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ORIGINAL ARTICLE **OPEN ACCESS**

In-Planta And *In-Vitro* Agrobacterium Mediated Genetic Transformation in Pigeonpea

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ABSTRACT

The major pest attacking on Pigeonpea is Helicoverpa armigera which is highly susceptible to the insecticidal proteins of Bacillus thuringiensis (Bt). A codon-optimized chimeric cry1Aabc gene of Bt driven by a constitutive promoter was introduced in pigeonpea (PKV Tara) by in-planta and in-vitro agrobacterium mediated genetic transformation. In the present investigation fixed parameters were used for in–vitro transformation experiment with aim to generate putative transformants. Explant (DCMEA) pre cultured on shoot induction medium (MS+ BAP 2 mg/lit) were allowed to colonize with bacterial culture of 0.8 OD followed by co-cultivation on shoot induction medium for 72 hrs and based on previous study acetosyringone at 100µM was used with Agrobacterium Culture. Similarly vacuum infiltration was also tried for 20min. The regenerated shoots transferred on the selection medium II and rooting medium. The observations were recorded for % shoot regenerated after 4 weeks, 8 weeks and 12 weeks on selection medium. The putative transformants obtained on kanamycin selection medium were transferred on rooting medium containing 1.5 mg IAA/lit. Out of 4 shoots survived on kanamycin medium after 12 weeks were subjected for confirmation using PCR, one shoot was found positive in both the npt II and Cry1Aabc gene. In the present study three different strategies were employed for in-planta transformation i.e. infection to germinating seeds, shoot tip infection, floral dip method. Observations were recorded for emergence, post emergence survival. In-planta inoculation of embryo axes of germinating seeds (48 hrs.) with vector pBI-121-GUS mobilized into Agrobacterium tumefaciens strains EHA 105 was carried out with addition of tobacco leaf extract or acetosyringone which resulted in higher positive GUS plants in tobacco leaf extract than acetosyringone. The T_0 seeds obtained from all three approaches of in-planta method were subjected to growth and molecular studies. The results for embryonic axis infection, floral dip method were positive when screened with PCR with both Cry1 Aabc and npt II gene specific markers and results were negative for shoot tip infection.

Keywords: Keywords *Transgenic Pigeonpea, In-Planta, In-Vitro, Genetic transformation, Helicoverpa armigera , Cry1Aabc gene.*

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INTRODUCTION

Pulses play an important role in rainfed and irrigated agriculture, among the pulses Pigeonpea [*Cajanus cajan* (L.) Millsp] is one of the major grain legumes of the semi-arid tropics[1-12]. It ranks fifth among the edible legume and caters the protein requirement of the majority of the Indian subcotinent (George et al.,1994). India is the largest pigeonpea producer country followed by Myanmar and Keniya. More than 80% of the world production occurs in India [20-23]. In India, red gram is prone to more than 200 species of insects, among which pod borer (*Helicoverpa armigera*) causes enormous losses (46.6-63.65%) which is a polyphagous lepidopteron insect, present throughout the year and can complete upto seven generations in a year under favourable condition. Indiscriminate use of pesticides to control *Helicoverpa armigera* has lead to series of consequences like, insecticide resistance, pest resurgence, outbreak of secondary pests, harmful residual effects, imbalances in natural ecosystem and higher production costs, which has been a concern in India and elsewhere. It is therefore necessary to develop more environment friendly approaches with minimum use of chemical pesticides. Development of resistant varieties is one such strategy. There are various methods through which genetic resistance against insect and pest can be achieved [13-16]. The most effective biotechnological move is toward the transfer of specifically constructed gene assembles through various transformation techniques. The plants obtained through genetic engineering contain gene or genes usually from an unrelated organism; such a genes are called

transgenes. Genetic transformation facilitates introduction of only specifically desirable genes without co-transfer of any undesirable genes from donor species, which normally occurs by conventional breeding methods. Genetic transformation has led to the possibility of transforming crops for enhanced resistance to insects and pathogens and has rapidly moved toward commercialization in various crops [19-27]. Development of transgenics expressing insecticidal proteins in productive cultivars is one of the strategies followed in many crop species including pigeonpea. The present study, reports two popular transformation systems i.e. *in-planta* and *in-vitro Agrobacterium* mediated genetic transformation for development of transgenic pigeonpea lines for improved plant protection against the pod borer.

