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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

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Summary

An efficient and reproducible protocol for the regeneration of shoots at high frequency was developed by using explants derived from the axillary meristems from the cotyledonary nodes of *in vitro*-germinated seedlings of chickpea (*Cicer arietinum* L.). Culture conditions for various stages of adventitious shoot regeneration including the induction, elongation, and rooting of the elongated shoots were optimized. The medium for synchronous induction of multiple shoot buds consisted of Murashige and Skoog basal medium (MS) with low concentrations of thidiazuron (TDZ), 2-isopentenyladenine (2-iP), and kinetin. Exclusion of TDZ and lowering the concentration of 2-iP and kinetin in the elongation medium resulted in faster and enhanced frequency of elongated shoots. Cultivation of the stunted shoots on MS with giberellic acid (GA_3) increased the number of elongated shoots from the responding explants. pH of the medium played a very crucial role in the regeneration of multiple shoot buds from the explants derived from cotyledonary nodes. A novel rooting system was developed by placing the elongated shoot on a filter paper bridge immersed in liquid rooting medium that resulted in rooting frequency of up to 90%. A comprehensive protocol for successful transplantation of the *in vitro*-produced plants is reported. This method will be very useful for the genetic manipulation of chickpea for its agronomic improvement.

Key words: chickpea; *Cicer arietinum*; shoot regeneration; tissue culture; rooting.

Introduction

Chickpea (*Cicer arietinum* L.) is one of the important grain legumes that plays a significant role in the nutrition of rural and urban poor in the developing world. Chickpea contributes 15% to the world's pulse harvest of about 58 million tonnes. Chickpea is traditionally grown in many parts of the world, including Asia, Africa, Europe, and North and South America, but the bulk of it is produced and consumed in South Asia and increasingly, the Middle East and some Mediterranean countries (Jodha and Subbarao, 1987). Despite significant gains in world pulse production during the past two decades, with an average annual growth rate of 1.9%, chickpea production growth has been slow. Chickpea yields worldwide have risen by only 80 kg ha^{-1}, 0.6% annually, and its area has remained virtually stagnant. There are several significant and refractory constraints to chickpea production. These include biotic constraints like *Ascochyta* blight, *Botrytis* gray mold, dry root rot, collar rot, *Fusarium* wilt, pod borer, and abiotic stresses like drought, salinity, and low temperature. The enhancement of insect and disease resistance in chickpea can increase its yield potential by as much as three times.

Modern biotechnology, including tissue culture, genetic engineering, and genetic transformation techniques, has provided new opportunities to enhance the germplasm of crop plants (Sharma and Ortiz, 2000). A reliable shoot regeneration protocol is a prerequisite for efficient application of genetic transformation strategies. Several regeneration protocols involving somatic embryogenesis and shoot organogenesis in chickpea have been reported with varying success (Rao and Chopra, 1987, 1989; Riazuddin et al., 1988; Rao, 1990, 1991; Dineshkumar et al., 1994; Sonia et al., 2002). Considerable work has been done on the induction of somatic embryogenesis from mature (Rao and Chopra, 1989) and immature leaflets (Barna and Wakhlu, 1993), mature (Suhasini et al., 1994) and immature embryo axes (Sagare et al., 1993), or cell suspension cultures (Prakash et al., 1994). However, the recovery frequency of plants has been very low which has limited genetic transformation studies.

Regeneration of shoot buds from various explants has also been reported to produce shoots, either directly (Shri and Davis, 1992; Kar et al., 1996; Sharma and Amla, 1998; Subhadra et al., 1998) or indirectly through a callus phase (Khan and Ghosh, 1984; Prakash et al., 1992; Barna and Wakhlu, 1994). However, to date effective chickpea regeneration has been possible only through the use of explants based on cotyledonary nodes or shoot apices derived from seedling explants (Sonia et al., 2002). In most of the instances,

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the shoots were formed as a result of proliferation of pre-existing meristems, making these systems inefficient for transformation studies. Such systems have been used for genetically transforming chickpea (Fontana et al., 1993; Kar et al., 1997; Krishnamurthy et al., 2000) but the success has been very low and often the protocols are not reproducible in different laboratories.

In our ongoing efforts to develop efficient tissue culture and transformation protocols for the genetic transformation of chickpea, we have carried out extensive work on various factors that can influence synchronous regeneration of multiple shoots and recovery of plants. These include the selection of suitable explant, age of the explant donor seedlings, and culture media. To facilitate the recovery of a large number of rooted plants, we also report a novel rooting and transplantation system that provides plants with high success rates. The protocols reported here should facilitate effective utilization of genetic transformation technology for the agronomic improvement of chickpea.

Materials and Methods

Plant material. Mature seeds of chickpea (*Cicer arietinum* L.) cultivar C-235, a widely grown cultivar in India, were surface-sterilized with 70% (v/v) ethanol for 1 min, 0.1% mercuric chloride for 10 min, and rinsed five times in sterile distilled water prior to soaking overnight. The de-coated seeds were kept for germination on MS medium (Murashige and Skoog, 1962), or as indicated for obtaining the explants of different seedling age. Mature embryo axes from overnight-soaked seeds were considered as 0-d-old seedlings.

