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Review

No more recalcitrant: Chickpea regeneration and genetic transformation

Firoz Anwar[2]*, P. Sharmila[1] and P. Pardha Saradhi[1]

[1]Department of Environmental Biology, North Campus, University of Delhi, Delhi, India.
[2]Plant Production Department, Biotechnology Division, King Saud University, Riyadh Kingdom of Saudi Arabia.

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Chickpea production is limited worldwide because of abiotic and biotic stresses. Efforts to overcome these production constraints through traditional breeding are difficult due to limited genetic variation. Novel regeneration is pre-requisite for genetic transformation offers the opportunity to overcome hybridization barriers and introduce novel genes for resistance. Although direct gene transfer via direct DNA transfer has been reported, *Agrobacterium* mediated transformation is the preferred method and standard protocols have been established for the production of transgenic plantlets derived from co-cultivation of embryonic axes. This was soon adopted due to difficulties associated with regeneration of whole plants from callus. Only few reports have been reported using genetic transformation/ transgene(s) against abiotic stress tolerance transgenic chickpea plants. Transgenic chickpea using bacterial *codA* gene tolerance against abiotic stresses have been developed. Chickpea improvement and application of genomics tools to the study of the chickpea genome will be enhanced through the use of genetic transformation.

Key words: *Cicer arietinum* L., TDZ, Indole butyric acid, (IBA), efficient rooting, aeration, transplantation, transformation.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important food crop in much of the developing world and ranks third in production among food legumes. India accounts for 75% of the world's production and is grown in rotation with cereal crops to break disease cycles, allow grassy weed control and improve soil nutrient status through the return of atmospheric nitrogen fixed through symbiosis. Chick-pea is good as a source of carbohydrate (48.2 - 67.6%), protein (12.4 - 31.5%), fat (6%) and nutritionally important minerals. Among the legumes, chickpea is the best hypo-cholesteremic agent, followed by black gram and green gram. Direct shoot organogenesis and establishment of plantlets from different explants of chickpea was reported earlier (Polisetty et al., 1996, 1997; Paul et al., 2000; Rizvi and Singh, 2000; Chauhan et al., 2003; Jayanand et al., 2003; Chakraborti et al., 2006; Table 1). Plantlets were developed through direct somatic embryogenesis and through callus from different explants of chickpea {Barna and Wakulu, 1993; Sagare et al., 1993; Suhasini et al., 1994; Kumar et al. 1994, 1995; Rizvi and Singh, 2000; Chauhan et al., 2002; Kar et al., 1996, 1997; Kiran et al., 2005 (Table 2)}.

Inspite of several reports of successful regeneration, chickpea is widely considered to be highly recalcitrant (Shri and Davis, 1992; Vani and Reddy, 1996; Rizvi and Singh, 2000; Polowick et al., 2004). Surprisingly, majority of published/reported chickpea regeneration protocols often are either not repeatable or work only in certain research laboratories, making researchers to believe that chickpea regeneration is highly recalcitrant. Two major hurdles that limit *in vitro* regeneration of chickpea are (i) induction and development of strong root system and (ii) establishment of *in vitro* raised plantlets in pots. In order to escape from these hurdles, researchers have preferred to go for grafting (Krishnamurthy et al., 2000; Sarmah et al., 2004; Senthil et al., 2004; Tewari-Singh et al., 2004; Sanyal et al., 2005). In general, grafting is tedious and time consuming requiring special skills. Moreover, graf-ting besides requiring additional seed lot, also might promote emergence of shoots/branches from axillary

*Corresponding author. E-mail: anfiroz@gmail.com.

Table 1. Regeneration in various genotypes of chickpea.

Cultivar	Medium	Growth regulators	Explants	Rooting	Responses	References
B115, C-235,	MMS	BAP, NAA, IAA	Single cotyledon with ½ embryo	Grafting	Establishment of plants	Chakraborti et al., 2006
C-235	MS	TDZ, 2-iP, Kinetin, GA_3, IBA	Embryonic axis	MS +IBA 5 µM/ 10 mM Pulse	Establishment of plants	Jayanand et al., 2003
C-235, k-850, BG-256, PGD- 84-10	MS MS salt+B_5	IAA, NAA, IBA Kinetin, BAP	a. Mature embryo b. Immature embryo	MS + 10.7 µM NAA + 9.8 µM IBA	Establishment of plants	Chauhan et al., 2003
P-256	MS	TDZ, Proline	Whole Seedling (cotyledonary notch region)	MS + 2.5 µM NAA	Establishment of plants	Rizvi et al., 2002
P-256	MS	TDZ, BAP, IBA, Spermidine, Putrescine, Potassium phosphate	Immature leaflet	MS + 2.5 µM IBA	Establishment of plants	Rizvi and Singh, 2000
C-235, BG-329	MS salts + B_5 vitamins	BAP	Excised root ip from two day old seedlings	Modified MS	Establishment of plants	Paul et al., 2000
BG-362,BG-329, BG-267, BG-256, C-235	MS Salts + B_5 Vitamins	Different concentrations of BA	a) Embryo axis with both cotyledons b) as in (a) except the tip of radicle c) As in (a) except one of the cotyledons d) as in (c) except the tip of radicle	$1/4^{th}$ MS	Multiple shoots and thin plants were developed	Polisetty et al., 1997
BG-362, BG-329 BG-267, BG-256 C-235	MS	NAA, IBA	Shoot tips	$1/4^{th}$ MS	Esablishment of plants	Polisetty et al., 1996
ICCV-1, ICCV-6 and Desi Variety	MS Macro + 4xMS Micro + B_5 Vitamins	BAP, NAA, IBA	Embryonic axis without apical root and shoot part	MS + 0.01 mg l^{-1} IBA	Formation of plants	Kar et al., 1996
JG-62 C-235, PGC1	B_5	BA, Kinetin, IAA	Epicotyl	B5 + 4 mg l^{-1}/l IAA + 0.5 mg l^{-1} KN	Multiple Shoots and shoots were rooted	Vani and Reddy, 1996
C-235	MS	2.4D, BAP, NAA, IBA, Picloram	Leaflet	MS + 1 µM IBA	Formation of plants	Barna and Wakhlu, 1994
Annigeri, ICCV6	B_5	BAP, IBA, Kin,	a. Meristem tips (apical meristem and leaf primordia), b. Cotyledonary-nodes	MS + 2.5 µM IBA	Multiple shoots and shoots were rooted	Brandt and Hess, 1993

buds that are often retained in the axils of cotyledons and the later might dominate over the grafted shoots.

The following section is a brief overview on chickpea regeneration and genetic transformation and confirmed

Table 1. Contd.

