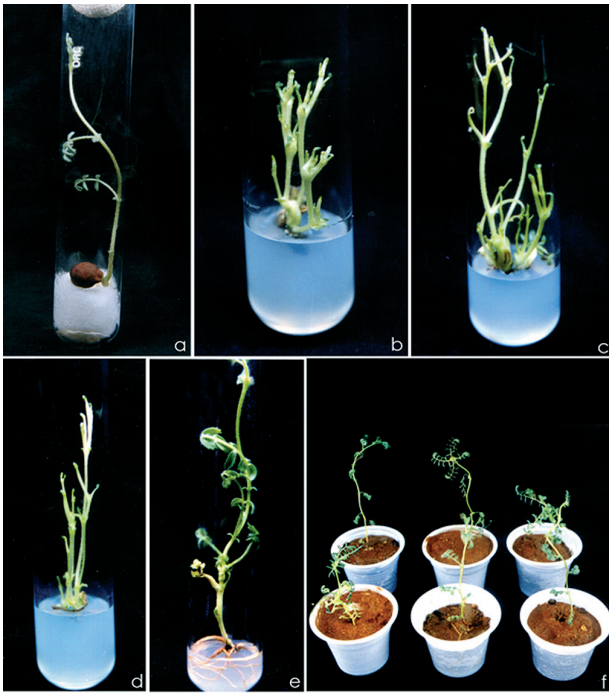

Fig 1. Rapid *in vitro* micropropagation of *Cicer arietinum* L. (a) *In vitro* raised seedlings (8-d old), (b) multiple shoot induction from shoot tip, (c) multiple shoot induction from cotyledonary node, (d) multiple shoot induction from node, (e) rooting of individual shoots, (f) hardening of plantlets

Table 1. Effect of different concentrations of BA on multiple shoot induction from shoot tip, cotyledonary node and node explants of *Cicer arietinum* L.

Explant type	BA concentration (μM)	Percentage of response (%) (mean ± SE)	Number of shoots per explant (mean ± SE)	Shoot length (cm) (mean ± SE)
Shoot tip	0.44	46.5 ± 0.42^{e}	6.6 ± 0.30^{d}	2.15 ± 0.16^{cd}
	2.22	63.5 ± 0.38^{c}	8.0 ± 0.25^{c}	3.7 ± 0.21^{b}
	4.44	**87.4 ± 0.45^{a}**	**9.6 ± 0.30^{a}**	**6.6 ± 0.16^{a}**
	6.66	80.4 ± 0.40^{b}	8.2 ± 0.20^{b}	2.7 ± 0.21^{c}
	8.88	53.2 ± 0.55^{d}	6.3 ± 0.15^{de}	1.7 ± 0.21^{d}
Cotyledonary node	0.44	52.3 ± 0.21^{de}	8.5 ± 0.22^{d}	6.4 ± 0.16^{d}
	2.22	76.1 ± 0.23^{b}	12.0 ± 0.21^{b}	8.2 ± 0.20^{b}
	4.44	**98.3 ± 0.19^{a}**	**15.1 ± 0.23^{a}**	**14.2 ± 0.32^{a}**
	6.66	70.9 ± 0.23^{c}	10.8 ± 0.24^{c}	7.5 ± 0.34^{c}
	8.88	55.7 ± 0.15^{d}	7.3 ± 0.21^{e}	5.7 ± 0.21^{e}
Node	0.44	45.1 ± 0.31^{d}	4.8 ± 0.24^{e}	2.6 ± 0.16^{cd}
	2.22	51.0 ± 0.25^{c}	5.6 ± 0.16^{c}	3.6 ± 0.16^{b}
	4.44	**72.0 ± 0.63^{a}**	**7.8 ± 0.13^{a}**	**4.7 ± 0.15^{a}**
	6.66	65.0 ± 0.25^{b}	6.4 ± 0.22^{b}	2.7 ± 0.15^{c}
	8.88	63.6 ± 0.26^{bc}	5.3 ± 0.15^{cd}	1.9 ± 0.17^{d}

Total number of explants taken for observation = 100
Values represent mean ± SE of ten replicates per treatment in three repeated experiments
Treatment means followed by different letters in their superscript are significantly differ from one another ($P < 0.05$) according to the Duncan's Multiple Range Test (DMRT)

0.1% (w/v) $HgCl_2$, 5 min treatment for maximum germination (98%) (Fig. 1a) and minimum contamination (Narashimhulu, Reddy 1983). A similar observation was also reported in *Vigna aconitifolia*, confirming the view that the pretreatment of seeds with specific surface sterilizing agents would predetermine the regenerating behavior of explant tissues (Godbole et al. 1984). The use of direct and large-sized explants had higher survival and growth rates than the smaller ones (Hu, Wang 1983).

