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Efficient protocol for break impasses of regeneration via callus for 20 genotypes of Chickpea

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Abstract

The results of studies on *in vitro* regeneration of chickpea (*Cicer arietinum* L.) have shown that indirect regeneration via callus was not successful. The introduction of an efficient and repeatable regeneration protocol is necessary for using transgenic technologies for the improvement of chickpea. The present study was undertaken to investigate the possibility of indirect regeneration in various chickpea genotypes. In this experiment, cotyledonary node explants of 20 chickpea genotypes were cultured on 6 callus induction media and were sub-cultured on 6 shoot induction media with different combinations of phytohormons that are introduced in other literatures as best combination for regeneration. The results showed that reduction in experimental time and cost was possible by only transferring green calluses to other processes of culture. It seems that the results of this study and the suggested protocol could be used in genotypes as MCC426, MCC495, MCC496, MCC724, MCC741, MCC763, MCC764, MCC769, MCC775, MCC 779, MCC780, MCC798, MCC805 and MCC814 in suggestive media for genetic transformation and other projects where organogenesis via callus formation is necessary.

Keywords: Chickpea (*Cicer arietinum* L.); Callus-mediated shoot formation; Zeatin; IBA; Organogenesis; Regeneration

Abbreviation

2,4-D: 2,4 Dichlorophenoxyacetic acid, B5: Gamborg medium, BAP: Benzylamino purine, CIM: Callus Induction Medium, IAA: Indole-3-acetic acid, IBA: Indole-3-butyric acid, Kin: Kinetin, MB: MS salts and B5 vitamins medium, MS: Murashige and Skoog medium, NAA: Naphtalenacetic acid, PGR: Plant Growth Regulator, RIM: Root Induction Medium, SE: Standard Error, SIM: Shoot Induction Medium.

Introduction

Chickpea (*Cicer arietinum* L.) as an ancient and major food legume crop is cultivated in over 40 countries. In spite of its large demand, global yield of chickpea has not remarkably

increased in the past few decades due to the damaged cause by several pathogen and susceptibility to abiotic stress (Sarmah et al., 2004; Sanyal et al., 2005; Chakraborti et al., 2006). Cultivars resistant to biotic and abiotic stresses which have better protein quality and quantity are needed. Chickpea, similar to other grain legumes, have narrow genetic base since they are essentially self-pollinated (although cross-pollination does take place, it is at very low frequency). Thus there is the need to widen the genetic base and incorporate desirable characters. There is an urgent need to use transgenic technologies for the improvement of chickpea. Routine transformation protocols are limited in chickpea. The low success has been attributed to poor regeneration ability (especially via callus) and lack of compatible gene delivery methods (Chandra and Pental, 2003). Transgenics are needed for genetic enhancement as part of plant breeding programs. The focus of transformation protocols should be on recovery of useful transgenics. Poor regeneration (compared to model system like tobacco, mustard) and especially poor regeneration via callus contributes to low success in the production of transformed chickpea (Chandra and Pental, 2003). A reliable shoot regeneration protocol is a prerequisite for efficient application of genetic transformation strategies (Jayanand et al., 2003). There are two major factors that clearly inhibit efficient transformation: 1. The shoot regeneration rate is genotype-dependent and genotype specificity affects transformation rate; 2. The very low efficiency of transformation indicates that gene transfer via *Agrobacterium* infection into cut or injured cotyledon or hypocotyls tissue hardly ever occurs for reasons that are not well understood (Lee et al., 2004).

In vitro selection of cell lines is now being used as an alternative tool to accelerate breeding programs in many crops. Earlier studies on this aspect resulted in isolation of NaCl adapted/tolerant cell lines of chickpea, but regeneration from these tolerant calluses could not be achieved (Jaiswal and Singh, 2001). Identification of possible somaclonal variants among callus regenerated plants at the early stages of development is considered to be very useful for quality control in plant tissue culture, transgenic plant production and in the introduction of variants (Soniya et al., 2001). There are several reports on somatic embryogenesis from immature or mature leaflets and immature or mature embryo axes (Sonia et al., 2003). Shoot organogenesis either directly (Singh et al., 2002; Jayanand et al., 2003; Sanyal et al., 2005; Chakraborty et al., 2006) or indirectly through callus formation has also been reported. Additionally, organogenesis preceded by formation of intermediate structure, the cotyledon like structure (CLS) from immature cotyledon was reported (Srivastava et al., 2001; Chandra and Pental, 2003).

