Research Article

An optimal protocol for *in vitro* regeneration, efficient rooting and stable transplantation of chickpea

Firoz Anwar, P. Sharmila and P. Pardha Saradhi

Department of Environmental Biology, University of Delhi, Delhi - 110 007, India

ABSTRACT

A rapid, reproducible and efficient regeneration method was developed for chickpea (*Cicer arietinum* L.) using single cotyledon with half embryonal axis as explants. MS medium supplemented with 4 ìM TDZ, 10 ìM 2-iP and 2 ìM kinetin induced 50-100 adventitious buds/shoots after 14 days of culture and elongated on MS medium supplemented with 5 ìM 2-iP and 2 ìM kinetin. Healthy, strong and 100 % rooting was achieved by exposing cut ends of the shoots to 10 sec pulse treatment with 100 ìmoles/ml IBA followed by their transfer to liquid MS basal medium within 10-14 d. 2-3 cm long shoots were most suitable for rooting. Potting-mixture with good aeration and lesser capacity to retain water was most suitable for achieving successful establishment of chickpea plantlets. Garden soil mixed with sand (gravel) and bio-manure in the ratio of 1:1:1 is most suitable for achieving cent percent transplantation success. Cent percent of plantlets got acclimatized, survived in the pots and showed normal growth, development, flowering followed by podding and seeds setting. Harvesting of seeds was done after the pods were fully matured and dry. In this communication, we have demonstrated for the first time that shoot length, pulse treatment of cut ends of shoots with 100 ìmoles/ml IBA and aeration of potting mixture are key factors for rapid micro-propagation and successful establishment of chickpea. **[Physiol. Mol. Biol. Plants 2008; 14(4) : 329-335]** *E-mail : ppsaradhi@gmail.com*

Key words : *Cicer arietinum*, Chickpea, Regeneration, Rooting, Transplantation, IBA

INTRODUCTION

Chickpea is the world's third most important pulse crop and India accounts for 75 % of the world's production. Chickpea 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 hypocholesteremic 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). Plantlets were developed through direct somatic embryogenesis and through callus from different explants of chickpea (Barna and Wakhlu, 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).

Correspondence and Reprint requests : P. Pardha Saradhi

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, grafting besides requiring additional seed lot, also might promote emergence of shoots/ branches from axillary buds that are often retained in the axils of cotyledons and the later might dominate over the grafted shoots.

Therefore, present investigations were carried to fine-tune the protocol for achieving strong root system and

successful establishment of the *in vitro* raised plantlets. In this communication we have been successful in furnishing a perfect protocol for in vitro production of plantlets with strong root system and their successful establishment.

MATERIAL AND METHODS

The seeds of chickpea (*Cicer arietinum* L.) cv. PG 114 (gifted by Dr. S.S. Yadav and Dr. Jitender Kumar, Indian Agricultural Research Institute, New-Delhi, India) were treated with 0.2 % cetrimide for 5 min, rinsed thoroughly with double distilled water followed by treatment with 70 % ethanol for 3 min and 0.1 % mercuric chloride solution for 5 min. Finally the seeds were washed 5-6 times with sterile double distilled water under aseptic conditions prior to soaking overnight. Fourteen hour imbibed seeds were de-coated (Figure 1A) and germinated on shoot induction medium (SIM), consisting of MS medium supplemented with 4 µM TDZ, 10 µM 2-iP and 2 µM kinetin, for 6 d. Explants consisting of single cotyledon with half embryonal axis with plumular and radicular ends were excised from resulting 6 d old seedlings (Figure 1B) were inoculated on SIM for further duration of 6 d. The explants were subsequently transferred to MS basal medium (i.e. devoid of growth regulators) for 10-15 d.

Elongation of Shoots

The adventitious buds/multiple shoot induced from the explants 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 d. They were then routinely sub-cultured at an interval of 10-15 d on SEM.

