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DEVELOPMENT OF EFFICIENT REGENERATION AND GENETIC TRANSFORMATION SYSTEMS IN LOCAL CULTIVARS OF CHICKPEA (*Cicer arietinum* L.)

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ABSTRACT

The yield of chickpea in Pakistan is unexpectedly low and unstable that may be due to biotic and abiotic stresses. To combat with these stresses, genetic make of chickpea should be improved incorporating important genes. For successful gene incorporation, an efficient tissue culture system may be established. Therefore, this study was conducted in two local chickpea genotypes namely Bittle-98 and Dasht-2000 for the establishment of an efficient tissue culture system. Among various concentrations of BAP, MS medium fortified with 3.0 mg/l BAP showed the highest multiple shoot formation (69.2 and 70.66%) in cvs Dasht-2000 and Bittle-98, respectively. Similarly, the highest elongation of regenerated shoots (58.33 and 91.66%) was recorded in Bittle-98 and Dasht-2000, respectively on MS media supplemented with 0.25 mg/l IAA. The highest rooting formation (60 and 80%) was recorded in Dasht-2000 and Bittle-98, respectively on MS media having 1.0 mg/l IBA. Half Embryo with single cotyledon was found to be the best explant for genetic transformation. During this study, hygromycin (75 mg/l), acetosyringone (100 µM) and cefotaxime sodium (500 mg/l) were found to be optimum for *Agrobacterium*-mediated transformation in chickpea. The putative transgenic plants were subjected to GUS and microscopic observations that confirmed the presence of *uidA* gene in both genotypes. The optimized protocols will provide a base line to improve transformation system and incorporate important genes in chickpea in future research programs.

Keywords: *Agrobacterium,*Acetosyringone, *Cicerarietinum*, Genetic transformation

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a major Rabi pulse crop grown on low fertility sandy loam soils in Pakistan. It occupied about an area of 0.975 million hectares with annual production of 475 thousand tons (Agriculture Statistics of Pakistan, 2013-14). In Punjab, 90% of chickpea crop is planted in rain-fed areas, out of which 75% is concentrated in Thal and 15% in Jhang. The yield of chickpea in Pakistan is unexpectedly low and unstable that may be attributed to biotic and abiotic stresses. Chickpea pod borer and weed borer infestation are the main constraints that severely affect yield losses, especially in irrigated areas (Ahmed and Awan, 2013). The chemical control for pod borer is expensive as well as for weeds is not possible due to susceptibility of chickpea itself to weedicides (Ahmed and Awan, 2013). The only solution to these problems lies in the development of resistant genotypes against pod borer and herbicides. But the sources of resistance against weeds and pod borer are not available in cultivated or wild chickpea. However, genetic engineering is an efficient tool for desired gene incorporation. Convention breeding is very tedious and time consuming job, while genetic engineering offers quick, labor saving and wider gene pool method of gene transfer (Sharma *et al.,* 2002). Gene transfer methods include particle bombardment, micro injection, electroporation and through *Agrobacterium*. The *Agrobacterium* own Ti-plasmid, and T-DNA region of the Ti-plasmid is excised, transformed into the host plant tissue and integrated into the genome of the plant (Ali *et al.,* 2016). The use of *Agrobacterium* is less labor intensive, does not require sophisticated equipment, cost effective and typically results in a low copy number of the introduced gene (Hadi *et al.,* 1996). Moreover, *Agro bacterium tumefaciens*-mediated transformation is a suitable technique to conduct complementation analysis in grain legume species (Nisha *et al.,* 2003). Chickpea has been found to be recalcitrant to tissue culture as well as for genetic transformation (Aasim *et al.,* 2013). *In vitro* morphogenesis is crucial for plant biotechnology because diverse genotypes exhibit different morphogenic potentials (Shah *et al.,* 2013; 2014a; 2014b). Plant growth regulators have direct effect on *in vitro* shoot regeneration (Ahmad *et al.,* 2012; Mehmood *et al.,* 2016). The apposite type and concentration of cytokinin significantly influences organogenesis (Uzma *et al.,* 2012; Shah *et al.,* 2015a).

We believe that it is the first report about *in vitro* morphogenesis and genetic transformation system in two local genotypes of chickpea namely Bittle-98 and Dasht-

2000 using various explants. Our optimized systems would prove beneficial for *in vitro* shoot regeneration and genetic transformation of recalcitrant germplasms of chickpea.

MATERIALS AND METHODS

Plant material and culture media: The present study was conducted at Genetic Transformation Lab, National Institute for Genomics and Advanced Biotechnology (NIGAB), NARC Islamabad-Pakistan. Two chickpea varieties "Bittle-98" and "Dasht-2000" were assessed for tissue culture and genetic transformation studies. Both of these varieties were bold seeded, desi type and blight disease resistant. Seeds were provided by Pulses Program, NARC, Islamabad-Pakistan.

Explant preparation: The mature and healthy seeds were first surface-sterilized with 70% ethanol for one minute then with 40% Clorox for 20 min followed by 4-5 times washing with sterilized water. Surface sterilized seeds were soaked overnight in sterilized water. The seed coats and radicals were discarded and a longitudinal slit was made. The dissected half embryos with or without cotyledon were chosen as explants to test their *in vitro* response.

Multiple shoot induction and shoot elongation: MS medium containing BAP (1, 2, 3 and 4 mg/l) were employed at different phases of plant regeneration, while MS medium enriched with IAA (0.25, 0.5 and 1.0 mg/l) was assessed for shoot elongation. The shoots having lengths of 3 cm or more per explant were recorded as elongated ones. The explants were incubated under a photoperiod for 16 h at 22 ± 2 ºC. Number of shoots formed from each explant was counted after two weeks.

