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Full Length Research Paper

In vitro regeneration and induction of multiple shooting in *Cicer arietinum* L. using cotyledonary nodal explants

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Chickpea, a temperate crop, is the world's third most important pulse crop and India produces 75% of world's supply. The two most common types of Chickpea are the white-seeded "*Kabuli*" and the "*Desi* "variety. *In vitro* regeneration was achieved using cotyledonary nodal explants using desi variety seeds of cultivar K850 of *Cicer arietinum*. The multiple shoot induction was observed with various concentrations of auxins and cytokinins. The maximum proliferation was found at 0.5 mg/L 6-benzylaminopurine (BAP). The effectiveness was observed at various combinations. Maximum efficiency of multiple shooting was obtained at 0.5 mg/L BAP and 0.05 mg/L 2,4-dichlorophenoxy acetic acid (2,4D). For root induction, well developed shoots were transferred into rooting medium supplemented with indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA); maximum response was found at concentrations 0.10 and 0.50 mg/L, respectively. Successful rooting was recorded on media supplemented with 0.1 mg/L IBA. The well rooted plants were transferred into the soil.

Key words: Explants, multiple shooting, phytohormones and acclimatization.

INTRODUCTION

Chickpea is a temperate crop, which probably originated from south-eastern Turkey and has spread to other parts of the world. Chickpea has significant economic importance as a source of food and fodder; its straw has forage value comparable to that of other straws commonly used for livestock feed (Singh, 1990). Crop improvement efforts like *Agrobacterium* mediated gene transfer methods have improved the adaptation of chickpea to the subtropic regions along with its disease resistance capacity. The two most common types of chickpea are the white-seeded "Kabuli" and the "Desi". The desi type of chickpea has small and colored seeds and the kabuli type have large and cream colored seeds (Millan et al., 2006). It is the third most important pulse crop in the world, grown in over 40 countries representing all the continents. Over 95% of the area, production and consumption are in the developing countries, where protein deficiency is common. Hence chickpea is considered as a good source of nutrition, particularly to the vegetarians and poor farmers of developing countries

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Abbreviations: BAP, 6-Benzylaminopurine; **KIN,** kinetin; **2,4-D,** 2,4-dichlorophenoxy acetic acid; **NAA,** 1-naphthaleneacetic acid; **IBA,** indole-3-butyric acid; **IAA,** indole-3-acetic acid.

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(FAO, 2009). Chickpea is well known for its use in cosmetics, herbal medicine and for the production of nutraceuticals (Neha, 2012). Among the dry edible legumes, chickpea possesses the top protein digestibility. The lipid fraction is high in unsaturated fatty acids, primarily linoleic and oleic acids (Mayer, 1976). Chickpea utilises symbiotic nitrogen fixation to meet 80% of its nitrogen requirement and can fix up to 140 kg N/ha from air. It leaves generous amount of residual nitrogen behind for ensuing crops and adds much needed organic matter to maintain and improve the soil health as well as the sustainability of ecosystems. Chickpea is well known for its vulnerability to flooding and excess moisture. Also under high moisture conditions, chickpea is prone to fungus and wilt diseases (Yadav et al., 2006). *Ascochyta* blight disease is a major disease seen in chickpea, affecting the yield about 50-80%. Other diseases affecting *Cicer areitinum* are bacterial blight, *Acrophialophora* wilt, dry root rot, Bushy stunt, distortion mosaic, *mystrosporium* leaf rot disease.

Molecular biology and plant biotechnology has emerged as a promising field and thus offering unparalleled opportunities and promises for the development of human resource as well as for the economic benefits (Sukapinda, 1993; Philip and Gamborg, 2005). The direct shoot organogenesis and establishment of plantlets from different explants of chickpea was reported earlier (Polisetty et al., 1996, 1997; Chauhan et al*.,* 2003; Jayanand et al., 2003; Chakraborti et al., 2006; Anwar, 2010).

The regeneration of *C. arietinum* via direct organogenesis has been reported by several researchers (Kartha et al., 1981; Islam et al., 2005; Anju, 2005). Plantlets were developed through callus from different explants of chickpea and through direct somatic embryogenesis (Barna and Wakulu, 1993; Rizvi and Singh, 2000; Chauhan and Singh, 2002). In the present study, high frequency of plant multiplication was standardized using appropriate amount of plant growth regulators. The study was performed using desi variety seeds of cultivar K850 of *C. arietinum* L.

