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Optimization of *In-planta* Method of Genetic Transformation in Pigeon Pea (*Cajanus cajan* L. Millsp.)

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A B S T R A C T

Pigeon pea (*Cajanus cajan* L. Millsp.) is one of the major grain legume crops of the tropics and sub-tropics grown in about 50 countries of Asia, Eastern and Southern Africa and the Caribbean for various uses such as food, fodder and firewood. The present study was conducted to develop and optimize tissue culture independent in planta transformation system in pigeonpea. This system can help in widening genetic base as well as solutions over various biotic and abiotic factors through engineering novel genes across the species. The various transformation parameters viz., Optical density of *Agrobacterium* suspension, virulence inducer and infection time were optimizad through 18 different treatment combinations. The plumular and inter cotyledonary meristem axes of 2-3 days old germlings of pigeon pea cv. BSMR 853 was exploited for Agroinfection by sewing needles. Out of 270 inoculated germlings 11.34 mean number of plantlets were recovered. The putative transformants were confirmed by GUS histochemical and PCR assay. Among 18 different treatment combinations, the treatment pertaining Agrobacterium suspension of O.D 1.0, virulence inducer (acetosyringone) at 250 µM/ml and infection time of 1.0 min was found optimum has shown significant impact on transformation efficiency. The treatment comprising bacterial O.D, 1.0 with 250 µM/ml acetosyringone and 1.0 infection time 1.0 min revealed 90.02% transformation efficiency. However, lowest transformation frequency i.e. 68.76% was reported in treatment of bacterial O.D. 1.5 with 150 µM/ml acetosyringone and 0.5 min. infection time. The present investigation revealed the optimization of in planta transformation parameters in pigeon pea and suitability of genotype BSMR853 for genetic transformation and further genetic improvement.

Introduction

Pigeon pea (*Cajanus cajan* L.) is an important grain legume of the semi-arid tropics and form a significant component of the diet of vegetarians. Pigeon pea is member of family fabaceae, order fabales and genus *Cajanus*. It is often cross-pollinated crop (20-70 per cent) having diploid chromosome number (2n = 22) with an estimated genome size of 833.07 Mb (Varshney *et al.*, 2011). It is short lived perennial, but traditionally, it is cultivated as an annual crop in Asia, Africa, Caribbean region and Latin America. Thus, it is

becoming one of the major grain legume crops of tropics and subtropics. Considering natural genetic variability in pigeon pea and presence of its wild relatives in the region, it has been postulated that, India is the primary center of origin of pigeon pea (Saxena *et al.*, 2008). Globally, it is cultivated on 4.92 mha with annual production of 3.65 mt and productivity is of 898 kg/ha. About 90% of the global pigeon pea area falls in India corresponding to 93% of global production/ (http://www.icrisat.org). Pigeon pea is second most important leguminous crop grown in India followed by chickpea. The area, Production and yield of pigeon pea during year 2012-13 in India is 3.38 mha, 2.27 mt and 671 kg/ha respectively (Kaur *et al.*, 2012). Pigeon pea seeds contain about 20-22% protein and appreciable amounts of essential amino acids viz., Methionine and Cysteine and minerals (Saxena *et al.*, 2008). It is a favorite crop of small holder dryland farmers, as it can grow well under subsistence level of agriculture and provides nutritive food, fodder and fuel wood. It is also a good source of fibers, vitamins and minerals. Pigeon pea is an excellent source of vitamin D and it also improves soil by fixing atmospheric nitrogen.

The production of pigeon pea is constrained by use of unfertile land, water logging or dry spells during critical stages of crop growth, pest and diseases problems, narrow genetic base and lack of drought-resistant, non-availability of high-yielding genotypes. The conventional plant breeding approach with improved agricultural practices is not found enough to improve the pigeon pea production over last 50 years. While the application of various advancements in molecular biology, genetic transformation and *in-vitro* techniques have significantly contributed to improve the production and quality of several crops. However, these modern tools have not been commercialised in pigeon pea to combat the severe losses caused by several biotic (i.e. pest and diseases like Pod borers, Root knot nematodes, *Fusarium* wilt, Sterility Mosaic etc.) and abiotic (i.e. drought, salinity, water logging etc.) stresses. The chief factor among them is pod borer (*Helicoverpa armigera*), becoming most serious and being infectious to all cultivated species of pigeon pea. Its larvae attack the flowers and pods of the pigeon pea, resulting in substantial damage and yield losses of over $300 million annually worldwide (Shanower *et al.*, 1999). Pod borer problems is complex and intractable, no single control strategy is successful in keeping its population below economic threshold level (ETL). On the other side, indiscriminate use of pesticides to control pests has led to series of consequences like insect resistance, pest resurgence, outbreak of secondary pest, harmful residual effects, imbalances in natural ecosystem and higher production costs which have been a concern in India and elsewhere. The wild relatives are available in pigeon pea but possess very narrow genetic base towards their improvement of this crop through conventional plant breeding techniques. Therefore, it is becoming important to develop a rapid transformation system for improvement of pigeon pea.

