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# *IN VITRO* STABLE PLANTLET REGENERATION FROM CALLUS CULTURE OF *CICER ARIETINUM* L.

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## ABSTRACT

*A rapid, simple and efficient protocol for plantlet regeneration was achieved through organogenic callus from internode explants of Cicer arietinum. Callus induction and organogenesis were significantly dependent on the presence/ absence and type of growth regulators. Internodes showed higher growth potential compared to leaf and node for callus induction. Callus induction was obtained after 15 days of inoculation in MS medium supplemented with 2,4-D 1mg/l. The highest shoot formation was obtained when BAP 1mg/l was used. The best response for rooting was obtained in 2,4-D 0.5 mg/l. Cytological study indicated that the chromosome number remain same (2n= 16 chromosomes) in callus and in vivo roots. Callus cytology revealed that callus tissues were diploid in nature. Karyotype analysis indicated that the number and type of both primary and secondary constrictions remain same in callus and in vivo roots. Thus, this can be an easier protocol for stable plant regeneration in Cicer arietinum. Rooted plantlets were successfully hardened under culture conditions and subsequently established in the field conditions. The recorded survival rate of the plants was 67.3%. Plants were without any detectable phenotypic variations.*

## KEY WORDS

*Callus, Cicer arietinum, organogenesis, regeneration.*

## INTRODUCTION

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. 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 di-nitrogen fixation.

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 and decreasing land resources, alternative means can be followed. Though *Cicer arietinum* is normally propagated through seeds, *in vitro* propagation can be of immense value, thereby conserving the species. 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. The *in vitro* propagated medicinal plants furnish a ready source of uniform, sterile, and

compatible plant material for mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants [1 and 2]. The loss of biodiversity and plantations due to deforestation in combination with the demand from both domestic and export markets have led to the utilization of *in vitro* methods of propagation as compensating tools to meet the commercial needs [3]. Furthermore, genetic improvement is another approach to augment the drug yielding capacity of the plant [4] which depends on the establishment of efficient adventitious shoot regeneration systems. *In vitro* techniques can provide reliable conservation and propagation of valuable germplasm as well as reduce or eliminate the load of microorganisms which may cause serious diseases [5 and 6]. 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 [7]. 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 organogenesis [8, 9, 10 and 11]. Up to date, there is no report on callus regenerates stability of *Cicer arietinum*. Only, a reliable protocol for micropropagation in *Cicer arietinum* from apical bud explants was reported [12], which presented the effects of several plant growth regulators, including BAP, IAA and IBA, in order to obtain high shoot and root regeneration rate and also considerale survive. Some factors as genotype, explant type and size, media composition have significant effect on micropropagation success [13].

The objective of this study is to introduce an efficient and reproducible regeneration protocol from callus cultures of this medicinal plant. For that purpose, the effect of different explant types like internode, node and leaf as well as plant growth regulators on organogenic callus induction and plantlet regeneration. Cytological study was studied for determination of chromosomal stability.

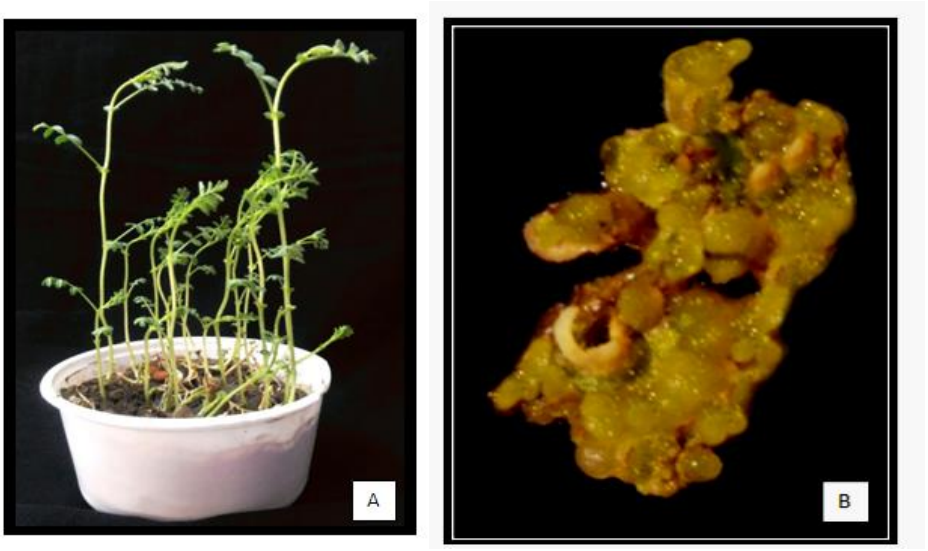
## MATERIALS AND METHODS

### Materials:

*Cicer* plants were grown and maintained in college garden. Plants were authenticated by the experts of University of Calcutta. Internodes, node and leaf were used for callus induction followed by subsequent regeneration (Table 1, Fig 1A).