Cultivar	Medium	Growth regulators	Explants	Rooting	Responses	References
Pusa-256	MS	NAA , IBA	Shoot tip	MS + 2 mg l^{-1} NAA + 2 mg l^{-1} IBA	Multiple shoots and individual shoots were rooted	Chandra et al., 1993
Local Ecotype	MS	Kinetin, BAP, NAA, IAA	Shoot tips	MS + 0.5 mg l^{-1} IAA + 0.05 mg l^{-1} Kin	Multiple shoos and individual shoots were rooted	Fontana et al., 1993
C-235	MS	2.4D, BAP, NAA	Leaflet	-	Multiple shoot	Barna and Wakhlu 1993
C-235, H-208	B_5	BAP, NAA	a) White Seed b) Shoot tip c) Cotyledon	-	Multiple shoots	Prakash et al., 1992
Kabuli	MS salts + B_5 Vitamins	TDZ, NAA	Mature Seeds	-	Spontaneous shoot differentiation on prolonged culture of seedlings	Malik and Saxena. 1992.
ICC 640	B_5	Zeatin, IAA, BA	Immature Cotyledons with excised embryonal axis	-	White cotyledon like structures (CLS) formed at the proximal end of the cotyledons; multiple shoots initiation near the base of CLS.	Shri and Davis 1992
ICCC-4 Annigeri, H-08	B_5	BAP, Kinetin, IAA	a) Hypocotyl b) Shoot tip	-	Multiple shoots	Neelam et al., 1986
G543, G130, L550	MS	2, 4 -D, IAA, NAA, Kinetin, BA	Shoot tip meristem	0.1 mg l^{-1} NAA + 0.02 mg l^{-1} BA	Shoots with root	Bajaj and Dhanju, 1979.

that chickpea is no more recalcitrant.

REGENERATION OF CHICKPEA

Direct shoot organogenesis from different explants of chickpea

A procedure to initiate shoot regeneration from various explants derived from mature seeds germinated on a medium containing cytokinins or cytokinins like substances and/ or auxin has been developed using *in vitro* culture of seeds in the presence of TDZ. Among the different cytokinins used in this study, a higher frequency of adventitious shoot buds/multiple shoots formation was observed in TDZ containing medium (Anwar et al., 2008). TDZ induces high frequency of somatic embryogenesis/ adventitious bud formation in some plant species, either alone or in combination with other growth regulator/s (Murthy et al., 1998, 1995; Chen and Chang, 2006; Delvin, 1989; Ganeshan et al. 2003; Huetteman and Preece, 1993; Onamu, 2003; Mroginski, 2004; Shan, 2000; Sharma, 2005; Tang and Newton, 2005, 2006 and reference cited in). A substituted phenyl urea derivative was found to be very effective for induction of multiple shoots from nodal and basal regions of the primary epicotyl (Malik and Saxena, 1992). MS salts and B_5 vitamins supplemented with 5 µM benzyl aminopurine (BAP) has been found to be a highly effective medium for multiple shoot formation from intact seedlings (Polisetty et al., 1997). This procedure excludes the selection and preparation of explants and complex manipulation of cultural condition. Multiple shoots have also been regenerated directly from immature cotyledons on B_5 basal medium containing zeatin (13.7 µM) and indole acetic acid (IAA) (0.2 µM) (Shri and Davis, 1992).

Regeneration of multiple shoots has been achieved from seedling. Shoot tips excised from field grown plantlets gave rise to multiple shoots when cultured on MS medium supplemented with IAA (2 mg l^{-1}) and Kinetin (0.5 mg l^{-1}) (Bajaj and Dhanju, 1979). Multiple shoots have been induced from shoots tips cultured on MS medium supplemented with naphthalene acetic acid (NAA) (2 mg l^{-1}) and indole butyric acid (IBA) (2 mg l^{-1}) (Chandra et al., 1993; Polisetty et al., 1996). Epicotyl explants gave rise to multiple shoots when cultured on B_5 medium containing BA (1 mg l^{-1}) and kinetin (1 mg l^{-1}) + IAA (0.5 mg l^{-1}) (Vani and Reddy, 1996). Various workers have achieved multiple shoots from embryo axis apical meristem cultured on MS containing kinetin (1 mg l^{-1})

Table 2. Somatic embryogenesis and plant development in various genotypes of chickpea.

Cultivar	Medium	Growth regulators	Explants	Responses	References
Direct Somatic Embryogenesis					
1. ICCV-10, Annigeri	MS	Picloram, 2, 4 -D, 2, 4, 5 -T, NAA, BAP, Kinetin	Hypocotyls	Plants developed from somatic embryos	Kiran et al., 2005
2. PG12, C-235	MS	2, 4, 5 -T, 2, 4 -D, BAP, Kinetin	a. Immature (IM)-cotyledon, b. IM embryo, c. Mature embryo, d. Leaf	Somatic embryo	Sagare et al., 1999
3. PG-5 PG-12 C-235	MS	2, 4, 5 -T, 2, 4 -D	Mature embryo	Plants developed from somatic embryo	Sagare et al., 1993
4. PG12, C-235	MS	2, 4, 5 -T, 2, 4 -D, BAP	Immature-cotyledon	Plants developed from somatic embryos	Sagare et al., 1993
5. PG12	MS	2, 4, 5 -T	Mature embryo axis	Plants developed from somatic embryos	Suhasini et al., 1994
6. Kabuli	MS salts + B_5Vitamins	TDZ , L-Proline	Mature seeds	Somatic embryos	Murthy et al., 1996
Somatic Embryogenesis via Callus					
C235, K-850, BG-256	MS MS + B_5	2, 4 -D, NAA, IBA, BAP, Kinetin,	Embryonal axis	Plants developed from somatic embryos	Chauhan et al., 2002
P-256	MS	TDZ, Proline	Whole Seedling (cotyledonary notch region)	Plants developed from somatic embryos	Rizvi et al., 2002
6153, CM72	MS	NAA, BAP, 2, 4 -D, Kinetin, IAA, IBA	Hypocotyls	Somatic embryo	Hussain et al., 2000
PG12	MS	2, 4, 5 -T	Mature embryo	Somatic embryo	Sagare et al., 1999
ICCV-4918	B_5	2, 4, 5 -T, BAP	Immature cotyledons	Somatic embryos	Ramana et al., 1996
BG256	MS, B_5	2, 4 -D, BAP, Picloram, IAA	Leaf	Plant from somatic embryo	kumar et al., 1995
C-235, JG-262 P-144, P-209	MS	2, 4 -D, Kinetin	Leaf	Plants developed from somatic embryos	Kumar et al., 1994
C-235	MS, B5, MS salt+B_5 vitamins	2, 4 -D, Kinetin, BAP, IBA	Leaf	Plants developed from somatic embryos	Kumar et al., 1994
Phule-G5	MS	2, 4 -D, Picloram, Kinetin	a. Root, b. cotyledonary nodes, c. epicotyl, d. nodes, internodes and leaf	Somatic embryo	Shanker and Mohanram, 1993
JG-62, Gaurav, Anngeri, BG-267 C-235	MS	2, 4 -D, BAP, Kinetin, TDZ	Immature leaflet	Plants developed from somatic embryos	Barna and Wakhlu, 1993
BG- 256	MS	2, 4 -D, BAP	Leaflet	Multiple shots/somatic embryos	Rao and Chopra VL, 1989

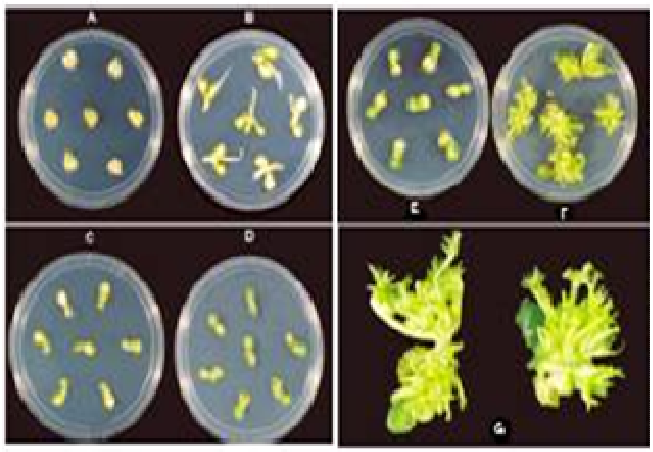

Figure 1. A: Seeds de-coated after 14 h imbibition, on shoot induction medium (SIM) (MS medium supplemented with 4 µM, 10 µM 2-iP and 2 µM Kin); B: Chickpea seedlings (6 d after incubation of de-coated seeds on SIM) used for obtaining explants for regeneration and transformation; D & E: Swollen embryonal axis attached to cotyledon showing the initiation of shoot induction {6 - 8 days after incubating the explants on SIM}; D: Two weeks old culture of embryonal axis with single cotyledon, showing the emergence of large number of adventitious buds {15 days after incubating 6 - 8 days old cultures from SIM on MS medium}.

