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Development of a simple and efficient protocol for chickpea (*Cicer arietinum* L.) transformation by floral injection method

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Abstract

Agrobacterium-mediated transformation has been widely used in various crops for development of varieties better in quality and adaptation against biotic and abiotic stresses. Chickpea plant shows limitation towards *in vitro* regeneration during course of transgenic plant development therefore it is the need of hour to develop a protocol independent of pre-culture, co-cultivation and regeneration steps. Therefore, in the present study a modification has been done in floral dip method of transformation in chickpea to examine the effect of genotypes, optical density of bacterial culture and floral bud stage. Six varieties (Parbat, Punjab-2008, Dasht, CM-2008, NCS-0709 and Noor-2009) were grown. Overnight culture of *Agrobacterium tumefaciens* strain LBA4404 harboring binary vector pCAMBIA1301 containing *GUS* gene was injected into the floral buds at two different stages. Transformed seeds were harvested at plant maturity and plants were subjected to GUS assay. Effect of genotypes, culture concentrations and floral bud stages on transformation efficiency was calculated. Analysis of Variance (ANOVA) and Tukey's Multiple Range Test (TMRT) were applied to data by using MSTAT-C software. Among all of the genotypes studied; DASHT genotype at hooded floral bud stage with *Agrobacterium* cell density of 0.5 OD_{600} has exhibited the highest transformation efficiency percentage.This method of plant transformation is more economical and feasible in chickpea because it doesnot involve regeneration stages.

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Introduction

Chickpea (*Cicer arietinum* L.) also known as gram is a self-pollinated, herbaceous, annual, bushy plant belonging to Fabaceaefamily. It is a 3rd most important pulse crop in the world (Anon. 1994). Its leaves, shoots, pods and seeds are consumed in raw, cooked or ground form in various cuisines worldwide. Chickpea seed is rich source of protein (25%), fiber, carbohydrates and minerals. The world largest producer is India with production of 8.83 million tones followed by Australia, Pakistan and Turkey. Pakistan is the third largest producer of chickpea with production of 0.75 million tonnes (FAO, 2013).

Factors that have limitized its production are biotic and abiotic stresses as well as low yield potential varieties (Hassan *et al.*, 2003). Conventional and non-conventional breeding approaches have been utilized for development of better yielding varieties against these constraints. However, non-conventional methods such as tissue culture and genetic engineering are less time consuming and avoid transfer of undesirable traits linked to desired ones (Clough and Bent, 1998; Akbulu *et al.*, 2008). Hence, trend is now increasing towards exploitation of these methods. But for implementation of plant tissue culture and genetic engineering to discover a gene, study its function and incorporate it into a new organism; a stable, efficient and reproducible transformation method is a prerequisite (Dobhal *et al.*, 2010). Transformation of a targeted gene can be achieved by *Agrobacterium*-mediated, particle bombardment, microinjection and electroporation method. But *Agrobacterium*-mediated transformation method is more reliable, cost effective, targeted and stable one (Feldmann and Marks, 1987; Gu *et al.*, 2008).

Agrobacterium-mediated transformation could be either tissue culture dependent or not. Tissue culture dependent transformation involves callus development followed by *Agrobacterium* infection. Regeneration from transformed calli results in the development of transgenic plants. However, regeneration is a big hurdle in legumes. All the legumes are recalcitrant to tissue culture regeneration after transformation process (Reddy. 2007). It urges to develop some strategy not involving regeneration steps (Larkin and Scowcroft. 1986). *In planta* transformation resolves this problem of rejuvenation in pulses. It offers various benefits such as cost effectiveness, devoid of tissue culturing, involves less time, labor and expertise, avoid somaclonal variations and mutagenesis. *Arabidopsis* was the first plant transformed through this technique (Clough and Bent. 1998; Feldmann and Marks, 1987). Numerous other crops have also been transformed through *in planta* transformation.