**Table 1 Different types of explant**

| Type of explant | Abbreviations |
|---|---|
| Node | A |
| Internode | B |
| Leaf | C |



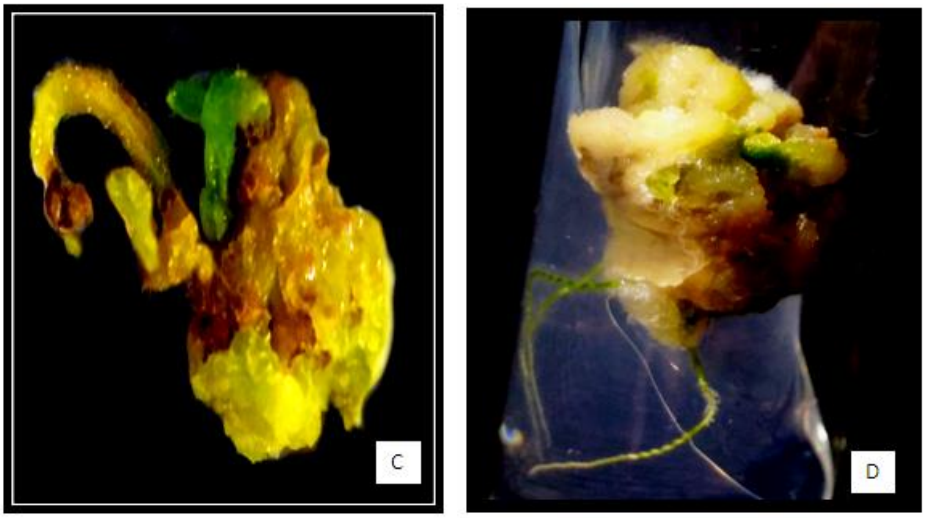



**Figure 1: 1A: *In vivo Cicer arietinum* plant; 1B: Callus induction; 1C: Shoot regeneration from callus; 1D: Root organogenesis.**

**Establishment of callus culture:**

Explants were washed under running tap water for about 25 minutes, then washed with the surfactant Tween 20 solution (2-6 drops in 100 ml solution) for 15 minutes to 20 minutes and finally rinsed with water till the surfactant was thoroughly removed. Further treatments were carried out inside a laminar air flow cabinet. The explants were surface sterilized with 0.1% (w/v) aqueous solution of Mercuric Chloride ($HgCl_2$) for about 10 minutes to 12 minutes and then thoroughly washed with sterile double distilled water for about 10 minutes [14]. Sterilized explants were cultured on MS medium supplemented with 2, 4-D in different concentrations (Table 2).

**Table 2 Callus induction in *Cicer arietinum***

| Type of explant | Medium used | Frequency (%) | Callus morphology |
|---|---|---|---|
| A | 2,4-D 0.5 mg/l | 13.00±1.25$^{a}$ | Thick green callus |
| | 2,4-D 1.0 mg/l | 30.00±2.50$^{b}$ | Thick compact yellow callus |
| | 2,4-D 1.5 mg/l | 27.00±1.90$^{c}$ | Thick green callus |
| | 2,4-D 2.0 mg/l | 25.00±1.80$^{c}$ | Thick green callus |
| B | 2,4-D 0.5 mg/l | 33.00±1.30$^{b}$ | Thick green callus |
| | 2,4-D 1.0 mg/l | 85.00±1.50$^{d}$ | Yellowish green soft callus |
| | 2,4-D 1.5 mg/l | 80.58±1.30$^{e}$ | Yellowish green soft callus |
| | 2,4-D 2.0 mg/l | 55.00±2.00$^{f}$ | Thick compact yellow callus |
| C | 2,4-D 0.5 mg/l | 03.00±1.20$^{g}$ | Yellowish green soft callus |
| | 2,4-D 1.0 mg/l | 13.00±2.00$^{a}$ | Thick green callus |
| | 2,4-D 1.5 mg/l | 07.00±1.95$^{h}$ | Thick green callus |
| | 2,4-D 2.0 mg/l | 05.00±1.85$^{h}$ | Thick compact yellow callus |
| LSD | | 1.44 | |