Several literature reviews have revealed few reports on shoot organogenesis indirectly through callus formation and those limited reports such as Barna and Wakhlu, 1994 and Altinkut et al., 1997 had problems on explant because shoots were produced from existing meristem not from calluses, and the results of Bajaj, 2000, Jaiswal and Singh, 2001, Huda et al., 2003 and Arora and Chawla, 2005 studies showed that the frequency of regeneration via callus in chickpea was too low. In general, the regeneration frequency through somatic embryogenesis and callus or CLS mediated organogenesis was low (Chakraborty et al., 2006). In most instances, 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 (Krishnamurthy et al., 2000), but the success has been very low and often the protocols are not reproducible in different

laboratories (Jayanand et al., 2003). In our ongoing efforts to develop efficient tissue culture protocols for the genetic transformation of chickpea and other projects that organogenesis via callus formation is necessary for them, we have carried out extensive work on various factors that can influence synchronous regeneration of chickpea. We report a novel and reproducible regeneration protocol through shooting of calluses tested on 20 genotypes of chickpea. The suggested protocol should facilitate effective utilization of genetic transformation technology for the agronomic improvement of chickpea.

Materials and Methods

Plant material

Mature seeds of 20 genotype of chickpea (*Cicer arietinum* L.) that are widely grown cultivars in Mashhad of Iran (Table 1), were surface-sterilized with 70% (v/v) ethanol for 30 s to 1 min and rinsed twice in sterile distilled water, then sterilized with 1% sodium hypochlorite solutions for 10-15 min, and rinsed five times in sterile distilled water prior to soaking overnight. The seeds were kept for germination on medium consists of 0.7% agar and sterile distilled water.

Table 1. Characteristics of the experimented genotypes.

Genotypes	Weight of 100 seeds (g)	Yield under Mashhad environment ($g.m^{-2}$)	Percentage of survey in winter culture in Mashhad condition
MCC 495	27.2	334.4	81.2
MCC496	25.6	196.4	100
MCC805	32.6	306.6	78.6
MCC814	32.6	267.8	100
MCC780	32.8	398.4	87.5
MCC781	33	188.9	100
MCC763	32.2	355.2	83.3
MCC791	33.2	622	100
MCC798	33.4	455.5	93.3
MCC771	34	463.8	77.3
MCC426	34.7	101.7	100
MCC769	35	372.7	95
MCC741	26.6	486.7	100
MCC779	35.4	339.4	86.7
MCC786	35.4	376	100
MCC724	35.8	218.6	93.3
MCC764	36.4	383.4	100
MCC770	36.4	277.5	84.6
MCC799	36.6	291.7	80
MCC775	40.2	374.1	100

Explants preparation

The seeds were germinated on water-agar and the seedlings were allowed to grow for 3-7 days until auxiliary buds were prominent. The auxiliary bud was carefully removed up to the base and two cuts were made through the auxiliary meristem where was hypocotyl and epicotyl region. Finally, the cotyledonary node explant was prepared.

Media and culture conditions

Unless mentioned otherwise, the following media were used during various stages of shoot regeneration. The callus induction media (CIM, Table 2), the shoot induction media (SIM, Table 2) and the root induction media (RIM, Table 3) consist of MS (Murashige and Skoog, 1962) salts and B5 (Gamborg et al., 1968) vitamins (MB) with different combinations of plant growth regulators (PGR), that were introduced in other literatures of chickpea and some other plants as best combinations for regeneration, were used. Culture media were used as liquid or solidified with 0.7% Difco-Bacto agar as required and 3% (w/v) sucrose and the pH was adjusted to 5.7-5.8 before autoclaving. All of the tissue cultures were maintained at 25±1 °C under 16 h photoperiod and under continuous cool white light provided by fluorescent lamps (30 $\mu Em^{-2}s^{-1}$).

Table 2. Combination and concentration of phytohormon used for callus induction and shooting.