Media and culture conditions. Unless mentioned otherwise, the following media were used during various stages of shoot regeneration. The shoot induction medium (SIM) consisted of MS along with 4 μ*M* thidiazuron (TDZ), 10 μ*M* 2-isopentenyladenine (2-iP), and 2 μ*M* kinetin. The shoot elongation medium (SEM) consisted of MS along with 5 μ*M* 2-iP and 2 μ*M* kinetin (SEM1) or MS + 2 μ*M* gibberellic acid (GA_3) (SEM2). The root induction medium (RIM) was used as liquid and consisted of MS with 5 μ*M* indolebutyric acid (IBA) at pH 5.9 or 6.0. Culture media were used as liquid or solidified with 0.8% (w/v) Difco-Bacto agar as required and the pH was adjusted to 5.2 for SIM and 5.5 for SEM1 and SEM2 prior to autoclaving. All the tissue cultures were maintained at 26 ± 1°C under continuous cool white light provided by fluorescent lamps (30 μE m^{-2} s^{-1}).

Explant preparation. The de-coated seeds were germinated on SIM and the seedlings were allowed to grow for 5–7 d until the axillary buds were prominent (Fig. 1*A*). Then the axillary bud was carefully removed up to the base and two cuts were made through the axillary meristem where the root and shoot tip were also removed up to the hypocotyl and epicotyl regions, respectively (Fig. 1*B*). This resulted in two axillary meristem explants (AME) per seedling (Figs. 1*C*, 2*A*) that were then cultured in SIM.

Induction of multiple shoots. The cultured AME were grown for 1 wk on SIM prior to transfer to MS. The induced multiple shoots were allowed to grow for 7–10 d on MS before their transfer to the SEM.

Elongation. The explants having multiple shoot buds were transferred to SEM1 for 10 d and subcultured for two or three passages on SEM2 at 7-d intervals. The multiple shoot clusters were separated into small bunches of shoots where each bunch grew thinner with respect to the number of shoots by every subculture, leaving at least three or four shoots per bunch in the final cycle. Stunted shoots, when placed on SEM2, elongated with increasing frequency and increased length of the internodes. Shoots that elongated to 5 cm in each passage were used for rooting.

Rooting. The elongated shoots were used to optimize their rooting in two phases, viz., phase 1 and phase 2. Both the phases were maintained in the culture room under aseptic conditions. The shoots that did not root in both the phases were processed further to phase 3. Dark green, healthy shoots less than 5 cm in length were ideal for the induction of adventitious roots. The rootable shoots were cultured in culture tubes (25 × 200 mm) containing filter paper bridges immersed in liquid root induction medium (RIM) that consisted of MS with modified levels of KNO_3 (9.4 m*M*; half of the concentration in MS). IBA was filter-sterilized and added at 5 μ*M*.

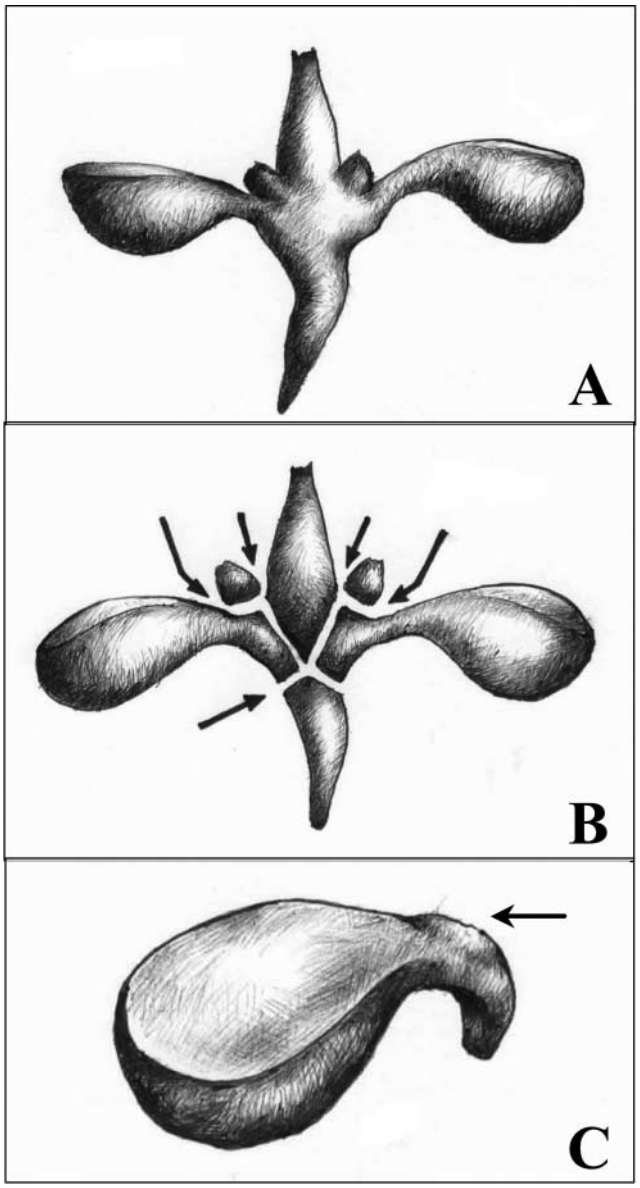

Fig. 1. Diagrammatic representation of the preparation of an axillary meristem explant (AME). *A*, Seedling explant after 5–7 d of *in vitro* germination. *B*, removal of shoot tip, axillary buds, and root from the seedling (*arrows* indicate surgical excision points). *C*, An AME ready for culture on shoot induction medium (*arrow* indicates the meristematic tissue).