Effect of BA on shoot regeneration

The meristem containing explants viz., shoot tip, cotyledonary node and node were excised from the surface sterilized, *in vitro* grown, 8-d old seedlings and cultured on MS medium augmented with BA (0.44–8.88μM) for multiple shoot induction. Of all the different concentrations of BA tested, 4.44μM BA was found to be more effective in inducing multiple shoots in the case of all three explants (Fig. 1b,c,d). It was observed that cotyledonary node explants produced the maximum number of shoots compared to shoot tip and nodal explants. The same medium supplemented with 4.44μM BA was used for further elongation of shoots. The maximum response notified in cotyledonary node explants was evaluated as the mean number of shoots of 15.1 and the shoot length of 14.2 cm. Whereas the mean number of shoots of 9.6 and the shoot length of 6.6 cm were observed in shoot tips, in nodal explants the mean number of shoots of 7.8 and the shoot length of 4.7 cm were encountered (Table 1).

In recent years, shoot tip and nodal explants have been preferred to produce large number of genetically identical clones (Bajaj, Dhanju 1979). Multiple shoot formation from shoot apices was obtained on MS medium supplemented with 20μM BA, 0.1μM NAA in pea (Griga et al. 1986). MS- solid medium fortified with BA and KIN alone and in combination increased the regeneration potential of shoot apical meristems of soybean, cowpea, peanut, chickpea and bean (Kartha et al. 1981). It was reported that BA was proved to be an ideal hormone for shoot multiplication of shoot tip culture in grain legumes (Sounder Raj et al. 1989). Nodal explants were also used to get higher rates of shoot multiplication of several plants (Shekawat, Galston 1983). In the present investigation nodal explants cultured on MS medium supplemented with 4.44μM BA showed maximum number of shoots; similar result were reported (Rao, Chopra 1989). Plant regeneration from cotyledonary node explant was observed in mungbean (Gulati, Jaiwal 1992) and peanut (Cheng et al. 1992). Similar results were also observed in cotyledonary node on MS medium supplemented with BA (Altaf, Ahmed 1986). In contradiction, high frequency of regeneration on

Table 2. Effect of different concentrations of IBA on rooting of microshoots of *Cicer arietinum* L.

IBA (μM)	Percentage of response (mean ± SE)	Number of roots (mean ± SE)	Root length (cm) (mean ± SE)
2.46	62.9 ± 0.43^{cd}	6.8 ± 0.20^{cd}	5.2 ± 0.24^{d}
4.92	75.2 ± 0.32^{b}	7.9 ± 0.23^{b}	6.2 ± 0.25^{c}
7.38	**95.5 ± 0.26^{a}**	**9.9 ± 0.23^{a}**	**10.4 ± 0.34^{a}**
9.84	66.8 ± 0.82^{c}	6.9 ± 0.23^{c}	7.2 ± 0.24^{b}
12.30	51.5 ± 0.63^{e}	5.5 ± 0.22^{e}	4.1 ± 0.23^{e}

For explanation see Table 1

MS medium with NAA and IBA was also achieved (Chandra et al. 1993). Thus cotyledonary nodes were reported as potential explants for the regeneration of shoots in grain legumes.

Rooting and hardening of regenerated plants

Rooting of shoots is the most critical step in the production of complete plants and their subsequent survival. The individual elongated shoots were isolated and transferred to MS medium fortified with IBA (2.46–12.30μM). IBA (7.38μM) was found to be more effective in the production of long, slender and healthy roots (Fig. 1e). For rooting of *in vitro* raised shoots, 0.1% IBA was used in soybean (Buising et al. 1994) and IAA in common bean (Kartha et al. 1981). It was reported that half-strength MS medium induced maximum rooting in cowpea (Kulothungan 1997), but in the present investigation root induction was maximum (95%) only in full-strength MS medium supplemented with 7.38μM IBA (Table 2). Rooted plantlets were initially hardened under culture conditions (Fig. 1f) and subsequently established in the field conditions. The survival rate of the plants in field conditions was recorded as 76.3% (data not shown).

CONCLUSION

From the above study, it was concluded that shoot tip, cotyledonary node and node explants are suitable for clonal propagation of chickpea. Cotyledonary node explants may be used for their higher rate of shoot multiplication. The protocol described in the present study is reproducible and can be used in future for further developments of the crop.

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References

ALTAF N., AHMED M.S., 1986. Proceedings of Symposium on Nuclear Techniques and *in vitro* Culture for Crop Improvement. FAO and IAEA, Vienna: 405–407.

ANJU A., CHAWLA H.S., 2005. Organogenic plant regeneration via callus induction in chickpea (*Cicer arietinum* L.). Role of genotypes, growth regulators and explants. Indian Journal of Biotechnology, *4*: 251–256.

BAJAJ Y.P.S., DHANJU M.S., 1979. Regeneration of plants from apical meristem tips of some legumes. Current Science, *48*: 906–907.

BARNA K.S., WAKHLU A.K., 1995. Modified single node culture method – A new micropropagation method for chickpea. *In vitro* Cell Developmental Biology – Plant, *31*: 150.

BUISING C.M., SHOEMAKER R.C., BENBOW R.M., 1994. Early events of multiple bud formation and shoot development in soybean embryonic axes treated with the cytokinin 6 – BAP. American Journal of Botany, *81*: 1435–1448.