MATERIAL AND METHODS

Plant material

Seeds of pigeon pea cultivar PKV-Tara were procured from SRS, Pulses Research Unit, Dr.PDKV, Akola. The seeds were surface sterilized first with 1% (v/v) laboline for 5 minutes, followed by washing with sterile distilled water for 5-6 times. The washed seeds were treated with 0.1 % (w/v) mercuric chloride for 5 minutes followed by rinsing with sterile distilled water for 5-6 times.

Transformation and co-cultivation

Agrobacterium tumefaciens strain EHA 105 harbouring *Cry*1 *Aabc* gene procured from National Research Center on Plant Biotechnology, New Delhi was used for genetic transformation. The mature embryo axes in which both shoot and root pole were removed referred to as decapitated mature embryonic axes (DCMEA) were used as explant. A colony of bacteria grown for 48 hours was taken and inoculated in 1 ml of liquid LB without agar medium containing 100 mg of Kanamycin and 10 mg/l Refampicin which was allowed to grow for 24 hr at 28^0C. The 24 hrs grown culture was transferred to AB medium and allowed to grow for more 18 hrs. Phenolic compound was added to the medium followed by standing period on shaker at 28^oC with 150 rpm. (O.D 0.8) The culture was then used for co-cultivation. YEMA, LB [24] and AB medium [27] were prepared as per standard protocol. Pre-cultured DCMEA were injured with the help of fine needle in LAF cabinet. The injured explant was dipped in bacterial culture so that their proximal cut ends were immersed into the suspension. Infected explants were placed on sterile Whatman's filter paper no1. The explants were allowed to co-cultivate with the *Agrobacterium* culture for 72 hrs and transferred to SIM supplemented with filter-sterilized cefotaxime (250 mg/l) and Kanamycin (100mg/l).

In-Planta Transformation

Infection to geminated seeds

Growing embryonic axis of germinated seeds was used as explants for infection with bacterial culture. The mature embryonic axis of seeds used by soaked for 24 hrs was pierced at meristematic region with the help of fine needle. The injured seeds were treated with *Agrobacterium* culture with acetosyringone or tobacco leaf extract for different durations.

Shoot tip infection

Primary shoot tip infection was carried out by giving a sharp cut at the growing tip of shoot to expose meristematic tissues to the *Agrobacterium* culture applied through cotton plug. The seedlings were then allowed to grow ex- vitro in to transgenic green house. The secondary infection was done when new branches grew by applying sharp cut at growing tip and inoculation with cotton plug dipped in *Agrobacterium* culture.

Floral dip method

The flower bud of pigeonpea was opened before pollination and *Agrobacterium* culture was applied to the pistil with help of brush. The bud was allowed to pollinate.

Confirmation of Putative Transformants

Screening through Kanamycin

For *in-vitro* studies primary screening was carried out on 100 mg/l Kanamycin at each step. Explants were sub-cultured on fresh medium with kanamycin at weekly interval. Observations on putative transgenic plants were recorded 8 weeks after inoculation on screening medium.

Molecular Screening

Total genomic DNA was extracted from the young leaves by Sambrook *et al.* [24]. Amplification of DNA extracted from plants was carried out by gene specific primers at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 60 s, 57 °C for 60 s for annealing and 72 °C for 5 min, with a final extension at 72 °C for 10 min. PCR products was resolved on 1.5% EtBr stained agarose gel in 1× TBE bufer. Gel images were documented using Gel Documentation system (BioRad Gel Doc, USA).