Floral injection method in which *Agrobacterium* is injected into the inflorescence through micropipette is a simple modification of floral dip method. It makes it more efficient and resourceful. Floral bud stage and concentration of bacterial culture (culture density) are the most crucial factors that can alter rate of transformation (Martinez-Trujillo *et al.,* 2004). *Agrobacterium* infection to soybean pods at different stages alters transformation efficiency percentage (Zia *et al.*, 2011).Therefore an effort was made in the current study to investigate effects of bacterial culture concentration, bud stage and genotype on *in planta* chickpea transformation method. The objectives were identification of most promising genotype for transformation, detection of best floral bud stage for *in planta* transformation and optimization of *Agrobacterium* culture concentration (OD) for highest transformation efficiency. It will facilitate future development of standardized and optimized protocol for obtaining high rates of transformants in shorter time period.

Materials and methods

Plant material and growth conditions

Six varieties (Parbat, Punjab-2008, Dasht, CM-2008, NCS-0709 and Noor-2009) were sown in pots following completely randomized design (CRD) with ten replications of each variety. Soil was prepared by mixing 33% of sand, 33% of silt and 33% of clay. Pre-treatment of *desi* type (Parbat, Punjab-2008, Dasht) and *kabuli* type (CM-2008, NCS-0709 and Noor-

2009) seeds was performed using captan and carbendazim fungicide at the rate of 1gkg^{-1} and 1.5gkg^{-1} respectively (Kaiser *et al.*, 1984).Pots were placed in controlled conditions at 25°C with 80% relative humidity and 15 hour photoperiod.

Bacterial strain and plasmid

The *Agrobacterium tumefaciens* strain LBA4404 harbouring pCAMBIA1301 vector was used for chickpea transformation. T-DNA region of plasmid carries *uidA* gene for β-glucuronidase (GUS) and Hyg R gene encoding hygromycine phosphotransferase resistance under the influence of CaMV 35*S* promoter.

Preparation of inoculum

Glycerol stock of *Agrobacterium* culture was streaked on Yeast Extract Peptone (YEP) media plate supplemented with antibiotics (25 mgml^{-1} kanamycin and 10mgml^{-1} rifampicilin) and incubated at 28°C for 48 hours. A single colony was picked and inoculated into 5ml Luria-Bertani (LB) media containing 25 mgml^{-1} kanamycin and 10 mgml^{-1} rifampicilin antibiotics (pH 7.2) with help of autoclaved micropipette tip. The culture was incubated overnight in incubator shaker at 28°C and 210 rpm followed by centrifugation in ultra-centrifuge at 10,000 rpm for 10 min to collect pellet. The pellet was re-suspended in antibiotic free LB medium. The optical density (OD_{600}) of culture was adjusted to 0.5 and 1.0 by using spectrophotometer.

Bud infection with bacterial culture

The Previously reported procedure of *in planta* transformation was used in this study with some modifications (Akbulut *et al.*, 2008). The bacterial culture of 50µl (OD_{600} of 0.5 and 1.0) was injected by syringe at stage 1 (closed bud, 7 mm and green) and stage 2 (hooded bud, 10 mm and pale) of chickpea floral buds of all mentioned genotypes (Table 1).

Histochemical GUS staining assay

Chickpea seeds were harvested from transformed floral buds at maturity and were sown in pots filled with sand, silt and clay mixture. The pots were placed under control conditions in growth chamber. After 10 days of germination plants were subjected to histochemical GUS staining assay to determine the rate of transformation (Jefferson, 1987). Dissected shoots of 0.5cm were dipped in GUS staining solution consisting of 2 mMX-Gluc, DMSO,triton X-100,100 mM Tris-HCl (pH 7.5), 50 mM sodium phosphate and 2mM potassium ferricyanide. After overnight incubation at 37°C explants were submerged in 70% ethanol solution for 4 hours for chlorophyll removal and clarity. Along with this a control reaction was also performed using segments from untransformed plants to check the possibility of any pre-existing *GUS* activity.