The development in biotechnology facilitates the transfer of cloned and well-defined genes across the plant species through methods of genetic transformation viz., microprojectile bombardment, *Agrobacterium*-mediated gene transfer, viral vectors, electroporation, sonication, etc. Among these, the most commonly used method for genetic transformation in re-calcitrant crop like pigeon pea is the *Agrobacterium* mediated gene transformation (Horsch *et al.*, 1985) although it is time consuming, regeneration system dependant and has difficulty while controlling the overgrowth of *Agrobacterium* etc. Therefore, it is becoming vital to develop

and optimize *In-planta* transformation system in pigeon pea which enables rapid gene transformation in pigeon pea. A key component of most of the functional genomics approaches is a high-throughput transformation system which is emerging as an important tool of crop improvement. Transformation technique also offers strategies for over expression or suppression of endogenous genes to generate new phenotypic variation towards investigation of gene function for crop improvement. Thus, it is imperative to have an efficient regeneration and transformation system in order to introduce novel traits in crop like pigeon pea. Therefore, in view of development of tissue culture independent rapid transformation system towards improvement of pigeon pea, optimization of *In-planta* transformation system was attempted to optimize *In-planta* transformation conditions and molecular analysis of pigeon pea transformants.

Materials and Methods

Plant material

Seeds of pigeon pea cultivar BSMR 853 procured from Agricultural Research Station (ARS), Badnapur were used during course of this investigation. The seeds were washed in distilled water initially and then rinsed in 70% ethanol for 5 minutes. This step was repeated twice. Then the seeds were treated with 0.1% $HgCl_2$ solution followed by washing in double distilled water for 5 minutes to remove the traces of surface sterilent. The sterilized seeds were placed in ½ MS seed germination medium for 3-5 days.

Procedure for *in planta* transformation of pigeon pea

Agrobacterium tumefaciens strain, EHA101 harbouring the binary plasmid *pBI121* containing *GUS* gene procured from NRCPB, New Delhi was used for transformation. The vector contains the *neomycin phosphotransferase II* (*nptII*) gene driven by the nopaline synthase promoter. *Agrobacterium* was grown overnight at 28^0C in 25 ml of YEM medium (pH 7.0) containing 50 µg/ml kanamycin. The bacterial culture was later pelleted at 6000 rpm for 5 min. The *Agrobacterium* pellets were resuspended in 50ml liquid ½ MS medium and stored at 4^oC till further use. The suspension culture approximately 15 ml of *Agrobacterium* strain EHA101 harboring *GUS* gene was taken separately in sterile petri-plate. Further acetosyringone was added at different concentrations (150, 200 and 250µM) to increase efficiency of transformation. The needle incised germinated seedlings were dipped into the suspension culture containing *GUS* gene in ½ MS media for 5-10 seconds and shake at 50 rpm for 5 minutes. The seedlings were removed from the suspension culture and dried on sterile filter paper. Further the inoculated seedlings/ germlings were sown in plastic cup containing sterile cocopeat. Further these plants were transferred into plastic pot containing sand, soil and FYM and grown upto maturity in green house.

Histochemical *GUS* analysis

The histochemical *GUS* assay was performed at shoot initiation and developmental stage of transformed pigeon pea plants. This assay was used to check the presence of *GUS* gene incorporation into transformed pigeon pea plants. Shoots of putative pigeon pea transformants were dipped into 20 ml assay solution and incubated at 37^0C overnight in the dark chamber wrapped in aluminium foil. Based on appearance of blue colour precipitate in pigeon cells qualitative analysis of pigeon pea transformation was performed. The histochemical *GUS* analysis to determine the β-glucuronidase activity in the putatively

transformed plantlets was carried out in accordance with Jefferson *et al.*, (1987).