Root formation: Twenty five elongated shoots were shifted to MS medium fortified with IBA (0.5 and 1.0 mg/l) and NAA (0.5 and 1.0 mg/l), 3% sucrose and 0.5% agar for rooting. Plantlets were investigated 10 and 14 days after culture.

Plantlets establishment and acclimatization: Long plantlets of Bittle-98 with well-developed roots were used for acclimatization. After 14 days of incubation in rooting medium, cotton plugs of culture tubes were loosened to about one week. After one week, plantlets were shifted in pots containing sand-soil-manure mixture (1: 1: 1). Pots were covered with polythene bags for one week and put into a growth chamber to maintain humidity and enhanced establishment. Finally after three weeks, pots were shifted in greenhouse.

Transformation vectors: PTCL-5-GUS harboring binary vector was employed for establishment of gene incorporation system. The *Agrobacterium tumefaciens* strain EHA105 was used as transformation vector carrier. The T-DNA region of PTCL-5-GUS vector contains the coding sequence of *hpt* and *uidA* gene.

Transformation of plasmid DNA into Agrobacterium: For transformation of plasmid DNA competent *Agrobacterium* cells were prepared as described by Sambrook and Russel (2001). The transformation of plasmid DNA was carried out employing liquid nitrogen technique as illustrated by Tzfira *et al.* (1997).

Transformation strategy: *Agrobacterium* strain EHA-105 harboring PTCL-5-GUS vector was splashed on agar solidified YEP medium containing rifampicin (20 mg/l), kanamycin (50 mg/l), and hygromycin (50 mg/l) and grown at 28 ºC. A single colony was sorted out and shifted to 3 ml YEP medium having same selectable antibiotics and culture was allowed to shake (250 rpm) at 28 ºC for 12-16 h. At 5000 rpm the bacterial cells were pelleted for 5 min then suspended in 100 ml flask (Pyrex) containing 25-30 ml YEP with different concentrations of acetosyringone (0, 50, 100 and 150 µM) to an A_{600nm} = 0.3-0.35. The 100 ml flak (Pyrex) containing the culture was allowed to shake for 12 h at 150 rpm until an $A_{600\ nm}$ = 0.4-0.6 was reached. Embryo axes were used for co-cultivation. One day sub-cultured explants were immersed in YEP liquid media for 10-15 min and then dried on sterilized filter paper to remove excess of bacteria. The infected explants were transferred to co-cultivation medium (MS basal salts supplemented with 0, 50, 100 and 150 µM acetosyringone) at 26 ºC for 2 d in dark. After co-cultivation, the explants were first washed 3-4 times with sterilized water followed by 2 times washing with MS medium containing 250, 500, 750 and 1000 mg/l cefotaxime sodium to kill excess bacteria. Explants were removed from shaking and placed on the filter paper for 15 min to blot excess of liquid. Subsequently, the infected explants were transferred to selection medium (MS basal salts + 3 mg/l BAP + 0, 50, 75 and 100 mg/l hygromycin) and incubated at 26 ºC in light. After one week, necrosis of explants started except for those which were resistant to the antibiotic (hygromycin). These explants were transferred to second selection medium (MS basal salts + BAP 3 mg/l + 75 mg/l hygromycin) for two weeks. The survived explants later on shifted to the elongation medium (MS basal salts, IAA 0.25 mg/l, hygromycin 75 mg/l).

GUS Histo-Chemical assay: The putative transgenic explants were subjected to GUS (X-Gluc staining) expression assay using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc). The tissues were directly incubated in the solution at 37 ºC for 12-16 h. When incubation was completed, explants were washed with double distilled water and incubated in 95% ethanol for 1-2 h for bleaching of chlorophyll contents. The material with GUS expression displayed blue coloration.

Microscopic observations of transformed cells were recorded.

Data analysis: Data collected for each parameter were analyzed using complete randomized design (CRD) and statistical differences among means were estimated at 5% level of probability by Duncan's Multiple Range Test (DMRT) using the Statistical Software v 5.0 Soft (1995). Each treatment was repeated three times

RESULTS

Culture conditions: Different concentrations of commercial bleach Clorox (10-60%) were tested on germination frequency culturing twenty five seeds of each variety. The highest germination frequency (80%) was recorded in Bittle-98 using 40% Clorox, while in cv. Dasht-2000, the maximum germination frequency (76%) was found using 40% Clorox signifying no contamination at these optimized concentration of Clorox. On the other hand, seed contamination was noticed below 30% and weed viability was affected at above 60% Clorox (Table 1).

Multiple shoot formation: The effect of BAP was tested on multiple shoot formation culturing half embryo with cotyledon, half embryo without cotyledon and cotyledonary node. Results revealed that the highest multiple shoot induction frequency (69.2 and 70.66%) was recorded in Bittle-98 and Dasht-2000, respectively on MS basal media supplemented with 3.0 mg/l BAP culturing half embryo with cotyledon (Table 2; Fig. 1A, C & D). Similarly, the best multiple shoot induction frequency (20 and 50%) was found in Dasht-2000 and Bittle-98, respectively on MS basal media supplemented with 3.0 mg/l BAP culturing half embryo without cotyledon (Table 2; Fig. 1E). While the highest multiple shoot induction frequency (70 and 80%) was secured in Dasht-2000 and Bittle-98, respectively on MS basal media enriched with 2 mg/l BAP culturing cotyledonary node (Table 2; Fig. 1B & F). Significance differences in terms of multiple shoot formation were also noted in Dasht-2000 variety for various hormonal concentrations. When cotyledonary node was used as explant, the results were quite different from the other two explants with regard to hormonal concentration. In this case, although varietal responses were there, 2.0 mg/l BAP generated good result i.e. 80 and 70% multiple shoot induction frequency for Bittle-98 and Dast-2000, respectively. It was found that selection of a proper explant was more important for multiple shoot induction frequency along with hormonal type and concentration.

