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In-vitro regeneration studies of an important legume, *Cicer arietinum*: Hurdles and future prospects

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ABSTRACT

There are several economically important grain legumes including chickpea that play significant role in nutrition of the rural and urban poor in developing world. Plants are subjected to a large number of stresses that may interfere with the normal growth and development. The model legumes are being developed as experimental systems to study a number of key biological questions using molecular tools including genomics and proteomics. Most of the functional genomics approaches rely upon the high-throughput transformation system useful for studying various gene identification strategies. The difficulty to transform a plant varies from species to species in legumes. There is limited success in exchange of the desirable characters by the classical and modern breeding technologies, in important pulse crop chickpea and biotechnological tools like plant tissue culture and genetic transformation techniques have emerged as a potential supplement. The major bottleneck is requirement of an in vitro manipulation of leguminosae members and the availability of reproducible, efficient and better plant regeneration methods. The regeneration and transformation of legumes particularly chickpea suffers due to recalcitrant nature towards rooting and transplantation of the in vitro regenerated plants. This becomes a limiting factor for the application of this technology towards designated mandate of crop improvement programs. This article discusses the hurdles and strategies for transformation of legumes in general and chickpea in particular.

INTRODUCTION

Leguminosae family members range from herbaceous annuals to woody perennials and have been domesticated for the production of food, forage, fibers, medicinal and industrial compounds etc. To understand the molecular basis of unique metabolic pathways that result in myriad use of legumes, is both a matter of scientific curiosity and of economic necessity for a country like India because of their importance in the sustainability of the mankind. The model legumes are being developed as experimental systems to study a number of key biological questions using molecular tools including genomics and proteomics. And most functional genomics approaches rely upon the high-throughput transformation system useful for studying various gene identification strategies.

Plant transformation is emerging as an important crop improvement tool. Most importantly transformation theoretically provides greater scope for the sources of genes for plant improvement, far beyond the available gene pool via sexual hybridization. Transformation also offers strategies for overexpression and silencing of endogenous genes and we can introduce new genes or manipulate endogenous gene expression by silencing. This generates new phenotypic variation useful for studying function of genes and for crop improvement.

The difficulty to transform varies from species to species in legumes. Like transformation in other organisms, legume transformation also requires development of:

(i) A source of totipotent cells acts as DNA acceptor
(ii) DNA delivery method into the target cells
(iii) Identification and selection of transformed cells

For recalcitrant legume transformation, regeneration is highly genotype specific and in vitro plant regeneration is considered to be an "art". This specifically requires substantial training and skills to produce transgenics which may be subsequently analyzed using biotechnological tools to produce a thesis or publication.

More often, the regeneration is slow and the transformation frequency (no. of transformed plants generated from each explant) is often low. Cocultivation of explants with Agrobacterium tumefaciens in species that are amenable to in vitro somatic embryogenesis, relatively rapid and efficient transformation methods have been developed. In many legume species, inducing somatic embryogenesis or organogenesis is difficult. Regeneration of shoots from the meristematic explants or from cotyledonary node after Agrobacterium infection is emerging as a rapid and relatively efficient method of transformation in a number of legume species including Lotus japonicas (Oger et al., 1996), Medicago truncatula (Trieu and Harrison, 1996) etc.

Another problem associated with legume transformation is production of low frequencies of tissue culture-induced phenotypic abnormalities in the transgenic plants. Thus, development of simple, rapid, reproducible transformation system that require the minimum amount of "art" like in planta transformation system for Arabidopsis (Clough and Bent, 1998), will have a similar impact on legume biology.

In the transformation of forage and pasture legumes, significant success has been achieved in the last decade. But, the progress in legume transformation is still underway especially in the case of pulses and grains. Progress in transformation of large-seeded legumes and more recent progress is presented in Table 1.