While 60–80% of the elongated shoots rooted in phase 1, the shoots devoid of roots were carried on to phase 2 where the shoot length should be a minimum of 8 cm. Such shoots were briefly dipped in filter-sterilized solution of 100 μ*M* IBA and placed on filter paper bridges in culture tubes containing hormone-free liquid MS. The effect of $\frac{1}{2}$ MS or MS devoid of any growth regulator and the effect of various concentrations of α-naphthaleneacetic acid (NAA), IBA, and sucrose on rooting were also studied.

Rooting in the hydroponic system. This system is termed the phase 3 of rooting. About 10–20% of the rootable shoots which did not root even after two or three subcultures on RIM were carried to the hydroponic system that was generally used for hardening during the transplantation process. Quarter-strength Arnon's solution was used to fill an 8-cm Magenta jar and the shoot was suspended with support (Fig. 2*G*) such that 1 cm of the shoot base was immersed in the solution that contained 3 μ*M* IBA. The medium was changed every 4–5 d until the root primordia appeared. The shoots with

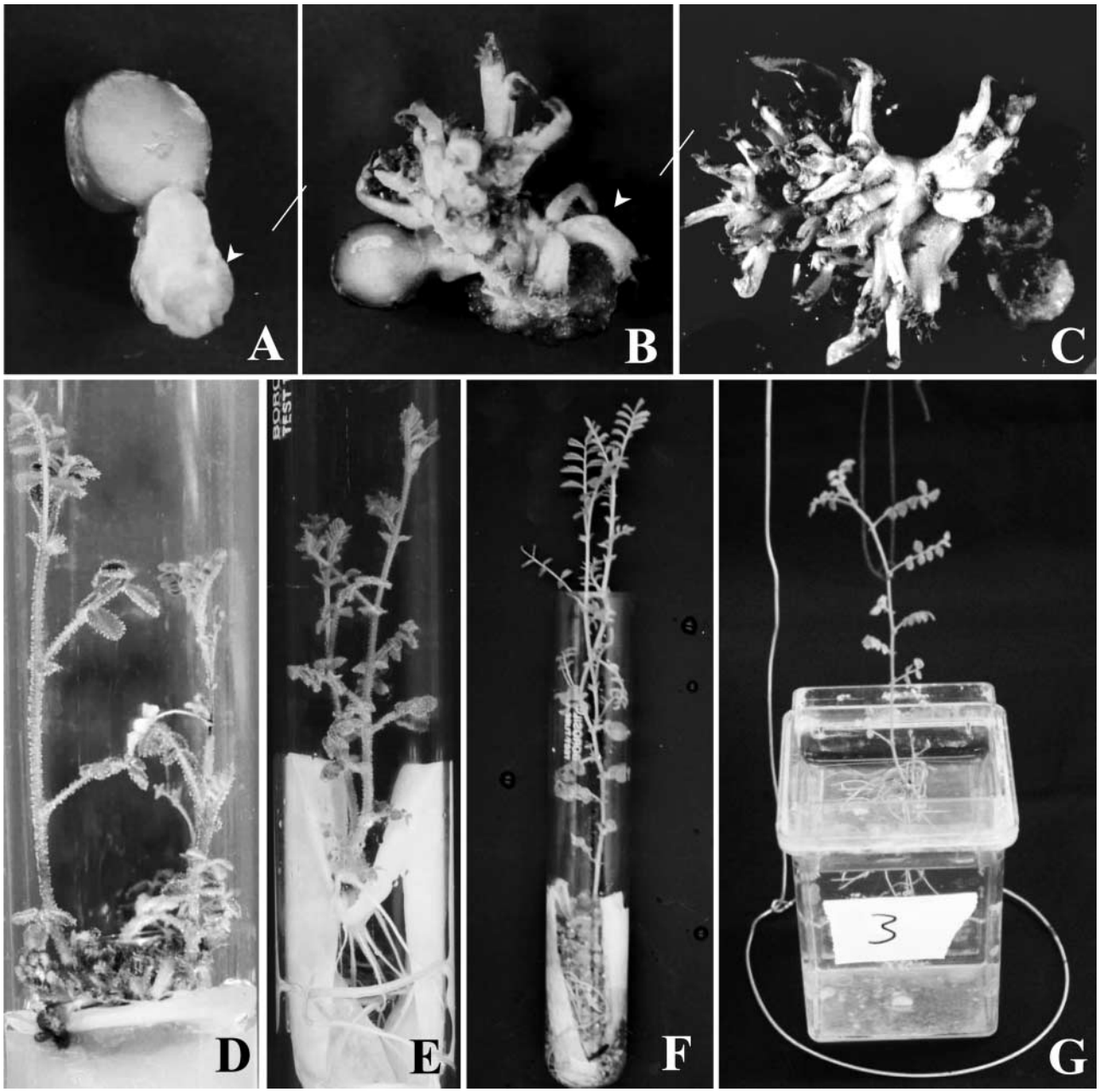

FIG. 2. Regeneration of multiple shoots from axillary meristem explants (AME) derived from *in vitro*-germinated seedlings of chickpea, *Cicer arietinum* L. *A*, AME derived from pre-soaked seeds at culture initiation (*arrow* indicates the meristematic tissue). *B*, Regeneration of multiple shoot buds (*arrow*) from AME after 5 d on shoot induction medium. *C*, Formation of multiple adventitious shoots from the meristematic tissue. *D*, Elongated shoot on SEM2 medium. *E*, Rooting of an elongated shoot on a filter paper bridge soaked in liquid medium. *F*, A rooted shoot from *E* in a culture tube containing fine gravel for the hardening of the root and shoot system prior to transplantation. *G*, Hardening of the rooted plant in static hydroponic culture containing quarter-strength Arnon's nutrient solution.

roots were transferred to the hormone-free Arnon's solution for further growth and hardening. Subsequently, after sufficient root growth, the plants were transferred to 20 cm diameter pots containing potting mix, comprising sand and black farm soil (3:2).

Hardening and transplantation of the rooted plants. The transplantation process was broadly divided into three stages; stage 1: initial transfer into small pots with covers (7–10 d); stage 2: acclimatization phase in which the plants were gradually exposed to the ambience by pinching holes and removal of the corners of the cover (15–20 d); and stage 3: transfer of the plants to the 20-cm diameter pots and maintenance in the glasshouse for further growth.

The hardening process started with the removal of cotton plugs from the culture tubes for 1–2 d. The plants were carefully taken out of the tube and the roots were thoroughly washed, dipped in diluted Thiram® (fungicide) solution and transferred to 8-cm diameter pots containing coarse sand (2–4 mm diameter) as the potting medium. They were completely covered with transparent polypropylene bags and allowed to grow for 7–10 d. Condensation on the inner surface of the plastic bag was removed twice daily. The plants were exposed to the ambient conditions gradually by pinching holes and cutting the corners of the polypropylene bags. Finally, after 10 d, the cover was opened on top and allowed to stay for about a week, following which the plant was carefully transferred to a 20-cm diameter pot containing potting mix. The potting mix comprised a mixture of smooth