Rooting

Two basic approaches were used for rooting elongated shoots varying in length from 3 to 8 cm in length based on several preliminary experiments in our laboratory. In the first approach, 3 to 8 cm long shoots were transferred to either semi-solid or onto Whatman filter paper bridges in liquid MS medium supplemented with 5 nmoles/ml (5 µM) IBA. In the second approach, the cut end of shoots were exposed to 10 sec pulse treatment with 100 µmoles/ml (100 mM) IBA and were transferred onto Whatman filter paper bridges in liquid MS basal medium.

All the cultures were maintained at 25±2 ºC with a light/dark cycle of 16/8 h. White fluorescent light with an intensity of 120 µmol m^{-2} s^{-1} was used for illumination.

Transplantation

The plantlets were carefully taken out from tubes, roots were thoroughly washed with tap water and transferred to small earthen pots (~8 cm diameter and 10 cm height) filled with potting mixture consisting of garden soil, sand and bio-manure (Khadi and Village Commission, Govt. of India) in equal proportion. Each pot with plantlet was initially covered with transparent polythene bag for 6 d. However, after 3 d the corners of the polythene bags were cut. Subsequently, the polythene bags were removed. After allowing the plants to grow further for another 6 d, the plants were carefully transferred to large earthen pots (30 cm diameter × 37.5 cm height) having garden soil, sand and bi-manure in equal proportion. Cent percent of plants got acclimatized, survived in the pots and showed normal growth, development, flowering followed by seed setting. Harvesting of seeds was done after pods were fully dried.

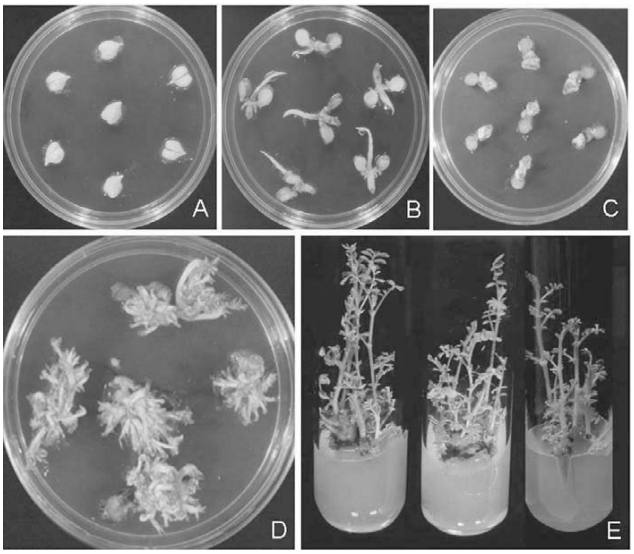

Fig. 1. Regeneration of chickpea PG-114. **A.** Seeds de-coated after 14 h imbibition, on shoot induction medium (SIM) (MS medium supplemented with 4 µM, 10 µM 2-iP and 2 µM Kin); **B.** Chickpea seedlings (6 d after incubation of de-coated seeds on SIM) used for obtaining explants for regeneration and transformation; **C.** Swollen embryonal axis attached to cotyledon showing the initiation of shoot induction {6 d after incubating the explants on SIM}; **D.** Two week old culture of embryonal axis with single cotyledon, showing the emergence of large number of adventitious buds {15 d after incubating 6 d old cultures from SIM on MS medium}; **E.** Shoots on shoot elongation medium (MS medium with 5 µM 2-iP and 2 µM kinetin) (14 d after transfer of shoots from SIM).

RESULTS AND DISCUSSIONS

TDZ (Thidiazuron) is a substituted phenyl urea [1-phenyl-3-(1,2,3-thidiazol-5-yl)urea] that has immense potential as a cytokinin in shoot organogenesis in a large number of plant systems including legumes (Murthy *et al*., 1998; Shan *et al*., 2000; Ganeshan *et al*., 2003; Onamu *et al*., 2003; Mroginski *et al*., 2004; Sharma *et al*., 2005; Chen and Chang, 2006; Radhika *et al*., 2006). Since, the observation made by Malik and Saxena (1992) TDZ has been noted to be the most promising cytokinin for shoot induction in chickpea by number of research teams (Barna and Wakhlu, 1993; Huetteman and Preece, 1993; Murthy *et al*., 1996; Rizvi and Singh, 2000; Jayanand *et al*., 2003; Senthil *et al*., 2004; Tewari-Singh *et al*., 2004; Kiran *et al*., 2005). TDZ was most effective in inducing a large number of healthier shoots when used in lower concentration (~10 μM) as compared to higher concentration (Malik and Saxena, 1992; Murthy *et al*., 1995, 1996, 1998; Jiang *et al*., 2005). Although exact mechanism of action of TDZ is not yet clear, it is believed to be involved in regulation of endogenous levels of various growth regulators (Malik and Saxena, 1992).