MATERIALS AND METHODS

Collection of disinfected plant material

In the present study, seeds of *C. arietinum* L. (vr. Desi of cultivar K850) were collected from National Germ Plasm Centre, Indian Agricultural Research Institute (IARI), Pusa, New Delhi. The plantlets were maintained in the Department garden during the months of July- December. The explants were obtained from the seedlings raised *in vivo* and *in vitro* (Figures 1 and 2)).

Surface sterilization of the explants

The cotyledonary nodal explants of chickpea were collected from the garden of K. S. Rangasamy College of Technology, Tiruchengode, Tamil Nadu, India (Figure 2). The excised explants were washed with tap water for 30 min followed by treatment with solutions of 2% (v/v) Teepol (Reckitt Benckinser - India) and 70% (v/v) ethanol for 1 min and thereafter washed three times with sterilized distilled water. The explants were then surface - disinfected with 0.1% (w/v) aqueous mercuric chloride solution for 5-6 min and finally rinsed with sterile water. The stem segments were then trimmed at both ends prior to inoculation on culture media.

Culture media and Inoculation

Macronutrients (50x) (Murashige and Skoogs media, 1962) which were composed of various constituents of nitrogen, phosphorus, potassium which helps for the growth of plant in higher rate. Micronutrients (100x) were prepared with trace elements which prevents necrosis of the plant. Additional constituents such as vitamins, myoinositol, iron source, amino acids were prepared and stored at -20°C. Carbohydrate source was provided to the media freshly during the preparation. Agar as a supporting media of 0.8% was added. The pH of the medium was adjusted to 5.6 to 5.8.

In all the experiments, the chemicals used for the experiment were of analytical grade (Himedia, Qualigens, Merkard and Sigma). The medium was dispensed into culture vessels (Borosil, Mumbai, India) and autoclaved at 105 kPa and 121°C for 15 min the surface disinfected explants were implanted horizontally on the culture medium test tubes (150 × 25 mm) containing 50 ml medium and plugged tightly with non-absorbent cotton. All the cultures were incubated at 25 ± 2°C under 16 h photoperiod of 45-50 mol m^{-2} s^{-2} irradiance provided by the cool white fluorescent tubes (Philips and Gamborg, 1995) and with 55-60% relative humidity. All subcultures were done at three week intervals.

Concentration and combinations of growth regulators and their effect on shoots and roots

The surface sterilized explants were cultured on MS medium supplemented with BAP, KIN, 2, 4-D and NAA each at five concentrations (0.05, 0.10, 0.50, 1.0 and 1.5) in individual as well as in combinations. A control treatment without cytokinins was also included for the experiment. For induction of rooting, IAA and IBA were supplemented with the MS medium for rooting (Table 1) (Figures 3, 4 and 5).

RESULT

In vitro seed germination was achieved on MS basal medium. The plants which were grown *in vitro* were used as explants source for the induction of multiple shooting (Figure 6). Multiple shoots were induced from the explants after four weeks of culture on MS medium supplemented with different concentrations of BAP at 0.50 mg/L (4.30 ± 1.10), KIN at 0.10 mg/L (2.30 ± 0.90) and NAA at 1.00 mg/L (1.3 ± 1.0) (Table 2). The maximum number of shoots were obtained with combination of BAP with NAA at 0.50 mg/L (5.0 ± 0.77) , 2,4 D at 0.05 mg/L (3.3 ± 1.10) and KIN at 0.50 mg/L (2.5 ± 0.80). By slightly increasing the concentration of BAP, it was observed that the shoot elongation decreases (Ault, 1994). It was also observed that there is an enhancement in callusing with increasing concentration of cytokinin. The shoots showed stunted growth in

Figure 1. Multiple shoot induction from the *C. arietinum* L. (vr. Desi of cultivar K850) cotyledonary node.

Figure 2. Fully regenerated *C. arietinum* L. (vr. Desi of cultivar K850) plants in trays.

Table 1. Concentrations of auxins and cytokinins in individual as well as in combinations used in the regeneration of *C. arietinum* L. (vr. Desi of cultivar K850).