Scope for optimization of legume transformation

The primary sources of totipotent cells used in legume transformation systems are organogenic and somatic embryogenic cells. Across the chickpea species there is a series of available genotypes including different cultivars which are amenable to a specific tissue culture type. But, in others, only a specific genotype is used for the initiation of tissue culture and, consequently transformation. Thus, increasing the range of chickpea genotypes that can undergo the required tissue culture process would be a major contribution in improving the transformation system of chickpea and may be accomplished by the conventional manual approach by moderating and manipulating the composition of tissue culture media, plant hormones, source of explants and culture conditions. Genomics may also act as a rescue by the ectopic expression of genes or homologs from leguminosae that promotes and shifts vegetative to embryogenesis stage like Baby boom (Boutilier et al., 2002) and Wuschel (Zuo et al., 2002) and enhance their regeneration and transformation capacity. Using meristems as a source of totipotent cells will also be productive for legume transformation eg. is of soybean transformation (Hinchee et al., 1988).

Transformation studies in Cicer species

Chickpea (Cicer arietinum L.) a member of leguminosae family, is an important pulse crop and grown as third most important legume in the world (Dhar and Gurha, 1998). Chickpea is a good source of carbohydrate (48.2-67.6%), protein (12.4-31.5%), starch (41-50%), fat (6%) and nutritionally important minerals (Geervani and Umadevi, 1989). Chickpea is a good source of protein for the people in developing countries.

Plants respond to environmental stresses, and the transduced signals cause expression of numerous genes associated with stress tolerance. A number of genes have been described that respond to environmental stresses such as drought, high salinity and low temperature in plants. In last few years, many research groups have initiated studies on the role of gene expression changes in response to stress and characterization of transgenic plants expressing the stress responsive genes. Genetic engineering and plant tissue culture is useful for improving the stress tolerance of plants. Several methods have been attempted

Table 1 Progress in transformation of some economically important legumes

Plant species, Genoty pe	Explant	Agrobacterium	Regeneration *via*	Citation
Pea (CDC-vienna)	Embryonic axis	*A.tumefaciens*	Organogenesis	Polowick *et al,.* (2000)
Mung bean (K-851)	Cotyledonary node	*A.tumefaciens*	Organogenesis	Jaiwal *et al.,* (2001)
Lupin (Unicrop)	Axillary shoot embryonic axis	*A.tumefaciens*	Organogenesis	Pigeaire *et al*., (1997)
Soybean (Bert)	Cotyledonary node	*A.tumefaciens*	Organogenesis	Olhoft *et al*., (2003)
Chickpea (PG-1)	Embryonic axis	*A.tumefaciens*	Organogenesis	Krishnamurthy *et al.*, (2000)
Pigeonpea (Pusa-855)	Embryonic axis	*A.tumefaciens*	Organogenesis	Lawrence and Koundal, (2001)
Peanut (JL-24)	Cotyledons	*A.tumefaciens*	Organogenesis	Sharma and Anjaiah, (2000)

to improve the stress tolerance by gene transfer through plant tissue culture approaches. Improvement in resistance by conventional breeding is limited due to the lack of sufficient and satisfactory levels of genetic variability within cultivated chickpea germplasm. An effective and alternative approach is to transfer genes via genetic engineering, which is otherwise difficult to introduce through conventional breeding. A very important factor for the success of gene transfer depends on the availability of an efficient, successful and reliable in vitro regeneration protocol. Chickpea, like other large-seeded legumes, is recalcitrant for in vitro regeneration and genetic transformation, thereby posing difficulty in generation of transgenic plants. Although, many of the economically important plants have been improved regarding yield and productivity against different stresses, through genetic transformation, however legumes generally proved notoriously recalcitrant due to the lack of reliable in vitro regeneration system (Barna & Wakhlu, 1994; Odutayo et al., 2005). So, developing an efficient, successful and reliable genetic transformation method for chickpea therefore holds promise to complement conventional breeding strategies and to transfer stress resistant genes from other sources.With increasing global population, methods for producing stress tolerant, high yielding and better quality chickpea crops will be needed. However, the available gene pool has not permitted the breeder to tailor the crop to the extent that it can tolerate various stresses and become a competitive crop. Classical and modern breeding technology has resulted in limited success in the transfer of desired traits in legumes. The insertion of desired DNA into the plant genome and the regeneration of plants from such cells by microbiological, molecular biology and tissue culture methods is referred to as plant genetic manipulation. This allows a selected plant variety to be manipulated and modified in a highly specific manner yet with low frequency and success rate is dismal. These genetic transformations for incorporation of foreign and agronomically desired traits to improve plants in terms of quantity and quality have been achieved in few grain legumes. But, very few reports exist about important gene transfer in orphan legume, chickpea. Few reports are- fertile transgenic plants (Pathak and Hamzah, 2008), fertile transgenic plants and glucuronidase gene expression upto 4th generation as reported by Polowick (Polowick et al., 2004) etc.