Despite its high efficiency to enhance shoot induction, use of TDZ has not become popular due to its immense toxicity resulting in the formation of deformed shoots, whenever it is used above threshold levels and/or for prolonged duration (Devlin *et al*., 1989; Onamu *et al*., 2003; Sharma *et al*., 2005).

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 d (Figure 1C). Use of TDZ with purine ring containing cytokinins such as kinetin, BAP and 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 our 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 i.e. 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 (Figure 1D). 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 d. They were then routinely sub-cultured at an interval of 10-15 d on SEM. (Figure 1E).

Gibberellins promote elongation of shoots (Jayanand *et al*., 2003), but during 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 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. 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 we can authentically state that exogenous application of neither auxins nor gibberellic acid is necessary and rather should not be encouraged. Therefore, the use of IAA in the medium for the elongation of shoot is not advisable.

Development of strong root system

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 the 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 were transferred to Magenta vessels containing B5 salts and vitamins and 0.7 % agar until root system 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 is 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 1/4th Arnon solution with 3 μM IBA for 2-3 weeks. Even during present investigations, shoots could be rooted in MS medium with 5 μM IBA, but the roots developed were relatively weaker and required 15-20 d. However, 10 sec pulse treatment of cut ends of shoots of chickpea with 100 mM (i.e. 100 μmoles/ml) IBA lead to root induction within 4 d and subsequently resulted in the development of strong root system within 10-12 d in liquid MS basal medium (Figure 2A-C). Some of the plantlets with strong root system showed flowering when retained in tubes for longer duration (Figure 2D).

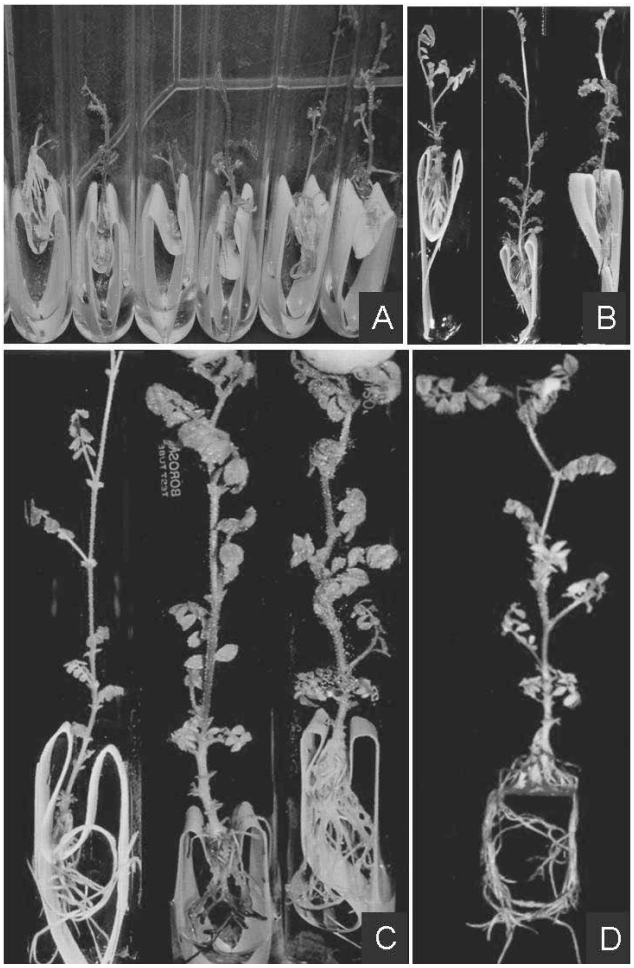

Fig. 2. Regeneration of chickpea PG-114. Shoots exposed to 10 sec 100 mM IBA pulse treatment, on liquid MS medium **A.** 8 d; **B.** 14 d; **C.** 28 d after transfer of the elongated shoots (please note strong root system). **D.** 28 d old plantlet showing strong root system and flowering.