Hormone	Concentration (mg/L)				
BAP	0.05	0.10	0.50	1.00	1.50
NAA	0.05	0.10	0.50	1.00	1.50
KIN	0.05	0.10	0.50	1.00	1.50
2,4 D	0.05	0.10	0.50	1.00	1.50
IBA	0.05	0.10	0.50	1.00	1.50
IAA	0.05	0.10	0.50	1.00	1.50
BAP + NAA	0.50 + 0.05	0.50 + 0.10	0.50 + 0.50	0.50 + 1.00	0.50 + 1.50
BAP + 2,4D	0.50 + 0.05	0.50 + 0.10	0.50 + 0.50	0.50 + 1.00	0.50 + 1.50
BAP + KIN	0.50 + 0.05	0.50 + 0.10	0.50 + 0.50	0.50 + 1.00	0.50 + 1.50

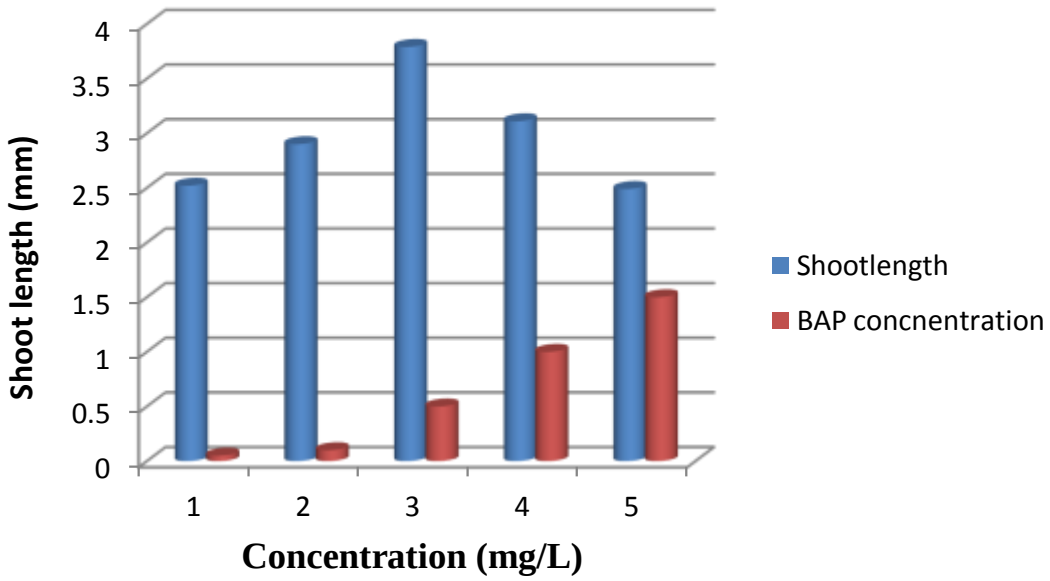

Figure 3. Effect of different concentrations of BAP on multiplication of *C. arietinum* L. (vr. Desi of cultivar K850) shoots.

the medium containing higher concentrations of IAA, with a lesser number of shoots (3.6 ± 0.8) produced. By counting the proliferated shoots, shoot multiplication was assessed after 2 weeks of culture. Individual shoots were excised and sub cultured in the same media composition for further elongation.

Rooting

The *in vitro* multiple shoots were sub-cultured to develop whole plants for root induction in media supplemented with different concentrations of IBA and IAA. When the rooting media were supplemented with IBA concentration (0.10 mgL^{-1}), the number and length of roots greatly increased. During autoclaving at room temperature, IBA was found to be more resistant than IAA to degradation in the tissue culture media (Nissen and Sutter, 1990). Majority of the roots developed two weeks earlier in the IBA medium than in the IAA (Figures 6 and 7). IBA concentration was beneficial for both root system development and shoot quality. The medium supplemented with IAA (0.05-1.50 mg/L) (Table 3) had poor rooting with an intervening callus.

DISCUSSION

There are two procedures for the regeneration in legumes including the shoot proliferation from the region adjacent to pre-existing meristems and the occurrence of unorganised callus phase giving rise to callus or embryos