and coarse sand mixed with black farm soil (3:2) with 10% organic matter (equal amounts of Cell Rich and rice straw compost). The effect of various factors such as potting medium composition, temperature, humidity, light intensity, and irrigation were also studied.

Hydroponics system. This system can also be used directly for hardening of the rooted shoots or at stage 1 of the above process, when little or no growth of the transplanted plants is observed. In some cases, especially with the plants rooted in phase 2 of rooting, there was little or no growth of the stem and leaves. This was found to be due to the lack of growth of the rooting system. Such plants at the end of stage 1 were removed from the small pots, the sand was washed off completely and they were placed into the hydroponics system as shown in Fig. 2*G*. After about 3 wk, when the root system attained sufficient growth, they were transferred to 20-cm diameter pots containing sand and black soil (3:2) for further growth and development of plants.

Results

Shoot Regeneration

Multiple shoots were observed by the end of the first week and morphogenic responses of the cultured explants were recorded at the end of the second week (Fig. 2*B*). The number of shoots was also counted at the end of the fourth week for standardization of the medium (Fig. 2*C*). Further subcultures of the explants on the same induction medium for about 6–7 wk resulted in over 100 shoots per explant. However, elongation frequency of the induced multiple shoots was brought down by prolonged culture of the explants on TDZ-containing medium. Hence, they were transferred to SEM in the third week and the results were recorded just before their transfer.

Effect of growth regulators on shoot induction. The results of various concentrations of TDZ, ranging between 2 and 100 μ*M*, on axillary meristem explant (AME) are shown in Table 1. A low level of TDZ (4 μ*M*; JCR2) was most effectives while levels of TDZ up to 10 μ*M* resulted in more or less similar numbers of shoots per responding explant. Increase in the TDZ concentration resulted in stunted growth of the shoot buds. Shoot buds that appeared at TDZ concentrations of 50 and 100 μ*M* failed to elongate. JCR13, which consisted of a combination of TDZ, 2-iP, and kinetin, showed best results by giving rise to 23 shoots at the end of 2 wk and 40 shoots at the end of 4 wk. The shoots were healthy and elongated well on SEM.

Effect of pH on multiple shoot regeneration. The pH of culture media proved to have an indispensable effect on regeneration from axillary meristems (Table 2). Clearly the acidic pH of 5.0 and 5.5 showed better multiple shoot induction when compared with the generally used pH of 6.0. The number of multiple shoots decreased at alkaline pH while the shoot buds induced on media with pH of 5.0–5.5 elongated well on the elongation medium.