Molecular analyses of the putative transgenic plants

Tissues from the progeny plants were analyzed for the presence of the introduced gene. Genomic DNA was isolated following the procedure of Lie *et al.,* (2007) from fresh leaf tissue from greenhouse-grown T_0 generation and that was used for polymerase chain reaction (PCR). PCR was performed to amplify a 750 bp *npt*II gene fragment. PCR was initiated by an initial denaturation at 94 ^{o}C for 4 min followed by 30 cycles of 1 min at 94 ^{o}C, 1 min at 56 ^{o}C and 1.5 min at 72 ^{o}C. The amplified PCR product was separated on 1.2 % agarose gel, stained with ethidium bromide dye and visualized under gel documentation System. Similarly, the PCR was also performed with DNA of non-transformed pigeon pea plant as a negative control. Based on number of plants recovered and *GUS* positive plant derived after PCR confirmation, transformation efficiency was calculated.

Results and Discussion

Seedling development and infection

Five days old seedlings (Figure 1a, 1b) grown on ½ MS medium were isolated aseptically under Laminar Air Flow cabinet. Each of *Agrobacterium* suspension of different treatment combinations, acetosyringone was taken into separate sterile petri-dish. Wherein, eighteen different treatment combinations were adopted towards optimization of three important transformation parameters *viz.*, O.D. of *Agrobacterium*, concentrations of virulence inducer, inoculation time etc. during *in planta* pigeon pea transformation. Fifteen plants were infected under each treatment. Seedlings with plumule just emerging were pierced at the apical meristem of axis and at the intercotyledonary region with a sterile needle and infected by immersion in the suspension of *Agrobacterium* for 0.5 to1.5 min. After infection, seedlings were washed briefly with sterile water and planted in plastic cup containing sterilized cocopit (Figure 2). The planted seedlings were maintained at control conditions in greenhouse. During this experiment the explant comprising axis of apical meristem and intercotyledonary region gave better response for *Agrobacterium* co-cultivation and further recovery of seedlings post co-cultivation. Similar procedure of agroinfection was adopted by Rao *et al.*, (2008) for Agroinfection of pigeon pea. In addition, Keshamma *et al.*, (2008) observed that, embryonic axes showed better response towards agro-infection and recovery of seedlings compared to other explants. While, in tomato embryonic apical meristem was found efficient during *in planta* transformation experiment (Supratna *et al.*, 2006).

Green house maintenance of transformed plants

Initially, a total of 270 plants of 18 different treatment combinations were grown in plastic cup containing sterilized coco-pit at green house conditions (Figure 3a). These plants were covered with polythene bags for 2-3 days to maintain high humidity. Further fully established seedlings were transferred into plastic pots containing sand, soil and FYM (2:1:1) at containment type green house (Figure 3b). These plants were allowed to grow upto the maturity stage and further covered with muslin cloth in order to harvest seed for next progeny (Figure 4). The survival rate of the plant at green house condition was calculated. On an average the maximum recovery of plantlets was observed upto 75.60% (Table 1b). It was revealed that, the

germination process of pigeon pea after *in planta* transformation remains unaffected. Similar type of results was recorded by Rao *et al.,* (2008) and described that, germination percentage and growth process of germinating embryos do not have any adverse effect of *Agrobacterium* during transformation. They reported fresh and healthy seedlings with germination frequency of 50% during prolonged time i.e. 1 h infection period.

Optimization of *in planta* method of pigeon pea transformation

The *in-planta* transformation parameters comprising, bacterial O.D (0.5, 1.0 and 1.5 min.), virulence inducer i.e. acetosyringone (150, 200 and 250 µM/ml) and inoculation period (0.5, 1.0 and 1.5 min) were assessed through 18 different treatment combination (Table 1a). The putative pigeon pea transformants grown at green house conditions were further assessed for confirmation of transgene integration. Further, the best treatment conditions were evaluated by adjudging optimal concentration of transforming parameters in genotype BSMR 853.

