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In vitro REGENERATION OF CHICKPEA (*Cicer arietinum L.*)

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ABSTRACT

A protocol was developed for shoot multiplication and regeneration for chickpea (*Cicer arietnum L.*). Three varieties (Hudas, FLIP03-147C and FLIP05-100C) and two landraces ("Ein -Elbyda" and "Baladi") were used in this study. For multiplication experiments, shoots tips of about 2 cm length from *in vitro* establishment seedlings of "Ein-Elbyda" and "Baladi" landraces were used. Explants were cultured on Murashige and Skoog (MS) medium supplied with 0.0, 2.2 and4.4 µM benzyl adenine (BA), or 0.0, 9.2 and 18.4 µM Kinetin, both cytokinins were combined with 0.0 or 2.7 µM naphthalene acetic acid (NAA). The highest shoot number (9.7, 6.6) was obtained at media supplied only with 2.2 µM BA for both landraces. Lower shoot number (5.3 and 4.5) was obtained at media supplied with 9.2 µM kinetin in both "Baladi" and "Ein-Elbyda" respectively. For regeneration experiments, leaf and stem, of all tested varieties and landraces were used. Explants were cultured on MS media supplied with different levels of NAA and N-1,2,3-Thiadiazol-5-yl-N-phenylurea (TDZ). Shoot regeneration from leaf explants was obtained on MS basal medium supplemented with TDZ at 1 and 2 µM in both landraces with 50 and 43% shoot regeneration for both "Baladi" and "Ein-Elbyda", respectively. Variation in regeneration ability among tested varieties was clear, the highest shoot percent (66.7%) was obtained at 1 µM TDZ with Hudas varietiy, other varieties exhibited lower regeneration percentages.

Keywords: Chickpea; *Cicer arietinum*; *in vitro*; regeneration; thidiazuron; growth regulators.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important grain legume cultivated worldwide on more than 12 million ha (FAOSTAT, 2012). However, the productivity of chickpea has not improved considerably over the years (Singh and Kataria, 2012). The production of chickpea is highly influenced by biotic and abiotic stresses (Hossain, 2009; Kiran et al., 2005). Cultivated chickpea has limited source of genetic variability, therefore, conventional breeding methods for improving productivity are limited. Recently, there has been a great interest in biotechnological methods (Gamborg, 2002; Mallikarjuna and Muehlbauer, 2011). Modern biotechnology has provided new opportunities including tissue culture, genetic engineering, and genetic transformation to enhance the germplasm of crops (Singh et al., 2008). Production of transgene and its stable expression through plant tissue culture supported by several genetic tools is again the most critical issue discussed nowadays (Bhatia, 2015). *In vitro* regeneration is an integral part of genetic transformation procedures. Many legumes have generally proved notoriously recalcitrant due to the lack of reliable *in vitro* regeneration system. (Barna and Wakhlu, 1993; Khawar et al., 2004; Yadav, 2009). Regeneration in chickpea *via* direct shoot induction and somatic embryogenesis has been reported from various explants (Batra et al., 2002; Richa and Singh, 2002; Kiran *et al.*, 2005; Krishna and Sanu, 2008). Indirect buds formation was accomplished on MS added with 0.5mg/l kinetin via callusing on mature embryos (Kadiri et al., 2014). Successful shoot regeneration of preconditioned mature embryo and embryonic axis explants was achieved with 10 mgl^{-1} benzylaminopurine (BA) (Aasim et al., 2011). Maximum number of shoots was produced with shoot tip and cotyledonary explants. (Sujatha et al., 2007; Rekha and Thiruvengadam, 2009;

Ugandhar et al., 2012). A combination of BAP and 2,4-D or NAA enhanced proliferation response. Of different combinations MS+1.0 2,4-D+0.5 NAA+0.5 BAP mg/l proved exceptionally good both for callus induction and proliferation (Naz et al., 2008). High shoot induction and multiplication from shoot tip of Chickpea was produced with MS medium fortified with thidiazuron (TDZ) (1.0-7.0 mg/L) (Anwar et al., 2008; Parveen et al., 2012).

In vitro regeneration of chickpea is difficult and there is an urgent need to develop a regeneration protocol that can ensure easy multiple and qualitative superior shoots that could be rooted and yield fertile plants (Aasim et al., 2013). Therefore, the aim of this study is to investigate the direct effect of various concentrations of TDZ, NAA, BA and Kinetin on shoot regeneration of different varieties and landraces of chickpea.

MATERIALS AND METHODS

Plant Material

Seeds of three varieties (Hudas, FLIP03-147C and FLIP05-100C) and two landraces ("Ein-Elbyda" and "Baladi") collected from the International Center for Agricultural Research in the Dry Areas (ICARDA) were used in these experiments. The seeds were surface sterilized using 40% of chlorox (sodium hypochlorite) for 15 minutes containing 0.1% (v/v) tween 20 as wetting agent, then were rinsed three time with sterile distilled water each for five minutes.