(Fontana et al., 1993) or on medium containing MS macro 4 X MS micro, B_5 vitamins, BAP (3 mg l^{-1}) and NAA (0.004 mg l^{-1}) (Kar et al., 1996, 1997). C-235 and BG-329 seedling explant were used in MS salt + B_5 vitamins + 5 µM BAP and checked for the effect of BAP induction period on shoot differentiation. With increase in the duration of culture, there was increase in the number of shoot buds with a simultaneous decrease in further elongation in the elongation medium (Paul et al., 2000). Direct regeneration from mature and immature embryo as explants led to maximum regeneration frequency as observed from mature embryo in MS salts + B_5 vitamins + 4% sucrose which was further elongated on MS + 0.6 µM IBA + 8.8 µM BAP + 4% sucrose (95% regeneration) followed by 50% on MS + 0.6 µM IBA + 4.4 µM BAP + 4% sucrose. Immature embryo regenerated better in liquid medium using filter paper bridge when compared to solid medium. Regeneration frequency of 52% was observed in liquid medium (MS + 2.8 µM IAA + 2.3 µM) (Chauhan et al., 2003). Over 100 multiple shoots were observed in embryonal axis explants from seedling (C-235) in induction medium (MS + 4 µM TDZ + 10 µM 2-iP + 2 µM kinetin, pH 5 - 5.5) for about 6 - 7 weeks, multiple shoots were elongated on MS containing 5 µM 2-iP + 2 µM kinetin, pH 5 - 5.5 (SEM1), non elongated shoots on SEM1 were transferred on SEM2 (MS + 2 µM GA_3, pH 5-5.5) (Jayanand et al., 2003; Sharma et al., 2007). Maximum 26 shoots were induced from single cotyledon with half embryonal axis in MS medium supplemented with 1.6 mg l^{-1} BA, 0.04 mg l^{-1} NAA and shoots elongate in the presence of 0.2 mg l^{-1} IAA (Chakrabarti et al., 2006); > 100 shoots per explant (single cotyledon with half embryonal axis) in MS medium supplemented with 4 µM, 10 µM 2-iP and 2 µM Kin (Anwar, 2007; Anwar et al., 2008, 2009) (Figure 1C).

Explants consisting of single cotyledon with half embryonal axis obtained from 6 day old seedlings (Figure 1B) on MS medium supplemented with 4 µM TDZ, 2 µM kinetin and 10 µM 2-iP was found to be good for producing optimal shoot/adventitious buds from the explants. Embryonal axis attached to cotyledon showed significant swelling and exhibited initiation of shoot induction within a duration of 6 - 8 days (Figures 1C - E). Use of TDZ with purine ring containing cytokinins such as kinetin, BAP and N6-[2-Isopentyl]adenine (2-iP) has been shown to promote the formation of a large number of healthy shoots (Eisinger, 1983; Mroginski and Kartha, 1984; Radhika et al., 2006). In order to avoid negative impact of TDZ in the formation of shoots, it was found wise to transfer the explants immediately after shoot induction event to a medium devoid of TDZ. Cytokinin such as 2-iP and kinetin are well known to promote rapid shoot multiplication (Jayanand et al., 2003; Kiran et al., 2005). Even in this investigation, TDZ at 4 µM in combination with 2-iP (10 µM) and kinetin (2 µM) was found to be optimal for the expansion of meristematic zone followed by shoot induction. Subsequent to the transfer of the cultures onto MS basal medium that is growth regulator free medium, emergence of a large number of adventitious shoots/ buds from all over the surface of the swollen embryonal axis was recorded within 7 - 15 days. In general, 50 - 100 shoots arose from each explant within a time period of 15 days (Figures 1F - G). The induced multiple shoot/adventitious buds were excised from the bunch, without any callus or globular structures and cultured on the shoot elongation medium (SEM) consisting of MS medium supplemented with 5 µM 2-iP and 2 µM kinetin for 10 days. They were then routinely subcultured at an interval of 10 - 15 days on SEM (Figure 2).

Gibberellins promote elongation of shoots (Jayanand et al., 2003; Sharma et al., 2007), but during the present investigation, it was realized that the shoots transferred to plant growth regulator free medium have potential to synthesize and maintain desired endogenous levels of gibberellins and other auxins. Although, elongation of shoots was promoted on MS medium supplemented with various cytokinins in combination with GA_3, stronger/ healthy elongated shoots were obtained on MS medium supplemented with kinetin and 2-iP in the absence of GA_3. GA_3 promoted elongation of the shoots but the shoots were weak with more inter-nodal elongation. Therefore, for subsequent experiments, shoot elongation was achieved on MS medium supplemented with kinetin in combination with 2-iP.

In general, it is widely accepted that apical meristems are strong zones for synthesis of auxins (IAA). Therefore, exogenous application is often found to be deleterious.

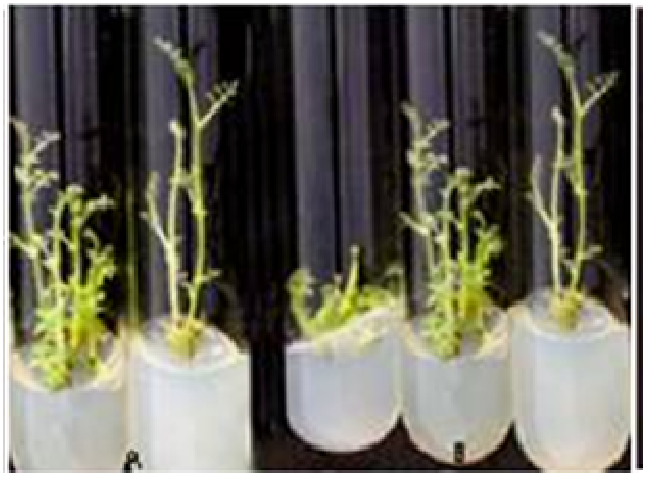

Figure 2. Shoots on shoot elongation medium (MS medium with 5 µM 2-iP and 2 µM kinetin) (10-14 d of different stage after transfer of shoots from SIM).

IAA is photosensitive and gets degraded under light. Interestingly, Chakraborti et al. (2006) have reported that IAA at low concentration can bring about shoot elongation. Based on the findings of the present investigation it can be authentically stated that exogenous application of neither auxins nor gibberellic acid is necessary and should not be encouraged. Therefore, the use of IAA in the medium for the elongation of shoot is not advisable.