PCR confirmation of putative transformants

For molecular analysis of transgenics, DNA extraction was performed from both transformed and non-transformed shoot tissues by CTAB method (Doyle and Doyle, 1990). To confirm for the presence of *GUS* reporter gene in genome of transformants PCR analysis was performed using *GUS* primers. A reaction mixture (20µl) comprising of 2.5 µl of 10X buffer, 0.5 µl of dNTPs, 1 µl of 50 Mm $MgCl_2$, 2 µl of each primer (forward and reverse), 10 µl of H_2O and 2 µl of DNA template was prepared. The mixture was incubated in thermal cycler with initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation step at 94°C for 1min, annealing at 56°C for 30 sec, extension at 72 °C for 1min and final extension step at 72 °C for 5 min. Along with this reaction a control reaction consisting of DNA template from untransformed plants was also subjected to PCR analysis. The PCR products were resolved on 1% agarose gel for detection of *GUS* fragment presence.

Statistical analysis

The experiment was performed in two factorial completely randomized designs (CRD). The experimental data was subjected to Analysis of Variance (ANOVA), Tukey's Multiple Range Test (TMRT) as used by Steel *et al.* (1997) using MSTAT-C software.

Results

There are several factors that affect *Agrobacterium*-mediated transformation such as genotype, type of explant, age of explant, bacterial strain, culture concentration and infection duration. Optimization of such factors and development of a standardized protocol is fundamental for successful transformation of any plant Therefore in the present research *in planta* transformation method was studied in chickpea transformation and effect of floral bud stage, bacterial culture density and genotype was investigated for the first time. There is no previous data available relevant to this aspect of *in planta* chickpea transformation.

Table 1. Floral bud stages and cultural densities used in the present study.

Treatment No.	Floral Bud Stages	Culture Density (OD_{600})
1	Closed	0.5
2	Hooded	0.5
3	Closed	1.0
4	Hooded	1.0

Table 2. Analysis of Variance of Effect of Culture Concentration and Floral Bud Stage on Chickpea Genotypes.

SOURCE	DF	SS	MS	F	P
Genotype	5	1453.37	290.674*	751.92	0.0000
Treatment	3	234.15	78.049*	201.90	0.0000
Genotype*Treatment	15	119.48	7.965	20.60	0.3641
Error	216	83.50	0.387		
Total	239	1890.50			

α = 5%, DF = Degree of Freedom; SS = Sum Squares; MS= Mean Squares; F = Calculated values; P = Tabulated value from ANOVA table, *Effect of culture density and bud stage.*

Six varieties each with ten replications were used in this study. At flowering, two types of floral bud stage were identified and selected for *Agrobacterium* injection. Stage I (closed bud stage) specifications were immature stigma and anthers at the base of the bud while stage II (hooded bud stage) was characterized by elongated corolla, anthers about half the length of style and receptive stigma.Immature anthers at these stages had miniature pores from which bacterium entered and caused infection. Each bud was injected by 50µl of bacterial culture with the help of syringe.

Table 3. Genotypes, treatments and transformation efficiency %

Genotypes	Culture Concentration (OD_{600})	Floral Bud stage	Mean Infected floral buds	Mean Transformed seed	Transformation efficiency (%)
CM-2008	0.5	Closed	10	3	30
		Hooded	10	6	60
	1.0	Closed	10	3	30
		Hooded	10	5.0	50
NCS-0709	0.5	Closed	10	0.0	0
		Hooded	10	0.0	0
	1.0	Closed	10	0.0	0
		Hooded	10	0.0	0
NOOR-09	0.5	Closed	10	4	40
		Hooded	10	7	70
	1.0	Closed	10	3	30
		Hooded	10	6	60
PARBAT	0.5	Closed	10	4	40
		Hooded	10	6.	60
	1.0	Closed	10	3	30
		Hooded	10	5	50
PUNJAB-08	0.5	Closed	10	0.0	0

		Hooded	10	0.0	0
	1.0	Closed	10	0.0	0
		Hooded	10	0.0	0
DASHT	0.5	Closed	10	5	50
		Hooded	10	8	80
	1.0	Closed	10	4	40
		Hooded	10	7	70

The comparison of mean values of genotypes by Tukey's test (Table 4) showed that DASHT (genotype no. 6, *Desi*-type) gives maximum mean value of 6 as compared to NCS-0709 (genotype no. 2, *Kabuli*-type) and PUNJAB-08 showing zero value.