Elongation of Regenerated Shoots

Elongation of shoots was found to be best when the SEM was adjusted to pH 5.5 (Fig. 2*D*; data not shown). Results of elongation experiments employing various growth regulators are shown in Table 3. Plant growth regulators were employed at low concentrations to increase the number of elongated shoots where 2-iP, kinetin, and GA_3 were the key factors for shoot elongation. Elongation on kinetin alone was found to be minimal while the elongation on hormone-free MS was not encouraging. However, prolonged culture on MS resulted in the rooting of 5–10% of the elongated shoots. All the tested combinations showed elongation of 0.1 to three shoots per explant, in the initial stages, that grew to about 5 cm in 2 wk. The remaining explant was placed again on the fresh elongation medium (SEM2), which resulted in the elongation of few more shoots. GA_3 played a crucial role at this juncture by elongating the stunted shoots. Application of GA_3 showed an increased internodal length and better leaf morphology. Initiation of elongation with SEM1 followed by culture on SEM2 showed a considerable increase in the elongation frequency (Table 3).

Rooting of Shoots

The differentiation of roots on the elongated shoots occurred over a period of 1–3 wk. About 20% of the shoots showed root formation within 3–4 d and another 40% produced roots by the end of 1 wk.

TABLE 1

EFFECT OF TDZ, 2-iP, AND KINETIN ON SHOOT REGENERATION FROM THE EXPLANTS DERIVED FROM AXILLARY MERISTEMS OF CHICKPEA

Media	Growth regulators (μ*M*)			No. of explants cultured	No. of shoots per responding explant (after 2 wk)	No. of shoots per responding explant (after 4 wk)
	TDZ	2-iP	Kinetin			
JCR1	2	–	–	32	7.3 ± 0.6	11.3 ± 0.6
JCR2	4	–	–	32	14.7 ± 2.1	26.3 ± 1.2
JCR3	6	–	–	32	13.7 ± 2.3	25.7 ± 1.2
JCR4	8	–	–	32	13.7 ± 1.5	22.0 ± 1.0
JCR5	10	–	–	32	12.3 ± 1.2	19.3 ± 1.5
JCR6	20	–	–	32	11.0 ± 2.0	19.3 ± 0.6
JCR7	30	–	–	32	6.7 ± 1.5	15.0 ± 2.0
JCR8	40	–	–	32	9.0 ± 1.0	11.7 ± 1.5
JCR9	50	–	–	32	5.3 ± 0.6	9.0 ± 1.0
JCR10	100	–	–	32	3.3 ± 0.6	4.7 ± 0.6
JCR11	4	5	2	32	13.0 ± 2.0	23.0 ± 2.0
JCR12	4	5	4	32	15.3 ± 1.5	20.3 ± 0.6
JCR13	4	10	2	32	23.3 ± 1.5	40.0 ± 2.6
JCR14	4	10	4	32	14.7 ± 2.5	25.3 ± 2.5

The results were recorded at the end of 2 and 4 wk and the values are means of three replicates.

TABLE 2

EFFECT OF pH ON MULTIPLE SHOOT REGENERATION FROM THE AXILLARY MERISTEM EXPLANTS OF CHICKPEA

Media	pH	No. of explants cultured	No. of explants with shoots	No. of shoots per explant	Percentage explants with shoots
JPH1	4.0	40	37	20.3 ± 1.3	92.5
JPH2	4.5	40	38	21.7 ± 0.6	95.0
JPH3	5.0	40	40	28.3 ± 1.5	100
JPH4	5.5	40	40	29.7 ± 0.5	100
JPH5	6.0	40	39	23.7 ± 0.6	97.5
JPH6	6.5	40	37	23.7 ± 1.2	92.5
JPH7	7.0	40	33	15.7 ± 1.5	82.5
JPH8	7.5	40	35	11.3 ± 0.5	87.5
JPH9	8.0	40	27	6.3 ± 1.2	67.5

Results were recorded at the end of 3 wk and the values are mean from three replicates.

The remaining 20% developed roots in the second to third week. These were termed late roots. The lateral roots appeared within 2–3 wk (Fig. 2*E*). On average, 70–80% of elongated shoots rooted well. Late roots showed slow growth and they contained numerous root hairs. In general, a pH of 5.9–6.0 was favorable for the initiation of roots (data not shown). Shoots that did not produce roots were carried to phase 2 where they were subjected to a pulse treatment with IBA. Rooting in this phase was adventitious with innumerable roots originating from different parts of the shoot. However, some abnormalities resulted as roots formed all over the shoot if the shoot length was less than 5 cm. When exposed to higher concentrations of IBA, the tissue at the basal part of the shoot showed browning and necrosis. Shoot growth slowed down until the newly formed roots started to elongate. Prolonged exposure (>5–10 min) to high concentrations of IBA killed the whole shoot. Rooting was found to be best on media of pH 5.9–6.0. Over 60% of the shoots transferred to the hydroponic system for root induction produced well-developed adventitious roots (Fig. 2*G*).

Effect of sucrose on rooting. Variations in the concentration of sucrose showed varied rooting frequencies (Table 4). Results were recorded at the end of the phase 1 of rooting. In general, all the tested sucrose levels showed positive results. Sucrose at 1.5% and 2% showed the best results in terms of frequency and root morphology. Little or no response was observed in the absence of sucrose.

Effect of media adjuvents on rooting. The effect of various concentrations of IBA and NAA, $\frac{1}{2}$ MS, and MS were tested and the results are shown in Table 4. IBA at 5 μM concentration was found to be best for rooting. A brief exposure of the shoots to CPR5 (Table 4) prior to culturing them on liquid MS showed faster and efficient rooting.