Agrobacterium tumefaciens and A. rhizogenes both have been employed for the genetic engineering in chickpea. A. rhizogenes produces hairy roots in “composite” plants i.e. an untransformed plantlet with hairy roots. Such composite plants find use in functional analysis related to root characteristics like root diseases & nodulation and do not transmit the transgenic trait to their offsprings, thus, are of little use in crop improvement (Somers et.al., 2003). The hairy roots were induced in some cultivars by few wild strains of A. rhizogenes (Seifkes-Boer et al., 1995). In all the protocols, except that reported by Pathak and Hamzah (2008), the transformation frequency was very low.

In vitro regeneration of cicer species

There is limited success in exchange of the desirable characters by the classical and modern breeding technologies, in this important pulse crop i.e. chickpea and biotechnological tools like plant tissue culture and genetic transformation techniques have emerged as a potential supplement. These have paved the way for creation of an elite germplasm and crop improvement (Sharma et al., 2006).

But the major bottleneck is requirement of an in vitro manipulation of leguminosae members and the availability of reproducible, efficient and better plant regeneration methods. To add to the woes, reliable transformation protocols are wanting, due to recalcitrant nature of chickpea (Polowick et al., 2004) and also the asynchronous shoot bud production (Krishnamurthy et al., 2000). In majority of the published work the regeneration procedures are mostly not repeatable or work only in few research labs, compelling us to believe that chickpea regeneration is extremely recalcitrant.

Till now many research groups are dealing with this objective, but several regeneration and transformation procedures involving organogenesis and somatic embryogenesis have different success rates (Singh et al., 2003). And effective chickpea regeneration has been possible only through the use of explants derived from shoot apices and cotyledonary nodes (Singh et al., 2003). There are two major hurdles that limit in vitro regeneration and transformation of chickpea

(i) induction and development of strong root system i.e. problems related to rooting

(ii) establishment of in vitro raised plantlets

The regeneration and transformation of legumes particularly chickpea suffers setback due to rooting and transplantation of the in vitro regenerated plants. This becomes a limiting factor for the application of this technology towards designated mandate of crop improvement programs. In order to escape from these hurdles, researchers have preferred to go for grafting (Krishnamurthy et al., 2000) but generally it is tedious and time consuming apart from special skills and art. The protoplast culture offers exciting possibilities in the field of somatic cell genetics and crop improvement and isolation and regeneration of chickpea protoplasts from hypocotyl derived protoplasts cultured in specialized medium supplemented with phytohormones produced microcalli, but microcalli were unable to undergo differentiation and organogenesis to yield plantlets (Sagare and Krishnamurthy, 1991). By optimization of various tissue culture variables to develop an efficient and reproducible transformation and regeneration protocol, better regeneration and transformation efficiency was achieved (Jayanand et al., 2003). In this method, there was use of axillary meristem explant formed by removing the axillary bud (Jayanand et al., 2003).

Organogenesis in chickpea

The process by which cell or any group of cells differentiates to form organs is called organogenesis. Organogenesis is commonly induced in agriculturally important plants by the manipulation of exogenous phytohormone levels. It occurs either directly from tissue explants or from unorganized mass of cells called callus. Organogenesis in legumes is controlled by explants type and age, different combination of auxin/cytokinin and genotypes.