Indirect shoot organogenesis via callus from different explants of chickpea

Regeneration of shoots via callus phase has been achieved from whole cotyledon and shoot tip explants by culturing on B_5 supplemented with BAP (1 mg l^{-1}) and NAA (1 mg l^{-1}) (Prakash et al., 1992). Multiple shoots were induced from calli derived from hypocotyls cultured on B_5 + 2% sucrose + BAP (1 mg l^{-1}) + Kinetin (1 mg l^{-1}) + IAA (0.5 mg l^{-1}) and subsequently transferred to MS + 2% sucrose + kinetin (2 mg l^{-1}) + NAA (2 mg l^{-1}) (Neelam et al., 1986b, 1986c). Induction of multiple shoots from cotyledonary node explants cultured on B_5 medium containing BAP (0.5 to 1 mg l^{-1}) or NAA (0.5 mg l^{-1}) has been reported (Khan and Ghosh, 1984). Callus derived from immature leaflets cultured on MS + 2, 4 -D (25 µM) or MS + 2, 4 -D (2, 4 -Dichlorophenoxyacetic acid) (5 µM) gave rise to multiple shoots on subsequent transfer to MS + BAP (10 µM) (Barna and Wakhlu, 1994). Immature leaflet explants of chickpea (P-256) developed callus on MS medium supplemented with 1.25 µM 2, 4 -D and 10 µM BAP and calli were regenerated on MS + 5 µM BAP + 10 µM TDZ + 0.1 µM IBA. The addition of spermidine enhanced the number of shoots by 73.8% (Rizvi and Singh, 2000). Up to date review of direct and indirect shoot organogenesis from different cultivar and explants of chickpea are listed in Table 1.

Somatic embryogenesis

Somatic embryogenesis involves *in vitro* formation of embryos from somatic tissues cultured on media supplemented with various hormones. These embryos develop from single cells and pass through globular, heart shaped, torpedo shaped, nodulated and cotyledonary embryo stages to give rise to complete plantlets. The whole process may proceed on a single medium or may require medium alterations specific for a specific developmental stage. Direct callus somatic embryogenesis and plant development in various genotypes of chickpea are listed in Table 2.

Somatic embryogenesis in chickpea has been involved directly or through an intervening callus phase in different explants that were recalcitrant. Some report on somatic embryogenesis has been reported. Direct somatic embryogenesis was achieved by culturing, mature seeds on MS salts + B_5 vitamins + TDZ (10 µM) + proline (1 mM) (Murthy et al., 1996). Immature cotyledons gave rise to somatic embryos when cultured on B_5 medium supplemented with 2, 4, 5 -T (2, 4, 5 -Trichlorophenoxyacetic acid) (7.8 µM) + BAP (4.4 µM) (Ramana et al., 1996). Somatic embryos obtained by culturing immature cotyledons and embryo axes on MS + 2, 4, 5 -T (3.0 mg l^{-1}), gave rise to complete plantlets on subsequent transfer to half strength MS medium supplemented with zeatin (1 mg l^{-1}) (Sagare et al., 1993). Mature embryo axes cultured on solid or liquid MS medium supplemented with 2, 4, 5 -T (3 mg l^{-1}), directly induced somatic embryos which were transferred to half strength MS medium containing 0.1 mg l^{-1} abscisic acid from maturation and subsequently sub cultured on half strength MS medium containing zeatin (1 mg l^{-1}) (Suhasini et al., 1994). Mature embryo axis of chickpea cultivar PG-12 was cultured on MS supplemented with 3 mg l^{-1} 2, 4, 5 -T (Sagare et al., 1999).

Complete plantlets have been regenerated through somatic embryogenesis via callus by culturing mature leaflets on MS + 2, 4 -D (0.5 mg l^{-1}) + BAP (0.5 mg l^{-1}) in dark and subsequent transfer to MS + IAA (0.1 mg l^{-1}) + BAP (3 mg l^{-1}) (Rao and Chopra, 1989) or immature leaflets on MS + 2, 4 -D (25 µM) during induction. MS + ABA during maturation and MS + GA_3 (15 µM) + IBA (1 µM) during conversion (Barna and Wakhlu, 1993), gave a maximum number (17) of somatic embryos from callus with 15 *de novo* shoots from whole seedling (hypocotyledonary notch region) on MS + 10 µM TDZ + 5 mM proline (Rizvi et al., 2002). Creamy callus was observed on MS medium supplemented with 1.25 mg l^{-1} 2, 4 -D and 0.25 mg l^{-1} kinetin and whitish callus was on MS + 0.5 mg l^{-1} 2, 4 -D and 1.4 mg l^{-1} 2, 4 -D + 0.25 mg l^{-1} kinetin. Medium with 1.25 mg l^{-1} 2, 4 -D and 0.25 mg l^{-1} kinetin was found to be best for embryo induction and somatic embryos were best matured on MS salt + B_5 vitamins + 2 mg l^{-1} BAP + 1.25 mg l^{-1} IBA for 4 weeks and elongated on MS. C-235 genotypes showed better maturation of somatic embryo followed by BG-256 and K-850.

Complete plants regeneration via somatic embryogenesis was carried out by culturing leaves on MS + 2, 4 -D (1.25 mg l^{-1}) + Kinetin (0.25 mg l^{-1}) in the dark for embryogenesis and subsequently transferred to light on medium containing MS salts + B_5 vitamins + IBA (0.125 mg l^{-1}) + BAP (2 mg l^{-1}) for maturation, and then to B_5 + BAP (0.25 mg l^{-1}) during conversion and B_5 basal for plantlet formation (Kumar et al., 1994). The same protocol was repeated for 3 more cultivars (Kumar et al., 1995).

Anther culture

Anthers or pollen grains on culture produce haploid plants, that is, plants with gametic chromosome number. These were particularly useful in plant breeding both for rapid production of homozygous lines following chromosome doubling to original ploidy level and for the detection and selection of recessive mutants. Another culture has also been reported (Gosal and Bajaj, 1979; Khan and Ghosh, 1983; Bajaj and Gosal, 1987; Gosal and Bajaj, 1988; Huda et al., 2001; Kennedy et al., 2002).

Protoplast culture

Plant protoplasts (single cell system) culture has been exciting possibilities in the field of somatic cell genetics and crop improvement. Isolated protoplasts serve as the field of somatic cell cloning and development of mutant lines (Bhojawani and Razdan, 1996). Isolation and regeneration of chickpea protoplasts from hypocotyl derived protoplasts cultured on V47 medium supplemented with NAA (1.5 mg l^{-1}) and BAP (0.5 mg l^{-1}) were able to produce microcalli, but microcalli failed to undergo differenttiation and organogenesis to produce plantlets (Sagare and Krishnamurthy, 1991).

***In vitro* rooting and grafting of shoots**

Several protocols of regeneration of chickpea have been suggested using shoot meristems through organogenesis and somatic embryogenesis but rooting in regenerated shoots was a problem. The shoots transferred to a medium containing auxin have been used for rooting. In a protocol provided by Prakash et al. (1992), shoots were transferred firstly to B_5 medium + gelling agent 8 g l^{-1}) + NAA (0.1 mg l^{-1}) and then to B_5 + gelling agent (8 g l^{-1}) + NAA (1 mg l^{-1}) and then B_5 + gelling agent (12 g l^{-1}) + NAA (2 mg l^{-1}). An increase in concentration of agar-agar (1.2%) and inclusion of activated charcoal (0.2%) in the second medium increased percentage of root differentiation. In another method suggested by Malik and Saxena (1992), shoots were cultured for 1 - 3 weeks on basal culture medium containing MS macro- and micronutrients, B_5 vitamins, 3% sucrose and 0.25% gelrite, then transferred to basal medium containing 2.5 µM NAA. *In vitro* regenerated shoots cultured on B_5 + 0.5 mg l^{-1} NAA also resulted in profuse rooting (Khan and Ghosh, 1984). Auxins, generally IBA were used in media for rooting. Shoots were cultured in a root induction medium having MS salts and vitamins and 0.05 mg l^{-1} IBA (Kar et al., 1996). B_5 medium containing 1 mg l^{-1} IAA in combination with 0.05 mg l^{-1} kinetin has also been used for root induction (Neelam et al., 1986).