Successful establishment of in vitro raised plantlets

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). During 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 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. We account failure in these particular cases to excess moisture, high degree of water holding capacity of potting mixture. During our investigations, potting mixture consisting of the garden soil mixed with sand (gravel) (Figure 3A) and bio-manure (Khadi and Village Commission, Govt. of India) in the ratio of 1:1:1 (Figure 3B) is most suitable for achieving cent 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, we did not find autoclaved soil mixture in any way to be an essential requirement for the establishment of in vitro raised plantlets of chickpea. Instead we 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, 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 Figure 3.

Another important factor that often limits regeneration and successful establishment of in vitro raised plantlets of chickpea is the season (i.e. month of the year) when these are attempted. Best and cent percent establishment success followed by good seed set is achieved when the *in vitro* raised plantlets of chickpea are transplanted during October and November months (i.e. beginning of winter). As is evident from the Figure 3, the plants transferred during January possessed lesser vegetative growth and branching (Figure 3 F) as compared to the ones transferred during the months of October and November (Figure 3 G and H). Over all vegetative growth including number branches followed by flowering, pod setting and seed production was superior in the plants obtained from the plantlets transferred to pots during October and November.

In summary, we have developed a simple and perfect protocol for successful rooting of shoots and establishment of *in vitro* raised plantlets of chickpea. 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 our investigations, cent percent