Culture Media

Chickpea seeds were transferred aseptically into (Murashige and Skoog, (1962) (MS) medium supplemented with 30 mgl^{-1} sucrose, 0.1 gml^{-1} myinistol and solidified with 8 gml^{-1} agar. The pH of the medium was adjusted to 5.6-5.8. The solidified medium was sterilized by autoclaving at 121°C for 21 minutes. After disinfestations, seeds of chickpea were planted in test tubes each containing 10 ml of MS basal medium with one seed per test tubes. The tubes were incubated in a growth chamber at 22±1°C for two weeks with 16 h of photoperiod illumination of 40 µmol m-$2s^{-1}$ supplied from cool white fluorescent.

Shoot Multiplication Experiment

In vitro shoot tips of about 2cm length form both landraces (Baladi and Ein-Elbyda) were cut and transferred into MS basal media supplemented with NAA 0.0, 2.7 µM combined with different levels of either BA (0.0, 2.2 and 4.4 µM) or kinetin at 0.0 ,9.2 and 18.4 µM (Tables 2 and 3). The cultures were incubated in a growth chamber at 22±1°C for two weeks with 16 h of photoperiod illumination of 40 µmol $m^{-2}s^{-1}$ supplied from cool white fluorescent. After four weeks of incubation, the numbers of shoots were recorded.

Regeneration Experiment

In vitro Leaf and stem explants of about 2 cm length from all the varieties and landraces were cultured onto MS media supplement with two different hormone levels (NAA at 0.0, 2.7 µM) and cytokinin (TDZ) at 0.0, 1, 2 µM) (Table 4). Each explants was cut into two pieces of and cultured into 9 cm diameter petri dishes (4 segments for each plate). All cultured plates were incubated under complete dark conditions for 3 weeks, then transferred to an incubator at 22C^{o}±1 and 16 h daylight. After one month each plate was tested for the shoot regeneration.

Statistical Methods and Data Analysis

The combinations of the different growth regulator levels in multiplication experiments and type of explants and growth regulators levels in regeneration experiments were considered as treatments. Treatments were arranged as factorial in a completely randomized design with 4 replicates in regeneration experiments and 10 replicates in multiplication experiments. Each experiment was repeated twice, the average of the two experiments was used for data analysis. Collected variable, were summarized and analyzed in one way analysis of variance, using SAS soft (SAS, 1990), comparative analysis were conducted for the significant results using LSD at .05 probability.

RESULTS

Seed Plantation

After 2 weeks, seeds of different varieties and landraces germinated successfully onto the basal

medium, however "Baladi" exhibited the best growth onto MS medium comparing with FLIP05-100C, Ein-Elbyda landrace, FLIP03-147C, and "Hudas" (Table 1). All of the seedlings were clean, no contamination was observed. There were significant differences in seed germination percentages among the 5 investigated chickpea types. 75% germination was observed at Baladi landrace; however, others exhibited 43%, 30%, and 30% 25% for Ein-Elbyda landrace, FLIP05-100C, HUDAS and FLIP03-147C, respectively.

Table 1. Germination % of chickpea seeds on MS- media

CV/Landrace	Germination % mean ±SE
FLIP03-147C	25±1.5b*
Hudas	30±2.7b
FLIP05-100C	30±1.7b
Baladi	75±5.5a
Ein-Elbayda	43±2.6b

*Number followed by the same letter or letters are not significantly differ at 5% level according to Least Significant Difference (LSD)

Shoot Multiplication Experiment

Effect of different levels of BA and NAA on shoot multiplication

The effect of both NAA and BA on the number of multiplied shoots of "Baladi" is shown in Table 2. The highest shoot number (9.7, 6.6) was obtained at media supplied with 2.2 µM BA for both landraces (Baladi and Ein-Elbyda) (Fig. 1), when NAA was combined with BA, lower shoot number was produced in both landraces. In "Baladi" only 1.3 shoots were produced when NAA was added at 2.7 µM only, however, in Ein-Elbyda, the lowest shoot number per plant were observed without hormone (1.3) with explants cultured into media without hormones. It was noted that all of shoots produced were axillary shoot.

The effect of Kinetin and NAA on shoot multiplication

Data on shoot multiplication on MS medium with both NAA and Kin is presented in Table 3. The highest shoot number was obtained at media supplied with 9.2 µM (5.3 and 4.5) in both "Baladi" and "Ein-Elbyda" respectively, however, these numbers were not significantly differ from that produced on media supplied with 18.4 Kin only and medium supplied with 9.2 and 2.7 µM Kin and NAA, respectively. No shoots were produced at 2.7 µM NAA only with both landraces.

Regeneration Experiment

Landraces

The effect of explants and growth regulators on the percentage of regenerated shoots and average number of shoot for both Baladi and Ein-Elbyda landraces is shown in Table 4. Significant interaction among the studied factors (explants and hormones) on shoot regeneration was observed. Shoot regeneration was obtained only with media contained 1 and 2 µM TDZ in both landraces, 50 and 43%, respectively. When NAA was used at 2.7 µM no regeneration was observed. On the other hand, no shoot regeneration was achieved using stem explants with all hormone combinations. Similar trend was observed with shoot number.