Elongation of induced shoots: During this study, it was observed that growth of more than six-week old regenerated shoots was inhibited in multiple shoot induction media (MS basal media supplemented with BAP). These shoots were too week and were unable to develop rooting system. Therefore, it was important to elongate shoots further for the development of profuse rooting system. For this purpose, MS media fortified with three concentrations of IAA (0.25, 0.5 and 1 mg/l) was investigated on shoot elongation in two chickpea genotypes. MS media supplemented with 0.25 mg/l was found to be the most effective for elongation of regenerated shoots yielding 58.33 and 91.66% shoot elongation frequency in Dasht-98 and Bittle-2000, respectively (Table 3; Fig. 1G & H). Normal elongated shoots were bright green with properly opened leaves, distinct nodes and internodes with average length of 3-5 cm. MS media having 0.5 mg/l IAA also showed positive response for shoot elongation, while MS media enriched with 1.0 mg/l IAA had the negative effect and reduced the elongation frequency in both the genotypes (Table 3).

Root formation: For full plant recovery, shoot (3-5 cm) obtained after 20 days of incubation in elongated media were shifted in rooting media (Table 4). Two concentrations of IBA (0.5 and 1 mg/l) were amalgamated with MS basal media. Dasht-2000 and Bittle-98 showed the best response i.e. 60 and 80% root formation frequency, respectively on 1.0 mg/l IBA (Table 4; Fig. 1K). IBA (0.5 mg/l) gave less rooting frequency in both cultivars (Table 4). Two concentrations of NAA (0.5 and 1.0 mg/l) were also supplemented with MS basal media and were assessed on root formation frequency in both the cultivars. Results presented in table 4 showed that NAA did not enhance the frequency of root formation. In this experiment, we noticed that IBA was the best growth regulator for root formation and Bittle-98 responded better for rooting because its roots were thick, long and strong than that of Dasht-2000 whose root formation frequency was low and roots were thin and weak.

Genetic transformation: Establishment of transformation protocol was the second part of present study. This was an attempt to establish transformation system in Pakistani chickpea genotypes. GUS assay and selectable marker genes were used for the assessment of transgene activity.

Effect of acetosyringone on transformation efficiency: In the virulence system of *Agrobacterium*, unit virA acts as sensor of phenolic compounds like acetosyringone. Four different concentrations of acetosyringone (0, 50, 100 and 150 uM) were assessed on virulence sensitivity of agropine strain EHA-105 that in turn affect plant transformation efficiency in chickpea (Table 5). The optimum level of acetosyringone (100 µM) was proved to be more efficient for stimulating the virulence system of *Agrobacterium* generating a maximum value of explants showing transient GUS activity for Bittle-98, being significantly higher than those efficiencies at 0, 50 and

150 µM (Fig. 1I). The same trend was noticed in case of Dasht-2000, although transformation efficiency was lower (Table 5; Fig. J).

Table 1. Effect of different concentrations of Clorox on *in vitro* plant germination

Clorox concentration (%)	Total seeds inoculated	No of germinated seeds		Germination frequency (%)	
		Dasht-2000	Bittle-98	Dasht-2000	Bittle-98
10	25	2	3	08	12
20	25	4	6	20	28
30	25	5	10	24	40
40	25	16	20	64	**80**
50	25	19	14	**76**	48
60	25	7	6	20	24

The bold letters demonstrate the highest germination frequency

Table 2. Effect of different concentrations of BAP on multiple shoot induction frequency culturing different explants in chickpea

Multiple shoot induction media	Multiple shoot induction frequency culturing half embryo With cotyledon		Multiple shoot induction Frequency culturing half embryo Without cotyledon		Multiple shoot induction Frequency culturing Cotyledonary node	
	Bittle-98	Dasht-2000	Bittle-98	Dasht-2000	Bittle-98	Dasht-2000
MS + 1.0 mg/l BAP	36.00	24.00	8.00	0.00	40.00	35.00
MS + 2.0 mg/l BAP	60.00	66.68	53.33	16.00	**80.00**	**70.00**
MS + 3.0 mg/l BAP	**69.2**	**70.66**	**56.00**	**20.00**	70.00	60.00
MS + 4.0 mg/l BAP	49.32	58.67	33.32	12.00	50.00	40.00

The concentrations of plant growth regulators were taken in mg/l. After six weeks of culture, data were collected. Each data is the average of three replicates.

Table 3. Effect of different concentrations of IAA on shoot elongation of two chickpea genotypes

	Bittle-98			Dasht-2000		
IAA conc	No of shoot explant	No of elongated shoot	Frequency of shoots elongation (%)	No of shoots as explants	No of elongated shoot	Frequency of shoots elongation (%)
0.25 mg/l	**24**	**22**	**91.66**	**24**	**14**	**58.33**
0.5 mg/l	24	18	75	24	11	45.83
1.0 mg/l	24	14	58.3	24	9	37.5

Table 4. Effect of different concentrations of IBA and NAA on root formation frequency culturing elongated shoots

PGR concentrations	Root formation frequency (%)	Root formation frequency (%)
	Bittle-98	Dasht-2000
MS + 0.5 mg/l IBA	40	20
MS + 1.0 mg/l IBA	**80**	**60**
MS + 0.5 mg/l NAA	20	20
MS + 1.0 mg/l NAA	**40**	**20**

PGR denotes plant growth regulators. Twenty five elongated shoots were used as explants sources for root formation. Bold letters denote the best root formation frequency.