RESULTS

In-vitro transformation

In the present study, a rapid and efficient shoot regeneration system was used for the transformation of pigeonpea. A total of 50 explants in three replications were co-cultivated. First set up experiment comprises of standardized parameter along with 100µM of acetosyringone. The data showed varied response in survivability at 4 weeks, 8 weeks and 12 weeks i.e. 48 %, 7.3%, and 2.6% respectively. The second experiment comprises of standardized parameter along with 20 min. vacuum infiltration with genotype PKV-Tara. The data revealed varied response i.e. 22.6%, 2%, 0%, respectively. Shoots survived on selection medium of kanamycin 100 mg/lit were transferred to rooting medium after 12 weeks. From shooted seedlings the 1-2 small leaves were taken for DNA extraction. Four seedlings from selection medium were checked through PCR for the presence of transgene using *Cry1 Aabc* and *npt II* specific primer along with plasmid as positive control. From the samples tested one sample was found to be positive. (Plate 2)

Table1. Percentage of co-cultivated explants regenerated after 4 weeks, weeks and 12 weeks

Treatment	% explants regenerated on selection medium after 4 week	% shoots survived on selection medium after 8 weeks	% shoot on rooting medium after 12 week
A. culture with acetosyringone (100 µM)	48.0	7.3	2.6
Vacuum Infiltration (20 Min)	22.6	2	00
Control	18.6	0.6	00

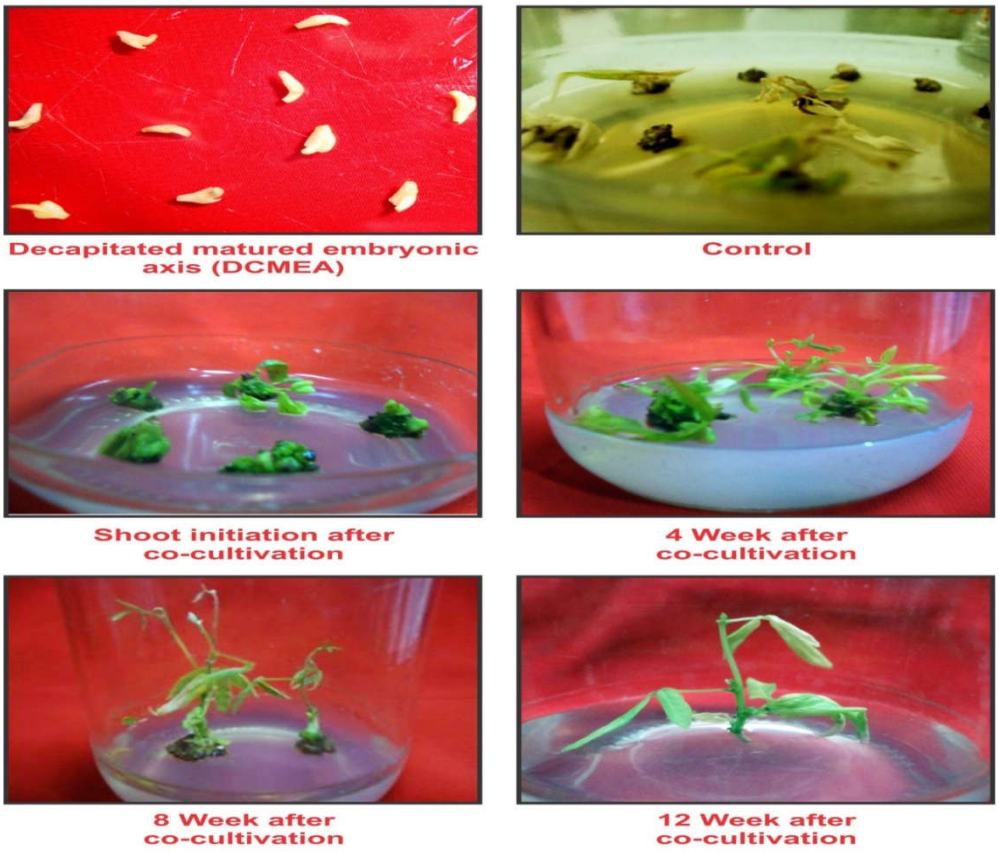

Plate 1 DCMEA Derived Shoot On Selection Media

Saharan *et al*. [23] in indica rice cultivars HKR-46 and HKR-126 reported improvement in *Agrobacterium*-mediated transformation using 100µM concentration of acetosyringone in the *Agrobacterium* culture. Droste *et al.* [10] used 100µM acetosyringone in order to increase the virulence of *Agrobacterium tumefaciens* in soybean. Hamid Rashid *et al.* [22] also reported transformation efficiency of 15.62% in wheat when acetosyringone concentration of 100µM was used.