***Data expressed as Mean ± S.E. of 3 replicates P ≤ 0.0001. Means followed by different superscript letters in the same column present significant difference. P ≤ 0.05.**

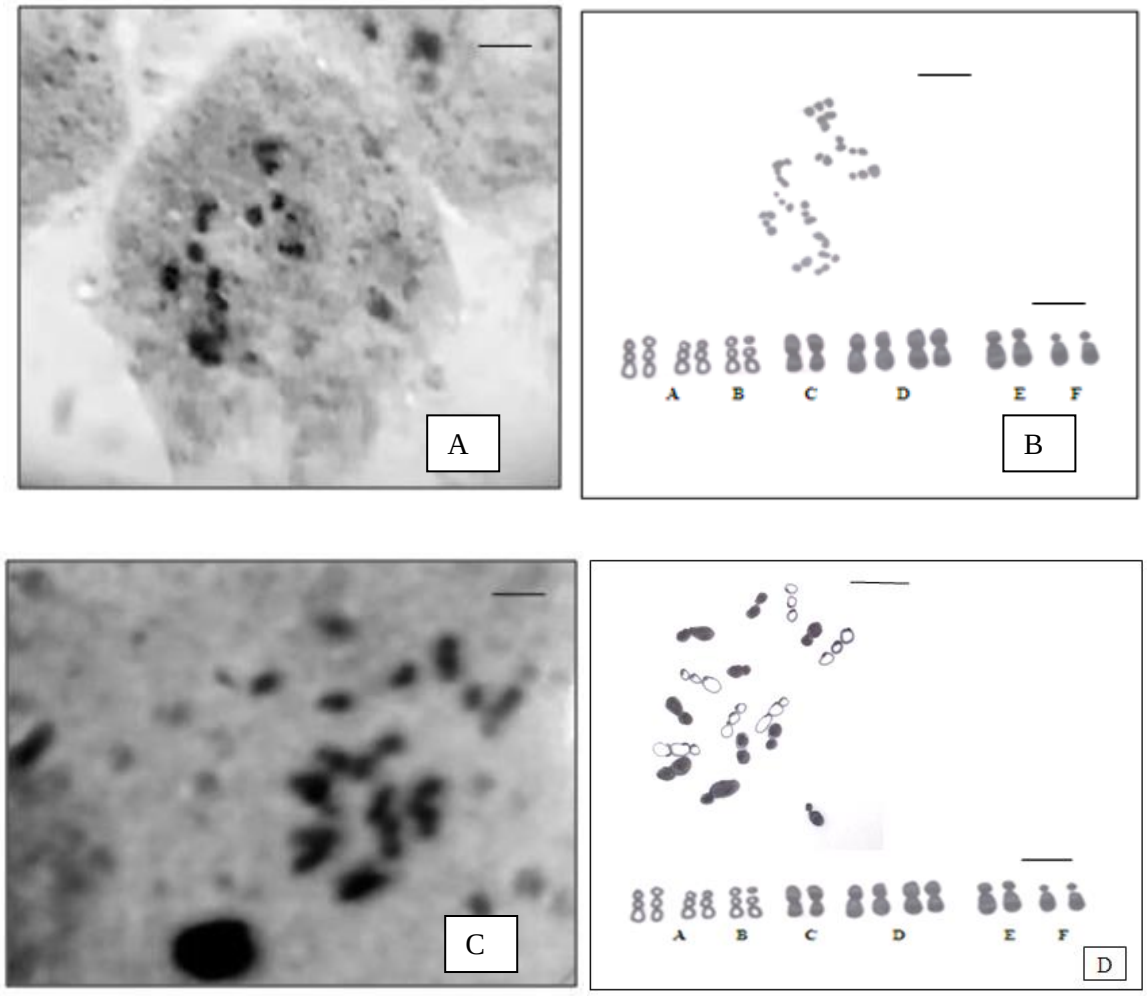



**Figure 2: Photographs of metaphase plate, drawing and karyotype analysis from *in vivo* root (A-C) and callus (D-F) (Bar= 5µm).**