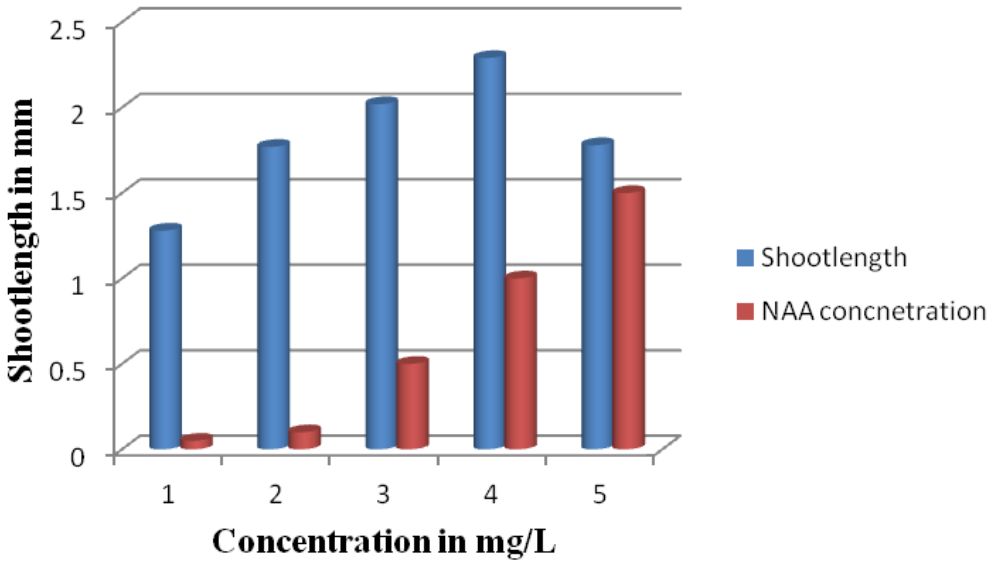

Figure 4. Effect of different concentrations of NAA on multiplication of *C. arietinum* L. (vr. Desi of cultivar K850) shoots

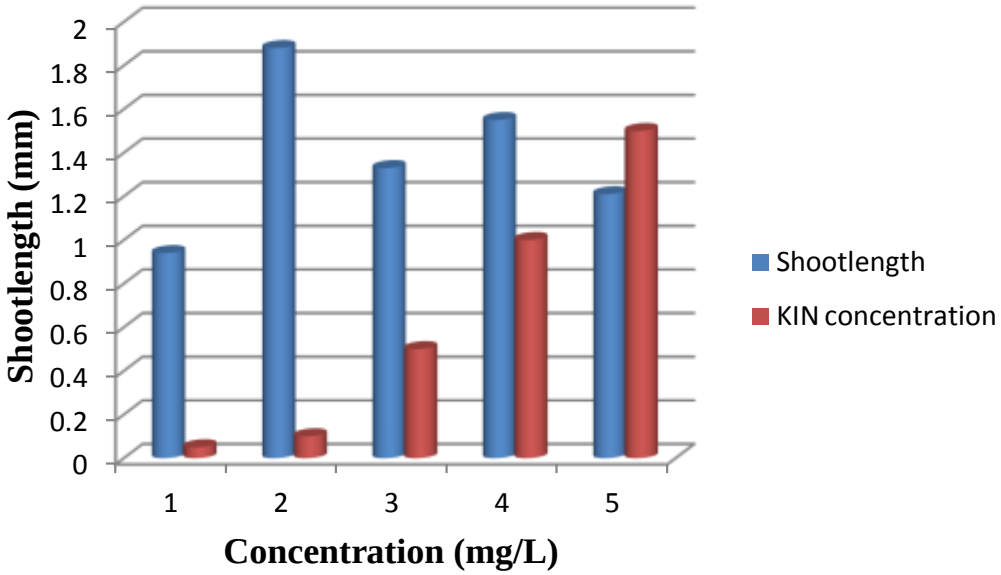

Figure 5. Effect of different concentrations of KIN on multiplication of *C. arietinum* L. (vr. Desi of cultivar K850) shoots.

(Davey et al., 1994). The later type of regeneration is extremely low in frequency and constancy in chickpea (Parrott et al., 1992). Importance of the present study was the standardisation of high frequency plant multiplication using appropriate amount of plant growth regulators. Depending on varying concentration of growth hormones, the capacity of shoot bud differentiation and shoot proliferation from shoot tip explants of chickpea varies. In the presence of cytokinins like NAA and KIN, there was good shoot bud induction and proliferation response compared to basal medium. This study shows that combination of 0.5 mg/L BAP and NAA were significantly more effective for inducing shoot organogenesis (Table 1). BAP was reported to be an ideal hormone for shoot multiplication of shoot tip culture in grain legumes (Shagufta et al., 2007). The maximum shoot length was obtained for combination of NAA and BAP (Figure 8) and was 4.13 mm and minimum shoot length was found to be

