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Rapid *in vitro* Micro Propagation of Chick pea (*Cicer arietinum* L.) From Shoot tip and Cotyledonary node explants

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Abstract

A rapid, simple and efficient protocol for *in vitro* multiple shoot induction and plantlet regeneration was achieved from two different explants of *Cicer arietinum cv* (ICCCR) (kranthi). The explants viz shoot tip and cotyledonary node were cultured on MS medium fortified with Benzyl Adeno Purine (BAP) (0.5-3.0 mg/L) and Kinetin (Kn) (0.5-3.0 mg/L) for multiple shoot induction. Multiple shoots proliferation was best observed at 2.0 mg/L BAP from all the two explants within three weeks of culture. Of the two different explants tested, BAP was found to be more effective than Kinetin for shoot multiplication. The highest number of shoots (12.0 ± 0.3) was achieved on MS medium augmented with 2.0 mg/L BAP. The medium supplemented with (2.0 mg/L) BAP better than all other media concentrations in cotyledonary node explants. Individual shoots were aseptically excised and sub cultured in the same media for shoot elongation. The elongated shoots were transferred to Indole Butyric Acid (IBA) (0.5-1.0 mg/L) and Indole Acetic Acid (IAA) (0.5-1.0 mg/L) for root induction. Rooting was observed within two weeks of culture. MS medium supplemented with (1.0 mg/L) IBA proved better with seventy percent rooting after 25 days of implantation. Most of the roots were long and healthy. 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; 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* L.) 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, fiber, 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 up gradation of economically important plants has been reported [1]. 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 [2-5]. Thus the objective of the present study was to induce maximum number of shoots and regenerate whole plants from shoot tips and cotyledonary node explants of *Cicer arietinum* L (ICCCR) (kranthi).

Shoot Tip Culture

The term shoot culture is now preferred for cultures started from explants bearing an intact shoot meristem, whose purpose is shoot multiplication by the repeated formation of axillary branches. In this technique, newly formed shoots or shoot bases serve as explants for repeated proliferation; severed shoots, explant size, shoot cultures are conventionally started from the apices of lateral or main shoots, up to 20 mm in length, dissected from actively-growing shoots or dormant buds. Larger explants are also sometimes used with advantage: they may consist of a larger part of the shoot apex or be stemming segments bearing one or more lateral buds; sometimes shoots from other *in vitro* cultures are employed. When apical or lateral buds were used almost exclusively as explants, the name 'shoot tip culture' came to be widely used for cultures of this kind. As the use of larger explants has become more common, the term shoot culture has become more appropriate.

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Large explants have advantages over smaller ones for initiating shoot cultures in them.

Shoot regeneration from shoot apex/shoot tip is direct, relatively simple and is not prone to somaclonal variation and chromosomal abnormalities. This is an elegant methodology of multiplying plants *in vitro* sterling from a single shoot with obvious potential when applied to crop plants [6].

Culture of shoot meristem, especially through enhanced branching, permits rapid clonal propagation land a high degree of genetic uniformity of the progeny [7]. This mericlone technology has been wide spread practical application in producing virus free plants *in vitro* in recent year [8-16]. Although mainly used for virus elimination meristem tip culture has also enabled plants to be freed from other pathogens including viroids, mycoplasms, bacteria and fungi, also meristem freeze preservation as a method of conservation of germplasm has made possible to utilize when needed [17]. Successful generation of entire plants from frozen meristems of *Arachis hypogaes* and *Cicer arietinum* has been reported [18].

The latest technology for delivery of genesiute plant tissues is the biolistic gun which requires regenerable tissues, so target tissues may be callus, suspension cells, leaves, meristem tips or any other regenerable explants.

Methodology

The seeds of *Cicer arietinum* L (ICCC-34) (kranthi) cultivar were obtained from ICRISAT Hyderabad A.P. 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/s2/s), 16 h light per day photoperiod for another 4-7 days and maintained at 25 ± 2°C and 55-60% relative humidity.