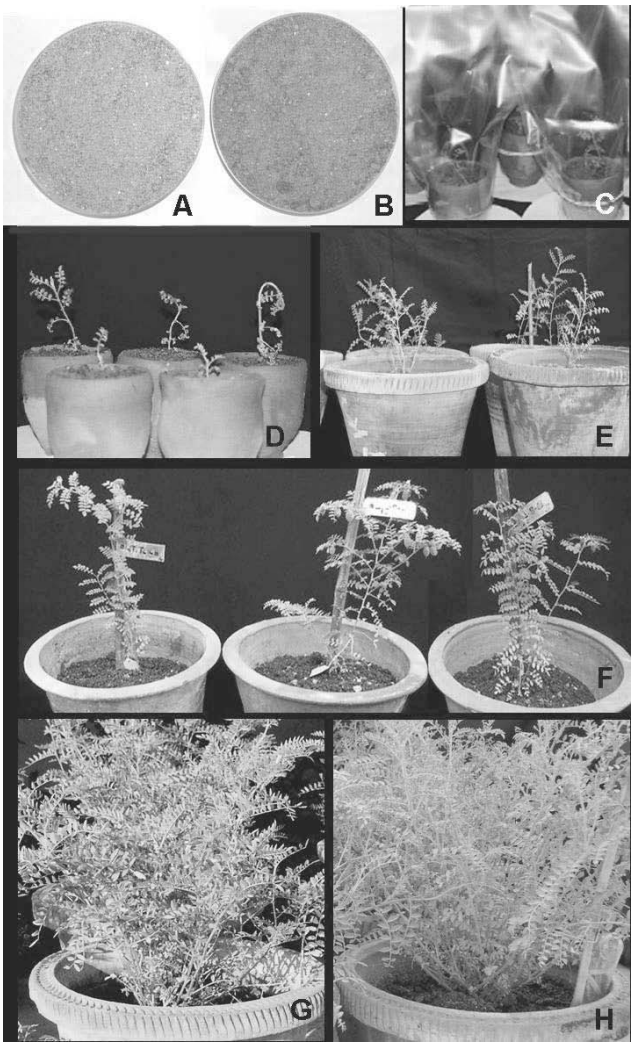

Fig. 3. Regeneration of chickpea PG-114. **A & B.** Sand and Potting mixture used for planting *in vitro* raised plantlets, respectively. **C & D.** Plantlets transferred to small earthen pots with a mixture of garden soil, sand and bio-manure in equal proportion. Freshly transferred plantlets covered with polythene bags (C) and acclimatized plantlets (D). **E.** Plants of chickpea established in large earthen pots (two week after transfer) with a mixture of garden soil, sand and bio-manure in equal proportion. **F.** Plants obtained from *in vitro* raised plantlets showing flowering and podding. **G & H.** Plants obtained from *in vitro* raised plantlets planted during the months of October and November (the main/actual season for sowing seeds). Please note vigorous growth/branching and flowering in G and H.

of the 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.

ACKNOWLEDGEMENTS

This work was supported by funds received from (i) the Swiss Agency for Development and Cooperation, Government of Switzerland and the Department of Biotechnology, Government of India under the Indo-Swiss Collaboration in Biotechnology. We are thankful to Dr. Katharina Jenny (Switzerland), Dr. S.R. Rao (DBT, New Delhi), Professor Barbara Hohn (Basel, Switzerland) and Dr. Gabriele Schachermayr (Lausanne, Switzerland) for their constant encouragement and support.

REFERENCES

Barna KS and Wakhlu AK (1993). Somatic embryogenesis and plant regeneration from callus cultures of chickpea (*Cicer arietinum L.*). *Plant Cell Rep.* 12: 521- 524

Chakraborti D, Sarkar A and Das S (2006). Efficient and rapid in vitro plant regeneration system for Indian cultivars of chickpea (*Cicer arietinum* L.). *Plant Cell Tiss. Org. Cult.* 86: 117-123

Chauhan R and Singh NP (2002). Plant regeneration via somatic embryogenesis in chickpea (*Cicer arietinum* L.). *Ind. J. Genet.* 62: 319-321

Chauhan R, Tiwari A and Singh NP (2003). Differential requirement of mature and immature embryo of chickpea (*Cicer arietinum* L.) for *in vitro* regeneration. *Ind. J. Plant Physiol.* 8: 28-33

Chen JT and Chang WC (2006). Direct somatic embryogenesis and plant regeneration from leaf explants of *Phalaeopsis amabilis*. *Biol. Plant.* 50: 169-173

Cournac L, Dimon B, Carrier P, Lahou A and Chagvardieff P (1991). Growth and photosynthetic characteristics of *Solanum tuberosum* plantlets cultivated in vitro in different conditions of aeration, source supply and CO_2 enrichment. *Plant Physiol.* 97: 112–117

Devlin RM, Zbiec II and Nowicka SE (1989). The effect of TDZ on some plant growth systems. *Proc. Plant Growth Regul. Soc.* 16: 99-103

Ebrahim MKH and Ibrahim AI (2000). Influence of medium solidification and pH value on in vitro propagation of Maranta leuconeura cv Kerchoviana. *Sci. Hortic.* 86: 211-221

Eisinger W (1983). Regulation of pea internode expansion by ethylene. *Ann. Rev. Plant Physiol.* 34: 225-240

Ganeshan S, Baga M, Harvey BL, Rossnagel BG, Scoles GJ and Chibbar RN (2003). Production of multiple shoots from thidiazuron-treated mature embryos and leaf-base/apical meristems of barley (*Hordeum vulgare*). *Plant Cell Tiss. Org. Cult.* 73: 57-64

Hazarika BN (2006). Morpho-physiological disorders in *in vitro* culture of plants. *Sci. Hortic.* 108: 105–120

Huetteman CA and Preece JE (1993). Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tiss. Org. Cult.* 33: 105-119

Indurker S, Misra HS and Eapen S (2007). Genetic transformation of chickpea (*Cicer arietinum* L.) with insecticidal crystal protein gene using particle gun bonbardment. *Plant Cell Rep.* 26: 755-63