Researchers	CIM ($mg.l^{-1}$)					SIM ($mg.l^{-1}$)						
		2,4-D	BAP	NAA	zeatin		BAP	NAA	Kin	IAA	IBA	zeatin
Altinkut (1997)	M1	0.5	0	0.1	0	RM1	0.2	0	0.5	0.2	0	0
Huda *et al* (2003)	M2	3	3	0	0	RM2	2	0.5	0	0	0	0
Singh *et al* (1997)	M3	2	0	0	0	RM3	1	1	0	0	0	0
Jaiswal and Singh (2001)	M4	0	0.5	0.5	0	RM4	2	0	0	0	0.125	0
Arora and Chawla (2005)	M5	0	1	2	0	RM5	2	0.05	0	0	0	0
Lee *et al* (2004)	M6	0	0	0.05	2	RM6	0	0.01	0	0	0	2

CIM: Callus Induction Medium; SIM: Shoot Induction Medium.

Induction of calluses

The cultured explants were converted to callus for 7-10 days on CIM. The induced calluses were allowed to grow for 10-14 days on CIM and sub-cultured for one or two passages on CIM.

Induction of shoots

One month old calluses were transferred to SIM for two weeks. The calluses having shoots sub-cultured for two or three passages on SIM. Shoots that elongated to 1.5 cm were separated from calluses and transferred into free hormone MB media for 1-2 weeks and increased their length. Shoots that elongated to 5 cm in each passage were used for rooting.

Rooting, hardening and transplantation of rooting plants

The elongated shoots were used for optimizing their rooting in RIM. All phases were maintained in the culture room under aseptic conditions. The shoots that did not root and rooted shoot in R1 were processed further to R2. Dark green and healthy shoots were ideal for the induction of adventitious roots. The rootable shoots were cultured in culture tubes (25×200 mm) containing filter paper bridge immersed in liquid RIM. IBA was filter-sterilized and added at 1 ($mg.l^{-1}$).

Table 3. Combination and concentration of phytohormon used for rooting.

RIM	Basic medium	IBA ($mg.l^{-1}$)	Sucrose ($g.l^{-1}$)	Agar ($g.l^{-1}$)
R1	1/2MB	1	10	0
R2	1/2MS	1	10	0.7

RIM: Root Induction Medium.

The ideal rooted shoots were carefully taken out of the tube and the roots were thoroughly washed and transferred to small pots containing pit and per light (1:3) as the potting medium. They were completely covered with transparent cover and were allowed to grow. The plants were exposed to the ambient conditions gradually by pinching holes on cover and removing the cover two days in a week. Transferred plants irrigated with distilled water and sprayed with Hoagland solution. After the acclimatization phase, plants were transferred to big pots and maintained in the glasshouse for further growth.

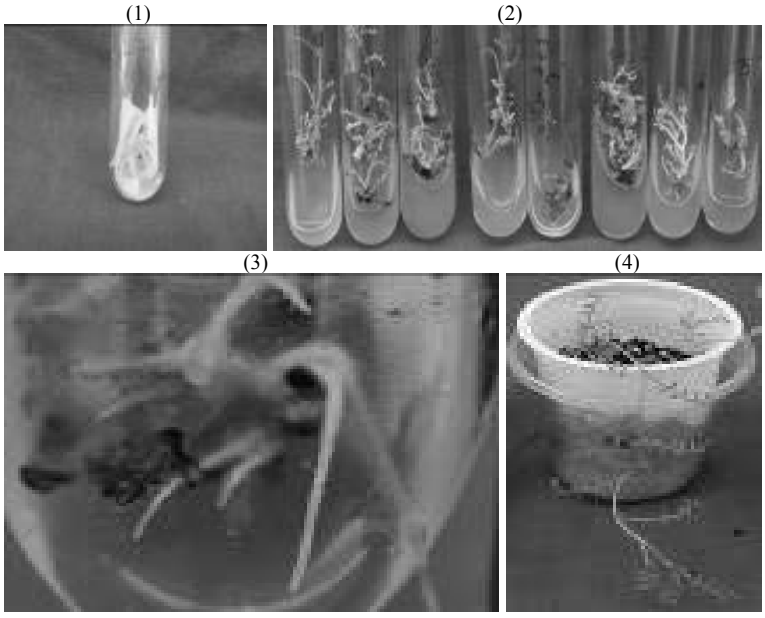


Figure 1. The processes of rooting shoots, (1) induction of root on R1 (2) induction and growth of roots on R2 (3) rooting of shoots (4) hardening of produced whole plants.