**Culture medium:**

Expalnts were placed on MS medium [15] supplemented with 2,4-D in different concentrations. The pH of the medium was fine adjusted to 5.8 before autoclaving. Media contained 3% (w/v) sucrose and solidified with gelrite 0.25% (w/v). Molten medium was dispensed 20 ml (approximately) into each culture tube (25×150mm) and plugged with non-absorbent cotton. The medium was autoclaved at 15 lbs/sq inch pressure and 121°C for 15 minutes. MS basal medium augmented with various plant growth regulators (pH 5.7-5.8) tested for callus induction, shoot initiation and root induction [16].

**Culture conditions:**

All the cultures were maintained at 23±2°C temperature and 60-70% relative humidity. The photoperiod is provided by 16 hours day light and 8 hours dark having 3000 lux light intensity by cool white fluorescent tubes (Phillips India Ltd.).

**Determination of chromosomal stability:**

Chromosome analysis involves somatic chromosome analysis of organogenic callus and *in vivo* root were carried out by aceto-orcein techniques [17] and compared with the *in vivo* roots of *Cicer* (Table 3). Karyotype analysis was carried out according to Levan *et al*., 1964 [18]. After determination of chromosomal stability, the calli were further cultured for shoot and root induction so that we can understand the ploidy status of regenerated plants.

**Table 3 Callus cytology showing ploidy level of callus**

| No of observation | Total no of div cells | No of cells with 2n chr no | No of cells with >2n chr no | No of cells with <2n chr no |
|---|---|---|---|---|
| 1 | 40 | 37 | 2 | 1 |
| 2 | 47 | 46 | 1 | 0 |
| 3 | 50 | 47 | 1 | 2 |
| 4 | 42 | 42 | 0 | 0 |
| 5 | 54 | 53 | 0 | 1 |

**Table 4 Karyotype analysis in *Cicer arietinum***

| Type of cell | Chr no | No of chr with sec cons | Type of chr present* | Karyotype formula | Chr size range (µm) |
|---|---|---|---|---|---|
| *In vivo* root | 16 | 6 | A (St,Sm)<br>B (St,m)<br>C (M)<br>D (m)<br>E (Sm)<br>F (St) | $A_4B_2C_2D_4E_2F_2$ | 2.0-1.2 |
| Callus | 16 | 6 | A (St,Sm)<br>B (St,m)<br>C (M)<br>D (m)<br>E (Sm)<br>F (St) | $A_4B_2C_2D_4E_2F_2$ | 0.9-1.7 |

***St= Sub terminal, Sm= submedian, M= Median, m= Median region. (Chr= Chromosome, sec cons= secondary constrictions)**

**Shoot regeneration from callus:**

Callus tissues were transferred to MS medium containing different concentrations of BAP (pH- 5.7) for shoot regeneration (Table 5). Culture conditions were same as in above.

**Root organogenesis of microshoots:**

Regenerated shoot buds were transferred to MS medium containing different concentrations of 2,4-D (pH- 5.8) for root development (Table 6). Culture conditions were same as in above.

**Table 5 Shoot regeneration from internodal callus in *Cicer arietinum***

| Growth regulators (mg/l) | % of explants showing response | No. of shoots/ explants* | Average length of shoots* |
|---|---|---|---|
| BAP 0.25 | 83 | $1.0\pm 0.3^a$ | $4.0\pm 0.3^a$ |
| BAP 0.50 | 87 | $3.0\pm 0.5^b$ | $4.2\pm 0.5^a$ |
| BAP 1.00 | 98 | $5.0\pm 0.6^c$ | $5.3\pm 0.4^b$ |
| BAP 1.50 | 82 | $4.0\pm0.5^d$ | $4.5\pm 0.6^a$ |
| BAP 2.00 | 80 | $3.0\pm0.4^b$ | $4.0\pm0.2^a$ |
| LSD | | 0.46 | 0.76 |

*** Data expressed as Mean ± S.E. of 3 replicates P ≤ 0.0001. Means followed by different superscript letters in the same column present significant difference. P ≤ 0.05.**