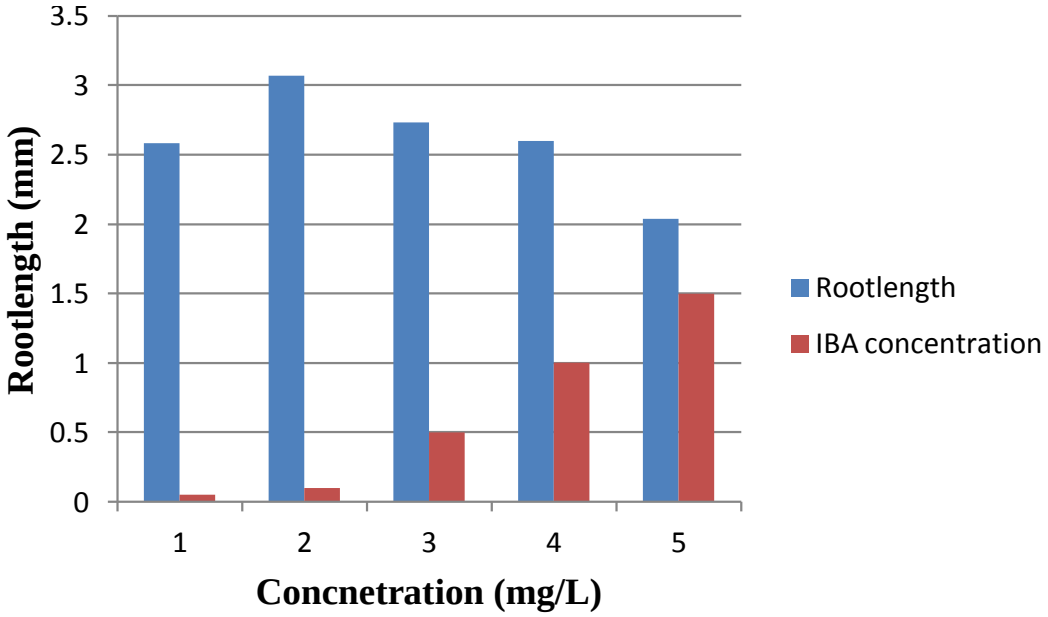

Figure 6. Effect of IBA on induction of roots in the *C. arietinum* L. (vr. Desi of cultivar K850) microshoots.

Table 2. Effects of BAP, KIN, and NAA concentrations on the percentage of reactive *C. arietinum* L. (vr. Desi of cultivar K850) explants, the number of shoots and the maximum shoot length per explant after four weeks of culture on MS medium.

Hormone	Concentration (mg/L)	Percentage of responsive explants	No of shoots per explants	Maximum shoot length (mm)
Control	-	70	1.3 ± 0.6^{lm}	0.80 ± 1.1^{mn}
BAP	0.05	69^{cd}	1.7 ± 0.9^{e}	2.52 ± 0.29^{d}
	0.10	74^{bc}	3.0 ± 1.18^{b}	2.90 ± 0.27^{c}
	0.50	98^{a}	4.3 ± 1.10^{a}	3.79 ± 0.39^{a}
	1.00	82^{b}	2.8 ± 0.97^{bc}	3.11 ± 0.35^{b}
	1.50	64^{cd}	1.6 ± 1.01^{ef}	2.49 ± 0.24^{de}
NAA	0.05	54^{f}	0.6 ± 0.66^{jk}	1.28 ± 0.51^{kl}
	0.10	62^{de}	1.1 ± 0.94^{gh}	1.77 ± 0.75^{ij}
	0.50	46^{gh}	1.1 ± 0.83^{gh}	2.02 ± 0.46^{g}
	1.00	78^{bc}	1.3 ± 1.0^{g}	2.29 ± 0.57^{ef}
	1.50	39^{i}	0.6 ± 0.48^{jk}	1.78 ± 0.79^{hi}
KIN	0.05	24^{l}	0.6 ± 0.48^{jk}	0.94 ± 0.7^{n}
	0.10	49^{fg}	2.3 ± 0.90^{d}	1.88 ± 1.06^{gh}
	0.50	30^{jk}	1.0 ± 0.77^{hi}	1.33 ± 0.54^{k}
	1.00	38^{ij}	1.2 ± 0.74^{gh}	1.55 ± 0.81^{k}
	1.50	34^{jk}	0.7 ± 0.64^{j}	1.21 ± 0.63^{lm}
BAP+NAA	0.50 + 0.05	64^{h}	1.4 ± 1.11^{f}	2.85 ± 0.63^{f}
	0.50 + 0.10	76^{e}	3.3 ± 0.90^{bc}	3.25 ± 0.46^{dc}
	0.50 + 0.50	100^{a}	5.0 ± 0.77^{a}	4.13 ± 0.26^{a}
	0.50+ 1.00	46^{l}	3.5 ± 0.80^{b}	3.36 ± 0.49^{cd}
	0.50 + 1.50	36^{m}	2.1 ± 1.13^{de}	2.49 ± 0.80^{h}