Jackson MB, Abbott AJ, Belcher AR, Hall KC, Butler R and Cameron J (1991). Ventilation in plant tissue culture and effects of poor aeration on ethylene and carbon dioxide accumulation, oxygen depletion and explants development. *Ann. Bot.* 67: 229–237

Jayanand B, Sudarsanam and 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

Jiang B, Yang Y, Guo Y, Guo Z and Chen Y (2005). Thidiazuron-induced *in vitro* shoot organogenesis of the medicinal plant *Arnebia euchroma* (Royle) Johnst. *In Vitro Cell Dev. Biol. Plant* 41: 677-681

Kar S, Basu D, Das S, Ramakrishnan NA, Mukherjee P, Nayak P and Sen SK (1997). Expression of *CryIA(C)* gene of *Bacillus thurigenesis* in transgenic chickpea plants inhibits development of pod borer (*Heliothis armigera*) larvae. *Transgenic Res.* 6: 177-185

Kar S, Johnson TM, Nayak P and Sen SK (1996). Efficient transgenic plant regeneration through *Agrobacterium*-mediated transformation of Chickpea (*Cicer arietinum*. L). *Plant Cell Rep.* 16: 32-37

Kiran G, Kaviraj CP, Jogeswar G, Kishor KVK and Rao S (2005). Direct and high frequency somatic embryogenesis and plant regeneration from hopocotyls of chickpea (*Cicer arietinum* L.), a grain legume. *Curr. Sci.* 89: 1012-1018

Krishnamurthy KV, Suhasani K, Sagare AP, Meixner M, Kathen de A, Pickardt T and Schieder O (2000) *Agrobacterium* mediated transformation of chickpea (*Cicer arietinum* L.) embryo axes. *Plant Cell Rep.* 19: 235-240

Kumar PA, Bisaria S, Pai RA and Sharma RP (1995). Comparative shoot regeneration in different genotypes of chickpea, *Cicer arietinum* L. *Ind. J. Exp. Biol.* 33: 77-78

Kumar VD, Kirti PB, Sachan JKS and Chopra VL (1994). Plant regeneration via somatic embryogenesis in chickpea (*Cicer arietinum* L.). *Plant Cell Rep.* 13: 468-472

Malik KA and Saxena PK (1992). Thidiazuron induces high frequency shoots regeneration in intact seedlings of pea (*Pisum sativum*), Chickpea (*Cicer arietinum*) and lentil (*Lens culinaris*). *Aust. J. Plant Physiol.* 19: 731-740

Mroginski E, Rey HY, Gonzalez AM and Mroginski LA (2004). Thidiazuron promotes *in vitro* plant regeneration of *Arachis correntina* (*Leguminosae*) via organogenesis. *Plant Growth Regul.* 23: 129-134

Mroginski LA and Kartha KK (1984). Tissue culture of legumes for crop improvement. *Plant Breed. Rev.* 2: 215-264

Munns R (2002). Comparative physiology of salt and water stress. *Plant Cell Environ.* 25: 239-250

Murthy BNS, Murch SJ and Saxena PK (1995). TDZ-induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea*): endogenous growth regulator levels and significance of cotyledons. *Physiol. Plant.* 94: 268-276

Murthy BNS, Murch SJ and Saxena PK (1998). Thidiazuron: a potent regulator of in vitro plant morphogenesis. *In Vitro Cell Dev. Biol. Plant* 34: 267-275

Murthy BNS, Victor J, Singh RP, Fletcher RA and Saxena PK (1996). *In vitro* regeneration of chickpea (*Cicer arietinum* L.): stimulation of direct organogenesis and somatic embryogenesis by thidiazuron. *Plant Growth Regul.* 19: 233-240

Onamu R, Obukosia SD, Musembi N and Hutchinson MJ (2003). Efficacy of thidiazuron in *in vitro* propagation of carnation shoot tips: Influence of dose and duration of exposure. *Afr. Crop Sci.* J 11: 125-132

Paul V, Chandra R, Khetarpal S and Polisetty R (2000). Effect of BAP induction period on shoot differentiation from seeding explants of chickpea (*Cicer arietinum* L.). *J. Plant Biol.* 27: 235-239