Analysis

The experiment was conducted as factorial in framework completely randomized design with four replications. Factors concluded genotypes in 20 levels (Table 1) and media in 6 levels (Table 2). For analysis data, the SPSS was used. By using the compare mean test (Duncan Multiple Range Test), the effect of genotype media and cross effect of genotype and media on factors such as wet and dry weight of calluses, number shoot produced and numbers of rooted shoot were compared.

Results

Callus induction

In most of the experimented genotypes, callus induction was observed after one week and morphogenesis response of the cultured explants were recorded after 4 weeks. Generally, specifics of the produced calluses on different media were large, green to yellow with a hard surface on M1, yellow with a hard surface on M2, large, yellow to white, moist and slightly transparent on M3, large green with a hard surface but a little bit moist on M4, small, green with a hard surface on M5 and dark-green with a hard surface on M6. In most of the genotypes, the color of the calluses turned into brown. This interaction in M1 and M3 was more eminent the other media.

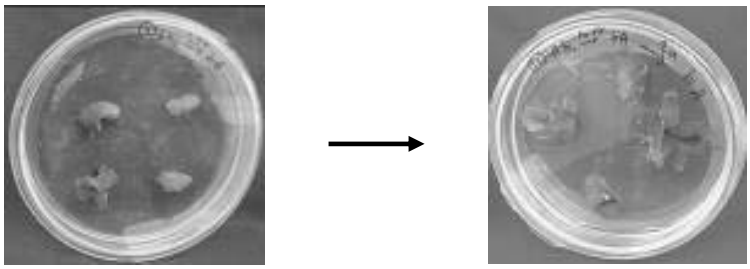
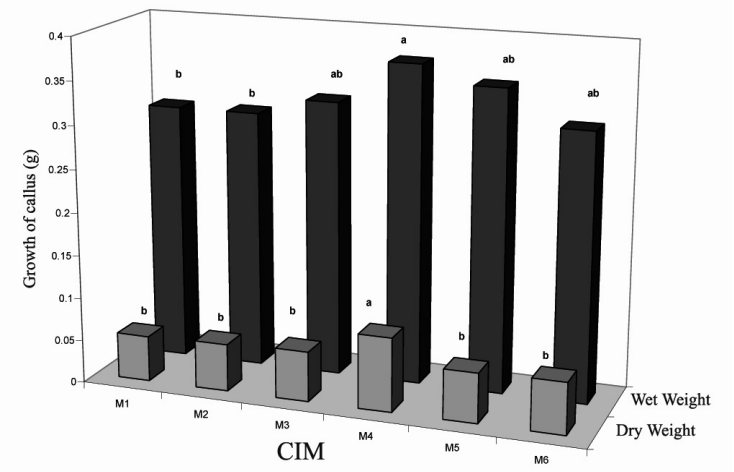


Figure 2. The growth of calluses of MCC496 of chickpea.

Effect of genotype: Variance segregation of wet and dry weight data showed that there was significant difference between genotypes in the 5% level. Results showed that MCC426 and MCC798 (0.38 g) had maximum and MCC 781 (0.23 g) had minimum of mean wet weight. MCC426 (0.1013 g) had maximum and MCC 781 (0.0421 g) had minimum of mean dry weight. It shows the effect of genotype on the growth of callus.

Effect of media: Significant difference between the media for growth of calluses at 5% level was observed. M4 with means of 0.3803 g wet weight and 0.086 g dry weight had maximum growth of callus. In this study, M4 consisting of MB along with 0.5 $mg.l^{-1}$ NAA and 0.5 $mg.l^{-1}$ BAP had the best response to induction of callus and produced most the heaviest calluses (Graph 1).



Graph 1. Compare mean of growth of callus in different media.
Mean followed by same superscript is not significantly different at P<0.05 level.