In the vacuum infiltration method of 20 min the percent explants regenerated on selection medium after 4 weeks, 8 weeks and 12 weeks i.e. 22.6%, 2% and 0% respectively, after 12 week the shoots were not survived on the rooting medium. Ikram-Ul-haq [15] reported upto 28.23 per cent transformation efficiency when two month old hypocotyls derived embryogenic calli of cotton (*Gossypium hirsutum* L.) cv. Cocker-312 were infected through agro-infiltration for 10 min at 27 psi units.

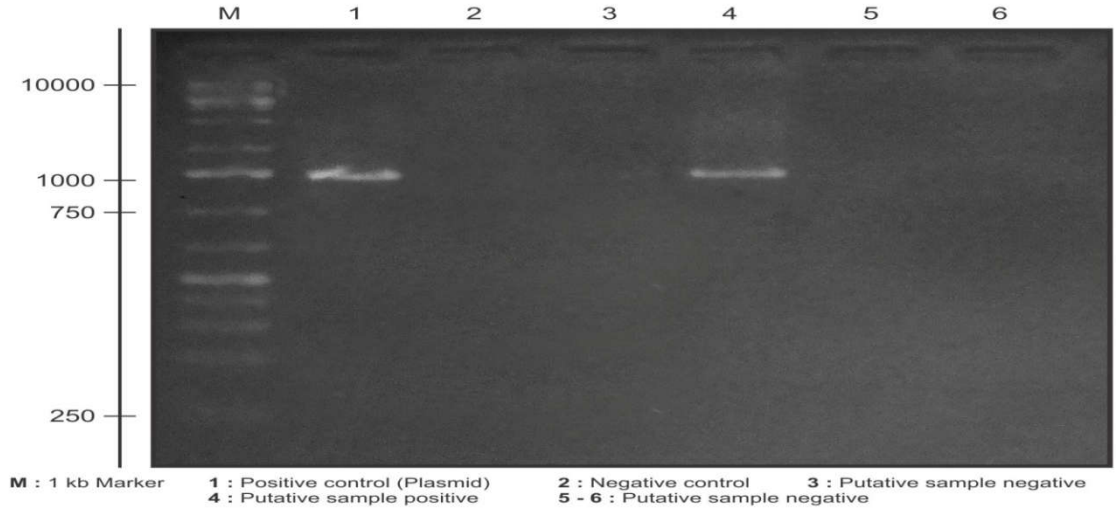

Plate 2 Screening of Putative Transformants using *Cry1Aabc* gene specific primers

Binary plasmid vector pBI-121-*GUS* mobilized into *Agrobacterium tumefaciens* strains EHA 105

In-planta inoculation of embryo axes of germinating seeds (48 hrs.) with vector pBI-121-*GUS* mobilized into *Agrobacterium tumefaciens* strains EHA 105 was carried out with addition of tobacco leaf extract or acetosyringone and the transient *GUS* expression was used as the base for identifying the most appropriate conditions for transformation. The pre-culturing, piercing and infection conditions tried resulted in 19.44 % of positive *GUS* plants when tobacco leaf extract was used along with *Agrobacterium* culture Table 2, Whereas 10.55 % of positive *GUS* plants were recorded when acetosyringone was used along with *Agrobacterium* culture. Table 3

Table 2. *GUS* histochemical assay of *in-planta* inoculated plants of pigeonpea with *Agrobacterium* strain EHA-105 harboring pBI-121-*GUS* with Tobacco leaf extract

Treatment duration	No. of infected seeds	No. of embryo axes showing expression of *GUS*	% of embryo axes showing Transient *GUS* expression
30 min.	**60**	**11**	**18.33**
60 min.	**60**	**16**	**26.67**
90 min.	**60**	**8**	**13.33**
Total	**180**	**35**	**19.44**