Polisetty R, Patil P, Deveshwar JJ, Khetarpal S and Chandra R (1996). Rooting and establishment of in vitro shoot tip explants of chickpea (*Cicer arietinum* L.). *Ind. J. Exp. Biol.* 34: 806-809

Polisetty R, Patil P, Deveshwar JJ, Khetarpal S, Suresh K and Chandra R (1997). Multiple shoot induction by benzyladenine and complete plant regeneration from seed explants of chickpea (*Cicer arietinum* L.). *Plant Cell Rep.* 16: 565-571

Polowick PL, Baliski DS and Mahon JD (2004). *Agrobacterium tumefaciens*-mediated transformation of chickpea (*Cicer arietinum* L.): gene integration, expression and inheritance. *Plant Cell Rep.* 23: 485-491

Radhika K, Sujatha M and Rao TN (2006). Thidiazuron stimulates adventitious shoot regeneration in different safflower explants. *Biol. Plant.* 50: 174-179

Rizvi SMH and Singh RP (2000). *In-vitro* plant regeneration from immature leaflet-derived callus cultures of *Cicer arietinum* L. via organogenesis. *Plant Cell Biotechnol. Mol. Biol.* 1: 109-114

Sagare AP, Suhasini K and Krishnamurthy KV (1993). Plant regeneration via somatic embryogenesis in chickpea (*Cicer arietinum* L.). *Plant Cell Rep.* 12: 652-655

Sanyal I, Singh AK, Kaushik M and Amla DV (2005). *Agrobacterium*-mediated transformation of chickpea (*Cicer arietinum* L.) with *Bacillus thuringiensis cry1Ac* gene for resistance against pod borer insect *Helicoverpa armigera*. *Plant Sci.* 168: 1135-1146

Sarmah BK, Moore A, Tate W, Molving L, Morton RL, Ress DP, Chiaiese P, Chrispeels MJ, Tabe LM and Higgins TJV (2004). Transgenic chickpea seeds expressing high level of a bean á–amylase inhibitor. *Mol. Breed.* 14: 73-82.

Senthil G, Williamson B, Dinkins RD and Ramsay G (2004). An efficient transformation system for chickpea (*Cicer arietinum* L.). *Plant Cell Rep.* 23: 297-303

Shan X, Li D and Qu R (2000). Thidiazuron promotes *in vitro* regeneration of wheat and barley. *In Vitro Cell Dev. Biol. Plant* 36: 207-210

Sharma VK, Hansch R, Mendel RR and Schulze J (2005). Influence of picloram and thidiazuron on high frequency plant regeneration in elite cultivars of wheat with long-term retention of morphogenecity using meristemic shoot segments. *Plant Breed.* 124: 242-246

Shri PV and Davis TM (1992). Zeatin-induced shoot regeneration from immature chickpra (*Cicer arietinum* L.) cotyledons. *Plant Cell Tiss. Org. Cult.* 28: 45-51

Subhan S, Sharmila P and Pardha Saradhi P (1998). *Glomus fasciculatum* alleviates transplantation shock of micropropagation *Sesbania*. *Plant Cell Rep.* 17: 268-272

Suhasini K, Sagare AP and Krishnamurthy KV (1994). Direct somatic embryogenesis from mature embryo axes in chickpea (*Cicer arietinum* L.). *Plant Sci.* 102: 189-194

Tewari-Singh N, Sen J, Kiesecker H, Reddy VS, Jacobsen HJ and Mukherjee SG (2004). Use of herbicide or lysine plus threonine for non-antibotic selection of transgenic chickpea. *Plant Cell Rep.* 22: 576-583

Vani AKS and Reddy VD (1996). Morphogenesis from callus cultures of chickpea, (*Cicer arietinum* L.). *Ind. J. Exp. Biol.* 34: 285-289

Yadav SS, Kumar J, Yadav SK, Singh S, Yadav VS, Turner NC and Redden R (2006). Evaluation of *Helicoverpa* and drought resistance in desi and kabuli chickpea. *Plant Genet. Resources* 4: 198-203