The rooting can be facilitated by another method which gave good results with reduction in amount of salts used in the medium. Polisetty et al. (1996) used shoots (2 - 3 cm) which were initially cultured with ¼th strength MS + 0.75% sucrose + 0.8% agar without hormonal supplement for 30 days and rooting was achieved in 90 - 100% shoots.

Rooting and successful transplantation were major hurdle for chickpea plantlet formation. Therefore, drafting was carried according to the method described by Pickardt et al. (1995) for the establishment of putative transgenics (Krishnamurthy et al., 2000; Senthil et al., 2004; Sarmah et al., 2004; Singh et al., 2004; Sanyal et al., 2005) with minor modification, hence it could not be referred to as a true transgenic.

Regenerated shoots were rooted in MS solid medium containing 2.5 µM IBA (Rizvi and Singh, 2000) and 2.5 µM NAA (Rizvi et al., 2002). 93.3 and 80% rooting were recorded in ¼th MS + 10.4 µM NAA + 2% sucrose and ¼th MS + 10.7 µM NAA + 1% sucrose (Chauhan et al., 2003). Shoots were rooted on liquid medium (MS + 9.4 mM KNO_3 + 2% sucrose + 5 µM IBA) for 2 weeks on filter paper bridge (60 - 70% rooted), un-rooted shoots were pulse treated with 100 µM IBA and culture on filter paper bridge in liquid MS for 2 weeks (10 - 20% rooted) and the remaining were transferred to hydroponic system containing ¼th Arnon solution + 3 µM IBA for 2 - 3 weeks (10-15% rooted). This is more time consuming, for growth chamber is required for rooting and maintenance (Jayanand et al., 2003).

Elongated putative transformed shoots were rooted and grafted; shoots were rooted according to Murfet (1971), Morton et al. (2001), for 1 week or shoots were dip in 1 mg l^{-1} IBA and cultured on MS medium supplemented with 1 mg l^{-1} IAA + 10 mM MES for 2 weeks (Sarmah et al., 2004). Elongated putative transformed shoots were rooted on B_5 + 1 µM NAA + 150 mg/l Kan for 1 - 3 weeks (Polowick et al., 2004). Elongated putative transformed shoots were rooted on MB (MS salt + B_5 vitamins) + 2.5 µM NAA for 3 - 4 weeks. Rooted shoot were transferred to ½ MB. Grafting also followed because of the inconsistent performance of *in vitro* rooted plants in the glass house and extended culture period. 65 - 75% of grafted shoots were established in glass house (Senthil et al., 2004). Selected shoots were rooted on MB + 4.9 µM IBA + 100 mg l^{-1} kan + 5 mg l^{-1} PPT or 2 mM of each of lycine and threonine. Shoots (3 - 5 cm) were also grafted onto

Figure 3. Shoots exposed to 10 sec 100 mM IBA pulse treatment, on liquid MS medium A: 14 d; B: 24 d after transfer of the elongated shoots (please note strong root system).

10 - 12 days old seedlings (Singh et al., 2004).

Putative shoots were rooted on ½ MS + 0.5 mg l^{-1} IBA + 0.02 g l^{-1} $AgNO_3$ + 1% sucrose in 0.5% (w/v) agar. Micrografting of individual shoots were performed on 7 - 8 days old rootstock of same genotype prepared by transversely cutting the epicotyl region of the germinated seedling (Sanyal et al., 2005).

Thus, increasing the auxin concentration could induce rooting. Normal rooting could be induced only when the shoots were hard and not fragile and succulent or else, they tended to form callus. Roots developing from the shoots should arise directly from the base of the shoot and intermediary callus should be avoided as this does not has well defined vasculature (vascular connection between shoot and root). Tap root is preferred over other kinds of roots.

One of the major hurdles that limit *in vitro* regeneration of chickpea is the induction and development of strong root system. This compelled several researchers to adopt to grafting (Krishnamurthy et al., 2000; Sarmah et al., 2004; Senthil et al., 2004; Sanyal et al., 2005; Chakraborti et al., 2006). However, grafting is time consuming, requires special skills and the success rates vary significantly. Roots induced from cut ends of shoots of chickpea were shorter in length in semisolid medium as compared to liquid medium, similar to earlier reports (Jackson et al., 1991; Cournac et al., 1991; Ebrahim and Ibrahim 2000; Hazarika, 2006). The retardation in root length in the semisolid medium may be attributed to the relatively lower aeration in agar-gelled medium as indicated by earlier researchers (Hazarika, 2006; Pati et al., 2006; Rout et al., 2006).

Kat et al. (1996, 1997) rooted chickpea shoots in rooting medium consisting of MS salts, MS vitamins and 0.05 mg/l IBA and subsequently transferred the plantlets to half-strength MS medium containing 20 g/l sucrose. Polowick et al. (2004) used rooting medium consisting of B5 basal salts and vitamins supplemented with 1 µM NAA for rooting chickpea shoots and subsequently shoots with short roots which were transferred to Magenta vessels containing B5 salts and vitamins and 0.7% agar until root system was well established in 1 - 3 weeks while those with roots longer than 3 cm were transferred directly to soil. However, frequency of rooting was only 10 - 60% (Polowick et al., 2004). Although, Jayanand et al. (2003) reported high frequency rooting and transplantation success, the protocol adopted by them was time consuming involving three specific phases viz. i) rooting of shoots on liquid MS medium with 9.4 mM KNO_3, 2% sucrose and 5 µM IBA for 2 weeks on filter paper bridge, ii) pulse treatment of un-rooted shoots with 100 µM IBA and culturing on filter paper bridges in liquid MS for 2 weeks and iii) transfer to hypotonic system containing ¼th Arnon solution with 3 µM IBA for 2 - 3 weeks. Even during the present investigations, shoots could be rooted in MS medium with 5 µM IBA, but the roots developed were relatively weaker and required 15 - 20 days. However, 5 - 10 s pulse treatment of cut ends of shoots of chickpea with 100 mM (that is, 100 µmol/ml) IBA led to root induction within 4 days and subsequently resulted in the development of strong root system within 10 - 12 day in liquid MS basal medium (Figure 3) (Anwar et al., 2008, 2009). This much high concentration of IBA is 100% effective and first time reported in any crop (Anwar et al., 2008, 2009). This idea can be applicable in case of other crop from which *in vitro* rooting is difficult. *In vitro* chickpea rooting and grafting of different cultivar are listed in Table 3.

Transplantation / establishment of plantlet

After *in vitro* rooting of shoots, the plantlets have to be established in soil and for this, various methods gave been employed. Prior to transfer to soil, hardening of the plantlets was done. In case of plants regenerated from leaflet callus via somatic embryogenesis, complete plantlets in tubes were subjected to the following hardening procedure.