Two culture concentrations of 0.5 and 1.0 at OD_{600} were used. Two floral bud stages and culture concentrations/ density makes four different treatments as shown in table 1.

0.5OD_{600} showed significant effect on the rate of transformation as compared to closed floral bud stage with culture concentration 1.0 OD_{600}At closed floral bud stage 20 floral buds of each genotype were infected, 10 buds with 0.5 OD_{600} and other 10 by OD_{600} of 1.0. Same procedure was performed for hooded bud stage of each variety. Harvested seeds were grown in next season for confirmation of transformation. Their shoots were subjected to *GUS* assay and expression was observed by visual examination for confirmation of blue stain presence. PCR was performed to confirm *GUS* gene fragment presence in the genome of plants. Transformation efficiency percentage was calculated on the basis of number of transformed plants (confirmed by GUS) per number of infected flowers.

Table 4. Mean Comparisons of Different Treatments for Transformation by Using Tukey's Test.

6	DASHT	6	A
3	NOOR-09	5.4750	A
4	PARBAT	4.7750	B
1	CM-2008	4.1250	B
5	PUNJAB-08	0	C
2	NCS-0709	0	C

Blue staining in (A) section represents GUS positive sample from *in planta* transformed floral buds of chickpea. Section (B) shows the sample from untransformed plant (control)*Effect of genotypes*
Effect of genotype and interaction among genotype, bud stage and culture concentration on *Agrobacterium*-mediated *in planta* transformation of chickpea was explored. Number of seeds harvested, GUS assay and PCR results were synchronized and compared for each genotype to find out most promising genotype among the all six (Parbat, Punjab-2008, Dasht, CM-2008, NCS-0709 and Noor-2009). Statistical analysis revealed that genotypes showed significant effect on the rate of transformation (Table 2). Comparison of results of all varieties shows that DASHT (genotype No.6) have high transformation efficiency as compared to other varieties studied as shown in table 4. Both GUS staining and PCR analysis shows that variety DASHT is best for transformation hence this variety can be used to achieve maximum transformation rate. Results are in consent with previous studies (Figure 1)which reported that genotype has significant effect on the rate of transformation

Analysis of variance indicated that *Agrobacterium* culture density and bud stage have highly significant effect on transformation efficiency as shown in Table 5. The mean values calculated on the basis of number of floral bud injected per treatment were compared by

using Tukey's test (Table 4). The treatment no.2 (OD_{600}: 0.5; late floral bud stage) showed maximum transformation efficiency (6.6667) followed by treatment no.4 (OD_{600}: 1.0; late floral bud stage). The minimum mean values were observed in treatment no.1 (OD_{600}:0.5, early floral bud stage) and 3 (OD_{600}: 1.0; early floral bud stage) with treatment no. 3 being the lowest result producing. These findings illustrate that late floral bud stage along with both types of culture densities (0.5 OD_{600} & 0.6OD_{600}) gave higher percentages of transgenics (Table 3 & 4, Figure 4) significantly affected by using culture concentration of 0.8 OD_{600} in oil palm.Culture concentration of 0.8 OD_{600} also gives significant results in winter jujuba, sugarcane and *Acacia crassic*.