Table 2. Contd.

Hormone	Concentration (mg/L)	Percentage of responsive explants	No of shoots per explants	Maximum shoot length (mm)
BAP+2,4D	0.50 + 0.05	96ab	3.3 ± 1.10bc	3.52 ± 0.19b
	0.50 + 0.10	72^{ef}	2.1 ± 1.22^{de}	2.71 ± 0.38^{fg}
	0.50 + 0.50	60^{ij}	1.1 ± 0.94^{gh}	2.10 ± 0.31^{j}
	0.50 + 1.00	84^{d}	1.1 ± 0.83^{gh}	2.10 ± 0.46^{j}
	0.50 + 1.50	59^{jk}	0.6 ± 0.48^{ij}	2.03 ± 0.34^{jk}
BAP+KIN	0.50 + 0.05	58^{jk}	0.8 ± 0.74^{hi}	1.5 ± 0.72^{mn}
	0.50 + 0.10	70^{fg}	1.30 ± 0.90^{fg}	1.65 ± 1.07^{lm}
	0.50 + 0.50	94^{bc}	2.50 ± 0.80^{d}	3.37 ± 0.41^{bc}
	0.50 + 1.00	72^{ef}	1.10 ± 0.83^{gh}	2.36 ± 0.76^{hi}
	0.50 + 1.50	63^{hi}	0.60 ± 0.48^{ij}	1.76 ± 0.67^{l}

Means and standard errors (±SE) are presented for each column. Means sharing at least one letter are not significantly different at the P≤ 0.05 level (Duncan's multiple range test).

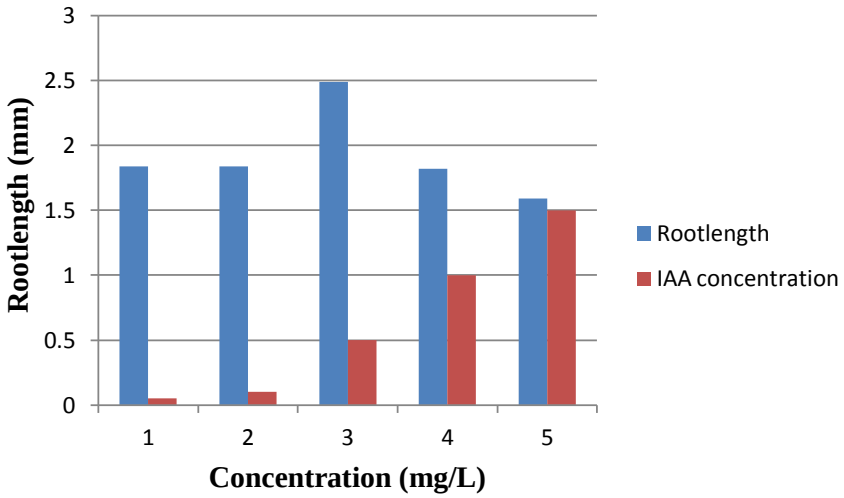

Figure 7. Effect of IAA on induction of roots in the *C. arietinum* L. (vr. Desi of cultivar K850) microshoots.

Table 3. Effects of auxin on *in vitro* rooting of *C. arietinum* L. (vr. Desi of cultivar K850) shoots cultured on MS medium.