Selection of Explants

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

Culture Media and Culture Conditions

MS media containing 3.0% sucrose and supplemented with various concentrations cytokinins such as BAP (0.5 - 3.0 mg/L) and Kn (0.5 - 3.0 mg/L) were used. The initial pH of the culture media was adjusted to 5.8 before addition of 0.8% (w/v) agar- agar. The medium was dispensed into culture tubes (25 + 150 mm) each containing 15 ml of the culture medium capable with non-absorbent cotton and was autoclaved at 121°C for 15 minutes. In each cultures tube one shoot tip explants was implanted. The cultures were maintained under 16h light provided with white fluorescent tubes (40 µmol m-2s-2) at 25 ± 2°C.

Results

Data on multiple shoot induction from shoot tip explants cultured on MS medium fortified with different concentrations of BAP and Kn alone is presented in table 1. 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 0.1% (w/v) $HgCl_2$ 5 min treatment for maximum germination (98%) and minimum contamination [19]. 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 [20]. The use of direct and large sized explants had higher survival and growth rates than the smaller [21].

Effect of BAP

The meristem containing explants shoot tip were excised from the surface sterilized, *in vitro* grown, 8-d old seedlings and cultured on MS medium augmented with BAP (0.5-3.0 mg/L) for multiple shoot induction of all the different concentrations of BAP tested, (2.0 mg/L) BAP was found to be more effective in inducing (6.0 ± 0.3 shoots / explants) (Figure 1a). But at high concentration of BAP (3.0 mg/L) considerably the number of shoot induction was found to be reduced. As the concentration of Kn was increased up to 2.0 mg/L the multiple shoots number was increased but as the concentration of BAP (2.0 mg/L) to (3.0 mg/L) BAP resulted the number of shoots were reduced.

Effect of Kn

Shoot tip explants were capable of directly developing multiple shoots on MS basal medium containing different concentrations of Kn (0.5 - 3.0 mg/L). Multiple shoot initiation from shoot tip explants was observed within 20 - 25 days after inoculation. Highest number of shoots (5.8 ± 0.3) was observed in the medium concentration of Kn was increased up to 2.0 mg/L the multiple number of shoots was also increased (Table 1) (Figure 1b).

Discussion

The results of present investigation show that the shoot tip explants from mature plants of *Cicer arietinum* L. (ICCC-34) (kranthi) could be induced to produce multiple shoots *in vitro*. Maximum number of shoots was induced on MS medium fortified with various concentrations of BAP and Kn. In recent years, shoot tip explants have been preferred to produce large number of genetically identical clones. Multiple shoot formation from shoot apices was obtained on MS medium supplemented with 20 µM BA, 0.1 µM NAA in pea [22]. MS-solid medium fortified with BAP and Kn alone and in combination increased the regeneration potential of shoot apical meristems of

Growth regulators (mg/L)	% of explants showing response	No. of shoots (cm) SE*	Average length of shoots SE*
BAP			
0.5	85	3.0 ± 0.4	2.4 ± 0.2
1.0	90	4.0 ± 0.3	5.4 ± 0.3
1.5	95	5.0 ± 0.6	5.2 ± 0.4
2.0	100	6.0 ± 0.3	8.7 ± 0.5
2.5	80	3.5 ± 0.3	6.9 ± 0.4
3.0	70	2.0 ± 0.3	5.3 ± 0.4
Kn			
0.5	70	2.0 ± 0.6	2.9 ± 0.4
1.0	80	3.2 ± 0.7	4.4 ± 0.3
1.5	90	4.0 ± 0.2	5.5 ± 0.4
2.0	95	5.8 ± 0.4	8.3 ± 0.6
2.5	75	4.9 ± 0.5	6.0 ± 0.3
3.0	70	4.0 ± 0.3	4.0 ± 0.4

* Mean ± Standard Error

Table 1: Effect of different concentration of BAP, Kn on multiple shoot induction from shoot tip explants of *Cicer arietinum* (ICCC-34) (kranthi).

soybean, cowpea, peanut, chickpea and bean. It was reported that BAP was proved to be an ideal hormone for shoot multiplication of shoot tip culture in grain legumes [23]. These results are also in agreement with those on *Tectona grandis* [24]. Abizzia lebbeck [25] multiple shoot induction was also observed in *Ziziphus manritiana* [12] and Vanilla plantifolia [26] shoot tips cultured on MS + cytokinin alone as it was observed in the present studies. Nasir et al. (1997) have studied the shoot meristem culture in 16 cultures of cotton using several media formation. They observed the best shoot developmentation MS media containing Kn alone compared to other media with NAA / IAA in combination with Kn. These results are to the present observation in *Cicer arietinum* L (ICCC) (kranthi) which contain with cytokinins showed the increased number of shoots/ explant have also observed the similar results when they have cultured the shoot tips of F1 hybrids of *Paulownia*.