Table 5. Effect of different levels of acetosyringone on transformation efficiency in chickpea

Acetosyringone (μM)	Explant cultured	GUS+ve		Transformation efficiency (%)	
		Bittle-98	Dasht-2000	Bittle-98	Dasht-2000
0	40	6	5	15	12.5
50	40	8	6	20	15
100	40	20	14	50	35
150	40	1	2	2.5	5

Table 6. Effect of different levels of cefotaxime sodium on bacterial growth in chickpea

Cefotaxime sodium (mg/l)	Explants cultured	Bacterial growth observed		Necrosis or Browning	
		Bittle98	Dasht2000	Bittle98	Dasht2000
250	40	22	19	0	0
500	40	12	7	0	0
750	40	5	2	24	15
1000	40	0	0	35	28

Table 7. Effect of hygromycin as selecting agent on transformation of chickpea

Bittle-98				Dasht-2000			
Hygromycin (mg/l)	Explant	Regenerated shoots	Explant regeneration %	Hygromycin (mg/l)	Explant	Regenerated shoots	Explant regeneration %
0	40	38	95.0	0	40	34	85.0
50	40	28	70.0	50	40	21	52.5
75	40	19	47.5	75	40	13	32.5
100	40	4	10.0	100	40	2	5.0

Optimization of cefotaxime sodium

In *Agrobacterium tumefaciens*-mediated transformation protocols, it is very important to control the growth of bacteria after co-cultivation period. Cefotaxime sodium is an antibiotic used to control the overgrowth of bacteria on explant. During this study, four different concentrations of cefotaxime sodium (250, 500, 750 and 1000 mg/l) were examined to identify the optimum dose. The results revealed that 500 mg/l proved to be the best level giving minimum bacterial growth and damage to explant was observed (Table 6). Whereas 250 mg/l does not control the bacterial growth and most of the explants damaged due to the overgrowth of bacteria. However, the higher doses of cefotaxime sodium (750 and 1000 mg/l) adversely affected the explant growth and regeneration and necrosis of explant occurred.

Optimization of hygromycin as selectable marker

For selection of transformants, hygromycin was added at the stage of selection. Four different concentrations of hygromycin (0, 50, 75 and 100 mg/l) were used to identify optimum dose. Hygromycin (50 mg/l) resulted in high shoot formation in both varieties (Table 7). 75 mg/l seemed to be the optimum lethal dose (Fig. 1N). Although 100 mg/l hygromycin provided a very strict selection pressure. Yet it also damages even the resistant shoots and could not be continued.

DISCUSSION

Genetic transformation offers the transfer of genes across the generic/specific barriers. The basic pre-requisite of genetic transformation of crop plants is that there must be reproducible regeneration system from single cells in particular crop species for which genetic manipulation is required. The current investigation in Pakistani chickpea varieties (Bittle-98 and Dasht-2000) has been focused on these two areas i.e. establishment of *in vitro* regeneration system and genetic transformation protocols.

Proper sterilization procedure is essentially required for seed germination. Clorox is used as a surface sterilizer and played an effective role in contamination free seeds under *in vitro* conditions. During this study, 40 and 50% Clorox was found to be the most appropriate for surface sterilization of chickpea seeds. Very high concentration of Clorox (more than 50%) had an inhibitory effect and the seeds lost their viability. These findings were confirmatory with those of Akbulut *et al.* (2008) in which they used similar concentration of Clorox for seed sterilization in chickpea.

In our studies, half embryo with cotyledon was found to be the best explants for multiple shoot regeneration in chickpea. Our results were in contrast with those of Sarker *et al.* (2003) who reported that cotyledonary nodes yielded the best multiple shoot regeneration in chickpea. These variations might be due to difference in genetic composition of genotypes. Polisetty *et al.* (1996) also reported that existence of cotyledon attached to embryo was necessary for shoot induction in chickpea. Various concentrations of BAP alone were tested in this study and successful multiple shoot formation was recorded on media supplemented with 3.0 mg/l BAP culturing half embryo with cotyledon and half embryo without cotyledon in both genotypes, while in case of cotyledonary node the highest multiple shoot regeneration frequency was recorded on media fortified with 1.0 mg/l BAP. Similar to our findings, Paul *et al.* (2008) and Yousefiara *et al.* (2008) reported that BAP alone showed the best response for multiple shoot formation in chickpea. Contrary to our findings, Chakraborti *et al.* (2006) concluded that combination of cytokinins with a relatively lower concentration of auxin was useful for efficient frequency of multiple shoot production in Indian origin chickpea cultivars. It might be due to difference in genotypes and explants used. From our experiments, we found that BAP was more efficient in producing *in vitro* shoot regeneration and multiplication.

During this study, it was found that further growth of regenerated shoots was inhibited at certain stage (more than six week old culture) in growth medium having 3.0 mg/l BAP. These shoots were very weak and not able to develop rooting system. Therefore, it was necessary to elongate shoots further for the development of profuse root system. The inclusion of 0.25 mg/l IAA in MS media with 3.0 mg/l resulted in elongation of strong shoots. Similar findings were reported by Chakraborti *et al.* (2006). For full plant recovery, shoot (3-5 cm) obtained after 20 days of incubation in elongated media were shifted in rooting media containing full strength MS and 1.0 mg/l IBA. Similar results were reported by Paul *et al.* (2008). In lentil, IBA also gave best results for rooting (Khawar *et al.,* 2002).