Direct organogenesis

The shoot proliferation from pre-existing meristem instead of de novo formation of a meristem is termed as direct organogenesis and it bypasses the callus phase and shoot morphogenesis occurs directly from the cultured explants. Majority of the researchers have used either MS medium (Singh et al., 1997) or Gamborg's B5 medium (Reddy and Vani, 1997) and combination thereof (Kar et al., 1996) are also being used. Auxin promotes rooting, cytokinin induces shooting, the low auxin : cytokinin ratio regenerates shoots and a high auxin-cytokinin ratio promotes rooting, from the already regenerated shoots (Skoog and Miller, 1957). Thus, identification of appropriate auxin-cytokinin balance, the art of modulation and application is more critical in promoting organogenesis of the legume in vitro. Benzyladenine (BA) promotes

and various concentrations of BA were tried to induce multiple shoots (Polisetty et al., 1997). At higher BA concentration i.e. 25, 37.5 and 50 µM, symptoms observed were browning, thin weak shoots and shoot tip decay. In chickpea, BAP (6-benzyl-aminopurine) is the most widely used cytokinin for shoot regeneration (Polisetty et al., 1997). The other cytokinins kinetin (6-furfuryl aminopurine) (Reddy and Vani, 1997) and zeatin (6-(4-hydroxy-3-methyl but-2-enylamino)-purine (Hita et al., 1997) are also being used for regeneration studies.

N-phenyl-N'-(1-2-3-thidiazol-5-yl) urea or thidiazuron (TDZ) has high cytokinin like activity, stimulates shoot proliferation and very useful for rapid regeneration protocol by direct organogenesis (Murthy et al., 1996). TDZ has potential for in vitro morphogenesis in many plant species including grain legumes (Murthy et al., 1998). TDZ produces higher frequencies of shoot regeneration as compared to BA and BAP (Rajender-Singh et al., 2002). However, higher concentration of TDZ (5µM) negatively affected shoot proliferation. Among the different cytokinins, effectiveness of cytokinin was in the order of TDZ>BAP>KIN>ZET (Sharma and Amla, 1998).

Now, to standardize the regeneration protocol, type of explants used holds importance. In chickpea, choice of explants includes cotyledon, seed explants, cotyledon segments, shoot or meristem tip, hypocotyls, epicotyl, embryo etc. The size, age and orientation of explants used are also equally important. Higher rate of regeneration was seen in larger sized cotyledons in comparison to small size and the chickpea cotyledons when cultured abaxially, showed high regeneration frequency (Batra et al., 2002). In most of the plants, the in vitro morphogenesis is highly species specific and also genotype specific, within species. This observation applies to chickpea also and many research groups are involved to standardize the regeneration protocol for the given desired variety. The organogenic response differs greatly within the genotypes. For eg. cotyledons of ICC-11525 gave greater number of shoots than ICC-4951 and ICC-10301. The variety BG-256 showed best response for direct regeneration followed by C235 (Singh et al., 1997). Amongst ICCV variety, ICCV-10 gave better response than ICCV-2 and ICCV-37 (Reddy and Vani, 1997).

Callus mediated organogenesis

Caulogenesis is a type of organogenesis by which only adventitious shoot bud initiation takes place in the callus tissue. It is essential for the application of in vitro culture technique in crop improvement. Caulogenesis is influenced by many factors such as genotype, explants, media, phytohormone combinations. The most commonly used MS medium and Gamborg B5 medium supplemented with various auxin cytokinin combinations was used to induce callus (Roy et al., 2001). In chickpea, callus induction is by many explants such as cotyledon, epicotyl, hypocotyl, new leaflets, meristem, internodes, cotyledonary nodes, immature embryos and root tips (Vani and Reddy, 1996). However, better callus induction was seen in source of explants was immature cotyledons, immature leaflets, epicotyls and embryo axes (Barna and Wakhlu, 1994). Differential genotypic response for callus regeneration into shoot emphasized the aim of standardization of protocol that is genotype specific (Rao and Chopra, 1989).