Cotton plugs were loosen and kept for 4 - 5 days prior to transferring to ¼th strength liquid MS medium without sucrose for 24 h and they were transferred to sterile soilrite (Kumar et al., 1994) or sterile vermiculite for one week (Barna and Wakhlu, 1993) and then transferred to pots in the ratio of 3:1:1; sterile soil: farmyard manure: sand (Kumar et al., 1994) or to pots containing garden soil (Barna and Wakhlu, 1993).

Plantlets regenerated via direct somatic embryogenesis induced from immature cotyledons and immature embryo axes were established in the soil of green house containing a mixture in the ratio of 1:1:1; peat: vermiculite: soil (Sagare et al., 1993).

Plantlets regenerated from callus cultures of hypocotyls were established in plastic pots containing vermiculite

Table 3. Current status of chickpea (*Cicer arietinum* L.) transformation.

Cultivar	Explant	*Agrobacterium* strain/gene/ vector	Mode of transformation /selection	Expression of transgene/s	Rooting/Grafting	Phenotype obtained	References
ICC10943 ICC10386	Embryo axis	*LBA4404/ pCAMBIA1305.2/p35SGUS*	Agrobacterium/ *Hyg*	PCR (*gus*), southern (*gus*)	MSB5 + 0.9 µM NAA + 1.2 µM IAA + 0.9 µM Kin	Fertile transgenic plants	Pathak and Hamzah, 2008
Gokce, Er, Akcin, Uzunlu, Kusmen	Seeds	*C58C1/EHA101/pTJK136/pTHW136/uidA/nptII*	Agrobacterium/ *Kan*	PCR (*nptII*), *gusassay*	-	-T_0 Direct gus assay and *nptII* PCR	Akbulut et al., 2008
ICCC37, PR-12	Epicotyls	*pHS 102/gus*	*Particle bombardment/ Kan*	PCR, Southern, gus assay of To plants	-	To plants	Shivani et al., 2007
C-235, BG-256, P-362, P-372	Cotyledonary nodes (20d old seedling)	*LBA 4404/cry1Ac/ p35SGUS-INT*	Agrobacterium/ *Kan*	PCR (*npt*II and *cry1Ac*), southern (*npt*II and *cry1Ac*) ELISA (*cry1Ac*), Insect bioassay	Grafting ½ MS + 0.5 mg/l IBA + 0.02 g l^{-1}Ag NO_3 + 1% sucrose	Fertile transgenic plants showed affective against Pod Borer, 14.5-23.5 ng/mg *cry1Ac* protein accumulation. ….	Sanyal et al., 2005
P-362, P-1042 P-1043	Decapitated embryo with one cotyledon (overnight socked seed)	EHA101/AK/ pIBGUS/ *pBI121-dsAK*	Agrobacterium/ *Kan* *Particle bombardment/ Kan*	*Gus*, LP assay, PCR (*gus*), southern (*npt*II probe), *AR* activity	Grafted MS+B5 org. + 4.9 µM IBA + 1.5% sucrose	Fertile transgenic plants; 1st chickpea transgenic report of AK/LT selecion system, over expression of lysine and threonine…	Singh et al., 2004
ICCV5, H208, ICCL87322, K850 Annigeri	Slices from plumule (2-5h socked seed)	*AGL1/uidA, bar/ pGIN*1 or *pGIP*	Agrobacterium/ *Kan, PPT*	PCR (*gus*), *gus* and LP assay, southern (*gus*)	MS+B5 org. + 2.5 µM NAA	Fertile transgenic plants; gus and LP assay confirmed upto T2 generation. ……	Senthil et al., 2004
CDC Yuma-kabuli	Longitudinal slide of embryonal axis	*LBG66/uidA, npt*II/*pPBI3010*	Agrobacterium/ *Kan*	PCR (*gus* and *npt*II), *gus* assay, southern (*gus*)	B5 + 0.18 mg l^{-1} NAA	Fertile transgenic plants; gus expression upto 4th generation….	Polowick et al., 2004
Semsen	Half embryonal axis with one cotyledon	*AGL1/α-AI*1/ *pRM50*	Agrobacterium/ *Kan*	PCR (*npt*II), *npt*II dot blot, southern (*α-AI*1 probe), insect bioassay and α-amylase activity	Grafting MS + 1 mg l^{-1} IBA	Fertile T1 and T2 transgenic, *α-AI*1 strongly inhibited the development of insect. …	Sarmah et al., 2004

and soil mixture (Neelam et al., 1986) and plantlets derived from callus culture of immature leaflets were established in pure vermiculite (Barna and Wakhlu, 1994). Plantlets regenerated from embryonal axis were transferred to 8 cm (diameter) pots and the plants were covered with polypropylene bags and gradually open after 7-10 days period or the rooted shoots were suspended in Magenta Jars containing ¼th Arnon solutions and in the second stage, the hardened plants were transferred to 20 cm (diameter) pots containing 3:2; sand: black soil + Cell Rich (5%) +

Table 3. Contd.

Cultivar	Explant	*Agrobacterium* strain/gene/ vector	Mode of transformation /selection	Expression of transgene/s	Rooting/ Grafting	Phenotype obtained	References
Gokce, Akcin91, Izmir92	One week old seedlings	*A. rhizogenes pRi15834*	*Agrobacterium*	Wild type	-	Callogenic mass	Khawar and Ozcan, 2004
PG1, PG12 Chafa	Embryo (from overnight socked seed)	**C58C1/uidA, nptII/ p35SGUSINT *GV2260/uidA, nptII/p35SGUSINT *EHA101/uidA, nptII/pIBGUS*	*Agrobacterium*/ Kan	PCR (*npt*II), gus assay, southern (*npt*II probe)	Grafting	Four t1 transgenic plants shown gus only not *npt*II amplicon. …….	Krishnamurthy et al., 2000
6153, CM72	Hypocotyl	*nptII/pBI121/ gus*	Particle bombardment/Kan	Gus assay	-	Somatic embryo	Hussai et al., 2000
Red chickpea Canitez 87 MB-10	Shoot primordial of mature embryo (from 24h seedlings)	*A. tumefacienc 4404/uidA, nptII/ pBI121 A. rhizogenes 9402 /uidA*, npt*II / pBI-121*	*Agrobacterium*/ Kan	Southern (*gus*)	-	Hairy root . …	Altinkut et al., 1997
ICCV1, ICCV6	Embryonal axes without root and shoot meristem	*Cry1Ac/ pcrylAc BDC2*	Particle bombardment/ Kan	Positive (*crylAc*), Southern blot (*crylAc*) and insect bioassay	MS + 0.05 mg l^{-1} IBA	Cry1Ac transgenic chickpea. ……	Kar et al., 1997
ICCV-1 ICCV-6 Desi (Local Variety)	Embryonal axes without root and shoot meristem	*LBA 4404/uidA, npt*II/*pBI121*	*Agrobacterium*/ Kan	PCR (*npt*II), *gus assay*, Southern (npt*II*)	MS + 0.05 mg l^{-1} IBA	Transgenic plantlets transplanted to greenhouse. …..	Kar et al., 1996
ICC-4918	Immature cotyledon	*LBA 4404/uidA/ pBin19,GUS int*)	*Agrobacterium*	*gus* assay	1 mg/l Zeatin	Plantlet. . …..	Ramana et al., 1996
Local ecotype (Italy)	Embryonal axis lacking apical meristem	*LBA 4404/uid, npt/pBI121*	*Agrobacterium/ Kan*	*nptII dot blot, Southern (nptII)*	0.5 mg l-1 IAA + 0.05 mg l-1 Kin	Transgene expression of plantlet	Fontanna et al. 1993
Pusa-256	Leaf and stem explants	*R1601/nptII/ pTVK291*	*Agrobacterium/ Kan*	*Southern (nptII probe)*	-	Formation of transformed callus	Srinivasan et al., 1991

rice straw compost (5%). Stage 1 and 2required a day and night temperature of 20 ± 2 and 15 ± 1°C, respectively, and 50% humidity during day time and 80% at night and 10,000 - 12,000 lux light intensity during hardening process. This is more time consuming and for maintenance, growth chamber will be required (Jayanand et al., 2003).