Hormone	Concentration (mg/L)	Percentage of responsive explants	Number of shoots per explants	Maximum shoot length (mm)
IBA	0.05	62^{fg}	1.2 ± 0.74^{gh}	2.58 ± 0.53^{cd}
	0.10	100^{a}	4.6 ± 0.91^{a}	3.07 ± 0.41^{a}
	0.50	73^{cd}	3.0 ± 0.74^{cd}	2.73 ± 0.54^{b}
	1.00	81^{b}	3.1 ± 0.94^{c}	2.60 ± .47^{bc}
	1.50	69de	1.5 ± 0.92^{fg}	2.04 ± 0.58^{fg}
IAA	0.05	42^{i}	0.8 ± 0.60^{jk}	1.84 ± 0.50^{h}
	0.10	53^{h}	1.9 ± 0.83^{ef}	1.84 ± 0.50^{h}
	0.50	76^{bc}	3.6 ± 0.80^{b}	2.49 ± 0.58^{de}
	1.00	64^{ef}	2.1 ± 1.04^{e}	1.82 ± 0.90^{hi}
	1.50	38^{ij}	1.1 ± 0.94^{ij}	1.59 ± 0.75^{g}

Means and standard errors (±SE) are presented for each column. Means sharing at least one letter are not significantly different at the P≤ 0.05 level (Duncan's multiple range test).

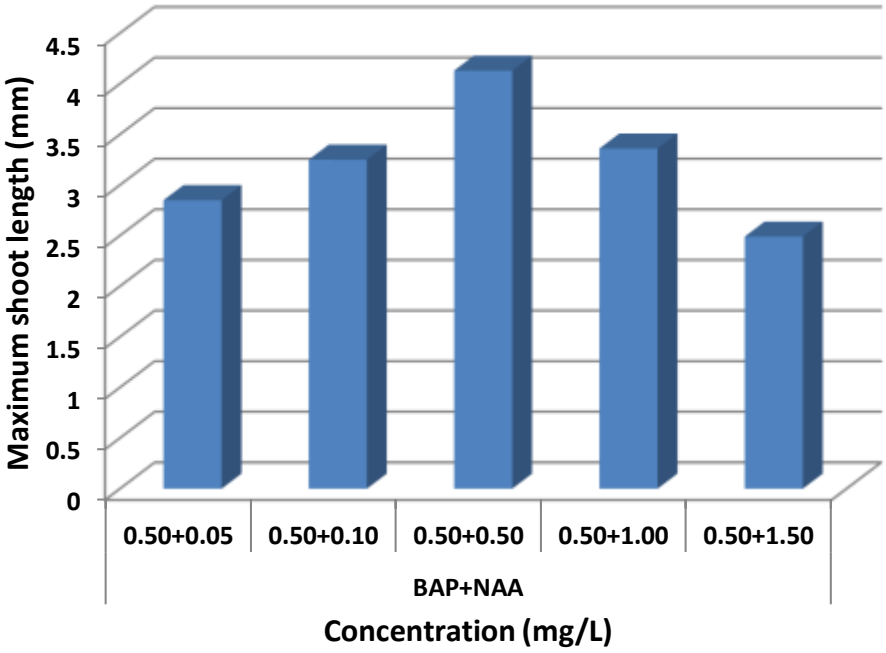

Figure 8. Effect of BAP and NAA on multiplication of *C. arietinum* L. (vr. Desi of cultivar K850) shoots per shoot tip as explant.