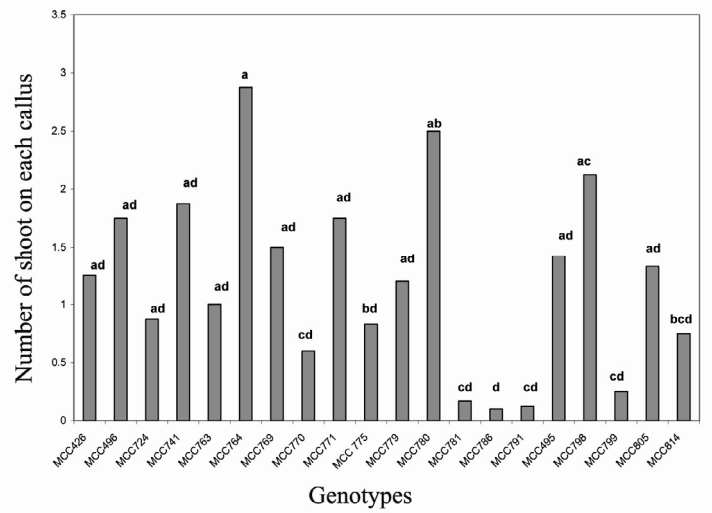
Cross effect of genotype and media: There was significant difference in 5% level in this case. It was observed in compare mean that MCC426 in M4 had maximum of dry and wet weight (0.55 and 1.2 g).

Shoot regeneration

Generally, different changes after transfer calluses to SIM was observed, changes as growth of calluses, leaves production, color change, shooting and whole plant production. Despite of various changes done to organogenesis in calluses, genotype shooting happened in all. Different genotypes showed different responses to SIM; therefore, it was not possible to report the same duration to induction of shoot and the same medium for all of 20 genotypes. In this study, some of the genotypes did not produce any shoots after 4 weeks, but after some more time shooting was observed on them. There was difference in morphology and frequency of the produced shoots in different genotypes.

Figure 3. Shooting of calluses in MCC 798 on RM4.

Effect of genotype: Results of compare mean of the produced shoot in different genotypes showed that there was significant difference between genotypes in the 5% level. The number of shoots in MCC 764 with 2.9 shoot on each callus was significantly more than other genotypes that produced 0.1-2.5 shoot on each callus (Graph 2). Results showed that genotype had effect on shooting of calluses. Arzani et al. (1999) reported that genotype was the most effective factor on induction callus and shooting in wheat.



Graph 2. Compare mean of the number produced shoot on each callus in different genotypes. Mean followed by same superscript was not significantly different at $P<0.05$ level.

Effect of media: Maximum mean of shooting observed on RM6 and had significant difference with other media in 5% level. Medium that was suggested by Lee et al. (2004) in shooting of pepper (RM6) in studied genotypes had the best shooting (3.2 shoots on each callus) compared to the other media. It seems that the use of zeatin (exist in RM6) effected the best shooting of calluses. Results showed that RM4 had significant difference with RM2, RM3 and RM5 in mean of shooting (Graph 3).

Cross effect of genotype and media: Results showed that MCC764 in M4 produced the maximum number of shoots (6 shoots on each callus).

Rooting of shoots

In this study, the same medium was used for rooting of shoots, but different rooting in different genotypes was observed. Frequency of rooting on shoots that imposed diversion on solid medium was better than the other situation. Generally, the needed duration to induction of root was more than the required time for the development of the root. Rooting of the shoots was observed on RIM, additionally rooting of shoots was observed in some of the shoots that were formed on RM5 and RM6 without transfer to RIM, also it was observed in some of the shoots that were transferred to free hormone medium for elongation.