CHANDRA R., CHATRATH A., POLISETTY R., KHETARPAL S., 1993. Differentiation of *in vitro* grown explants of chickpea (*Cicer arietinum* L.). Indian Journal of Plant Physiology, *36*: 121–124.

CHENG M., HIS D.C.H., PHILLIPS G.C., 1992. *In vitro* regeneration of Valencia type peanut (*Arachis hypogaea* L.) from cultured petioles, epicotyl sections and other seedling explants. Peanut Science, *19*: 82–87.

GODBOLE D.A., KUNACHI M.N., POTDER U.A., KRISHNAMURTHY K.V., MASCARENHAS M.F., 1984. Studies on a drought resistant legume: The moth bean, *Vigna aconitifolia* (Jacq) Marechal. II. Morphogenetic studies. Plant Cell Reports, *3*: 75–78.

GOMEZ K.A., GOMEZ K.A., 1976. Statistical procedures for agricultural research with emphasis on rice. Los Bans, Philippines International Rice Research Institute.

GRIGA M., TEJKLOVA E., NOVAK F.J., KUBALAKOVA M., 1986. *In vitro* clonal propagation of *Pisum sativum* L. Plant Cell, Tissue and Organ Culture, *6*: 96–104.

GULATI A., JAIWAL P.K., 1992. *In vitro* induction of multiple shoots and plant regeneration from cotyledons of *Vigna radiata* (L.) Wilczek. Plant Cell, Tissue and Organ Culture, *23*: 1–7.

HU Y.W., WANG P.J., 1983. Meristem, shoot tip and bud culture. In: EVANS D.A., SHARP W.R., AMMIRATO P.V., YAMADA E. (eds.), Handbook of Plant Cell Culture. Vol. I. New York, Macmillan: 177–227.

ISLAM R., RIAZUDDIN S., FAROOQUI H., 1995. Clonal propagation from seedling nodes and shoot apices of chickpea (*Cicer arietinum* L.). Plant Tissue Culture, *5*: 53–57.

KARTHA K.K., PAHL K., LEUNG N.L., MROGINSKI L.A., 1981. Plant regeneration from meristems of grain legumes – soybean, cowpea, peanut, chickpea and bean. Canadian Journal of Botany, *59*: 1671–1679.

KULOTHUNGAN S., 1997. *In vitro* culture studies on cowpea (*Vigna unguiculata* (L.) Walp.). [Ph.D. Thesis.] Tiruchirappalli, South India, Bharathidasan University.

MURASHIGE T., SKOOG F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, *15*: 473–497.

NARASHIMHULU S.B., REDDY G.M., 1983. Plantlet regeneration from different callus cultures of *Arachis hypogaea* L. Plant Science Letters, *31*: 147–153.

RAO B.G., CHOPRA V.L., 1989. Regeneration in chickpea (*Cicer arietinum*) through somatic embryogenesis. Journal of Plant Physiology, *134*: 637–638.

SCOWCRAFT W.R., RYAN S.A., 1985. Tissue culture and plant breeding. [Manuscript prepared: YEOMAN M. (ed.), Plant Culture Technology.] Oxford, Blackwell Scientific.

SHEKAWAT N.S., GALSTON A.W., 1983. Isolation, culture and regeneration of mothbean, *Vigna aconitifolia* leaf protoplasts. Plant Science Letters, *32*: 43–51.

SOUNDER RAJ V., TELAVATHI D.H., NIJALINGAPPA B.H.M., 1989. Shoot tip culture in *Dolichos biflorus* L. Current Science, *58*: 1385–1388.

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Rychlé klonování rostlin *in vitro* u *Cicer arietinum* L.

ABSTRAKT: Pro tři různé typy explantátů *Cicer arietinum* byl vypracován rychlý, jednoduchý a efektivní protokol pro indukci výhonů a regeneraci rostlin *in vitro*. Pro indukci mnohonásobných výhonů byly kultivovány apikální meristémy stonků, děložní a stonkové nody na médiu MS doplněném benzyl adeninem v koncentraci 0,44μM až 8,88μM. Tvorba mnohonásobných výhonů byla pozorována při koncentraci 4,44μM benzyl adeninu u všech tří typů explantátů během dvou týdnů kultivace. Děložní nody produkovaly nejvyšší počet výhonů ze všech tří testovaných typů explantátů. Jeden explantát produkoval sedm až patnáct výhonů. Jednotlivé výhony byly asepticky odděleny a jejich dalšího růstu bylo dosaženo na médiích stejného složení. Pro zakořeňování, k němuž docházelo v průběhu dvou týdnů, byly vyvinuté výhony přeneseny na médium s kyselinou indolylmáselnou v koncentraci 2,46μM až 12,30μM. Zakořeněné rostliny byly úspěšně otužovány a postupně přesazovány do polních podmínek. Přežívání rostlin dosahovalo 76,3 %. U rostlin nebyly pozorovány žádné viditelné fenotypové odchylky.

Klíčová slova: apikální meristém; děložní nodus; nodus; mnohonásobné výhony; zakořeňování; otužování

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