Confirmation of transformed plants by histochemical GUS assay

Leaf samples of greenhouse grown transformed pigeon pea plants were collected at different developmental stages viz., seedling, branching and maturity stage and further tested for histochemical GUS analysis. The histochemical GUS assay discriminated the transformed and non-transformed pigeon pea plantlets of cv. BSMR 853. The transformed plantlets showed blue color precipitate at midrib area of leaf, stem and on younger leaves (Figure 5a, 5c). The plantlet developed through each treatment was screened through histochemical GUS assay. However, the histochemical *GUS* assay was also adopted with non- transformed /control plantlets of the same cv. BSMR853 wherein, they did not show the blue color precipitates on tested leaf sample (Figure 5b). The histochemical *GUS* assay method described by Jefferson *et al.,* (1987) is simple, rapid and require less expertise. Many of researchers used this method for confirmation of transgene as Rao and Rohini (1999) utilized this method for confirmation of pigeon pea transformants, Keshamma *et al.,* (2008) in cotton; Ombori *et al.,* (2013) in maize; Ching *et al.,* (1997) and Razzaq *et al.,* (2011) in wheat; Lee *et al.,* (2011) in soybean crop plants. They revealed that, *GUS* gene expressed plants grew normally and remain fertile. Similarly, *GUS* is very stable and tissue extracts continue to show high levels of *GUS* activity after prolonged stage of harvested samples (Jafferson *et al.,* 1987). It could help to make simplicity in histochemical analysis via. collecting and preserving samples for longer duration. Thus, during this course of investigation an attempt have been made to optimize *in planta* transformation protocol in pigeon pea by using *GUS* reporter gene.

Confirmation of transformed plant by PCR analysis

The genomic DNA extracted from putative pigeon pea transformants of genotype BSMR 853 was subjected to PCR amplification with *GUS* gene specific primer. The PCR amplified product was resolved on 1.2 % agarose gel. The 9.25 mean number of putative transformants showing 750 bp amplicon (Figure 6) was considered as *GUS* gene positive pigeonpea plants. While those lacking were designated as non-transformed plants. Based on histochemical *GUS* assay and PCR confirmation the transformation efficiency of genotype BSMR 853 was calculated. The *GUS* histochemical based early detection of transformants was not

recommended in pigeon pea, as it gave false positive due to endogenous *GUS* like activity exhibited by pigeon pea (Rao *et al.,* 2008) and other crops (Sudan *et al.,* 2006). Hence in present investigation, PCR based confirmation of transformants was adopted. Similarly, this kind of approach was adopted by many of the researchers in different crops namely, Lin *et al.,* (2009) in rice; Supartana *et al.,* (2006) and Razzaq *et al.,* (2011) in wheat; Lee *et al.,* (2011) in soybean etc.

Optimization of transformation parameters

Efficient transformation systems using readily available explants are in high demand for agronomically important plants. Though fertile transgenic plants have been generated from a greater number of plants, yet the transformation frequency for most species is still low. *Agrobacterium*-mediated transformation technology has not been routinely applied to pigeon pea because of recalcitrant approach (Rao *et al.,* 2008). However, the *in vitro* regeneration systems available in pigeon pea limited to few genotypes and morphogenetic response of the pigeon pea is known to be a genotype specific phenomenon described by Mohan & Krishnamurty (1998). Hence, further optimization of the transformation parameters such as bacterial OD, inoculation time and virulence inducer would be useful to increase *in planta* transformation efficiency.

During this investigation, the average transformation efficiency was calculated based on mean number of survived plantlets and actual transformed plantlets under each treatment of transformation experiment. The *Agrobacterium* OD, 1.0 was found more effective for transient expression of *GUS* gene (Table 1b). The maximum numbers of transformed plants were obtained at OD 1.0 under each treatment compare to OD 0.6 and 1.5. The transformation efficiency of genotype, BSMR 853 was ranged between 68.76 to 90.02%. It was also observed that, increase in concentration of O.D i.e. of 1.5 as well as decrease in concentration of O.D. i.e. of 0.6 laid impact on decrease in transformation efficiency of genotype BSMR 853. Moreover, higher density of *A. tumefaciens* could increase the transient *GUS* expression but could not give stable transformation frequency. Similar findings were also reported by Cheng *et al.,* (1997) & Supatana *et al.,* (2006).

The second transformation parameter, i.e. inoculation time of 1.0 min, 3min and 5 min were assessed through different treatment combinations with other parameters. The effect of inoculation time was not much correlated with percent transformation efficiency. It was highest at lower inoculation time (0.3min). However, it was also found highest at higher inoculation time (1.5 min). The influence of lower as well as higher inoculation time was found at par with each other. The range of *GUS* expression and transformation efficiency among transformed pigeon pea plants were ranged between 68.76 to 90.02%. Further, it was noticed that, the hardened seedlings of pigeon pea remained fresh and healthy after infection with Agrobacterium. Thus, it was concluded that, there is no more effect of Agroinfection to the germination of seedlings in pigeon pea. Maximum number of plantlets gets recovered after infected with *Agrobacterium*. Thus, inoculation time had not much influence in-terms of *GUS* expression, seedlings germination and transformation efficiency.