Genetic transformation for incorporation of foreign traits has been accomplished in some grain legumes. But there are very few reports on agronomically important gene transfer in chickpea. (Jiang *et al.,* 2005; Akbulut *et al.,* 2008). *Agrobacterium*-mediated gene transformation of chickpea have been reported in the previous research studies by Krishnamurthy *et al.* (2000); Tewari *et al.* (2004); Sarmah *et al.* (2004); Sanyal *et al.* (2003). Establishment of transformation protocol was the second part of present study after tissue culture. This was an attempt to establish transformation system in chickpea genotypes released in Pakistan.

The transfer of T-DNA from bacterium to plant cell is tightly regulated process and multiple factors from both plant & bacterial cells are simultaneously required for the transformation process (Tzfira *et al.,* 1997). At bacterial level, it is mediated by a set of virulence genes such as Vir A, Vir G, Vir E, Vir D, and a complex of Vir B (Park *et al.,* 2000; Shah *et al.,* 2016) with optimal expression in the presence of phenolic inducers such as acetosyringone that are released by wounded plant cells and certain sugars (Stachel *et al.,* 1985; Cangelosi *et al.,* 1990). During this study, a series of experiments were conducted for better induction of the virulence genes by modification of acetosyringone. The recalcitrance of plant species to *Agrobacterium* transformation is due to absence or deficiency of phenolic inducer compound like acetosyringone. In the virulence system of *Agrobacterium,* Vir A acts as sensor of phenolic compound like acetosyringone and subsequently acts as kinase of Vir G phosphorylation which in turn, regulates the activity of other vir operons (Turk *et al.,* 1991; Shah *et al.,* 2015b). However the sensitivity of Vir to acetosyringone concentration is variable with respect to plasmid type and bacterial strain (Aldemita and Hodges, 1996). In our study, 100 Um acetosyringone was found to be optimum concentration giving maximal transformation efficiency irrespective of chickpea genotypes tested.

In *Agrobacterium tumefaciens*-mediated transformation system, it is very important to control the growth of bacteria after co-cultivation period. Cefotaxime sodium is an antibiotic that controls the overgrowth of bacteria on explant. The antibiotics which are commonly used to eliminate *Agrobacterium tumefaciens* from plant tissues have been shown to influence morphogenesis either positively or negatively (Ling *et al.,* 1998). In chickpea transformation, different levels of cefotaxime sodium were used to optimize the standard level that control the overgrowth of *Agrobacterium tumefaciens* on explants and do not inhibit plant regeneration. In present study, at 250 mg/l cefotaxime sodium, the regeneration frequency was high but transformation efficiency was low because it didn't control the overgrowth of bacteria and most of the explants damaged.

During our study, selectable marker gene and GUS assay were used for the assessment of transgene activity in chickpea plants. Hygromycin (75%) was found to be optimal lethal dose that didn't affect chickpea plant regeneration. Similar to our findings, Mogensen *et al.* (2005) used *Agrobacterium tumefaciens* to transform Ascochytarabiei, which is the pathogen of chickpea blight. T-DNA containing a hygromycin resistance gene was employed. 908 transformants were obtained from germinated pycnidiospores on a selection medium which contained hygromycin. In our study, qualitative transient *GUS* expression analysis was done with optimized bacteria culture to detect transgene in chickpea genotypes. From our results, we found that histochemical

staining strong *GUS* expression was detected in transgenic plants. Our findings were consistent with the results by Husnain *et al.* (2000) who optimized conditions for biolistic transformation. *GUS* gene was introduced in hypocotyls tissues for transient expression and frequency obtained was 58%. These results provided the basic information for chickpea transgenic confirmation via *GUS* expression analysis. Hence, MS basal media supplemented with 3.0 mg/l BAP and 0.25 mg/l IAA was found to be optimum for multiple shoot regeneration in chickpea. Our standardized protocol for efficient regeneration in Pakistani chickpea genotypes would be beneficial for other recalcitrant chickpea cultivars in order to improve their genetic potential. The optimized genetic transformation protocol mediated by *Agrobacterium tumefaciens* would also be helpful in transforming various genes against biotic and abiotic stresses in chickpea.