**Table 6 Root organogenesis from callus in *Cicer arietinum***

| Growth regulators (mg/l) | % of explants showing response | No. of roots / explants* | Average length of roots * |
|---|---|---|---|
| 2,4-D 0.25 | 85 | $12.0\pm 0.6^a$ | $3.0\pm0.2^a$ |
| 2,4-D 0.50 | 90 | $15.0\pm 0.5^b$ | $4.5\pm 0.6^b$ |
| 2,4-D 1.00 | 81 | $11.0\pm 0.3^a$ | $3.1\pm 0.6^a$ |
| 2,4-D 1.50 | 78 | $10.0\pm0.7^a$ | $3.5\pm0.4^a$ |
| 2,4-D 2.00 | 80 | $07.0\pm0.3^c$ | $3.3\pm 0.4^a$ |
| LSD | | 0.76 | 0.48 |

*** Data expressed as Mean ± S.E. of 3 replicates P ≤ 0.0001. Means followed by different superscript letters in the same column present significant difference. P ≤ 0.05.**

**Acclimatization:**

Rooted plantlets were removed from the culture medium followed by washing under running tap water to remove gelrite. Then the plantlets were transferred to a pot containing pre- soaked vermiculite and maintained inside a growth chamber set at 27$^{o}$C and 70-80% relative humidity. After 25 days they were transplanted to poly bags containing mixture of soil + sand + manure in 1: 1: 1 ratio and kept under shade house for a period of three weeks and subsequently established in the field conditions [19]. The survival rate of the plants in field conditions was recorded as 67.3%.

**Data and statistical analysis:**

Data were analysed statistically to determine the least significant difference (LSD). 3 replications of each set of experiment were taken for study and an average was calculated.

## RESULTS AND DISCUSSION

This research was undertaken to establish optimal chickpea *in vitro* regeneration from callus. Results showed that callus induction, shoot and root regeneration are influenced by explant type and culture medium including type of hormone [20, 21].

Callusing started at the cut ends of all the explants after 15 days of inoculation with varying frequency irrespective of the concentration of growth regulators used in this experiment (Table 2). Very little callus growth was seen in leaf and node explants. The calli were green to light yellowish in color with soft structures (Table 2, Fig 1B). The induction of organogenic callus was usually promoted by an auxin, especially 2,4-D [22, 23 and 24]. The results of our experiment showed a simple protocol using only single type of hormone for callus induction instead of using different hormones, thus this protocol was more cost effective than other reports. Using 2,4-D 1mg/l , 85% response of callus formation from intermodal explants was observed. Callus induction through a perfect hormonal concentration has been reported for several plants including *Astragalus adsurgens* [25], lavandin (*Lavandula* × *intermedia* [26], *Valeriana edulis* [27], *Lathyrus sativus* [28], *Diffenbachia* sp. [29] as well as the current study which is an auxin (2,4-D) induced organic calli.

Cytological study showed that calli was stable in respect to their chromosome number, types and karyotype formula (Table 3, 4).

The calli derived from internode were subcultured on shoot induction media i.e MS medium augmented with BAP (0.25–2.0 mg/l) for multiple shoot induction. The capacity of shoot bud differentiation and shoot proliferation depended on hormonal variation (Table 5, Fig 1C). One week after transferring to regeneration medium, green spots appeared on the surface and green shoot buds started emerging. There was good shoot bud induction and proliferation response only in the presence of cytokinin and no response in the basal medium. Of all the different concentrations of BAP tested, 1.0 mg/l BAP was found to be more effective in inducing multiple shoots (98% regeneration frequency). The same medium supplemented with 1.0 mg/l BAP was used for further elongation of shoots. The mean number of shoots of 3 and the shoot length of 4.2 cm. BAP (2.0 mg/l) resulted the number of shoots were reduced (Table 5).

Rooting of shoots is the most critical step in the production of complete plants and their subsequent survival. The individual elongated healthy shoots were isolated and transferred to MS medium fortified with 2,4-D (0.25–2.0 mg/l). 2,4-D (0.5 mg/l) was found to be more effective in the production of long, slender and healthy roots (Fig 1D, Table 6). Profuse rhizogenesis was observed on 0.5 mg/l 2,4-D, compared to( 0.25 - 2.0 mg/l) 2,4-D. In MS medium containing (0.5 mg/l) 2,4-D root regeneration frequency was 90% of plants produced roots with 15 ± 0.5 roots/explants (Table 6).