Somatic embryogenesis

The formation of bipolar embryo like structure from the somatic cell is known as somatic embryogenesis and of great importance in plant tissue culture and regeneration studies. Somatic embryogenesis is useful for production of somaclonal variants, clonal multiplication and genetic transformation studies (Hartweek et al., 1988). In chickpea, induction of globular-stage somatic embryos from young leaflets (Ghanti et al., 2002), embryonic axis (Richa-Chauhan and Singh, 2002) and mature seeds (Rizvi et al., 2002) have been reported. Different mode of somatic embryo initiation was due to the use of different cultivars, explants and in-vitro conditions (Sagare et al. 1995).

Rhizogenesis and establishment of plantlets

The initiation and formation of root system in plants is known as rhizogenesis. The plant's survivability depends on the number and type of roots produced and for better establishment of plants in the field, well developed rooting system is needed. Likewise shoot regeneration, rooting is also dependent on the phytoregulators, media variability, in-vitro conditions photoperiod, genotype etc. The quality and number of roots produced per shoot is determined by the concentration and type of auxins (Mallikarjuna et al., 1993). Auxins such as IAA, IBA, NAA and cytokinins like kinetin and BAP were the chosen rooting inducers in chickpea. For root induction, IBA was proved to be the best rooting factor in chickpea (Brandt and Hess, 1994). A new method, by placing the regenerated shoots on a filter paper bridge immersed in MS liquid medium supplemented with IBA was developed by Jayanand et al., (2003). The response of rooting also depends on the nature of media i.e. between liquid and solid rooting induction (RIM) medium supplemented with auxins (Romero et al., 1998). As compared to solid medium, the induced roots were thicker and longer in liquid medium.

Recalcitrant nature of chickpea has posed problems in development of efficient regeneration protocol and ultimately establishment of plantlets in soil. The acclimatization of plantlets was better on pure vermiculite and vermiculite perlite mixture. There are various potting media like black and red soil, smooth and coarse sand, and vermiculite either alone or in combination with each other and better survival was seen in coarse sand.

CONCLUSION

Plants are subjected to a large number of stresses and these adverse conditions may interfere with the normal growth and development which ultimately results in lower food quality and yield. There are several economically important grain legumes including chickpea that play significant role in nutrition of the rural and urban poor in developing world. With increasing global population, methods for producing stress tolerant, high yielding and better quality chickpea crops will be needed. Genetic engineering and plant tissue culture is useful for the genetic manipulations of plants. There are several reports for the in vitro regeneration of the chickpea plants from different explants as we have seen above. But very few reports on successful regeneration after genetic transformation of chickpea is available, due to the recalcitrant nature of this crop towards commonly applied tissue culture technique. Since, it is highly recalcitrant plant there is an urgent need to study and design a regeneration protocol that must ensure easy qualitative multiple shoots with effective root system.