Table 3. *GUS* histochemical assay of *in-planta* inoculated plants of pigeonpea with *Agrobacterium* strain *EHA*-105 harboring pBI-121-*GUS* with Acetosyringone

Treatment duration	No. of infected seeds	No. of embryo axes showing expression of *GUS*	% of embryo axes showing Transient *GUS* expression
30 min.	60	07	11.66
60 min.	60	08	13.33
90 min.	60	04	06.67
Total	180	19	10.55

Putu Supartana [26] *in-planta* transformation method for rice (*Oryza sativa L*) using *A. tumefaciens* strains M-21 and LBA 4404 harboring pIG121-Hm binary vector. Embryo of a soaked seeds were inoculated with *Agrobacterium tumefaciens* by piercing embryonic apical meristem with needle dipped in Agro-inoculum. Inoculated seeds were incubated at 22^0C for two days thereby washed with cefotaxime and allowed to grow till maturity. The pIG121-Hm binary vector contains a *GUS* gene with an intron as well as hygromycin resistance gene in its T-DNA. Therefore they were able to use a histochemical assay for *GUS* and resistance to hygromycin to investigate the transformation of plants by strain. Among 30 plants (T_1)

tested, 13 plants (43%) showed a positive reaction in *GUS* assay, while all 30 non transformed plants showed a negative reaction.

The results of experiment with pBI-121-*GUS* are very encouraging and indicative of the appropriate conditions followed for *in-planta* transformation using pBinAR-*Cry1 Aabc* mobilized into *Agrobacterium tumefaciens* strain EHA 105.

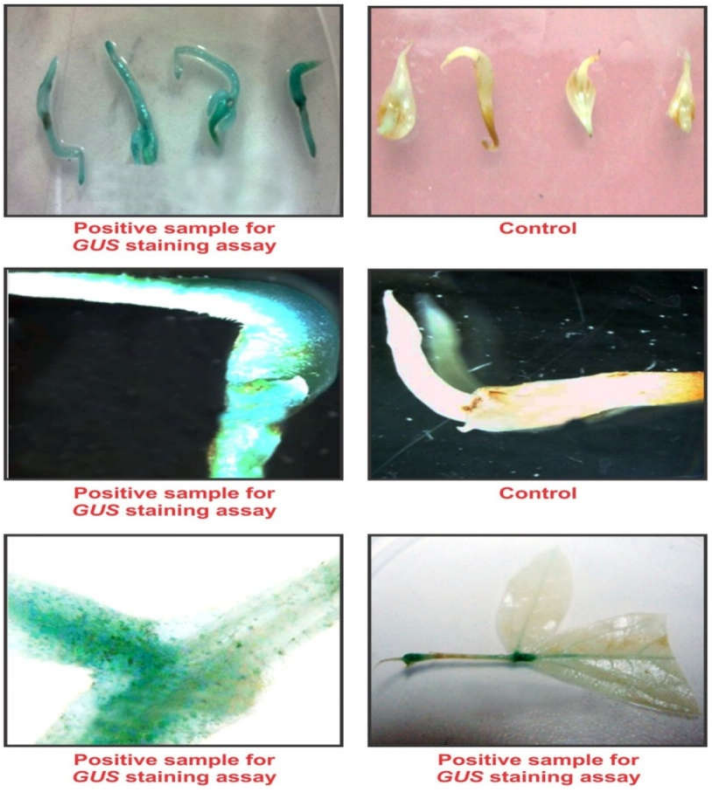

Plate 3 Positive Samples of FUS Assay Along With Control

***In-planta* transformation**

Infection to germinating seeds

The results of survivability of infected germinating seeds were significant at 5% level of probability and 1% level of probability.