TABLE 3

EFFECT OF MEDIA COMPOSITION ON ELONGATION OF REGENERATED SHOOTS

Culture media	Plant growth regulators (μM)				Elongation I	Elongation II
	2-iP	BA	Kinetin	GA_3		
CEL1	2.0	–	2.0	–	1.2	2.3
CEL2	5.0	–	2.0	–	3.1	4.2
CEL3	–	2.0	2.0	–	1.1	2.5
CEL4	–	5.0	2.0	–	1.7	3.1
CEL5	–	–	2.0	–	0.3	0.5
CEL6	–	–	5.0	–	0.5	0.6
CEL7	–	–	–	2.0	1.2	7.5
CEL8	–	–	–	5.0	1.1	4.8

Results were recorded from three replicate experiments taking shoots induced on JCR13 medium (see Table 1). Each replicate experiment consisted of a total of 40 explants with induced shoot buds at 18–23 per explant and the number of shoots elongating per explant was the average of all the 40 explants. Data compiled from three replicated experiments.

Elongation I: number of shoots elongated per explant by the end of second week. Elongation II: number of shoots elongated in the next two or three subcultures.

Transplantation and Hardening of Plants

Effect of potting medium. Various potting media such as black soil, red soil, smooth sand, coarse sand (2–4 mm particle size), and vermiculite, individually and in combination with each other, were tested in stage 1. Experiments with coarse sand (2–4 mm particle size) showed the best results for stages 1 and 2 of transplantation. There was about 80% survival at the end of stage 1 and 60% by the end of stage 2. In stage 3, sand (smooth and coarse) and black soil mixed at a 3:2 ratio showed the best results. Approximately 5–10% organic matter, comprising a mixture of Cell Rich and rice straw compost, was added to the potting medium.

Temperature. Stages 1 and 2 required a day temperature of 20 ± 2°C and a night temperature of 15 ± 1°C. Stage 3 required a slightly higher temperature of 25 ± 3°C and 15 ± 3°C for day and night, respectively.

Humidity. This played a crucial role in the hardening process. The hardening process was done in a walk-in type growth chamber (Conviron®) which had a relative humidity of 50% during the day and over 80% at night. The plants were initially exposed to humidity as high as over 90% by covering them with polypropylene bags. They were slowly acclimatized to the ambient humidity by gradually opening the cover. The entire acclimatization process required about 1 mo. before stage 3.

Light intensity and photoperiod. Plants were maintained at a light intensity of 2500–3500 lux up to the rooting stage. However, continuation of this condition in the hardening process resulted in initial long and abnormal growth and premature death of the plants. Hence, the entire hardening process was carried out at a light intensity of 10 000–12 000 lux. With regards to photoperiod, stage

TABLE 4

EFFECT OF CULTURE MEDIUM ON THE ROOTING OF *IN VITRO*-FORMED AND ELONGATED SHOOTS OF CHICKPEA

Rooting media	Medium constituents[a]			No. of shoots with roots[b]		Percentage shoots with roots
	Sucrose (%)	IBA (μM)	NAA (μM)	Phase 1	Phase 2	
CPR1 ($\frac{1}{2}$MS)	2.0	–	–	4.1	–	10.25
CPR2 (MS)	3.0	–	–	3.3	–	8.25
CPR3	0.0	5.0	–	0.1	0.0	0.25
CPR4	1.0	5.0	–	17.0	3.2	50.50
CPR5	1.5	5.0	–	30.5	8.2	96.75
CPR6	2.0	5.0	–	25.9	10.1	90.00
CPR7	2.5	5.0	–	19.9	5.7	64.00
CPR8	3.0	5.0	–	16.8	7.0	59.50
CPR9	2.0	5.0	–	27.2	12.4	99.00
CPR10	2.0	10.0	–	17.1	3.5	51.50
CPR11	2.0	–	5.0	3.9	–	9.75
CPR12	2.0	–	10.0	6.4	–	16.00

[a] All media were used as liquid containing a filter paper bridge. CPR3 to CPR8 contained sucrose at various concentrations with IBA as the rooting hormone added at 5 μM concentration. CPR1, CPR2, CPR11, and CPR12 did not contain phase 2 as there was no pulse treatment with IBA.

[b] Results were recorded at the end of 3 wk for phase 1 and 5 wk for phase 2. Each combination tested under three replications. Total number of shoots per replicate was 40.

1 was maintained at 10 h light and 14 h dark, stage 2 at 12 h light and 12 h dark, and stage 3 at 14 h light and 10 h dark.

Irrigation. Chickpea was found to be very sensitive to the irrigation process. Optimal moisture was maintained throughout the transplantation process by irrigating with 10–25 ml of irrigation medium as and when required.