Plantlets derived from direct regeneration of shoot tip explants where cultured on 1/4th strength MS without agar for elicitation of root growth. Root portion was then washed with 0.1% mercuric chloride and tap water. These plantlets were transferred to ¼th strength Hoagland solution for 20 days and then established in vermiculite (Polisetty et al., 1996). Rooting was a major hurdle for chickpea plantlet formation, therefore, drafting was carried out according to method described by Pickardt et al. (1995) for the establishment of putative transgenics(Krishnamurthy et al., 2000; Senthil et al., 2004; Sarmah et al., 2004; Sanyal et al., 2005) with minor modification, hence we could not say it was true transgenic.

In vitro rooted shoots were transferred to plastic pots

Figure 4. Sand and Potting mixture used for planting *in vitro* raised plantlets, respectively. Plantlets transferred to small earthen pots with a mixture of garden soil, sand and bio-manure in equal proportion. Freshly transferred plantlets covered with polythene bags.

containing soil mixture (Sunshine No. 4. Sun Gro Horticulture, Bellevus, Wash.), 20/15°C (day/night) and 16/8 h (light/dark) photo period with 200 µ mol quanta/m^2/s, established plants were transferred into bigger pots of same soil mixture and grown under green-house, 16/8 h (light/dark) photo period. Young plantlets were covered with transparent beakers to maintain humidity for 3 - 4 days and slowly acclimatized (Polowick et al., 2004). Sarmah et al. (2004) reported that *in vitro* shoot were grafted and grown under glass house condition. 65-75% of grafted shoots were established in glass house (Senthil et al., 2004). *In vitro* rooted and grafted shoots were transferred to pots containing soil: vermiculite; 1:1 and covered with transparent plastic bags. A cut was gradually extended over a 3 week period until the bag was completely open. After 15 - 20 days of transplantation, the plants were transferred to glass house (Singh et al., 2004).

Micrografting of individual shoots were performed on 7 - 8 days old rootstock of same genotype prepared by transversely cutting the epicotyl region of the germinated seedling. The scion of putative shoots was inserted into vertical incision made in the epicotyl region of the stock. The resulting grafts were initially wrapped with micropore tape and cultured in pots containing soilrite that were irrigated with ½× MS for 15 days in culture room and then transferred into the pots containing soilrite: loam: leaf manure; 1:1:1 in green house maintained at 22 - 24°C, 60 - 80% humidity and 14 h photoperiod (Sanyal et al., 2005).

Another major hurdle limiting chickpea regeneration is the establishment of *in vitro* raised plantlets in pots/field. This is another major reason behind preference for grafting. The plantlets with shorter length have higher potential to withstand transplantation shock and establish better in pots. It has been reported earlier that the root to shoot ratio has an important role in the successful establishment of *in vitro* raised plantlets (Subhan et al., 1998). Understandably, shorter shoot area will reduce excessive loss of water leading to rapid loss in turgidity of the plantlets (Munns, 2002). In the present investigations, plantlets with shoot length of 3 to 5 cm survived better than the plantlets with longer shoot length. Many of the plantlets with longer shoot length failed to establish even if the shoots were stouter, as they tend to collapse/ lodge and die within few days after transplantation to pots.

For transplantation of plantlets, various potting mixtures were used. These include autoclaved/sterile (Indurker et al., 2007) and non-autoclaved/non-sterile soil, soilrite, manure, vermiculite, soilrite + garden soil (in the ratio of 1:1), soilrite + garden soil + manure (in the ratio of 1:1:1), commercial soil mixture (Sunshine No. 4, Sun Gro Horticulture, Bellevue) (Polowick et al., 2004). In all these cases, the successful establishment rate was very low. Failure was accounted in these particular cases of excess moisture and high degree of water holding capacity of potting mixture. In the present investigations, potting mixture consisting of garden soil mixed with sand (gravel) and bio-manure (Khadi and Village Commission, Govt. of India) in the ratio of 1:1:1 is most suitable for achieving percent transplantation success. Chickpea is well known to be susceptible to flooding and excess moisture (Yadav et al., 2006). Under high moisture condition, chickpea is prone to fungus and wilt diseases (Yadav et al., 2006). During the present investigations, autoclaved soil mixture was not found in any way to be an essential requirement for the establishment of *in vitro* raised plantlets of chickpea. Instead it was realized that improving aeration/porosity of potting mixture by mixing sand is highly beneficial. Chickpea grows best on fertile sandy, loam soils with good internal drainage (Yadav et al., 2006). Good drainage is necessary because even short period of flooding, water logging soil and moisture reduces growth and increases susceptibility to root and stem rots and heavy rainfall season shows reduced yields due to disease outbreaks and stem lodging problem from excessive vegetative growth (Yadav et al., 2006). Some of the stages involved in transplantation of *in vitro* raised plantlets and their successful establishment in smaller and larger earthen pots is shown in Figures 4 - 8.

Another important factor that often limits regeneration and successful establishment of *in vitro* raised plantlets of chickpea is the season (that is, month of the year) when these are attempted. Best and percentage establishment success followed by good seed set is achieved when the *in vitro* raised plantlets of chickpea are transplanted during the months of October and November (that is, beginning of winter). Over all, vegetative growth including number of branches followed by flowering, pod setting

Figure 5. Acclimatized plantlets.

Figure 6. Plants of chickpea established in large earthen pots with a mixture of garden soil, sand and bio-manure in equal proportion.

Figure 7. Plants of chickpea established in large earthen pots with a mixture of garden soil, sand and bio-manure in equal proportion.

and seed production (Figures 9 - 10) (Anwar et al., 2008, 2009) was found to be superior in the plants obtained from the plantlets transferred to pots during October and November.

Figure 8. Plants of chickpea established in large earthen pots with a mixture of garden soil, sand and bio-manure in equal proportion.

Figure 9. Plants showing pods and seed setting.

Figure 10. Harvesting stage of seeds after drying of pods and plants.

In summary, a simple and perfect protocol for successful rooting of shoots and establishment of *in vitro* raised plantlets of chickpea has been developed. Successful establishment of *in vitro* raised well rooted plantlets depends upon two basic factors viz., (i) shoot length of the plantlets and root to shoot ratio and (ii) potting-mixture with good aeration and lesser capacity to retain water. During the investigations, the percentage of plantlets with shoot length of 3 to 5 cm potted in garden soil mixed with sand (gravel) and bio manure in equal proportion, established perfectly and set seed (Anwar et al. 2008, 2009) (Figure 9).