Table 2. Effect of different levels of BA and NAA on shoot multiplication of two landraces of chickpea

BA level (µM)	NAA level (µM)	Number of shoots (mean±SE)	
		"Baladi "	"Ein-Elbyda"
0.0	0.0	1.7±0.37b*	1.3±0.15c
0.0	2.7	1.3±0.26b	2.2±0.19c
2.2	0.0	9.7±0.97a	6.6±1.6a
2.2	2.7	1.9±0.59b	1.9±0.35c
4.4	0.0	6.3±1.17a	5.3± 1.4 ab
4.4	2.7	1.7±0.35b	3.5±0.45bc

*Number followed by the same letter or letters are not significantly differ at 5% level according to LSD test

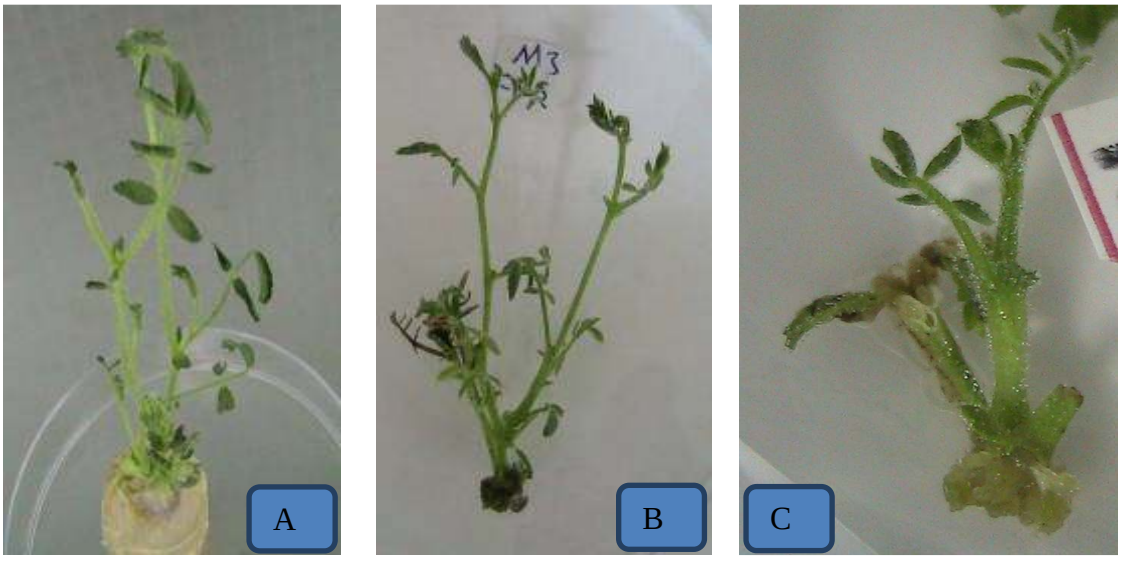

Fig. 1. Shoot multiplication and regeneration of chickpea varieties: A) Shoot multiplication of Ein-Elbyda landrace on MS media, B) Shoot multiplication of Baladi landrace. C) Shoot regeneration of FLIP05-100C on 2 µM TDZ

Table 3. Effect of different levels of Kin and NAA on shoot multiplication of two landraces of chickpea

Kinetin level (µM)	NAA level (µM)	Number of shoots (mean±SE)	
		"Baladi "	"Ein-Elbyda"
0.0	0.0	1.6±0.05b*	1.3±0.06c
0.0	2.7	0.0±0.0c	0.0±0.0c
9.2	0.0	5.3±0.56a	4.5±0.25a
9.2	2.7	2.3±0.42b	2.8±0.17ab
18.4	0.0	2.9±0.37ab	3.7±0.35a
18.4	2.7	2.3±0. 19b	1.7±.09c

*Number followed by the same letter or letters are not significantly differ at 5% level according to LSD test

Table 4. Effect of explants, NAA and TDZ on shoot percent and average number of shoot of Baladi and Ein-Elbyda chickpea landraces

Explant	NAA (µM)	TDZ (µM)	"Baladi"		"Ein-Elbyda"	
			Shoot% mean±SE	Av. shoot no mean±SE	Shoot% mean±SE	Av. shoot No mean±SE
Stem	0.0	0.0	0.0±0.0b*	0.0±0.0	0.0±0.0c	0.0±0.0
	2.7	0.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0
	0.0	1.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0
	2.7	1.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0
	0	2.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0
	2.7	2.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0
Leaf	0.0	0.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0
	2.7	0.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0
	0.0	1.0	50.0±2.5a	2.5±1.4	43±2.9a	2.0±0.55
	2.7	1.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0
	0.0	2.0	50.0±4.7a	2.0±0.5	29.5±2.5ab	1.3±0.33
	2.7	2.0	0.0±0.0b	0.0±0.0	0.0±0.0 c	0.0±0.0
Sig interaction			Sig	NS	Sig	NS