Similar type of observations was reported by Rao *et al.,* (2008), wherein they revealed that, seedlings of pigeon pea cv. TTB7 remained unaffected after prolonged inoculation time. They also stated that, there was no effect of agroinfection on germination frequency and

infected seedlings. It remained fresh and healthy even the infection time is prolonged to 1h. In addition, the study in rice by Wagiran *et al.,* (2010) reported that, inoculation time did not have any effect on *GUS* expression and transformation efficiency. They added, the inoculation time was different in different plant species and type of explant dependent, and it might be due to susceptibility of explant to *Agrobacterium* infection.

The addition of virulence inducer i.e. acetosyringone during transformation experiment showed significant influence on transient expression of *GUS* gene. Inclusion of 250 µM/ml acetosyringone in *Agrobacterium* suspension during infection results in the highest *GUS* activity i.e. 90.02% for pigeon pea genotype, BSMR 853. While, the acetosyringone at concentration 150 µM/ml showed minimum transformation efficiency i.e. of 68.76% of the genotype. This result is supported with evidence reported earlier by Wagiran *et al.,* (2010) in rice cultivars wherein, they stated as increase in concentration of acetosyringone beyond 300 µM resulted into decline of percentage of *GUS* activity. In present study, virulence inducer i.e. acetosyringone played a crucial role towards enhancing the transformation of pigeon pea. The addition of acetosyringone in co-cultivation media activates the induction of *vir* genes and extends the host range of *Agrobacterium* strains (Saharan *et al.,* 2004; Zhao *et al.,* 1998). They have also stated that, the optimum concentration of acetosyringone in view of induction of highest transformation efficiency was varied from genotype to genotype. Thus, in present investigation the treatment combination comprising bacterial O.D 1.0, 250 µm/ml acetosyringone and inoculation time 1.0 min was found optimum for transformation of genotype BSMR 853 (Table 1b).

Earlier, similar type of experiments on optimization of transformation parameters in different crops have been attempted by several researchers viz., in pigeon pea (Rao *et al.,* 2008); cotton (Keshamma *et al.,* 2008); buckwheat (Kojima *et al.,* 2000); mulberry (Ping *et al.,* 2003); soybean (Chee *et al.,* 1989); rice (Supartana *et al.,* 2005) etc. The present investigation could result in the standardization of an efficient *in planta* transformation protocol in pigeon pea which gave 68.76-90.02% transformation efficiency in genotype BSMR 853 (Table 1b). This protocol optimized in this study is found efficient and does not involve any tissue culture regeneration procedure. Also, the protocol could generate relatively large number of T_0 transgenic in a short time. Similar findings were also reported earlier by Rao *et al.,* (2008) in pigeon pea and Rohini *et al.,* (1999) by producing 50-76.60% transformation efficiency in sunflower genotype Morden while Lucas *et al.,* (2000) reported 45-62 % transformation efficiency in sunflower *cv.* LSF 8.

Table.1a Optimized transformation parameters *viz.*, bacterial OD, virulence inducer and inoculation period

Transformation parameters	Value of parameter taken	Optimized value of parameters
Bacterial density (OD600)	0.5, 1.0, 1.5	0.5
Virulence inducer, Acetosyringone (µM/ml)	150, 200, 250	250
Inoculation period of Agrobacterium (min)	0.5, 1.0, 1. 5.	1.0