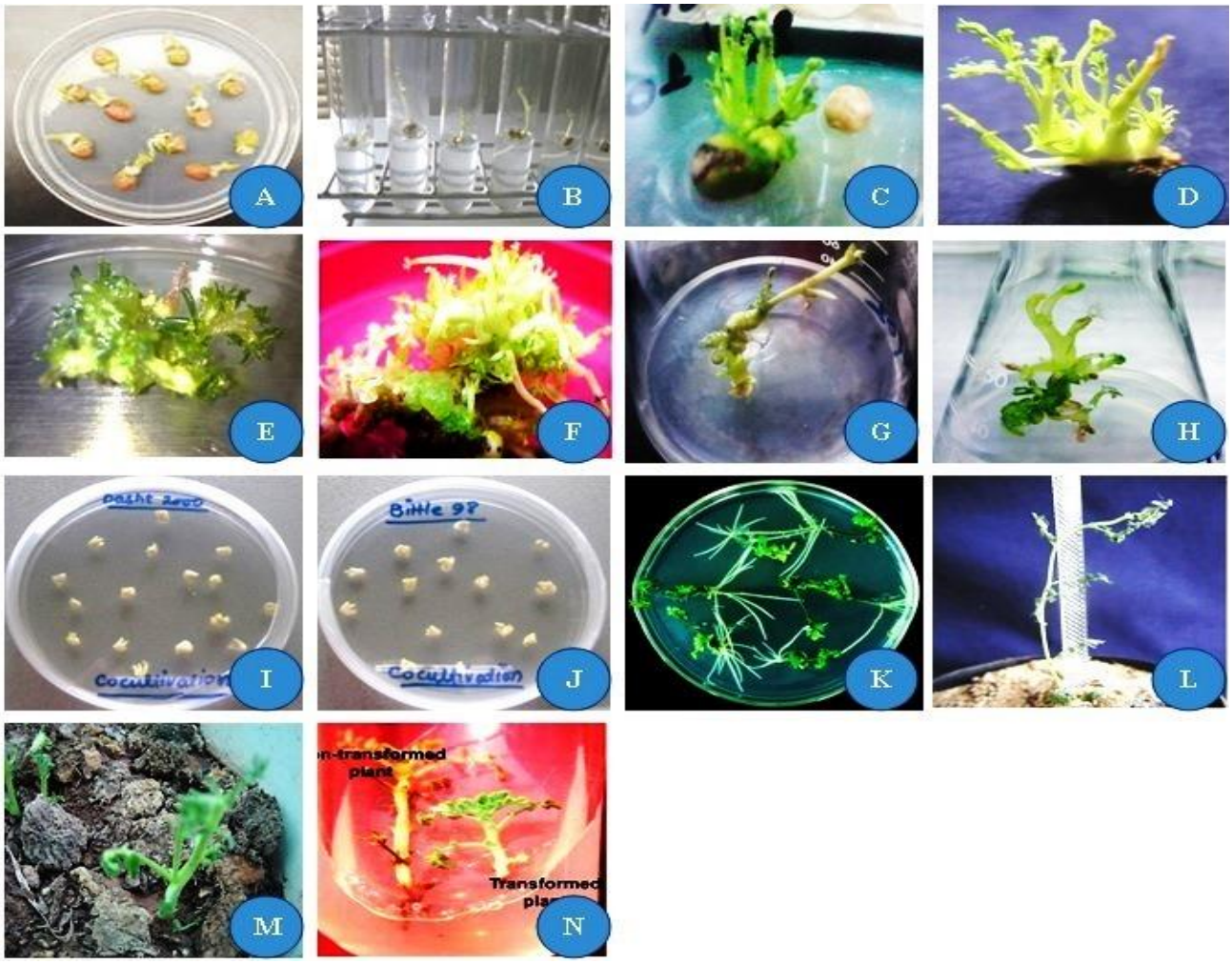

Figure 1. Different stages of chickpea transformation (A) Regeneration of half embryo with single cotyledon (B) Regeneration from cotyledonary node (C) Regeneration of multiple shoots culturing half embryo with cotyledon after 2 weeks of incubation (D) Regeneration of multiple shoots culturing half embryo with cotyledon on MS media enriched with 3.0 mg/l BAP after 21 days of incubation (E) Regeneration of multiple shoots culturing half embryo without cotyledon on MS media enriched with 3.0 mg/l BAP after 21 days of incubation (F) Regeneration of multiple shoots culturing cotyledonary node on MS media enriched with 2.0 mg/l BAP after 21 days of incubation (G, H) Elongation of multiple shoots on MS media containing 0.25 mg/l IAA (I) Co-cultivation stage of Dasht-2000 culturing half embryo with cotyledon explants (J) Co-cultivation stage of Bittle-98 culturing half embryo with cotyledon explants (K) Root formation on MS media containing 1.0 mg/l IBA (L) Hardening of plantlets (M) Established plants of Bittle-98 (N) Effect of 75 mg/l hygromycin on non-transgenic plants showing death through necrosis.

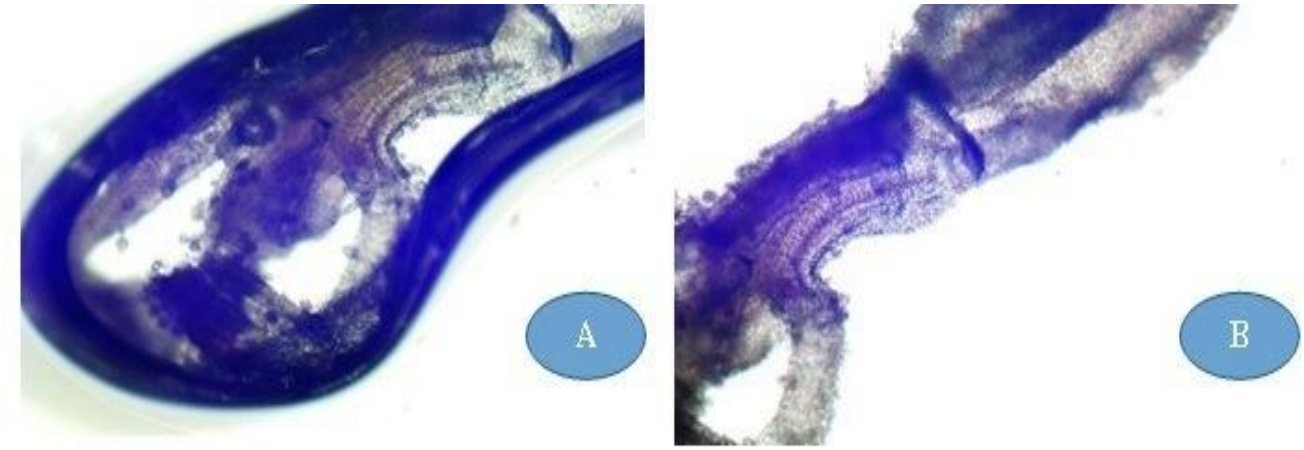

Figure 2. Histochemical staining of GUS activity was conducted in two chickpea varieties "Bittle-98" and "Dasht-2000". The putative transgenic plants showed blue colour. Observations of transformed plants were done by microscope. A. Bittle-98 B. Dasht-2000. In figure 2 the blue colour for transgenic plants is showing difference from colour for non-transgenic plants.