*Number followed by the same letter or letters are not significantly differ at 5% level according to LSD test

Table 5. Effect of explants, NAA and TDZ on shoot percent and average number of shoot of three chickpea varieties

Explant	NAA (µM)	TDZ (µM)	"FLIP05-100C"		"FLIP03-147C"		"Hudas"	
			Shoot% mean±SE	Shoot number mean±SE	Shoot% mean±SE	Shoot number mean±SE	Shoot% mean±SE	Shoot number mean±SE
Stem	0.0	0.0	0.0±0.0b*	0.0±0.0	0.0±0.0c	0.0±0.0	0.0±0.0c	0.0±0.0
	2.7	0.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0	0.0±0.0c	0.0±0.0
	0.0	1.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0	0.0±0.0c	0.0±0.0
	2.7	1.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0	0.0±0.0c	0.0±0.0
	0.0	2.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0	0.0±0.0c	0.0±0.0
	2.7	2.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0	0.0±0.0c	0.0±0.0
Leaf	0.0	0.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0	0.0±0.0c	0.0±0.0
	2.7	0.0	0.0±0.0b	0.0±0.0	16.7±3.2b	0.3±0.05	20.0±2.9b	2.0±0.33
	0.0	1.0	42.0±2.4a	1.6±0.33	33.3±4.4a	1.2±0.20	66.7±4.7a	3.6±0.58
	2.7	1.0	0.0±0.0b	0.0±0.0	0.0±0.0c	0.0±0.0	25.0±2.7b	1.6±0.33
	0.0	2.0	60.0±3.1a	3.0±0.83	16.7±2.2b	0.2±.01	61.7±4.7a	2.3±0.33
	2.7	2.0	8.3±0.94b	0.3±0.33	0.0±0.0c	0.0±0.0	55.5±3.0a	2.6±0.33
Sig interaction			Sig	NS	Sig	NS	Sig	NS

*Number followed by the same letter or letters are not significantly differ at 5% level according to LSD test

Varieties

Significant interaction between explants and growth regulators on shoot regeneration was exhibited (see above Table 5). Regeneration was observed only with leaf explants in all studied varieties, the highest shoot number was obtained at 1 and 2 µM TDZ with both FLIP05-100C" and "Hudas", but without significant difference between the two TDZ levels, however, significant different was observed with percentage of regenerated shoots between 1 and 2 µM of TDZ with "FLIP03-147C". The highest percentage of shoot formation (66.7, 61.7%) was obtained when TDZ was used at 1 and 2 µM with Hudas variety, when NAA was combined with TDZ, regeneration percentage was reduced.

Percent was obtained with "FLIP03-147C" and "Hudas" (16.7 and 20%) respectively. Regarding shoot number per regenerated leaf, similar trend was observed among the three varieties, however without significant different from other treatments.

DISCUSSION

In vitro Seed Development

Seed sterilization is an important step, which affects growth and regeneration. Sodium hypochlorite used as a surface sterilization agent, played an important role in germination of seeds (Chaudhry et al., 2007). In our study, seeds of all chickpea varieties and landraces were treated with 40% Chlorox, this level prevented contamination, however, seed germination percentage was not the same in all varieties. FLIP03-147C chickpea exhibited low growth after the initiation of germination, phenol metabolites was exudates from the seeds, this could explain the low germination % associated with this variety. Oxidized phenol compounds inhibit enzyme activity and darken the culture medium, subsequently the explants brown or blacken and die (Laukkanen et al., 1999; Arnaldos et al., 2001). Several authors have suggested solutions to minimize the lethal browning or blackening of explants caused by phenol compounds in plant tissue culture. Among these protocols are treating explants with antioxidants, such as cysteine, ascorbic acid, PVP or silvir nitrate (Lainé and David, 1994; Sanyal et al., 2005). Lopez-Amoros et al., (2006) reported an increase in hydroxybenzonic phenol production during seed germination of chickpeas.

Shoot Multiplication

The number of shoots produced in the multiplication experiments was not high, plants belong to the Fabaceae are difficult to regenerate through *in vitro* propagation, in particular, chickpea is considered a recalcitrant plant

(Aasim et al., 2013). Other researcher obtained higher multiplication rate using cotyledon as an explants (Chakraborti et al., 2006).

Multiple shoot formation *via* organogenesis could occur directly from the explants or indirectly from the dedifferentiated callus. Both approaches are controlled by plant hormones and other factors added to the medium (Tang and Chen, 2011). In our study, multiplied shoots were of axillary bud origin. The importance of BA in multiple shoot formation in chickpea and other legumes is widely reported (Barik et al., 2004; Odutayo et al., 2005). Similar to the findings of Shagufta et al., (2007), the result of present study indicates that BA level in the media is an important factor influencing the shoot number, when BA was used alone at 2.2 µM, the shoot number was high, when BA was used at 4.4 µM, shoot number was reduced in both landraces, therefore, our findings are in agreement with that of other investigators (Saleem et al., 2010; Rehman et al., 2004 and Veraplakorn et al., 2012), who reported low shoot multiplication rate with higher BA levels in the media. Other's results disagreed with our findings, who demonstrate that shoots number per explants increases with increasing BA level (Sujatha et al., 2007), however, during their studies, other varieties were used as well as different explants.