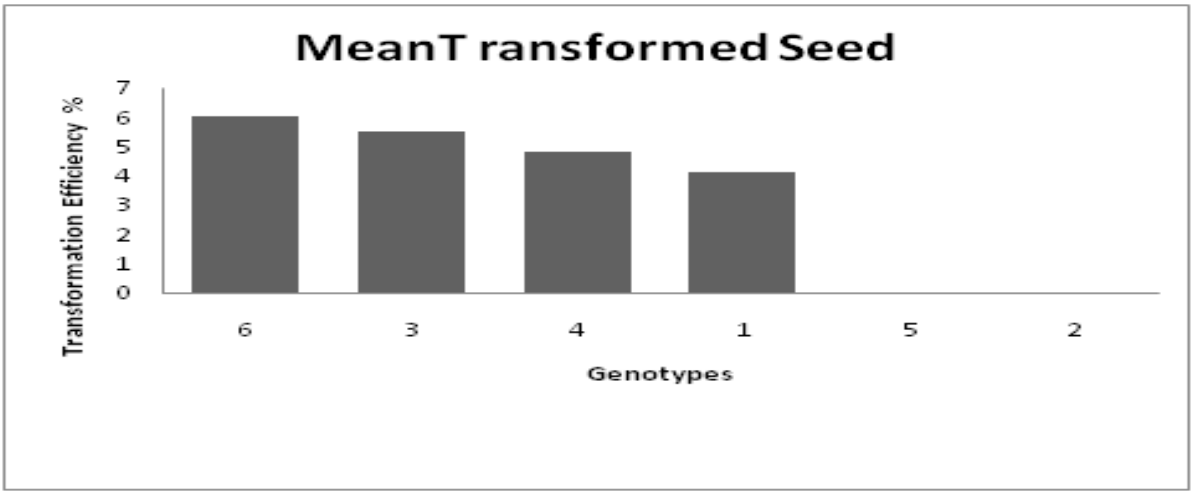

Fig. 1. Mean transformed seeds of each genotype.
Genotypes on X-axis (1-CM-2008, 2-NCS-0709, 3-NOOR-09, 4-PARBAT, 5-PUNJAB-08, 6-DASHT).

Discussion

It has been reported that floral bud stage and concentration of bacterial culture can affect the rate of transformation (Martinez-Trujillo, 2004).The results have shown that late floral bud stage has significant effect on rate of transformation with less concentrated culture as compared to early floral bud stage with more concentrated culture. The studies on *Arabidopsis* showed no significant impact of concentration on transformation efficiency (Clough, S. J. and A. F. Bent. 1998). While transformation in *Nicotiana tabacum* was significantly affected by bacterial concentrate.

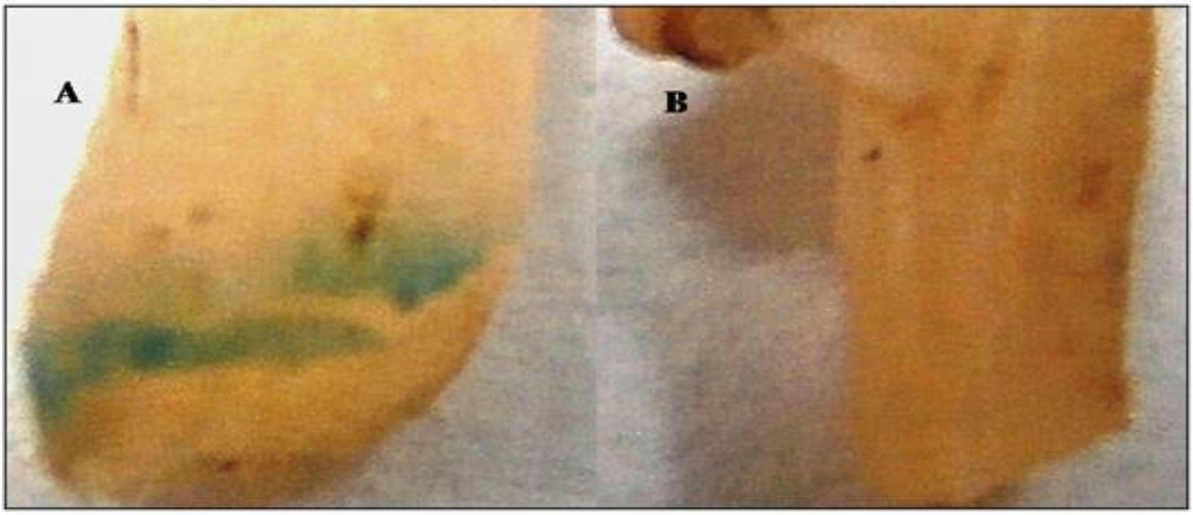

Fig. 2. GUS assay of shoot segments from chickpea plant.