The effect of different infection period with phenolic compounds viz. tobacco leaf extract and acetosyringone on the survivability are as follows embryonic axis of germinating seeds showed the varied response that the range obtained was 40.0 % to 73.3% when tobacco leaf extract was used along with the *Agrobacterium* culture and 30.0% to 60% when acetosyringone was used with the *Agrobacterium* culture. The treatment with tobacco leaf extract showed the highest survivability than acetosyringone. The treatment of 30 min. and 60 min. showed the significantly higher survivability as compare to the 90 min. of time duration and also showed good response for survivability after 45 days of germination.

Out of 180 seeds treated, 90 plants survived under transgenic house. The seeds of all the plants were collected and representative seeds samples were used to grow plants under contained facilities. Seeds were collected from each plant. The DNA sample of one representative seed from each plant was extracted and subjected for PCR confirmation with *npt II* and *Cry1 Aabc* gene specific primers. Out of 54 samples tested 2 samples were found to be positive with both *npt II* and *Cry1 Aabc* specific primers. One positive sample was found in the treatment of 60 min with tobacco leaf extract (Lane 3 of Plate 4) and other found in the treatment of 30 min with tobacco leaf extract (Lane 4 of Plate 4) and over control. Therefore the treatment of 60 min and 30 min were found to be superior as compare to 90 min. and also tobacco leaf extract was found to be superior as compare to acetosyringone.

Table 4.Effect of tobacco leaf extract and acetosyringone on survivability of co-cultivated germinating seeds with *Agrobacterium* strain EHA-105 harbouring *Cry1 Aabc* gene

Treatment	Duration of treatment	No. of seedlings Survived after infection (mean % of replicated trial)			
		15 days	30 days	45 days	75 days
Agrobacterium culture with Tobcco leaf extract	30 min.	73.3	73.3	66.7	50.0
Agrobacterium culture with Acetosyringone		60.0	60.0	56.7	40.0
Agrobacterium culture with Tobcco leaf extract	60 min	80.0	80.0	76.7	53.3
Agrobacterium culture with Acetosyringone		66.7	63.3	56.7	46.7
Agrobacterium culture with Tobacco leaf extract	90 min	60.0	56.7	53.3	40.0
Agrobacterium culture with Acetosyringone		50.0	50.0	46.7	30.0
Control		70.0	66.7	66.6	63.0
SE(m)		5.1	5.0	3.3	3.1
CD 5%		15.4	14.9	9.9	9.2

Supartana [26] developed simple and efficient *in-planta* transformation method for wheat (*Triticum aestivum L*) using *A. tumefaciens* strains M-21 and LBA 4404 harboring pIG121-Hm binary vector. Embryo of a soaked seeds were inoculated with *Agrobacterium tumefaciens* by piercing embryonic apical meristem with needle dipped in Agroinoculum. Inoculated seeds were incubated at 22°C for two days and sterilized by cefotaxime and are allowed to grow till maturity. Further T_0 are grown to T_1 and confirmed for transgene by PCR analysis and southern hybridization. Transformation efficiency of 29% and 38% was estimated by PCR detection. Keshamma *et al*. [17] produced transgenic cotton plants by tissue culture independent *Agrobacterium* mediated transformation system. *Agrobacterium* strain LBA 4404 harboring the binary vector pKIWI 105 and apical meristem of differentiated embryo of germinating seedlings were used for this experiment. First screening of transformation in T_0 generation was indicated by *GUS* histochemical assay and T_1 transformant were identified by PCR analysis and confirmed by southern hybridization. Survived 25 plants maintained in a green house and 2 plants out of 25 were PCR positive.

Infection to shoot tip

Shoot tip infection by *Agrobacterium* culture with tobacco leaf extract acetosyringone showed the varied survivability response and the range obtained was 33.3% to 63.3% when tobacco leaf extract was used with *Agrobacterium* culture and 43.3% to16.6% when acetosyringone was used with *Agrobacterium* culture. Treatment *Agrobacterium* culture with the tobacco leaf extract show the higher survivability.

Out of 60 seedlings treated, 12 plants survived under the transgenic house. The seeds of all the plants were collected and representative seeds sample were used to grow plants under contained facilities. Seeds were collected from each plant. The DNA sample of one representative seed from each plant was extracted and subjected to PCR confirmation with gene specific primers. All the tested plants found to be negative with both *npt II and Cry1 Aabc* specific primers.