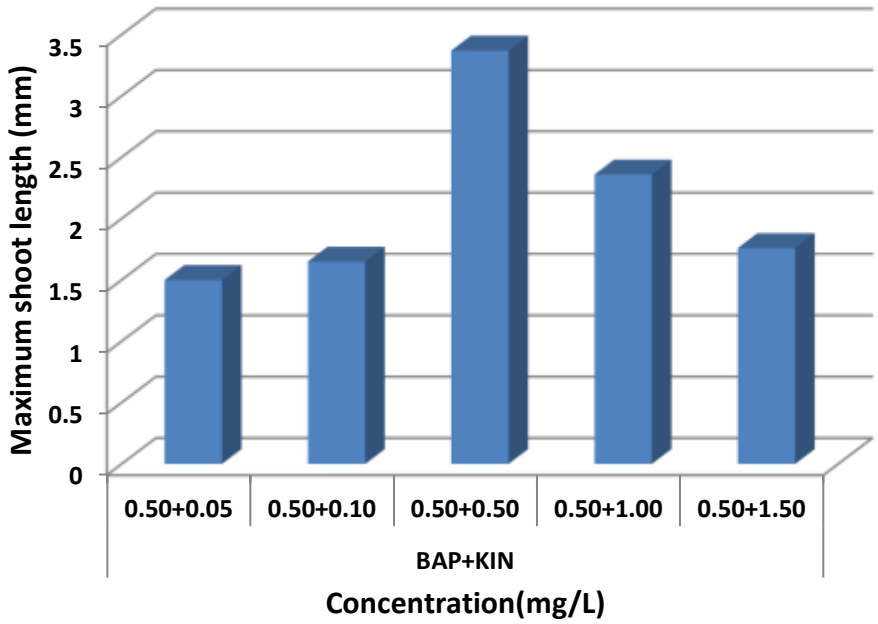

Figure 9. Effect of BAP and KIN on multiplication of *C. arietinum* L. (vr. Desi of cultivar K850) shoots per shoot tip as explant.

0.94 mm in the medium supplemented with KIN (Figure 9). BAP supplemented media showed good response at 3.79 mm shoot length. Singh et al. (2002) has also reported a similar response to BAP in multiple shooting. A combination of BAP and KIN gives a maximum shoot length of 3.37 mm and a minimum growth of 1.50 mm. The combination of BAP with 2,4 D gives a maximum shoot length of 3.72 mm and a minimum of 2.03 mm, respectively (Figure 10). Medium supplemented with NAA shows growth with maximum shoot length of 2.29 mm and

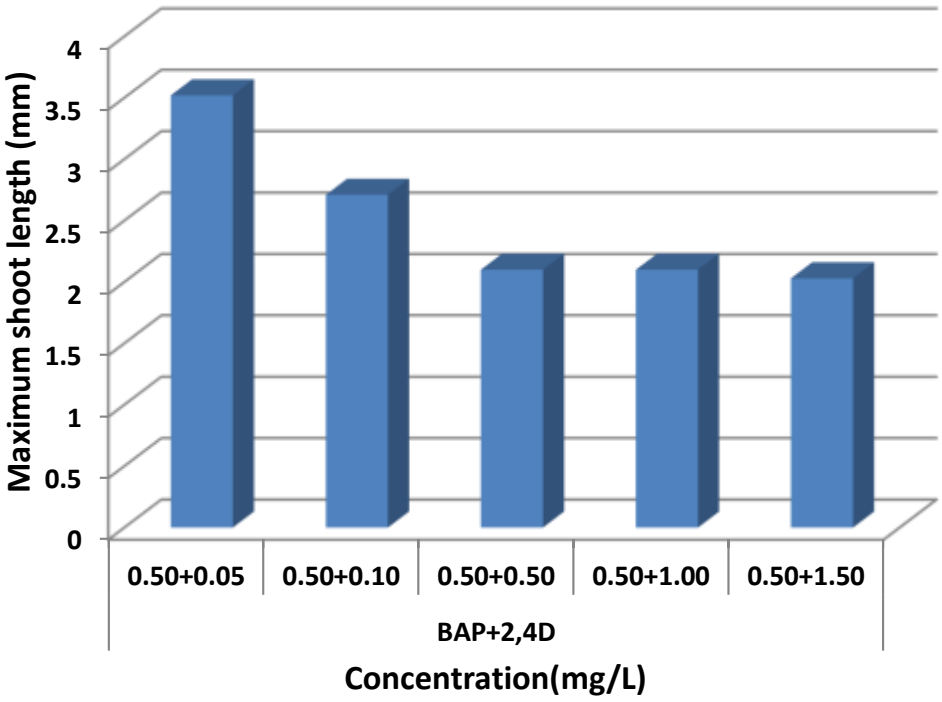

Figure 10. Effect of BAP and 2,4D on multiplication of *C. arietinum* L. (vr. Desi of cultivar K850) shoots per shoot tip as explant

a minimum of 1.28 mm. The data in Table 2 shows that the maximum root length was obtained for IBA (3.07 mm) and minimum shoot lengths (1.59 mm) were recorded for IAA respectively. The IBA concentration was found to be beneficial for both shoot as well as root system development. It was reported earlier that the half strength MS medium induced maximum rooting in cowpea (Kulothungan et al., 1995). Thus, it is evident from our studies that the combination of NAA and BAP are best suited for inducing multiple shoots in *C. arietinum* and high frequency of plant multiplication were standardised.

Conclusion

A reproducible protocol is developed for raising plantlets and clonal propagation for the cultivar K850 of *C. arietinum* L. The results of the present investigation are reproducible and can be used for future developments of the crop.

Conflict of interests

The authors did not declare any conflict of interest.

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