Chromosome study from root tips of *in vivo* plant and callus grown in 2,4-D 1mg/l showed stability. Nearly 97% cells are in same ploidy level (Table 3) indicating that there were no somaclonal variations occurred [30]. No variation occurred in chromosome number (2n = 16 chromosomes in both callus and *in vivo* root tips), number of secondary constrictions, types of chromosome and karyotype formula, all these clearly indicated that the calli remain stable in respect to chromosomal parameters (Fig 2 A-D). So the regenerated plants were genetically stable.

Thus, plantlet production was observed from internodal callus 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. Our result on callus regeneration internode culture shows the considerable importance for large scale propagation. The present study on callus induction and regeneration of *Cicer arietinum* may help conservation

of the species and possibly will lead to the production of secondary metabolites and extraction of active compounds from callus sources.

## CONCLUSION

From the above study, it was concluded that internodes were best explant for callus induction of chickpea. Callusing started at the cut ends of the explants after 15 days of inoculation in MS Basal media with 2,4-D. The highest amount of callus developed in 2,4-D 1mg/l media. Vigorous growth of calli was obtained after 50 days of inoculation. Stable plant regeneration was established from the organogenic calli has been seen here. Chickpea tissue culture morphogenetic response depends on plant growth regulators. Mature shoot and root development obtained in appropriate medium. The stability was determined by cytological analysis. Somatic chromosome number remains the same in seeds (2n = 16 chromosomes) and callus. 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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## ABBREVIATIONS

2,4-D- 2,4-dichlorophenoxy acetic acid, BAP- 6-Benzyl amino purine, MS- Murasige and Skoog's basal medium.

## REFERENCES

1. Anis M. and Faisal M. (2005). *In vitro* regeneration and mass multiplication of *Psoralea corylifolia* - An endangered medicinal plant. Ind. J. Biotech. 4: 261-264.
2. Uppendra D., Sumit M. and Meena J. (2005). Organogenesis, embryogenesis, and synthetic seed production in *Arnebia euchroma* - A critically endangered medicinal plant of the Himalaya. *In Vitro* Cell Dev. Biol. Plant. 41: 244-248.
3. Shaik S., Singh N. and Nicholas A. (2011). Cytokinin-induced organogenesis in *Lessertia* (Sutherlandia) *frutescens* L. using hypocotyl and cotyledon explants affects yields of L-canavanine in shoots. Plant Cell Tiss. Org. Cult. 105: 439-446.
4. Karuppusamy S., Kiranmai C., Aruna V. and Pullaiah T. (2006). Micropropagation of *Vanasushava pedata* - An endangered medicinal plant of South India. Plant Tissue Cult. Biotech. 16(2): 85-94.
5. Sharma A. and Agrawal V. (2012). Tissue culture aspects of ornamental plants (Review). CIBTech. Journal of Biotechnology 1(1): 40-48.
6. Kolomiets T.M., Malyarovskaya V.I., Gvasalia M.V., Samarina L.S. and Sokolov R.N. (2014). Micropropagation of subtropical fruit, ornamental crops and endemics of Western Caucasus: Original and optimized protocols. Agricultural biology 3: 49-58. [in Russian].
7. Scowcraft W.R., Ryan S.A. (1985). Tissue culture and plant breeding. [Manuscript prepared: Yeoman M. (ed.), Plant Culture Technology. Oxford Blackwell Scientific.
8. Kartha. K.K., Pahl K., Leung N.L. and Mroginski L.A. (1981). Plant regeneration from meristem of grain legumes soybean, cowpea, peanut chickpea, and bean. Can J Bot. 59: 1671-1679.
9. Islam R., Riazuddin S. and Farooqui H. (1995). Clonal propagation from seedling nodes and shoot apices of chickpea (*Cicer arietinum* L.). Plant Tissue Cult. 5: 53-57.
10. Barna K.S. and Wakhlu A.K. (1995). Modified single node culture method a new micropropagation method for chickpea. In vitro Plant 31: 150-152.
11. Anju A. and Chawla S. (2005). Organogenic Plant regeneration via callus induction in chickpea (*Cicer arietinum* L.) Role of genotypes, growth regulators and explants. Indian J Biotech 4: 251-256.
12. Salehi M., Hosseini B. and Jabbarzadeh Z. (2014). High-frequency in vitro plantlet regeneration from apical bud as a novel explant of *Carum copticum* L. Asian Pac. J. Trop. Biomed. 4(1): 424-428.
13. Malyarovskaya V.I. (2014). Factors influencing Camellia japonica clone micropropagation. Materials of Scientific Conference "Prospects and achievements of modern floricultural sciences" 15(6): 52-55.
14. Mukherjee, A. Lahiri, K. Samanta D. Mukhopadhyay M.J. and Mukhopadhyay S. (2011). Effects of Cyatokinin on *in vitro* Shoot Bud Multiplication and Subsequent Regeneration of *Solanum melongena* L. J Bot. Soc. Beng. 65*:* 173-177.
15. Murashige T.and Skoog F. (1962) A revised medium for rapid growth and bioassay with Tobacco tissue cultures. Physiol Plant 15: 473-497.
16. Mukhopadhyay S. and Samanta D. (2015). A review on in vitro propagation of *Centella asiatica* L.: an important medicinal herb. J Bot. Soc. Beng. 69(2)*:* 77-89.
17. Samanta D., Lahiri K., Mukhopadhyay M J. and Mukhopadhyay S. (2016). Cytomorphological analysis and *in situ* 4C nuclear DNA estimation in species and varieties of *Tabernaemontana* (fam. Apocynaceae) Nucleus 59: 99–105.
18. Levan A., Fredga K. and Sandberg A.A. (1964). Nomenclature for centromeric position on chromosome. Hereditas 52: 201–220.