In both Baladi and Ein -Elbayda landraces the addition of NAA at 2.7 µM reduces shoot production; similar finding in chickpea was reported by (Aasim et al., 2011). BA was the commonly used cytokinin followed by kinetin, in our study the shoot number was increased when 9.2, 18.4 µM Kinetin without NAA were used. In many reports, 2 mgl^{-1} BAP only or in combination with auxins was the best growth regulator in shoot induction (Shalini et al., 2001), hence it is proved that the concentration of BAP can increase up to certain limit otherwise, it causes undesirable effects. Sawardekar (2007) indicate that BA produced limited number of multiple shoots; they suggest that BAP can produce shoots more than 12 but BAP at higher concentration causes swelling of explants and shoots become watery.

In our study, stunted shoots were obtained with 2.2 µM BA experiment in "Baladi" chickpea. The occurrence of stunted shoots on unconditioned explants cultured on medium containing BA with NAA might be due to inhibition of active cell division in these explants (Aasim et al., 2009a). Abdelwahd et al., (2008) reported high shoot induction (26) obtained on shoot elongation medium consisting of MS medium supplemented with 6 µM 2-ip and 3 µM kin for 10 days.

In our study the highest number of shoots was obtained without NAA in both "Baladi" and Ein-Elbyda", this finding was in agreement of Anwar et al., (2010) who reported that MS basal medium without the addition of plant growth regulators resulted in a large number of shoot production in chickpea. This could be due to the ability of shoots transferred to plant growth regulator free medium to synthesize and maintain desired endogenous levels of gibberellins and other auxins.

Shoot Regeneration

In this study, regeneration was achieved with leaf explants only. This result agreed with the findings of Hofmann (2004) who reported that leaf explants is fairly good source for shoot induction through callus in chickpea. On the other hand, Aasim et al., (2011) reported successful shoot regeneration from mature embryo and embryonic axis explants of chickpea. The higher shoot regeneration ability from leaf explants compared to stem could be due to meristem activity (Hinchee et al., 1988). In our study, stem explant induced callus formation only, no other differentiation was observed. Aasim et al., (2010) showed that cotyledon node was more responsive when compared to hypocotyl explants.

In our study, shoot regeneration was observed when lower levels of TDZ were used, this result agreed with the findings of other researchers, who reported that TDZ at lower concentration is better than BA, Kinetin or 2iP (Saini and Jaiwal, 2002; Jayanand et al., 2003; Yoshida, 2002 and Anwar et al., 2010). On the other hand, Huda et al., (2003) reported that maximum percentage (40%) of shoot bud formation was obtained on MS medium with 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA. In our study, shoot vitrification was observed with Baladi and Ein-Elbyda landraces, similar finding was reported with (*Aasim et al.,* 2008;

Asaim et al., 2009b). Thidiazuron has been reported as a potent growth regulator for *in vitro* morphogenesis in many plant species including grain legumes (Wang et al., 2008). Thidiazuron has been demonstrated as a better induction factor for organogenesis and somatic embryogenesis in chickpea (Rizvi and Singh, 2000; Parveena et al., (2012). Although the exact mechanism of action of TDZ is not clear, it is believed to be involved in regulating endogenous levels of various growth regulators (Malik and Sexena, 1992). The high activity of a low concentration of TDZ has not been investigated, TDZ interacted with endogenous hormones, releasing, synthesizing, protecting or even inhibiting auxins *in situ* in combination with other sub culture metabolic change (Gill and Saxena, 1992).

In this study stem explants showed no response of shoot regeneration in both landraces and varieties, callus was only formed, this finding disagree with Arochiasamy et al., (2000). Internode explants was found to be the best for callus induction on medium containing 2 mgl^{-1} kinetin and 2 mgl^{-1} IAA (Huda et al., 2003). Organogenic differentiation in cell and tissue culture is due to hormonal manipulation of the culture medium, morphogenesis of shoot *via* organogenic is highly affected by the plant hormones ((Aloni et al., 2006 and George et al., 2008).

In our experiment, the media supplied with 1 and 2µM TDZ, showed variation response among the 5 genotypes used. The organogenic response varied greatly with the genotypes, the significance of the genotype in determining cultures response was recognized (Cardinale et al., 2007; Banu et al., 2011). Our findings agree with other reports (Mirkabad et al., 2010; Sayem et al., 2010) who reported different shoot regeneration ability among different chickpea genotypes.

CONCLUSION

In vitro multiplication and regeneration protocol for chickpea was established, two landraces and three common varieties were used, high variability in *in vitro* growth and development among the varieties was observed. BA at 2.2 µM and without auxin was the best for shoot multiplication of chickpea landraces**,** Leaf explant were better than stem sections for shoot regeneration. TDZ at 1 and 2 µM without auxin was the best for shoot regeneration from leaf explants. However, more investigations are needed to improve the multiplication and regeneration chickpea cultivar. *Ex vitro* studies are also needed to evaluate the growth and performance of the regenerated plants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

Aasim, M., Khawar, K.M., Ozcan, S. 2008. *In vitro* micropropagation from shoot meristems of Turkish Cowpea (*Vigna unguiculata L.*) cultivar Akkız. Bangladesh J. Bot. **37**:149-154.