The capacity of shoot bud differentiation and shoot proliferation from shoot tip explants of *Cicer arietinum* (ICCC) (kranthi) depended on hormonal variation. There was good shoot bud induction and proliferation response only in the presence of cytokinin and no response in the basal medium. Similar results are well documented in several medicinal plants [27], *Emblila officinale* [28] and *Withania somnifera* [29]. From our study it was clear that 2.0 mg/L BAP and Kn were significantly more effective for inducing shoot organogenesis. Well-developed shoot lets from our experimental data, it is evident that BAP and Kn are the best suited for inducing multiple shoots In conclusion, this communication describes an efficient rapid propagation system of *Cicer arietinum* L. (ICCC-34) (kranthi).

Cotyledonary Node Culture

The cotyledonary node induction is one of the most efficient method of micropropagation in plants since the emerging buds, especially from meristematic organs and tissues posses a great potential for vigorous development [30]. Axillary buds have been found to be most suitable for clonal propagation in several species *Morus niger* [30] *Compmiphora wightii* [31] *Ocimum sanctum* [32]. The present investigation describes a micropropagation technique using nodal culture as the source of direct production of multiple shoots in *Cicer arietinum* L (ICCC-34) (kranthi).

Methodology

The seeds of *Cicer arietinum* L (ICCC-34) (kranthi) cultivar were obtained from ICRISAT Hyderabad A.P. 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/s2/s), 16 h light per day photoperiod for another 4-7 days and maintained at 25 ± 2˚C and 55-60% relative humidity.

Selection of Explants

Cotyledonary node segments of 8-d old *in vitro* raised seedlings were selected as explants for direct shoot multiplication. The Cotyledonary node segments of 5-8 mm in length were excised aseptically.

Culture media and culture conditions

MS media containing 3.0% sucrose and supplemented with various concentrations cytokinins such as BAP (0.5 - 3.0 mg/L) and Kn (0.5 - 3.0 mg/L) were used. The initial pH of the culture media was adjusted to 5.8 before addition of 0.8% (w/v) agar- agar. The medium was dispensed into culture tubes (25 + 150 mm) each containing 15 ml of the culture medium capable with non-absorbent cotton and was autoclaved at 121^{0}C for 15 minutes. In each cultures tube one shoot tip explants was implanted. The cultures were maintained under 16 h light provided with white fluorescent tubes (40 µmol $m^{-2}s^{-2}$) at 25 ± 2^{0}C.

Results

The results of the Cotyledonary node explant cultures on the development of multiple shoots are shown in table 2. The Cotyledonary node explants of *Cicer arietinum* L. (ICCC-34) (kranthi) cultured on different hormonal combination showed varied results. The axillary buds become active within week after inoculation and new shoots become distinct by the second and third week with leaves and internodes.

The size of the Cotyledonary node explants was found to play an important role in initiation and elongation of shoots. The smaller (1 cm) explants could initiate more multiples than the longer (2.0 cm) Cotyledonary node explants. Although 1.5 cm long segments produced less number of shoot buds. They showed better elongation. Whereas the biggest Cotyledonary node segment (2.0 cm) tried to give multiples only a single shoot developed from each node.

Effect of BAP

The results on Cotyledonary node culture of *Cicer arietinum* L (ICCC-34) (kranthi) on MS medium + BAP (0.5-3.0 mg/L) alone are presented in table 2 and shown in figure 1c. The medium containing (1.0 mg/L) BAP induced maximum number of shoots (12.0 ± 0.5 shoots/ explant)) with (2.4 ± 0.29 cm) and also showed high percentage (90%) of responding cultures. As the concentration of BAP was increased up to 1.0 mg/L gradually the shoot bud proliferation was found to be decreased and when BAP concentration was increased above 1.0 mg/L the rate of shoot multiplication and elongation was reduced (Table 2).