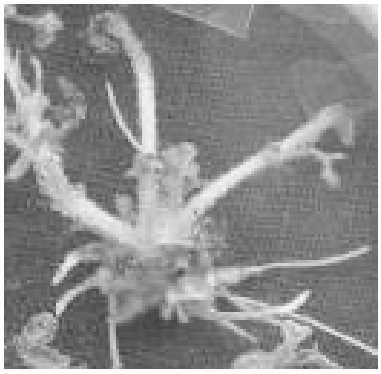
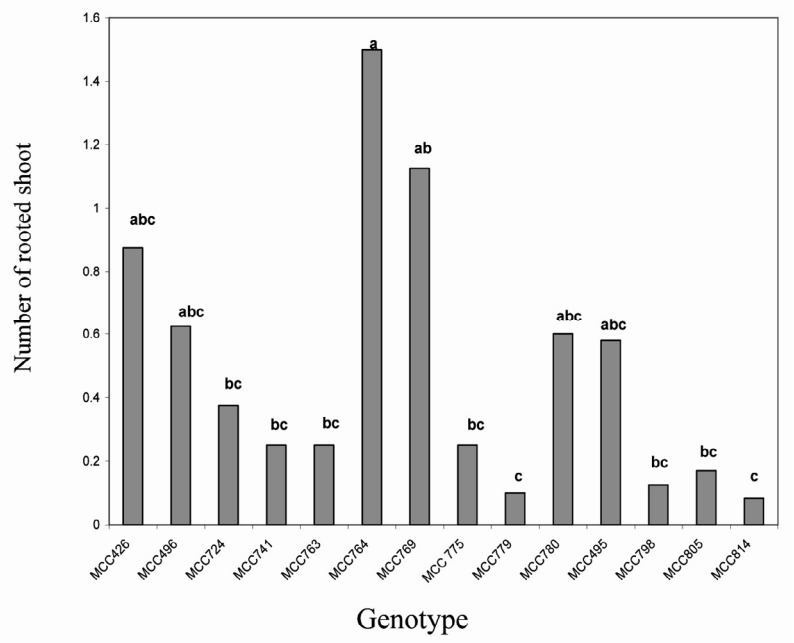


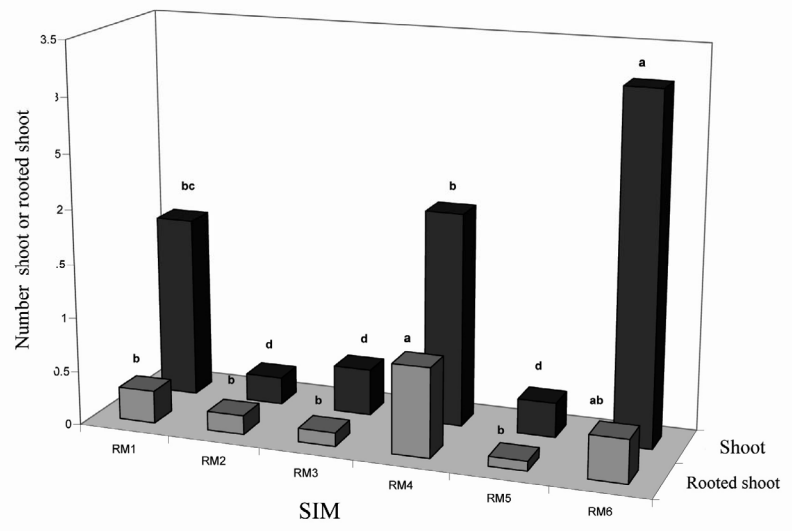
Figure 4. Production of whole plant from callus of MCC741 on RM6.

Effect of genotype: Results of compare mean of the number of rooted shoots in different genotypes showed that there was significant difference ($P<0.05$). MCC 764 (mean of 1.5 rooted shoot) had significant difference with other genotypes. Results showed that genotype had significant effect on rooting of shoot (Graph 3).



Graph 3. Compare mean of the number of rooted shoots in different genotypes.
Mean followed by same superscript is not significantly different at $P<0.05$ level.

Effect of shooting media on rooting of shoots: Results showed that maximum mean of the rooted shoot was 0.8 shoots that produced on RM4 consist of 2 $mg.l^{-1}$ BAP and 0.125 $mg.l^{-1}$ IBA. RM4 had significant difference with RM1, RM2, RM3 and RM5 in the 5% level to produce rooted shoot. It seems that callus induction media and shooting media such as rooting media had effect on rooting of the shoots. Means of rooted shoot on selected media was 0.8235-0.909. Results showed that the best results (whole plant production) would happen by culture explants on M4 or M6, transfer calluses to RM4 or RM6 and finally transferring shoots to rooting media (Graph 4).



Graph 4. Compare means of produced shoot and rooted shoot on different SIM.
Mean followed by same superscript is not significantly different at P<0.05 level.