Aasim, M., Khawar, K.M., Ozcan, S. 2009 a. *In vitro* micropropagation from plumular apices of Turkish cowpea (*Vignaun guiculata* L.) cultivar Akkiz. Sci. Hortic. **122**:468-471.

Aasim, M., Khawar, K.M., Ozcan, S. 2009b. Comparison of shoot regeneration on different concentrations of TDZ from shoot tip explant of cowpea on gelrite and agar containing medium. Not. Bot. Hort. Agrobot. Cluj. **37**:89-93.

Aasim, M., Khawar, K.M., Özcan, S. 2010. Efficient *in vitro* propagation from preconditioned embryonic axes of Turkish cowpea (*Vignaun guiculata L.*) Cultivar akkiz. Arch. Biol. Sci. **62**:1047-1052.

Aasim, M., Day, S., Rezaei, F., Hajyzadeh, M., Tahir, S., Ozcan, S. 2011. *In vitro* shoot regeneration from preconditioned explants of chickpea (*Cicer arietinum L.*) cv. Gokce. Afri. J. Biotechnol. **10**(11):2020-2023.

Aasim, M., Day, S., Rezaei, F., Hajyzadeh, M. 2013. Multiple shoot regeneration of plumular apices of chickpea. Turk J Agric For. **37**:33-39.

Abdelwahd, R., Hakam, N., Labhilili, M., Udupa, S.M. 2008. Use of an adsorbent and antioxidants to reduce the effects of leached phenolic *in vitro* plantlet regeneration of faba bean. Afr. J. Biotechnol. **7**:997-1002.

Aloni, R., Aloni, E., Langhans, M., Ullrich, C.I. 2006. Role of cytokinin and auxin in shaping root architecture: Regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. Annals of Bot. **97**:883-893.

Anwar, F., Sharmila, P. Saradhi, P. 2008. An ideal protocol for *in vitro* regeneration, efficient rooting and stable transplantation of chickpea. Physiol. Mol. Biol. Plants. **14**:329-335.

Anwar, F., Alghamidi, S., Ammar, H., Siddique. K.H.M. 2010. An efficient *in vitro* regeneration protocol for faba bean (*Vicia faba L.*). J. Med. Plants Res. **5**(28):6460-6467.

Anwar, F., Sharmila, P. Saradhi, P. 2010. No more recalcitrant: Chickpea regeneration and genatic transformation. Afri. J. Biotechnol. **9**(60): 782-797.

Arachiasamy, S., Varghese, G., Ignarcimuthu. S. 2000. High frequency regeneration of chickpea (*Cicer arietinum L.*) plantlets from leaf callus. Phytomorphology. **50**(3-4):297-302.

Arnaldos, T.L., Munoz, R., Ferrer, M.A., Calderon, A.A. 2001. Changes in phenol content during strawberry (*Fragaria x ananasa*, cv. Chandler) callus culture. Physiol. Plant. **113**:315-322.

Banu, T.A., Saker, R.S., Hoque, M.L. 2011. *In vitro* regeneration of four local varieties chickpea grown in Bangladish. Bangladish J. Sci. Ind. Res. **46**(3):379-384.

Barik, D.P., Naik, S.K.U., Nudgal, A., Chand, P.K. 2004. Rapid plant regeneration through *in vitro* axillary shoots proliferation of butterfly pea (*Clitoria ternatea L.*). *In vitro*. Cell. Dev. Biol. Plant. **43**:144-148.

Barna, K. S. Wakhlu, A.K. 1995. Modified single node culture method – a new micropropagation method for chickpea. *In vitro* Cell Dev Biology – Plant. **31**:150.

Batra, P., Yadav, N.R., Sindhu, A., Yadav, R.C., Chowdhury, V.K. 2002. Efficient protocol for *in vitro*direct plant regeneration in chickpea (*Cicer arietinum L.*). Indian J. Exp. Biol. **40** (5):600-602.22.

Bhatia, S. 2015. Application of plant biotechnology in: Modern Applications of Plant Biotechnology in Pharmaceutical Sciences (Bhatia, S., Sharm, K., Dahiya, R., Bera, T.) eds. Elsevier Inc. 157-207.
DOI: 10.1016/J.CBPA.2009.04.596

Cardinale, B.J., Wright, J.B., Cadotte, M.W., Carroll, I.T., Hector, A., Srivastava, D. S., Loreau, M., Weis. J.J. 2007. Impacts of plant diversity on biomass production increase through time because of species complementarity. Proc Natl Acad Sci USA. **104**:18123–18128.