Therefore, the effect of concentration of bacteria (*Agrobacterium*) seems to be species specific (Dobhal *et al.,* 2010). Basavanna (2003) reported that culture concentration of 0.6 OD_{600} significantly affect the transformation in pigeon pea. The rate of transformation, interaction of genotypes, treatments and transformation efficiency percentage (Figure 5) clearly shows that DASHT variety has dominance over the all others. Treatment comparison analysis (Figure 4) showed that treatment number 2 (hooded bud stage and 0.5 OD_{600})caused maximum transformation efficiency achievement.

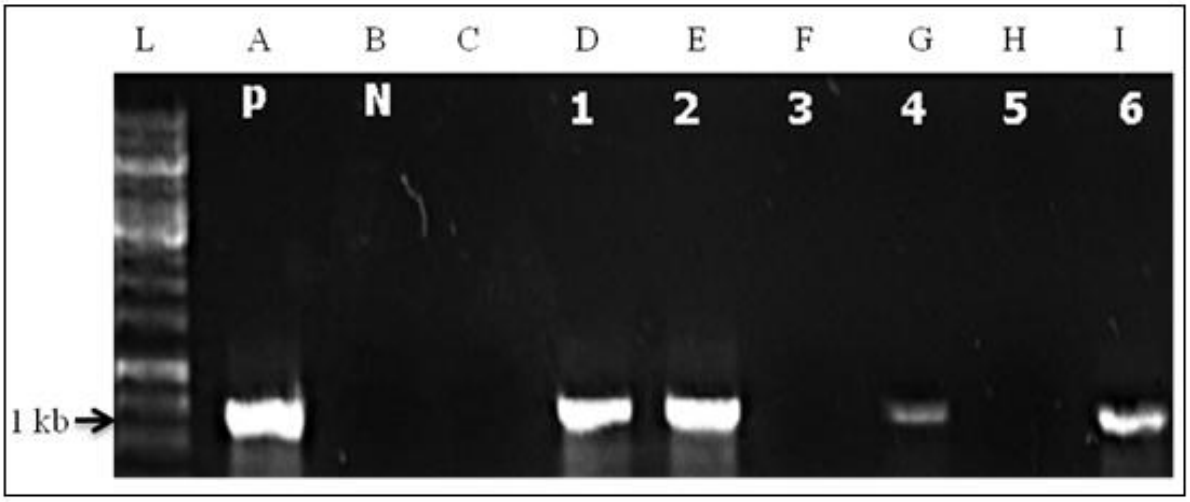

Fig. 3. PCR analysis of transformed chickpea plants for *GUS* resistant transgene.
PCR analysis of transformed chickpea plants to detect presence of *GUS* gene: Lane# 1=V-6, 2=V-3, 3=V-2, 4=V-1, 5=V-5, 6=V-4 transformed chickpea, P= Bacterial culture (positive control) and N= Negative control.

Therefore it can be concluded from this study that DASHT variety is most promising towards *in planta* transformation method. Contrary to transformation of early floral bud stage which showed decreasing trend towards percentage of transformants achieved. This shows that late floral bud has significant influence on success rate of obtaining higher percentages. Culture concentration of 0.5 OD_{600} (less dense bacterial culture) produced highest transformation efficiency percentage in all genotypes than with the 1.0 OD_{600} (more concentrated) (Table 3).