Cross effect of genotype and media: It was observed that MCC764 in RM4 produced maximum mean of rooted shoot (5.5 shoots). Analysis of genotype and media effects in dry and wet weight, number shoot and number of rooted shoot showed that genotype and media had significant effect on these factors.

Frequency of shooting and rooting in different genotypes

Maximum shooting (39.3%) happened in MCC496 and mean of produced shoot in each callus was 1.44 shoot that 0.75 of them rooted. Then 35.7% shooting in MCC763 happened in which the means of shoot in each callus in MCC763 was 1.77 shoots and 0.78 of them rooted. Also in MCC 798 35.7% shooting happened, but mean of shoot in each callus was 2.35 shoot and 0.41 of them rooted.

Table 4. Frequency of shooting and rooting in different genotypes.

Genotypes	Shooting (%)	Rooted shoot (%)	Shoot/callus±SE	Rooted shoot±SE
MCC426	30.4	57.2	0.69±0.63	0.75±0.29
MCC496	39.3	18.1	1.44±1.27	0.75±0.35
MCC724	20	50.0	1.64±1.27	0.7±0.42
MCC741	33.3	33.3	0.56±0.31	0.75±0.35
MCC763	35.7	59.9	1.77±1.31	0.78±0.38
MCC764	26.7	74.9	2.42±1.06	0.66±0.45
MCC769	15.4	100.0	1.25±1.06	0.75±0.59
MCC770	4.8	0.0	3±1.12	-
MCC771	17.6	0.0	1.85±1.1	-
MCC775	13.6	33.1	2.06±1.23	0.6±0.35
MCC779	23.3	9.9	2.8±1.87	1±0.32
MCC780	30.4	57.2	0.89±0.8	0.68±0.37
MCC781	5.7	0.0	0.67±0.47	-
MCC786	12.5	0.0	0.82±0.45	-
MCC791	11.1	0.0	0.5±0.37	-
MCC495	25.7	44.4	1.22±1.13	0.73±0.36
MCC798	35.7	19.9	2.35±1.8	0.41±0.25
MCC799	11.1	0.0	0.5±0.27	-
MCC805	23.5	50.2	2.61±2.5	0.59±0.58
MCC814	17.5	14.3	1.38±0.99	0.31±0.25

Frequency of shooting and rooting in different SIM

Maximum frequency of shooting (52.8%) in RM4 happened where mean of the produced shoot in calluses was 1.72 shoot and 0.7 of them rooted. Then in RM6 50% shooting in calluses was observed. In RM6 2.49 shoots produced in calluses and 0.48 of them rooted.

Table 5. Frequency of shoot and root in different SIM.

SIM	Shooting (%)	Rooted shoot (%)	Shoot/callus±SE	Rooted shoot±SE
RM1	28	26.8	1.59±1.51	0.64±0.34
RM2	5	16.0	1.23±1.17	0.73±0.69
RM3	10.8	29.6	0.47±0.23	0.83±0.29
RM4	52.8	50.0	1.72±1.43	0.7±0.33
RM5	17.9	59.8	0.46±0.33	0.78±0.38
RM6	50	20.0	2.49±1.67	0.48±0.36

Frequency of shooting and rooting in different callus color

Results (all data not shown) showed that only green calluses had capability to be transferred to other processes of culture (Table 6).

Table 6. Frequency of shooting and rooting in different callus color.

SIM	percentage of calluses with this color that produced whole plant	Color of callus that produced whole plant
RM1	50	Green-yellow
RM2	100	Yellow
RM3	66.7	Yellow
RM4	100	Green
RM5	100	Green
RM6	50	Dark-green

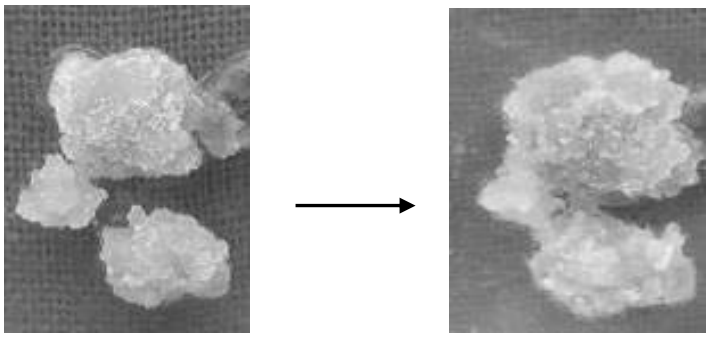


Figure 5. The conversion of yellow callus to green in MCC 426 on RM2.