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

Chaudhry, Z., Afroz, A., Rashid, H. 2007. Effect of variety and plant growth regulatorson callus Proliferation and regeneration response of three tomato varieties (*Lycopersicon esculentum L.*) Pak. J. Bot. **39**(3):857-869.

FAO. 2012. Food and Agricultural Organization Statistical Database. Rome: FA Food and Agricultural Organization.
Available: www.faostat.org

Gamborg, O.L. 2002. Plant tissue culture biotechnology milestones. *In vitro* Cell Dev. Biol. Plant. **38**:84–92.

George, E.F., Hall, M.A., Klerk, G.J. 2008. Plant propagation by tissue culture 3rd edition, 1. Springer, Dordrecht, The Netherlands. 501.

Gill, R., Saxena, P.K. 1992. Direct somatic embryogenesis and regeneration of plant from seedling explant of peanut (*Arachis hypogae L*): Promotive role of thidiazuron. Can J Bot. **70**:1186-1192.54.

Hinchee, M.A.W., Connor-Ward, C.A., Mcdonnell, R.E., Sato, S.J., Gasser, C.S., Fischhoff, D.A., Re, D.B., Fraley, R.T., Horsch, R.B. 1988. Production of transgenic soybean plants using *Agrobacterium tumefaciens-mediated* DNA transfer, Biotechnology. **6**: 915-922.

Hofmann, N., Nelson, R.L., Korban, S.S. 2004. Influence of media components and pH on somatic embryo induction in three genotypes of soybean. Plant Cell Tiss. Org. Culture. **77**: 157-163.

Hossain, M.D.A. 2009. Management of chickpea pod borer, *Helicover paarmigera* (Hubner) through intercropping and insecticide spraying. Thai. J. Agri. Sci. **36**(1):51-56.

Huda, S., Islam, R., Bari, M.A. 2003. Shoot regeneration from internode derived callus of chickpea (*Cicer arietinum L.*). International Chickpea and Pigeon pea Newsletter. **7**:28-29.

Huda, S., Islam, R., Bari, M.A. 2003. Shoot differentiation from cotyledon derived callus of chickpea (*Cicer arietinum* L.). Plant Tiss. Cult. 13(1):53-59.

Jayanand B., Sudarsanam, G., Sharma, K.K. 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.

Kadiri, A., Halfaoui, Y., Bouabdallah, L., Ighilhariz, Z. 2014. Chickpea (*Cicer arietinum* L.) *in vitro* micropropagation. Tur. J. Agri and Nat. Sci. **1**:1304-1309.

Khawer, M.K., Sancak, G., Uranbey, S., Ozan, S. 2004. Effect of thidiazuron on shoot regeneration from different explants of Lentil (*Lens cutlinaris* Medik) via organogenesis. Turk. J. Bot. 2.

Kiran, G., Kaviraj, C.P., Jogeswar, P.B.K., Kishor, S. 2005. Direct and high-frequency somatic embryogenesis and plant regeneration from hypocotyls of chickpea (*Cicer arietinum L.*), a grain legume. Curr. Sci. **89**:1012–1018.

Krishna, K., Sanu, D. 2008. Rapid multiplication of *Rauvolfia serpentine* Benth. Ex. Kurz through tissue culture. Scientific World. 6:6.

Lainé, E., David, A. 1994. Regeneration of plants from leaf explants of micropropagated clonal Eucalyptus grandis. Plant Cell Rep. **13**: 473-476.

Laukkanen, H., Häggman, H., Kontunen-Soppela, S., Hohtola, A. 1999. Tissue browning of *in vitro* cultures of Scots pine: Role of peroxidase and polyphenol oxidase. Physiol. Plant. **106**: 337-343.

Lopez-Amoros, M., T. Hernandez, L., Estrella, I. 2006. Effect of germination on legume phenolic compounds and their antioxidant activity. J. Food Comp. Anal. **19**:277-83.

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

Mallikajuna, N., Muehlbauer, F. C. 2011. Chickpea hybridization using *in vitro* techniques. Methods. Mol. Biol. **710**:93-105. DOI: 10.1007/978-1-61737-988-8_8

Mirakabad, Z.H., Baghrei, R.A., Mehrjerdi, Z.M. 2010. Efficient protocol for break impasses of regeneration via callus for 20 genotypes of chickpea. In. J. Plant Prod. 4:2.

Murashige, T., Skoog, F. 1962. A revised medium for rapid growth tobacco and bioassays with tissues cultures. Physiol Plant. **15**:473-497.

Naz, S., ALI, A., Siddique, F., Iqbal, J. 2008. Somatic embryogenesis from immature cotyledons and leaf calli of chickpea. Pak. J. Bot. **40**(2):523-531.

Odutayo, O.I., Akinirinusi, F.B., Odunbososye, I., Oso, R.T. 2005. Multiple shoot induction from embryo derived callus cultures of cowpea (*Vigna ungimculata L.*). Walp. Afri. J. Biotechnol. **4**:1214-1216.

Parveen,S.,Venkateshwarlu, M., Srinivas, D., Jagan, K., Ugandhar, T. 2012. Direct *in vitro* shoots proliferation of chick pea (*Cicer arietinum L.*) from shoot tip explants induced by thidiazuron. Bioscience Discovery. **3**(1): 01-05.