CM-2008

Variety CM-2008 after infection with bacterial culture of 0.5 OD_{600} at closed bud stage produced 4 seeds in total 10 infected buds. In next growing season only 3 seeds were germinated. Its calculated transformation efficiency was 30% (Table 3) as thirty per cent of samples showed positive GUS analyses result (Figure 2). In case of hooded bud infection with culture density of 0.5 OD_{600}; 7 seeds were produced out of which 6 were germinated and calculated transformation efficiency was 60%. The infection of closed and hooded bud with 1.0 OD_{600} exhibited 30% and 50% transformation efficiency respectively. A 1kb band appeared in lane number 7of gel picture depicts presence of *GUS* gene in CM-2008 *in planta* transformed chickpea plants (Figure 3). Genotype has shown good response with OD_{600}0.5 and hooded bud.

NCS-0709

No transformed seed of variety NCS-0709 was obtained and it displayed no respond towards GUS assay (Table 3). Expected 1 kb *GUS* band did not appear in lane 6 (representing sample from NCS-0709). Results show that NCS-0709 genotype has zero transformation efficiency %. This zero transformation efficiency was due to burning and dropping of buds after injection.

Noor-09

Variety NOOR-09 presented 40% and 70% transformation efficiency on infecting closed and

hooded bud with 0.5 OD_{600} culture concentrations respectively (Table 3). Infection of both types of buds with 1.0 OD_{600} resulted in 30% and 60% transformation efficiency. Lane E contain very sharp and thick 1 kb band of *GUS* gene which illustrates that NOOR-09 variety has been efficiently transformed with this method and have responded well (Figure 3). Maximum transformation efficiency has been achieved with 0.5 OD_{600} culture concentrations and hooded bud.

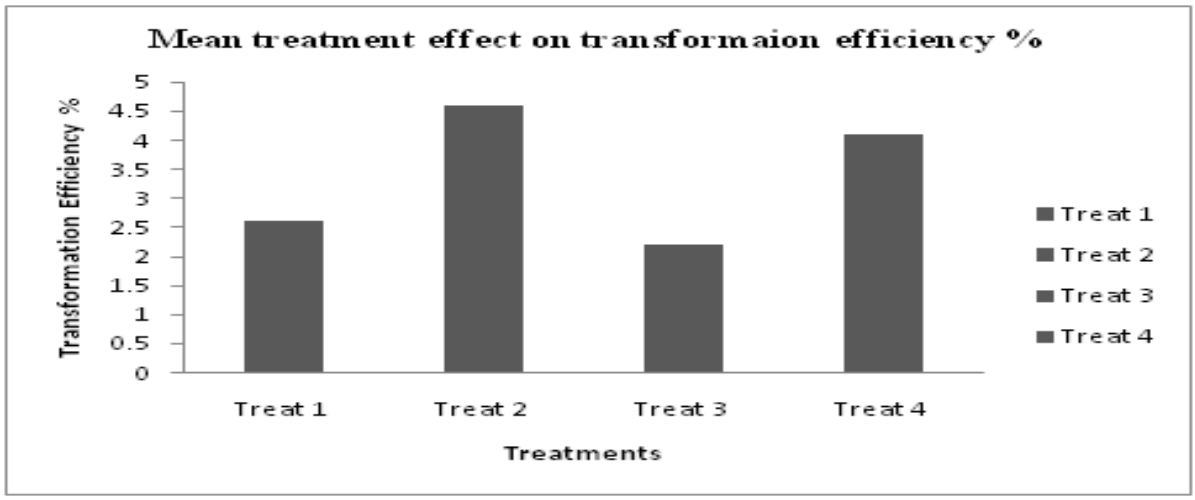

Fig. 4. Effect of treatments on transformation efficiency.
(Treat No.1-Culture conc.0.5OD_{600}, Early floral bud stage), (Treat No.2-Culture conc.0.5OD_{600}, Late floral bud stage), (Treat No.3-Culture conc.1.0OD_{600}, Early floral bud stage), (Treat No.4-Culture conc.1.0OD_{600}, Late floral bud stag.