19. Sujatha L.G., Jayabalan N., Ranjitha Kumari B.D. (2007). Rapid *in vitro* micropropagation of *Cicer arietinum.* Hort. Sci. (Prague), 34, (1): 1–5.
20. Yadav V., Malan L. and Jaiswal V.S. (1990). Micropropagation of Morus niger from shoot tip and nodal explants of mature trees. Scientia Hort. B 61-64.
21. Narashimhulu SB. and Redddy G.M. (1983). Plantlet regeneration from different callus cultures of Arachis hypogaea L. Plant Sci Lett. 31: 157-163.
22. Carman J.G. (1990). Embryogenic cells in plant tissue cultures: occurrence and behavior. In Vitro Cell Dev. Biol. Plant. 26: 746-753.
23. Bennici A. and Bruschi P. (1999). Stability of habituation in *Nicotiana bigeloii* var. *bigelovii* shoots regenerated from habituated callus. Plant Sci. 143: 195-199.
24. Kamo K., Jones B., Bolar J. and Smith F. (2005). Regeneration from longterm embryogenic callus of the *Rosa hybrida* cultivar kardinal. *In Vitro* Cell Dev. Biol. Plant. 41: 32-36.
25. Luo P.J., Jia J.F., Gu Y.H. and Liu J. (1999).. High frequency somatic embryogenesis and plant regeneration in callus cultures of *Astragalus adsurgens* Pall. Plant Sci. 143: 93-99.
26. Dronne S., Jullien F., Caissard J.C. and Faure O. (1999). A simple and efficient method for in vitro shoot regeneration from leaves of lavandin. Plant Cell Rep. 18: 429-433.
27. Castillo P., Ma´rquez J., Rubluo A., Herna´ndez G. and Lara M. (2000). Plant regeneration from callus and suspension cultures of *Valeriana eduliss* sp. *procera* via simultaneous organogenesis and somatic embryogenesis. Plant Sci. 151: 115-119
28. Zambre M., Chowdhury B., Kuo Y.H., Montagu M.V., Angenon G. and Lambein F. (2002) .Prolific regeneration of fertile plants from green nodular callus induced from meristematic tissues in *Lathyrus sativus* L. (grass pea). Plant Sci. 163: 1107-1112.
29. Shen X., Chen J. and Kane M.E. (2007). Indirect shoot organogenesis from leaves of Dieffenbachia cv. Camouflage. Plant Cell Tiss. Org. Cult. 89: 83-90.
30. Bhojwani S.S. and Rajdan M.K. (1996). Plant tissue culture: theory and Practice, a revised Edition. Elsevier, Netherland, 47-48.

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