Conclusions: This study had been successful in *in vitro* regeneration of the latest commercial chickpea varieties of Pakistan i.e. Bittle-98 and Dasht-2000. Half embryo along with cotyledon was found to be the best explant for tissue culture of chick pea. The study confirmed that Bittle-98 had good potential for genetic transformation in future research programs. Based upon present findings, it is recommended that this variety may be used in breeding programs for the control of blight in chickpea genotypes. There is still needed to work for proper acclimatization method. It is hoped that genes for specific traits like insect resistance, herbicide resistance, drought tolerance which are lacking in prevailing chickpea germplasm would be transformed into Bittle-98 using this optimized protocol.

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REFERENCES

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

Ahmad, M. Z., I. Hussain, S. Roomi, M.A. Zia, M.S. Zaman, Z. Abbas, and S.H. Shah (2012). *In vitro*response of cytokinin and auxin to multiple shoot regeneration in *Solanum tuberosum*L. American-Eurasian J. Agric. & Environ. Sci. 12(11): 1522-1526.

Ahmed K, and M. S. Awan (2013). Integrated management of insect pests of chickpea (*Cicer arietinum* L.Walp) in South Asian countries: Present status and future strategies – A review. Pakistan J. Zool. 45(4): 1125-1145.

Akbulut, M., M. Yusel, and H. A. Oktem (2008). Analysis and optimization of DNA delivery in chickpea (*Cicer arietinum* L.) seedlings by *Agrobacterium tumefaciens*. African J. Biotech. 7(8): 1011-1017.

Aldemita, R. R., and T. K. Hodges (1996). *Agrobacterium tumefaciens*–mediated transformation of japonica and *indica* rice varieties. Planta. 199: 612-617.

Ali, N., A. Zada, M. Ali, and Z. Hussain (2016). Isolation and identification of *Agrobacterium tumefaciens* from the galls of peach tree. J. Rural. Dev. Agric. 1(1): 39-48.

Cangelosi, G. A., R. G. Ankenbauer, and E. W. Nester (1990). Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proc. Natl. Acad. Sci. 87: 6708-6712.

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

Hadi, M. Z., M. D. McMullen, and J. J. Finer (1996). Transformation of 12 different plasmids into soybean via particle bombardment. Plant Cell Rep. 15: 500–505.

Husnain, T., T. Fatima, R. Islam, and S. Riazuddin (2000). Plant regeneration and expression of *beta-glucuronidase* gene in hypocotyle tissue of chickpea (*Cicer arietinum* L.). Pakistan J. Biol. Sci. 3: 842-845.

Hu, W., and G. C. Phillips (2001). A combination of overgrowth control antibiotics improves *Agrobacterium tumefaciens* mediatd

transformation efficiency for cultivated tomato (*Lycopersicon esculentum* M.). In Vitro Cell Dev-Pl. 27: 12-18.

Jiang, B., Y. Yang, Y. Guo, Z. Guo, and Y. Chen (2005). Thidiazuron-induced in-vitro shoot organogenesis of the medicinal plant Arnebiaeuchroma, RoyleJohnst. In Vitro Cell Dev-Pl. 41: 677-681.

Khawar, K. M., and S. Ozean (2002). Effect of indole-3-butyric acid on *in vitro* root development in Lentil (*Lens culinarismedik*). Turk. J. Bot. 26: 109-111.

Krishnamurthy, K. V., K. Suhasini, A. P. Sagare, M. Meixner, A. Dekathen, T. Pickardt, and O. S. Ladizinki (2000). *Agrobacterium* mediated transformation of (*Cicer arietinum* L.) embryo axes. Plant Cell Rep. 19(3): 235-240.

Ling, H. Q., D. Kriseleit, and M. G. Ganal (1998). Effects of ticarcillin/potassium clavulanate on callus growth and shoot regeneration in Agrobacterium-mediated transformation of tomato (*Lycopersicon esculentum* Mill). Plant Cell Rep. 17: 843–847.

Mehmood, K., M. Arshad, S. Ali, M. Qayyum, and G. M. Ali (2016). Comparative study of tissue culture response of some selected basmati rice cultivars of Pakistan. J. Rural Dev. Agric. 1(1): 30-38.

Mogensen, E. G., M. P. Challen, and R. N. Strange (2006). Reduction in solanapyrone phytotoxin production by *Ascochyta rabiei* transformed with *Agrobacterium tumefaciens*. FEMS Microbiol. Lett. 255(2): 255–261.

Nisha, K. K., K. Seetha, K. Rajmohan, and M. G. Purushothama (2003). *Agrobacterium tumefaciens*-mediated transformation of Brahmi [(*Bacopa monniera* (L.) Wettst.], a popular medicinal herb of India. Curr. Sci. 85: 85-89.

Pakistan Economic Survey (2013-14). Government of Pakistan, Pakistan Bureau of Statistics.