Alternative methods and troubleshooting. These observations were required to enhance the survival frequency of the tissue culture-grown chickpea. Fig. 2*F* shows an alternative method of embedding the rooting system in the coarse sand by its direct addition to the tubes containing liquid medium. In this method the top portion of the shoot was exposed to the ambient conditions and slowly acclimatized during plant growth. Fig. 2*G* shows another method of hydroponics in which the rooting system is completely immersed in the quarter-strength Arnon's solution. In this method the plant was directly exposed to the ambient conditions from the first day. However, drying was prevented because of the liquid nature of the nourishing medium. This method can supplement stages 1 and 2 described above and the plants can go directly to stage 3. The transplanted plants exhibited normal growth in the glasshouse and produced morphologically normal flowers and pods that contained viable seeds.

Discussion

A prerequisite for the *in vitro* manipulation of a plant species is the availability of an efficient and reproducible plant regeneration system. Several shoot regeneration protocols have been reported for chickpea (Sonia et al., 2002). These include the regeneration of shoots either directly from the explants through pre-formed or newly formed meristems, or also indirectly via a callus phase followed by the regeneration of shoot buds or somatic embryos. The success rates for the recovery of mature somatic embryos are rather low, making it an inefficient system for any meaningful studies on genetic transformation. To date, effective chickpea regeneration has been possible mainly through the use of explants based on embryo axis either from pre-soaked mature seeds or germinating seedlings. In most instances, the shoots developed as a result of proliferation of pre-existing meristems in cotyledonary nodes, shoot tips, and epicotyl (Shri and Davis, 1992; Kar et al., 1996; Subhadra et al., 1998) are often not reproducible. Hence, the present study considered the optimization of various tissue culture variables in the pursuit of developing efficient and reproducible shoot regeneration procedures.

The explant used in the present work differs from those previously used, due to the absence of any apical meristem (shoot and root tips) and axillary bud. This is achieved by removing the axillary bud prior to culture on shoot induction medium. This not only results in the exclusion of already differentiated tissue of the axillary bud but also results in wounding that is crucial for *Agrobacterium*-mediated transformations. The presence of cotyledonary tissue at the time of shoot bud induction has been shown to play an important role in induced morphogenesis from the target cells (Sharma et al., 1991).

Most of the previous reports used 6-benzyladenine (BA) as a principal phytohormone for the induction of multiple shoot buds. However, its applicability for chickpea tissue culture was restricted to micropropagation. Since 1988, TDZ has been reported to induce adventitious shoot buds in a number of plant species (Briggs et al., 1988; Henny and Fooshee, 1990). However, there are several disadvantages in using TDZ, including stunted shoots, abnormal leaf morphology, etc. To overcome these problems, explants should be induced with the lowest but effective TDZ concentration and kept on TDZ medium for the least duration that is specific for each species. Shoot quality can be considerably improved by employing a purine cytokinin in combination with TDZ (Briggs et al., 1988). The shoots originated on TDZ were short, but elongated after transfer to the medium containing IBA and 2-iP (Preece and Imel, 1991). In chickpea, multiple shoot regeneration was reported after the application of TDZ to the whole seed (Malik and Saxena, 1992). The use of pre-existing meristems such as shoot tip and cotyledonary nodes, and pretreatment of whole seed with lower concentrations of TDZ and BA, though encouraging, have limited applicability for genetic transformation

studies (Sharma and Amla, 1998). In contrast, the regeneration system described in our studies uses tissues that produce fresh meristematic cells that are more amenable to gene incorporation. Incorporation and standardization of appropriate concentrations of TDZ in germination and induction media is a significant modification over the existing protocols.

Standardization of the induction medium was done involving all stages of plant regeneration such as elongation and rooting. Firstly, the lowest but effective concentration of TDZ was found to be between 4 and 10 μ*M*. In view of the fact that higher concentrations of TDZ negatively interfere with the number of shoot buds and their elongation, 4 μ*M* was considered to be optimal. Inclusion of 2-iP and kinetin was found to be very effective by providing a faster regeneration of shoot buds that were adventitious. TDZ was completely excluded while lowering the concentration of 2-iP and kinetin in the shoot elongation medium (SEM1) so as to enhance the frequency of elongated shoots. One to four shoots elongated per explant, which could be increased even more by effecting the elongation in a stepwise manner. Initial exposure to 2-iP and kinetin, which elongated one or two shoots, followed by treatment with GA_3, produced the best results.

pH of the external medium is known to play a significant role in tissue culture as it affects uptake of various nutrient ions. Uptake of nitrate and ammonium are primarily affected by the medium pH (Behrend and Mateles, 1975; Hyndman et al., 1982; Raven, 1986). It was observed that nitrate uptake is favored at low pH and ammonium at higher pH. Axillary bud multiplication in shoot cultures of *Castanea* was most satisfactory when the pH of MS was reduced to 4 (Chevre et al., 1983). We have observed that pH 5.0–5.5 was very favorable for the regeneration of multiple shoot buds.