Growth regulators (mg/L)	% of explants showing response	No. of shoots (cm) SE*	Average length of shoots SE*
BAP			
0.5	60.0	09.0 ± 0.6	2.1 ± 0.6
1.0	95.0	10.0 ± 0.5	2.4 ± 0.2
1.5	90.0	11.9 ± 0.5	2.3 ± 0.2
2.0	70.0	12.0 ± 0.3	2.0 ± 0.3
2.5	65.0	08.0 ± 0.4	1.5 ± 0.3
3.0	50.0	05.0 ± 0.5	1.0 ± 0.4
Kn			
0.5	50.0	6.2 ± 1.6	1.3 ± 0.3
1.0	65.0	7.0 ± 0.5	1.5 ± 0.3
1.5	75.0	8.0 ± 0.4	2.0 ± 0.3
2.0	70.0	7.9 ± 0.5	1.8 ± 0.2
2.5	60.0	6.5 ± 0.4	1.5 ± 0.3
3.0	55.0	5.0 ± 0.5	1.0 ± 0.5

* Mean ± Standard Error

Table 2: Effect of different concentration of BAP, Kn alone in MS medium for multiple shoot induction from cotyledonary node explants of Cicer arietinum (ICCC-34) (kranthi).

Effect of Kn

The result on Cotyledonary node culture of *Cicer arietinum* L. (ICCC-34) (kranthi) on MS medium + Kn (0.5 - 3.0 mg/L) was observed. High percentage (75) of responding cultures were found at (1.5 mg/L) Kn compared to all other concentrations tested. Whereas more number of shoots were regenerated from Nodal explants at (1.5 mg/L) Kn (8.0 ± 04 shoots/explant) followed by 2.0 mg/L Kn at 2.0, 2.5 and 3.0 mg/L Kn (7.9 ± 0.5), (6.5 ± 0.4) and 5.0 ± 0.5 shoots/explant. With 70.0, 60.0 and 55.0 cultures response was recorded (Table 2) (Figure 1d).

In vitro Rooting

Fully elongated healthy shoots were transferred on to half strength MS root induction medium (RIM) [33] fortified with different concentration of IAA (0.5 - 2.0 mg/L) and IBA (0.5 - 2.0 mg/L) (Figure 1e).

Profuse rhizogenesis was observed on 1.5 mg/L IAA, compared to 0.5 - 2.0 mg/L) IAA/ IBA on MS medium containing (1.0 mg/L) IBA whereas 73% of plants produced roots with 8.3 ± 0.87 roots/explants. (Table 3).

Acclimatization

Rooted plantlets were removed from the culture medium and the roots were washed under running tap water to remove agar. Then the plantlets were transferred to polypots containing pre- soaked vermiculite and maintained inside a growth chamber set at 28^{0}C and 70-80% relative humidity. After three weeks they were transplanted to poly bags containing mixture of soil + s and + manure in 1: 1: 1 ratio and kept under shade house for a period of three weeks. The potted plantlets were irrigated with Hogland's solution every 3 days for a period of 3 weeks (Figure 1f).

Discussion

We were successful in shoots regenerating plants from cotyledonary node cultures on MS medium fortified with different concentrations of cytokininin i.e. BAP and Kn individually maximum number of shoots were induced at 1.0 mg/L BAP in comparison to 1.5 mg/L Kn as a role of growth regulators. However the shoot bud proliferation was found to be more on 1.0 mg/L BAP compared to1.5 mg/L Kn might have triggered the action of BAP in proper way for inducing more number of plant let regeneration among all hormonal combinations and concentrations used Influence of explanting season on culture establishment was also noted in *Tridax procumbense* [34] as we have observed in *S. melongena*. Similarly this was sown in other medicinal herbs including *Ocimum species* [35,27] Nodal explants were also used to get higher rates of shoot multiplication of several plants [36].

Growth Hormones (mg/L)		Percentage of response	Average no of roots (S.E)*
IAA	IBA		
00	00	23	1.0 ± 0.12
0.5	-	60	2.3 ± 0.37
1.0	-	70	3.2 ± 0.38
2.0	-	73	5.6 ± 0.38
-	0.5	54	4.3 ± 0.36
-	1.0	73	8.3 ± 0.87
-	2.0	70	6.3 ± 0.36

* Mean ± Standard Error.