Parbat

Transformation efficiency of 40% and 60% obtained when hooded and closed buds were infected with bacterial culture of 0.5 OD respectively on the other hand infection of buds with 1.0 density resulted in 30% and 50% transformation efficiencies (Table 3). Gel electrophoresis of PCR samples also displayed *GUS* band of 1 kb in lane I which confirms successful transformation of variety PARBAT with *GUS* gene (Figure 3).

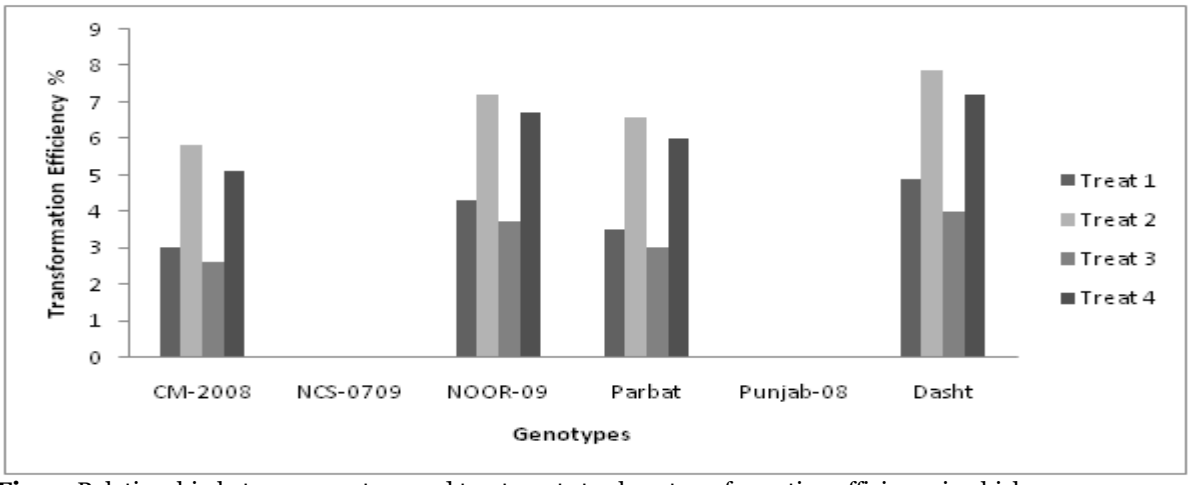

Fig. 5. Relationship between genotype and treatments to show transformation efficiency in chickpea.
(Treatment No. 1=Closed bud stage and 0.5 OD_{600}, Treatment No. 2=Hooded bud stage and 0.5 OD_{600}, Treatment No. 3= Closed bud stage and 1.0 OD_{600}, Treatment No. 4=Hooded bud stage and 1.0 OD_{600}). Therefore it can be concluded that hooded floral bud stage with culture concentration of 0.5OD_{600} (Treatment no.2) has high mean percentage of transformation efficiency (Table 5).

Punjab-08

Punjab-08 variety did not respond well towards all treatments. GUS assay showed no blue color in samples. Lane H in the gel picture did not display any *GUS* band which confirms that genotype is not responsive towards this method of transformation. The poor transformation efficiency was due to stunted growth, suffering from disease blight and dropping of buds after injection.

Dasht

Maximum transformed seeds of DASHT variety were obtained (Figure 1). Transformation efficiency of DASHT was 50% and 80% when closed and hooded buds were infected with bacterial culture of 0.5OD_{600}. Infection of closed and hooded floral buds with culture concentration of 1.0 OD_{600} resulted in 40% and 70% transformation efficiency respectively. All the plants of DASHT genotype showed blue stain in GUS assay. A *GUS* band of 1 kb appeared in gel electrophoresis analysis of its PCR sample as shown in lane D of Figure 3.

Conclusion

In planta transformation method is more efficient, easy and reproducible as compare to laborious, costly and time consuming tissue culture method. Using the floral bud for transformation gives more results as compare to other. This method is for the first time applied on chickpea for transformation. Among six different varieties of chickpea DASHT showed best response towards the floral dip method.

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