Park, S. H., B. M. Lee, M. G. Salas, M. Srivatanakul, and R. H. Smith (2000). Shorter T-DNA or additional virulence genes improve *Agrobacterium*-mediated transformation. Theor. Appl. Genet. 101: 1015-1020.

Paul, V., R. Polisetty, R. Chandra, and K. Suresh (2008). Age of seedling explant and regeneration potential in chickpea (*Cicer arietinum* L). Phytomorphol. 58(1): 41-48.

Polisetty, R., P. Patil, J. J. Deveshwar, S. Khetarpal, and R. Chandra (1996). Rooting and establishment of *in vitro* grown shoot tip explants of chickpea (*Cicer arietinum* L.). Ind. J. Exp. Biol. 34(8): 806-809.

Sambrook, J., and D. W. Russel (2001). Molecular cloning: A Laboratory Mannual. 3rdEd., Cold Spring Habour, New York.

Sanyal, I., I. Singh, A. K. Meetu, and D. V. Amla (2003). *Agrobacterium tumefaciens* mediated transformation of chickpea (*Cicer arietinum* L.) using mature embryonic axes and cotyledonary nodes. Ind. J. Biotech. 2: 524-532.

Sarker, R. H., B. M. Mustafa, A. Biswas, S. Mahbub, M. Nahar, R. Hashem, and M. I. Hoque (2003). *In vitro* regeneration in lentil (*Lens culinaris* M.). Plant Tiss. Cult. 13: 155-163.

Sarmah, B. K., A. Moore, W. Tate, L. Molvig, R. L. Morton, D. P. Rees, P. Chiaiese, M. J. Chrispeels, L. J. Tabe, and T. J. V. Higgins (2004). Transgenic chickpea seeds expressing high level of bean amylase. Mol. Breeding. 4: 73-82.

Shah, S. H., S. Ali, and G. M. Ali (2013). A novel approach for rapid *in vitro* morphogenesis in tomato (*Solanum lycopersicum* Mill.) with the application of cobalt chloride. European Acad. Res. 1: 2702-2721.

Shah, S. H., S. Ali, S. A. Jan, and G. M. Ali (2014a). Assessment of carbon sources on *in vitro* shoot regeneration in tomato. Pakistan J. Agri. Sci. 51: 197-207.

Shah, S. H., S. Ali, S. A. Jan, J. Din, and G. M. Ali (2014b). Assessment of silver nitrate on callus induction and *in vitro* shoot regeneration in tomato (*Solanum lycopersicum* Mill.). Pakistan J. Bot. 46: 2163-2172.

Shah, S. H., S. Ali, S. A. Jan, J. Din, and G. M. Ali (2015a). Callus induction, *in vitro* shoot regeneration and hairy root formation by the assessment of various plant growth regulators in tomato (*Solanum lycopersicum* Mill.). The J. Anim. Plant Sci. 25(2): 528-538.

Shah, S. H., S. Ali, S. A. Jan, J. Din, and G. M. Ali (2015b). Piercing and incubation method of *in planta* transformation producing stable transgenic plants by overexpressing *DREB1A* gene in tomato (*Solanum lycopersicum* Mill.). Plant Cell Tiss. Org. 120: 1139–1157.

Shah, S. H., S. Ali, Z. Hussain, S. A. Jan, J. U. Din, and G. M. Ali (2016). Genetic improvement of tomato (*Solanum lycopersicum* Mill.) with *AtDREB1A* gene for cold stress tolerance using optimized *Agrobacterium*-mediated transformation system. Int. J. Agric. Biol. 18: 471–482.

Sharma, H. C., J. H. Crouch, K. K. Sharma, N. Seetharama, and C. T. Hash (2002). Applications of biotechnology for crop improvement: Prospects and constraints. Plant Sci. 163: 381-395.

Stachel, S. E., E. Messens, M. M. Van, and P. C. Zambryski (1985). Identification of the signal molecules produced by wounded plant cells that activate DNA transfer in *Agrobacterium tumefaciens*. Nature. 18: 624-629.

Tewari, T., N. Singh, J. Sen, M. Kiesecker, V. S. Reddy, H. J. Jawbsen, and S. Guha-Mukherjee (2004). Use of a herbicide or lysine plus threonine for

non-antibiotic selection of transgenic chickpea. Plant Cell Rep. 22: 576-583.

Turk, H. J., L. S. Melchers, H. den Dulk-Ras, A. J. S. Rengenberg-Turk, and P. J. J. Hookaas (1991). Environmental conditions differently affect vir gene induction in different *Agrobacterium* strains. Role of the VirA sensor protein. Plant Mol. Biol. 16: 1051-1059.

Tzfira, T. C., S. Jensen, W. Wang, A. Zuker, B. Vinocur, and A. Vainstein (1997). Transgenic populastremula. A step by step protocol for its mediated transformation. Plant Mol. Biol. Rep. 15: 219-235.

Uzma, M. R. Khan, A. Muhammad, I. Hussain, S. H. Shah, T. Kumar, S. Inam, M. Zubair, H. U. Rehman, A. Sher, N. Rehman, S. Ahmed, and G. M. Ali (2012). Rapid *in vitro* multiplication of sugarcane elite genotypes and detection of sugarcane mosaic virus through two steps RT-PCR. Int. J. Agric. Biol. 14(6): 870–878.

Yousefiara, M., A. Bagheri, and N. Moshtaghi (2008). Optimizing regeneration conditions in chickpea (*Cicer arietinum* L.). Pakistan J. Biol. Sci. 11 (7): 1009-1014.