Discussion

It seems that the basic medium consisting of MS salts and B5 vitamins is the best for induction of callus and shooting of chickpea, but the medium consisting of ½ MS for rooting is the best. Plant growth regulators had effective role on the induction of callus, shooting and rooting of them. There was significant difference between each of the 20 genotypes for production of callus and shooting in media with different combinations. Generally, green and large calluses produced in medium with 0.05 $mg.l^{-1}$ NAA and 2 $mg.l^{-1}$ zeatin, and medium with 0.5 $mg.l^{-1}$ NAA and 0.5 $mg.l^{-1}$ BAP (M4). It was observed that the most shooting of produced calluses was in medium with 0.01 $mg.l^{-1}$ NAA and 2 $mg.l^{-1}$ zeatin (RM6) and medium with 0.125 $mg.l^{-1}$ IBA and 2 $mg.l^{-1}$ BAP (RM4). It seems that medium with 1 mg/l IBA and 1% sucrose are better than other media for rooting of shoot with less concentration of sucrose for the avoidance to produce necrotic roots.

The suggested media create a new progress in frequency of indirect regeneration of these genotypes. In this study 39.3% shooting for MCC496 and 18% root production of this produced shoots was observed. After that 35.7% shooting in MCC763 and MCC798, it was observed that 60% and 20% of them rooted, respectively. Success in regeneration via callus formation that was achieved in this study had not been reported in other similar studies such as Barna and Wakhlu, 1994; Altinkut et al., 1997 that had problems with explants, because shoots were produced from existing meristem not from calluses, and results of Bajaj, 2000; Jaiswal and Singh, 2001; Huda et al., 2003 and Arora and Chawla, 2005 reveal that frequency of regeneration via callus in chickpea was too low.

This study showed that different genotypes in various media produce calluses with different colors, similar to what Lee et al. (2004) studied on callus types for shoot formation. Thus color of callus can show result of experiment in other processes after callus induction; therefore, only green calluses have the capability to transfer to other processes of culture. Thus, it can reduce in time and cost of the experiment. It is noticeable that in some of repeats of experiment calluses that produced on M6, inserted to shooting phase without transfer to SIM, even in some case rooting of shoots observed after subculture of shoots on prior medium. It seems that the use of experiment hormone as zeatin is economic because of the reduction in culture duration and no need for repeatable subculture compared to other hormones. Generally, with respect to different responses of each of the other genotypes to indirect regeneration, it seems that the results of this study and the suggested protocol (Table 7) could be used for genetic transformation projects in some of chickpea genotypes as MCC426, MCC495, MCC496, MCC724, MCC741, MCC763, MCC764, MCC769, MCC775, MCC 779, MCC780, MCC 798, MCC805 and MCC814.

Table 7. Protocol for in direct regeneration of chickpea.

process	Conditions and PGR combination	Duration
germination	Sterile distilled water + 0.8%agar	3-7 days
Explant	Cotyledonary node	
Callus induction	Basic media (MB+ 3% sucrose+ 0.8% agar,pH 5.8) 0.05 $mg.l^{-1}$ NAA+ 2 $mg.l^{-1}$ zeatin or 0.5 $mg.l^{-1}$ NAA+ 0.5 $mg.l^{-1}$ BAP	Callus formation: 2-3 weeks Callus development: 2-3 weeks
Shooting	Basic media 0.01 $mg.l^{-1}$ NAA + 2 $mg.l^{-1}$ zeatin or 0.125 $mg.l^{-1}$ IBA + 2 $mg.l^{-1}$ BAP	Shoot formation:1-2 weeks Shoot elongation: 2-5 weeks
rooting	½ MS + 1 $mg.l^{-1}$ IBA + 1% sucrose + 0.7% agar	Root formation: 4-5 weeks Root elongation: 1-2 weeks

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