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REFERENCES

1. Barna KS and Wakhlu AK (1994) Whole plant regeneration of Cicer arietinum from callus cultures via organogenesis. *Plant Cell Reports* 13(9):510-513
2.bBatra P, Yadav NR, Sindhu A, Yadav RC, Chowdhury VK, Chowdhury JB (2002) Efficient protocol for in vitro direct plant regeneration in chickpea. *Indian Journal of Experimental Biology* 40:600-602
3. Boutilier K, Offringa R, Sharma VK, Kieft H, Ouellet T, Zhang L, Hattori J, Liu CM, van Lammeren AAM, Miki BLA et al. (2002) Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. *Plant Cell* 14:1737-1749
4. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium mediated transformation of Arabidopsis thaliana. *Plant J* 16:735-743
5. Dhar V and Gurha SN (1998) Integrated Management of Chickpea Diseases. Integrated Pest and Disease Management. Upadhyay RK, Mukerji KG, Chamola BP and Dubey OP (eds.) APH Pub. Co., New Delhi, India pp. 249
6. Geervani P and Umadevi T (1989) Effect of maturation of nutrient composition of selected vegetable legumes. *J Sci Food Agric* 46:243-248
7. Ghanti KS, Rao S, Venugopal RB, Rao SR, Kaviraj CP, Barad A (2002) Somatic embryogenesis and Agrobacterium-mediated transformation in chickpea (Cicer arietinum L.). *Plant Cell Biotechnology and Molecular Biology* 3(1-2):51-58
8. Hartweek LM, Lazzeri PA, Cui D, Collins GB, Williams EG (1988) Explant orientation effects on somatic embryogenesis from immature soybean cotyledons. *In vitro Cell Division and Biology* 24:821-828
9. Hinchee MAW, Connor-Ward DV, Newell CA, McDonnell RE, Sato SJ, Gasser CS, Fischhoff DA, Re DB, Fraley RT, Horsch RB (1988) Production of transgenic soybean plants using Agrobacterium-mediated DNA transfer. *Nat Biotechnol* 6:915-922
10. Hita O, Lafarga C, Guerra H (1997) Somatic embryogenesis from chickpea (Cicer arietinum L.) immature cotyledons: The effect of zeatin, gibberllic acid and indole-3-butyric acid. *Acta Physiologiae Plantarum* 19:333-338
11. Jayanand B, Sudarsanam G, Sharma KK (2003) An efficient protocol for the regeneration of whole plants of chickpea (Cicer arietinum L.) by using axillary meristem explants derived from in vitro germinated seedlings. In Vitro Cell. Dev. Biol. Plant 39, 171-179
12. Jaiwal PK, Kumari R, Ignacimuthu S, Potrykus I, Sautter C (2001) Agrobacterium tumefaciens-mediated genetic transformation of mungbean (Vigna radiata L. Wilczek): a recalcitrant grain legume. *Plant Sci* 161:239-247
13. Krishnamurthy KV, Suhasini K, Sagare AP, Meixner M, de Kathen A, Pickardt T, Schieder O (2000) Agrobacterium mediated transformation of chickpea (Cicer arietinum L.) embryo axes. *Plant Cell Rep* 19:235-240
14. Lawrence PK, Koundal KR (2001) Agrobacterium tumefaciens-mediated transformation of pigeon pea (Cajanus cajan L. Millsp.) and molecular analysis of regenerated plants. *Curr Sci* 80: 1428-1432
15. Mallikarjun N, Moss JP, Mallikarjun N (1993) Regeneration from elite chickpea genotypes. International Chickpea Newsletter 29:4-6
16. Murthy BNS, Murch SJ, Saxena PK (1998) Thidiazuron: A potent regulator of in vitro plant morphogenesis. In Vitro Cell Dev. *Biol Plant* 34:267-275
17. Murthy BNS, Victor J, Singh RP, Fletcher RA, Saxena PK (1996) In vitro regeneration of chickpea (Cicer arietinum L.) stimulation of direct organogenesis and somatic embryogenesis by thidiazuron. *Plant Growth Regulation* 19:233-240
18. Odutayo OI, Akinirinusi FB, Odunbososye I, Oso RT (2005) Multiple shoot induction from embryo derived callus cultures of cowpea (Vigna ungimculata L.). Walp. *African J. of Biotechnology*, 4:1214-1216
19. Oger P, Petit A, Dessaux Y (1996) A simple technique for direct transformation and regeneration of the diploid legume species Lotus japonicus. *Plant Sci* 116:159-168
20. Olhoft PM, Flagel LE, Donovan CM, Somers DA (2003) Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. Planta 216(5):723-35
21. Pathak MR, Hamzah RY (2008) An effective method of sonicated assisted Agrobacterium-mediated transformation of chickpea. *Plant Cell Tissue Organ Cult* 93:65-71
22. Pigeaire A, Abernethy D, Smith PM, Simpson K, Fletcher N, Lu CY, Atkins CA, Cornish E (1997) Transformation of a grain legume (*Lupinus angustifolius L.*) via Agrobacterium tumefaciens-mediated gene transfer to shoot apices. *Mol Breed* 3: 341-349
23. Polisetty R, Paul V, Devashwar JJ, Khetarpal S, Suresh K, Chandra P (1997) Multiple shoot induction by benzyladenine and complete plant regeneration from seed explants of chickpea (Cicer arietinum L.) *Plant Cell Report* 16(8):565-571
24. Polowick PL, Baliski DS, Mahon JD (2004) Agrobacterium tumefaciens mediated transformation of chickpea (Cicer arietinum L.): gene integration, expression and inheritance. *Plant Cell Rep*. 23: 485-491
25. Polowick PL, Quandt J, Mahon JD (2000) The ability of pea transformation technology to transfer genes into peas adapted to western Canadian growing conditions. *Plant Sci* 153:161-170
26. Rajender-Singh Jat, Sahoo RS, Srinivasan PD, Singh R (2002) Thidiazuron induced multiple shoot formation in chickpea (Cicer arietinum L.). *Journal of Plant Biochemistry and Biotechnology* 11(2):129-131