GENETIC TRANSFORMATION OF CHICKPEA

Plant genetic manipulation is the insertion of functional/ desired DNA directly into the genome of plant cell and the regeneration of whole plants from such cells by plant tissue culture techniques, molecular biology and microbiological methods which allows a chosen plant variety to be modified in a small but highly specific manner (Fenning and Gartland, 1995). Up to date genetic transformation of chickpea has been listed in Table 3. Genetic transformation of plants can be carried out using any one of several methods available. Indirect methods involve the use of a vector, which may be a bacterium; *Agrobacterium* or a virus; CaMV or Gemini virus. Direct methods of gene transfer are particle bombardment, electroporation, PEG mediated DNA uptake and microinjection (Potrykus, 1990).

Genetic transformations for incorporation of foreign traits have been achieved in some grain legumes. Success has also been achieved in the transfer of agronomically important traits to improve plants in terms of quantity and quality. But there are very few reports on agronomically important gene transfer in chickpea (Table 3). Both particle inflow gun (PIG) and *Agrobacterium* medicated gene transfer have been employed for engineering chickpea (Srinivasan et al., 1991; Fontanna et al., 1993; Seifkes-Boer et al., 1995; Kar et at., 1996; Kar et al., 1997; Husnain et al., 1997; Krishnamurthy et al., 2000; Sarmah et al., 2004; Singh et al., 2004; Senthil et al., 2004; Polowick et al., 2004; Sanyal et al., 2005: Jiang et al., 2005; Akbulut et al., 2008; Pathak and Hamzah, 2008).

Transgenic callus were raised through inoculating 12-15 days old leaf and stem explants with wild as well as disarmed strains of *Agrobacterium tumefaciens* (Srinivasan et al., 1991). Regeneration of transformed chickpea plants was done using disarmed *A. tumefaciens* strain *LBA4404* containing *pBI121* carrying *npt*II and *gus* genes (Fontanna et al., 1993). 24 h old embryonic axis deprived of shoot apex were inoculated with *Agrobacterium* (5×10^8 cells ml^{-1}) for 20 min and cultured on MS + kinetin (1 mg l^{-1}). Shoots arising from epicotyl region of embryo axes were selected on medium containing Kanamycin (50 mg l^{-1}) and *gus* expression was observed in leaves and roots of transformed plants. Kar et al. (1996) also used *LBA* 4404 strain to inoculate embryo axes deprived of root and shoot meristems and obtained multiple shoots by culturing explants on a medium containing MS macro, 4x MS micro, B_5 vitamins, BAP (3 mg l^{-1}), NAA (0.04 mg l^{-1}) and 3% (W/V) sucrose. Putative transformed shoots were selected on Kanamycin (50 mg l^{-1}) containing medium and rooted on MS supplemented with IBA (0.05 mg l^{-1}). Leaf and roots of transformants were positive for *gus* expression and southern blot for *npt*II gene.

Several groups have reported *Agrobacterium rhizogenes* mediated transformation systems for some chickpea cultivars (Srinivas et al., 1991; Prakash et al., 1992b; Seifkes-Boer et al., 1995; Altinkut et al., 1997; Khawar and Ozcan, 2004). Hairy roots have been induced in 4 chickpea cultivars by infection with 7 different wild strains of *A. rhizogenes* (Seifkes-Boer et al., 1995). Wild type *A. rhizogenes* by direct inoculation showed a callogenic mass instead of hairy root (Khawar and Ozcan, 2004). Expression of marker genes *npt*II and *gus* was studied employing particle inflow gun (PIG) and *Agrobacterium* to effect gene transfer into zygotic embryos of chickpea (Husnain et al., 1997). Efficiency of transient expression of maker genes by PIG was shown to be dependent on DNA to tungsten ratio, distance between the filter disk and plant tissue and use of a nylon screen. *GUS* under control of Actin promoter gave better results as compared under CaMV and Win promoters. *Agrobacterium* stains A 281 was founded to be more virulent than C58. Use of PIG in combination with *Agrobacterium* has been suggested for chickpea transformation.

An agronomically important *crylAc* gene conferring resistance towards pod borer *Heliothis armigera* and *Helicoverpa armigera* has been reported to be transferred to chickpea by Kar et al. (1997), Sanyal et al. (2005) and Shivani et al. (2007). Embryo or embryonal axes deprived of root and shoot apices were co-transformed with *crylAc* and *npt*II gene using a biolistic 1000/He particle gun. Explants gave rise to transformed multiple shoots on medium containing MS macro, 4X MS micro, B_5 vitamins, 3 mg l^{-1} BAP, 0.004 mg l^{-1} NAA, 3% w/v sucrose and 50 mg l^{-1} Kanamycin. molecular analyses by Southern and Northern blots revealed the presence of *crylAC* gene and its expression was confirmed by inhibition or larval development on feeding transgenic shoots. The co-transformation frequency of *npt*II and *crylAc* was estimated to be 45.8%. Inheritance of transgene/s was shown by PCR of T_1 (first filia) plants. Even the insect bioassay for pest resistance has been performed using stem of the plant instead of pods/seeds (Kar et al., 1997). Embryonal axis explants were used from four accessions of chickpea treated with *A. tumefaciens* strains *C58C1/p35SGUSINT/ GV2260* and *pIBGUS/EHA101*. For multiple shoot formation and elongation of shoots, MS medium supplemented with 0.5 mg l^{-1} Kanamycin or 10 mg l^{-1} phosphinothricin were selected. Putative transformed shoots were grafted according to Pickardt. T_0 (parent) plants showed PCR positive for both *npt*II and *GUS and* southern positive. Four T_1 plants showed PCR positive for *npt*II not for GUS (Krishnamurthy et al., 2000). It could be due to improper/unstable integration of T-DNA into the plant genome.

Longitudinal slices from embryonal axis of imbibed, mature seeds were used for *Agrobacterium* transformation (Schroeder et al. 1995) vector *LBG66* (*pPBI3008*) containing binary vector *pPBI3010* (as described by Polowick et al., 2000). B_5 medium was used for co-cultivation and for induction as well as for selection B_5

medium supplemented with 3 mg l^{-1} BAP and 50 mg l^{-1} kanamycin. For elongation of shoots, B_5 medium containing 1 mg l^{-1} BAP and 50 and 75 mg l^{-1} kanamycin for the two subsequent cycle and putative shoots were rooted in B_5 medium containing 0.18 mg l^{-1} NAA and 150 mg l^{-1} kanamycin. Putative transformed plantlets were established in green house. Molecular analyses of T_0 and T_1 by *GUS* activity, MUG assay and Southern blots revealed the presence of *npt*II gene, single insert which showed the 3:1 Mendelian inheritance pattern in T_1 population. Same patter of integration in T_0 and T_1 plants were found that confirmed that T-DNA was stably inherited to plant genome (Polowick et al., 2004). The transformation efficiency was almost 60% higher in the SAAT method than a simple *Agrobacterium* infection without sonication and sonicated wounding of the plant tissue and use of actively growing young decapitated embryo axis as an explant for *Agrobacterium* transformation play an important role in increasing the efficiency of transformation (Pathak and Hamzah, 2008).

In all the protocols, except that reported by Pathak and Hamzah (2008), the transformation frequency was very low. An efficient method to be employed for routine transformation experiments has been developed. Genetic transformations of a plant have to be achieved only if the transgene/s is stably inherited and expressed in subsequent progenies of plant. Only few report has been published on abiotic stress tolerance genetic transformation in chickpea. Hence, a lot still remains to be worked out in chickpea in order to get an economically superior germplasm with respect to the existing cultivated varieties.

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