Table 5.Effect tobacco leaf extract and acetosyringone on survivability of co-cultivated seedlings with *Agrobacterium* strain EHA-105 harbouring *Cry1 Aabc* gene

Treatment	No. of seedlings Survived after infection (mean % of replicated trial)			
	15 days	30 days	45 days	75 days
Agrobacterium culture with TLE	63.3	56.6	50.0	33.3
Agrobacterium culture with Acetosyringone	43.3	43.3	33.3	16.6
Control	76.6	76.6	73.3	70.0

On the other hand in chickpea, in *rafp2* gene transfer through *Agrobacterium* infection with 0.01% of Triton-X 100 for different incubation period of shoot tips, only one transformant was found in T_1 generation, out of 100 shoot tips, treated at 10 min incubation period in T_0 generation. However, infection to shoot tips resulted in no transformation events in *cry1Ac* gene transformation [22].

Floral dip method

Out of 60 floral buds infected, only three flowers set the pod. The harvested seeds were sown under transgenic house and DNA was extracted from the plants. The DNA sample from 3 plants was extracted and subjected to PCR confirmation with gene specific primers. Out of 3 sample tested one sample was found to be positive with both *nptII* and *Cry1 Aabc* specific primers when the floral bud infected with *Agrobacterium* culture added with tobacco leaf extract (Lane 5 of Plate 4). Tobacco leaf extract was also found to be superior as compare with acetosyringone.

Table 6.Effect of tobacco leaf extract and acetosyringone on pod formation from the co-cultivated floral buds with *Agrobacterium* strain EHA-105 harbouring *Cry1 Aabc* gene

Treatment	Total no. of floral bud infected	No. of Pod formation
Agrobacterium culture with TLE	30	2
Agrobacterium culture *with* Acetosyringone	30	1
Control	30	30

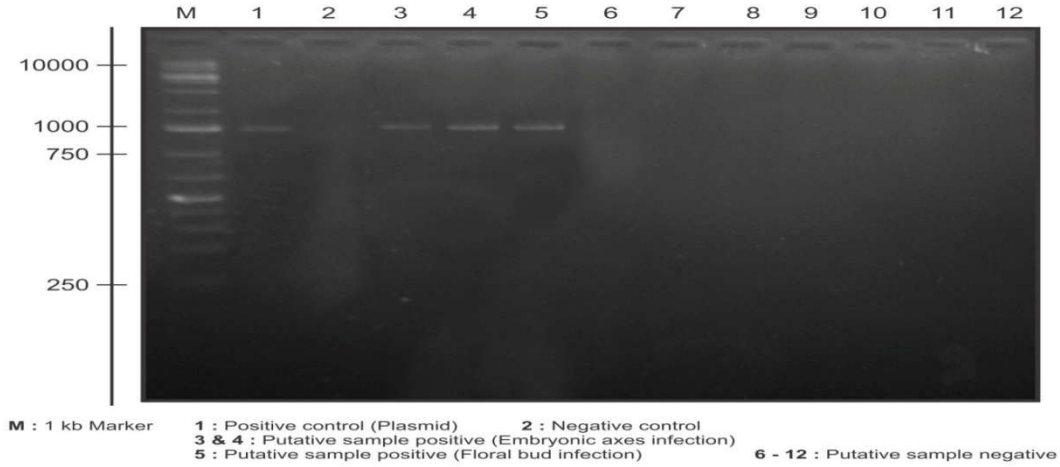

Plate 4 Screening of Putative Transformants using *Cry1Aabc* gene specific primers

Ye *et al*. [28] also examined *Agrobacterium* mediated floral dip transformation of *Arabidopsis.* In a smaller crossing study that did not monitor timing of floral development, they observed 15 transformants when *Agrobacterium* was applied to the pollen recipient flower and zero transformants when *Agrobacterium* was applied to the pollen donor.

From the three different strategies of *in-planta* transformation in the present investigation of transformation study embryonic axis infection method found to be superior among the floral dip method and shoot tip infection.

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