27. Rao BG, Chopra VL (1989) Morphogenesis in callus cultures of chickpea. *International Chickpea Newsletter* 21:7-11
28. Reddy KJ and Vani KD (1997) Differential response of excised embryo in induction of multiple shoots in different genotypes of *Cicer arietinum L. Advances in Plant Sciences* 10:79-82
29. Richa-Chauhan, Singh NP, Chauhan R (2002) Plant regeneration via somatic embryogenesis in chickpea (Cicer arietinum L.) . *Indian Journal of Genetics and Plant Breeding,* 62(4):319-321
30. Rizvi SMH, Jaiwal PK, Singh RP (2002) A possible involvement of cellular polyamine level in thidiazuron induced somatic embryogenesis in chickpea. Role of Plant Tissue Culture in Biodiversity Conservation and Economic Development 163-175
31. Romero MD, Moreno MT, Cubero JI, Gil J (1998) An efficient in vitro rooting method for chickpea. *International Chickpea Newsletter* 5:18-19
32. Roy PK, Lodha ML, Mehta SL (2001) In vitro regeneration from intermodal explants and somaclonal variation in chickpea (*Cicer arietinum L.*). *Journal of Plant Biochemistry and Biotechnology* .10(2):107-112
33. Sagare AP, Krishnamurthy KV (1991) Protoplast regeneration in chickpea (*Cicer arietinum L.*). *Indian J. Exp. Biol.* 29:930-932
34. Sagare AP, Suhasini K, Krishnamurthy KV (1995) Histology of somatic embryo initiation and development in chickpea (*Cicer arietinum L.*). *Plant Science,* 109(1):87-93
35. Seifkes-Boer HJ, Noonan MJ, Bullock DW, Conner AJ (1995) Hairy root transformation system in large-seeded grain legumes. I*sr. J. Plant Sci.* 43:1-5
36. Sharma KK, Anjaiah V (2000) An efficient method for the production of transgenic plants of peanut (Arachis hypogaea L.) through Agrobacterium tumefaciens-mediated genetic transformation. *Plant Sci* 159:7-19
37. Sharma KK, Bhatnagar-Mathur P, Jayanand B (2006) Chickpea (Cicer arietinum L.). In: Kan W (ed) Agrobacterium protocols (Methods in molecular biology), vol 44. Humana Press Inc., Totowa, pp 313-323
38. Singh A, Singh NP, Asthana AN (1997) Callus induction and direct regeneration from immature embryo in chickpea. *International Chickpea and Pigeonpea Newsletter* 4:39-40
39. Skoog F, and Miller CO (1957) Chemical regeneration of growth and organ formation in plant tissue culture in vitro. *Symposium on Society of Experimental Biology* 11:118-131
40. Somers DA, Samac DA, Olhoft PM (2003) Recent advances in legume transformation. *Plant Physiol.* 131:892-899
41. Trieu AT, Harrison MJ (1996) Rapid transformation of Medicago truncatula: regeneration via shoot organogenesis. *Plant Cell Rep* 16:6-11
42. Zuo J, Niu QW, Frugis G, Chua NH (2002) The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. *Plant J* .30:349-360