Previous studies on chickpea showed that rooting with any of the media combinations resulted in a very low frequency of root formation where most of the non-rooted shoots died due to desiccation. We have developed a novel technique of rooting the elongated shoots on paper bridges immersed in liquid medium

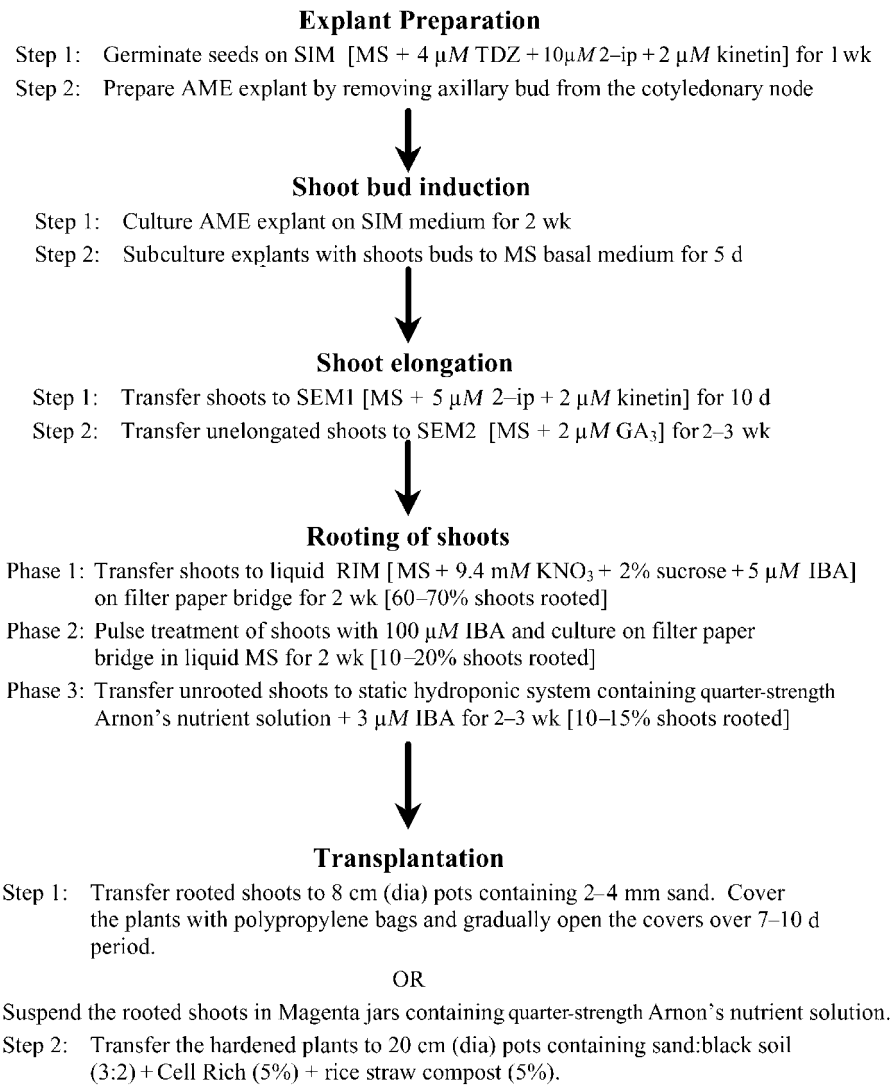

FIG. 3. Schematic representation of the protocol for *in vitro* regeneration of whole plants from axillary meristem explants (AME) of chickpea.

which has several advantages. Rooting frequency was very high, at 70–90% when compared to that of 5–10% in the semisolid medium (unpublished results). However, care should be taken to achieve this. Firstly, dark green, healthy shoots with well-spread leaflets with length not exceeding 5 cm were found to be ideal for rooting. Shoots that do not root should not stay on the same rooting container for a long time and they should be transferred to the fresh medium after the first batch has rooted. Care should be taken not to allow the shoots to acquire hyperhydricity because such shoots do not root well and have to be carried on to phase 2 of rooting. This system prevents the shoots from excessive desiccation resulting in the recovery of over 90% of the shoots that can be efficiently rooted. The transfer to the hardening phase should be started within 10 d of the root primordia being observed.

Very few reports discussed the problems involved in establishment of chickpea plants in soil (Dineshkumar et al., 1994; Polisetty et al., 1996). The difficulties prompted some workers to resort to inefficient *in vitro* grafting by utilizing scions from pre-germinated seedlings (Krishnamurthy et al., 2000). However, such methods are not only time consuming but technique-specific and the success rates vary among different laboratories. The rooting method proposed in this paper not only increased the frequency of rooted shoots but also resulted in very high recovery of plants after transplantation to the glasshouse. We have also derived a comprehensive method for the hardening and transplantation procedures. Meticulous observation of morphology of leaf size, color, stem inter-nodal length, and especially the root system growth was very much necessary during this process. Alternative hardening methods based on *ex vitro* static hydroponics were found to be very important in increasing the frequency of transplantable plants. The successful recovery of established plants becomes important while recovering the invaluable transgenic plants.

In conclusion, the comprehensive protocol reported here (Fig. 3) is efficient and reproducible. The induction of adventitious shoot buds was obtained with 4 μM TDZ at pH 5.0, their elongation in the presence of 5.0 μM 2-iP and 2.0 μM kinetin at pH 5.5, followed by a passage on 2.0 μM GA_3. Root induction was obtained in liquid medium containing 5.0 μM IBA at pH 6.0. The plants thus formed could be readily hardened in hydroponic cultures before their establishment in the glasshouse, thus providing an average of five to seven plants from a single explant. This protocol should provide an efficient means for *in vitro* manipulation of chickpea, an important legume crop of the semi-arid tropics.

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