Rehman, M.M., Amin, M.N. Jahan, H.S., Ahmed, R. 2004. *In vitro* regeneration of plantlets of *Curcuma longa* Linn. valuable spice. Asian J. Plant Sci. **3**:306-309.

Rekha, K.T., Thiruvengadam, M. 2009. Efficient micropropagation of chickpea (*Cicer arietinum L.*). Philippine Agric. Scientist. **92**(3):322-326.

Richa, C., Singh, N.P. 2002. Plant regeneration *via* somatic embryogenesis in chickpea (*Cicer arietinum L.*). The Ind. J. Genet. Plant Breeding. 62:4.

Rizvi, S.M.H., Singh, R.P. 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.

Saini R, Jaiwal, P.K. 2002. Age, position in mother seedling, orientation, and polarity of the epicotyl segments of black gram (*Vigna mungo* L. Hepper) determines its morphogenic response. Plant Sci. **163**:101–109.

Saleem, K., Mehboob, A., Mehnaz, G., Mehvish, F., Zahoor, A. 2010. *In vitro* micro-propagation of indigenous chick pea (*Cicer arietinum L.*) varieties. Afri. J. Agri. Res. **5**(21):2934-2938.

Sanyal, L., Singh, A.K, Meetu, K., Alma, D.V. 2005. Agrobacterium-mediated transformation of chickpea (*Cicer arietinum L.*) with *Bascillus thuringiensis* Cry1Ac gene for resistance against pod borer insect *Helicover paarmigera*. Plant Sci. **168**(4):1135-1146.

SAS Institute User"s guide Statistics. 1990. Ver. 6. 4th ed. SAS Inst. Cary. N. C.

Sawardekar, S. V. 2007. Transformation studies in chickpea (*Cicer arietinum* L.) Department of genetic and plant breeding college of agriculture. Dharwad University of Agriculture Sciences, Dharwad – 580005.

Sayem, M.A., Siddique, S.S., Zaman, M. 2010. *In vitro* regeneration through anther culture of Brassica SPP. Bangaladish J. Agri. Res. **35**(2): 331-341.

Shagufta, N., Amir, A., Fayyaz, S., and Javed, I. 2007. Multiple shoot formation from different explants of chick pea (*Cicer arietinum L*.) Pak J. Bot. **39**(6):2067-2073.

Shalini, S., Batra, P., Sindhu, A., Chowdhuray, V.K. 2001. Multiple shoots induction and complete plant chickpea (*Cicer arietinum L*.). Crop Res. **21**(3):308-311.

Singh, N., Kataria, N. 2012. Role of potassium fertilizer on nitrogen fixation in Chickpea (*Cicer arietinum L*.) under quantified water stress. J. Agr. Technol. **8**(1):377-392.

Singh, R., Sharma P., Varshney., RK**.**, Sharma., SK**.**, Singh., NK. 2008. Chickpea improvement: role of wild species and genetic markers. Biotechnol Genet. Eng. Rev. **25**:267-313.

Sujatha, G.N., Jayabalan, B.D., Kumari. R. 2007. Rapid *in vitro* micropropagation of *Cicer arietinum L*. Department of plant science, school of life Sciences, Bharathidasan University, India. Hortic. Sci. **34**(1):1-5.

Tang, X.M., Chen, L. 2011. A review on regeneration in cowpea (*Vigna unguiculate L*. Walp). Journal of Agriculture Science and Technology. 5:4. ISSN 1939-1250, USA.

Ugandhar, T., Venkateshwarlu, M., Sammailah, D., Jagan, M.R.K**.** 2012. Rapid *in vitro* micro propagation of chick pea (*Cicer arietinum* L.) from shoot tip and cotyledonary node explants. J Biotechnol Biomater. 2:148.

DOI: 10.4172/2155-952X.1000148

Veraplakorn V, Nakorn, M.N. Kaveeta, L. Suwanwong, S. Bennett, L. 2012. Variation and long term regenerative capacity of two important tropical forage legumes: Cavalcade (*Centrosema pascuorum* cv. Cavalcade) and stylo 184 (*Stylosanthes guianensis* CIAT184) *in vitro*. Afri J. Biotechnol. **11**(92):15843-15851.

Wang, H.M., Liu, M.H. Wang, M.H., Zu, Y.G. 2008. Effects of Thidiazuron, basal medium and light quality on adventitious shoot regeneration from in vitro culture stem of *Populus alba* x P. berolinensis. J. Forestry Res. **19**(3):257-259.

Yadav, A. 2009. Induction of organogenesis and somatic embryogenesis in seedling explants of some novel genotypes of chickpea (*Cicer arietinum* L.) A recalcitrant crop. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. **153**(2):214.

DOI: 10.1016/J.CBPA.2009.04.596

Yoshida, T. 2002. Adventitious shoot formation from hypocotyls sections of mature soybean seeds. Breeding Sci. **52**:1–8.