Table.1b Transformation efficiency of pigeonpea genotype BSMR 853

Treatment combination	Pigeon pea cv. BSMR 853		
OD600 + Virulence inducer (µM) + Inoculation (min)	Mean No. of plants recovered	Mean No. of plants transformed	Transformation efficiency (%)
0.6+150+0.5	12.33	9.33	75.66
0.6+150+1.0	12.00	9.00	75.00
0.6+200+1.5	11.30	8.66	70.40
0.6+200+0.5	12.66	10.00	78.98
0.6+250+1.0	12.00	10.00	83.33
0.6+250+1.5	11.66	9.66	82.84
1.0+150+0.5	10.33	9.00	87.12
1.0+150+1.0	10.33	9.00	87.12
1.0+200+1.5	9.00	7.00	77.77
1.0+200+0.5	13.00	11.33	87.15
1.0+250+1.0	13.33	12.00	90.02
1.0+250+1.5	12.66	11.33	89.49
1.5+150+0.5	10.66	7.33	68.76
1.5+150+1.0	10.33	7.66	74.15
1.5+200+1.5	10.66	9.00	84.42
1.5+200+0.5	10.00	8.33	83.30
1.5+250+1.0	11.33	9.33	82.34
1.5+250+1.5	10.66	8.66	81.23
Total Mean	**11.34**	**9.25**	**81.06**
SD	**1.15**	**1.34**	**6.33**
CV	**10.15**	**14.49**	**7.8**
SE	**0.27**	**0.31**	**1.49**

Transformation efficiency was calculated based on mean number of regenerated plantlets and mean number of transformed plantlets. Each treatment was performed in triplicates with 15 number of seedlings treatment

Fig.1a-1b a, Five days old seedlings of pigeon pea cv. BSMR 853 germinated on ½ MS medium; b, washed seedlings used for agroinfection

Fig.2 Recovery of agroinfected seedlings of each treatment grown in plastic cup

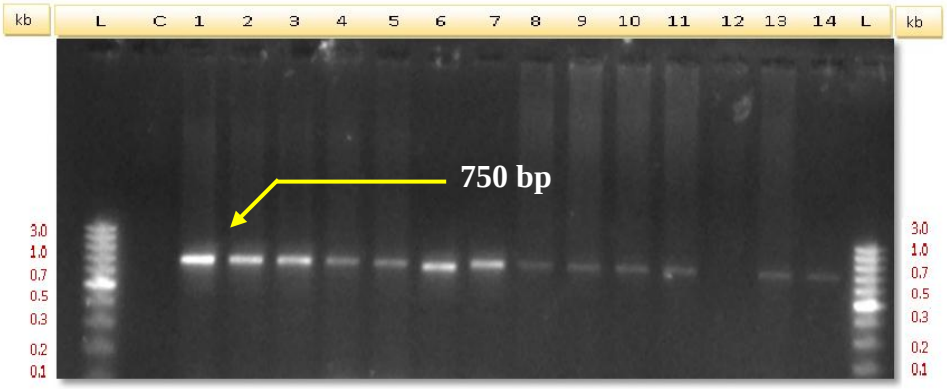
Fig.3a-3b a, putatively transformed seedlings of pigeon pea grown in plastic cup containing coco peat; b putatively transformed seedlings of pigeon pea grown in plastic pot containing Sand, Soil and FYM under greenhouse conditions

Fig.4 Transformed pigeon pea plantlets were maintained under greenhouse conditions for further selfing and generation of T1 progeny

Fig.5a Transformed pigeon pea plantlets showing histochemical *GUS* gene expression

Fig.5b-5c b, Histochemical *GUS* assay of control plant; c, Histochemical *GUS* assay of transformed plant

Fig.6 PCR based confirmation of transformed plantlets of pigeon pea by amplifying 750 bp GUS gene amplicon; L, 100 bp DNA ladder; C, Control plant; 1-14, putatively transformed plantlets under screening



Preliminary experiments suggested that, germination and growth processes of the germinating embryos of pigeon pea remained unaffected in the transformation procedure adopted here. Further steps of hardening of transformed seedling also did not adversely affect the development of the seedlings. The seedlings remained fresh and healthy and the germination frequency was not affected after inoculation of *Agrobacterium.*

In conclusion, the present investigation revealed the suitability of pigeon pea genotype BSMR853 for future transformation and integration of variety of transgenes towards insect pest, disease resistance and quality improvement etc. through tissue culture independent method developed in this study.

During this study, suitability of growing embryonic axis of cv. BSMR853 towards transformation efficiency was also assessed.

The explant growing embryonic axis provided 11.34 mean numbers of recovered plantlets after co-cultivation. The transformation efficiency based on *GUS* and PCR assay was found as high as 90.02% by using embryo as explants for direct Agroinfection. Thus, this investigation concluded with, optimization of tissue culture independent *in planta* transformation systems in pigeon pea genotype BSMR 853. This protocol would be exploited in genetically as well as qualitatively improvement of pigeon pea through rapid development of transgenic.

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