Table 3: Rooting ability of regenerated shoots from leaf explants culture of Cicer arietinum. cv (ICCC-34) cultured on MS medium supplemented with IAA and IBA.

During the present investigations multiple shoots were induced on MS Medium supplemented with various concentrations of cytokinins such as BAP and Kn alone. Similarly Sudharshan et al. [12] have observed the multiple shoot bud induction from Cotyledonary node segments of *Ziziphus mauritiana* on MS medium supplemented with BAP alone. It was also recorded the same results in *Vanilla planifolia* on MS + BAP alone [10]. When BAP and Kn concentration was increased (above 2.0 mg/L) the rate of shoot multiplication and elongation was reduced in the present investigation. Similar results were obtained in *Canavalia nirosa* [37], *Vigna radiata* [38] and *Pisonia alba* [39]. Shoot tip and nodal were found to be the best explants for multiple shoot formation. In accordance with this in the present study also the shoot tip and nodal explants were found to be suitable for multiple shoot regeneration in *Cicer arietinum* L (ICCCR) (kranthi). Although the effect of BAP on *in vitro* regeneration of multiple shoot regeneration in *Ocimum sanctum* has been reported earlier [27,40]. At high concentration of cytokinin lateral bud break was suppressed but callus proliferation improved as observed by Shahzad et al. [41] and also reported by Ahuja et al. [35] Patnaik and Chand [27] and Sahoo and Chand [34].

Direct regeneration of multiple shoots form nodal explants as observed on IBA + BAP / Kn supports the finding of Shahzad et al., [41] on *Ocimum sanctum*, Gulati and Jaiwal [38] on *Vigna radiata* and Varisai mohamed et al. [42] on *Macrotyloma uniforum*, Bais et al. [43] have also observed the maximum number of shoots on MS medium supplemented with auxin + cytokinin combination in nodal culture of *Decalepis hamiltonii* as it was found in *Mentha arvensis* (L.) The same synergistic effect was also recorded in *Plumbago indica* inducing maximum number of (17) of shoots / nodal explants on MS + IAA (0.1 mg/L) + BAP (3.0 mg/L). Gill et al. (1996) have also obtained multiple shoots of *Azadirachta indica* on MS + IBA + BAP. Similarly, it was reported in *Morus indica* [39]. Similarly this stimulatory effect of a single supplement of cytokinin was reported earlier in other medicinal species including *Curcuma* spp and *Zingiber officinale*, *Chlorophytum borinilianum* [27] and *Tridax procumbens* [34].

Figure 1: in vitro Micropropagation of Cicer arietinum L. cv (ICCCR) (kranthi) (a) Formation of multiple shoots on MS+BAP (2.0) mg/L from shoot tip, (b) Proliferation of multiple shoots on MS+Kn (2.0mg/L) from shoot tip, (c) Multiple shoot induction on MS+ BAP (2.0mg/L) from cotyledonary node, (d) Multiple shoot induction from cotyledonary node on MS+ Kn (2.0mg/L) (e) Rooting of individual shoots, (f) Hardening of plantlets

Thus, direct multiple shoot production was observed from nodal segments of the species studied. This type of clonal propagation has advantage, by producing true to type plants from a single individual in a relatively short time (Figure 1). Our result on Micro propagation using meristem / Cotyledonary node culture shows the considerable importance for large - scale propagation of *Cicer arietinum* L (ICCC-34) (kranthi) an import ant pulses plant.

In the present investigation nodal explants cultured on MS medium supplemented with 4.44 µM BA showed maximum number of shoots; similar result were reported [44]. Plant regeneration from cotyledonary node explant was observed in mungbean [38] and peanut [45]. Similar results were also observed in cotyledonary node on MS medium supplemented with BA [46]. In contradiction, high frequency of regeneration on MS medium with NAA and IBA was also achieved [47]. Thus cotyledonary nodes were reported as potential explants for the regeneration of shoots in grain legumes.

Conclusion

From the above study, it was concluded that shoot tip and cotyledonary 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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