Agrobacterium-mediated Genetic Transformation in Cucumber (*Cucumis sativus* L.)

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Abstract: The present study was undertaken to develop efficient transformation protocol for cucumber cv. Poinsett 76 using *Agrobacterium* strain EHA 105. Five-day-old mature cotyledon explants was used for transformation study. The infected explants were co-cultivated for 2 days in MS medium containing BA (1.0 mg L^{-1}). The selection of transformed shoots was carried out in MS medium fortified with BA (1.0 mg L^{-1}), Cefotaxime (300 mg L^{-1}) and PPT (2.0 mg L^{-1}). The transformed shoots were elongated in MS medium containing BA (1.0 mg L^{-1}), Cefotaxime (300 mg L^{-1}), PPT (2.0 mg L^{-1}) along with GA$_3$ (0.5 mg L^{-1}). The rooting of elongated shoots was achieved in MS medium with BA (1.0 mg L^{-1}), Cefotaxime (300 mg L^{-1}), PPT (2.0 mg L^{-1}) and IBA (0.6mg L^{-1}). The transient GUS expression assay and leaf disc assay were carried out in order to find transformed shoots. The molecular confirmation of transformed shoots revealed the foreign gene integration into cucumber genome.

Key words: cucumber, *Agrobacterium*, cotyledon, GUS expression

INTRODUCTION

Genetic engineering has many potential applications in fields such as medicine, agriculture and industries. In agriculture, the new application of genetic engineering includes the development of transgenic plants. The transgenic plants carry desirable traits like disease resistance, insect resistance and herbicide resistance. Eventually transgenic technology may be used for increasing photosynthetic efficiency, nitrogen-fixing ability and production of hybrid crops for food processing and molecular farming[1-4].

In order to establish a successful programme of practical plant genetic engineering, it is important to develop systems for the recovery of whole plants in large members from primary explants. This needs to be done while optimizing the procedure for introducing foreign genes into the species of interest. Tissue culture techniques can be extended to regenerate cucumber plants in large number at a reasonably shorter period. The unique ability of isolated plant cells to regenerate into whole plant[5] (totipotency) means that there is a wide range of potential target cell types for transformation. However, there is no universally applicable method of culture, regeneration and transformation systems for all species, as tissues from different genotypes will differ in their response to culture. A procedure to produce shoots through regeneration from one cultivar may be very different from that of another cultivar within the same species[6]. Therefore culture and regeneration protocols must be modified appropriately for cultures of each species[7].

Cucumber has a narrow genetic base and several crossing barriers hamper the introduction of desired traits from the related species[8]. The development of gene transfer technology for cucumber can be used to transfer engineered genes for improving its genotype. Among the cucumber cultivars, Poinsett 76 have been widely cultivated in India. The development of reproducible tissue culture and genetic transformation protocols in this cultivar and their applications would improve the cucumber cultivation and benefit cucumber industry in the years to come.

Among the available gene transfer systems, *Agrobacterium*-mediated gene transfer is considered as more efficient for the stable integration of genes into plant genome. So far, a few reports are available on gene transfer studies of cucumber[9-16]. Based on these reports, the present study was focused on *Agrobacterium*-mediated transformation via direct regeneration using cotyledon explants in an important cultivar, Poinsett 76 with an important *Agrobacterium tumefaciens* strain EHA 105[17].

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MATERIALS AND METHODS

Plant material: Seeds of cucumber cultivar Poinsett 76, (Indo-American Hybrid Seeds (India) Pvt Ltd., Bangalore, India) was procured and used for transformation experiments. The seeds were sterilized by washing in Teepol (commercial bleach solution, 0.6% sodium hypochlorite, Reckitt Benckiser (India) Ltd., Kolkatta, India) for 15 min., rinsed with distilled water three times, followed by soaking in it for 8hr. The soaked seeds were treated with 70% ethyl alcohol for 30sec, then rinsed with sterile water for three times and surface sterilized in 0.1% (w/v) mercuric chloride solution for 3 min. Then the seeds were rinsed with sterile water three times to remove the surface sterilant.

Pre-culture of explants: The sterilized seeds were kept in sterile moist cotton for 24hr. Then their seed coats were separated and aseptically removed without disturbing the cotyledons. The cotyledons were carefully dissected from the embryonic axis. The distal end of the cotyledon explants (0.5 cm in length) was cut or injured slightly in such a way that the distal end touched the medium. MS medium[18] containing BA (6-benzylamino purine) (1.0 mg L^{-1}) and L-Glutamine (20 mg L^{-1}) was used for shoot regeneration. The cultures were kept at 25 ± 2 ºC with a 16h photoperiod with the light intensity of 30 µmol m^{-2} s^{-1} under cool white fluorescent lamps.

Sensitivity of cotyledon explants to PPT: The sensitivity test of PPT was carried out in order to find the inhibitory concentration which arrests cotyledon growth. The sensitivity of cotyledon explants to PPT was determined by culturing the explants in shoot induction medium [MS + BA (1.0 mg L^{-1}) + L-Glutamine (20 mg L^{-1})] along with PPT (Hoechst, Germany) from 0.5 to 2.0 mg L^{-1}. The minimum inhibitory concentration (MIC) of the selection marker was used throughout the selection procedure of transformed shoots from explants. A positive control without selection agent was also maintained.

Agrobacterium strain and plasmid vector: Transformation was performed using *Agrobacterium tumefaciens* strain EHA 105. This strain harbored the plasmid pGA492GL (kindly provided by Rafael Perl Treves, Bar Ilan University, Israel), carrying *npt* II gene regulated by *nos* promoter and the *bar* and *gus* (uid A) genes regulated by CaMV 35S promoter (Fig. 1). The *gus* has an intron in the N-terminal region of the coding
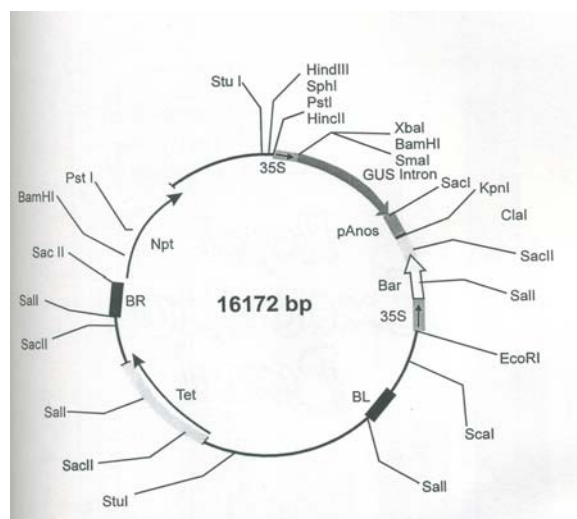

Fig. 1: pME524 showing map of plasmid pGA492GL-Bar (+) carrying GUS, *npt* II, *bar* and location of restriction sites

sequence. The *bar* gene confers resistance to phosphinothricin.

Agrobacterium culture: A single colony of *Agrobacterium tumefaciens* strain, EHA 105 suspended in 5ml of Luria-Bertaini (LB) medium containing 50 mg L^{-1} kanamycin (Sigma, USA) and 10 mg L^{-1} tetracycline (Sigma, USA), was incubated at 28 ºC on a shaker (Orbitek, India) at 200 rpm for 24 hr. The suspension of the *Agrobacterium* strains was diluted with a liquid half strength medium to obtain 1.0 OD (600 nm) concentration (5×10^8 cells L^{-1}). One hour before the co-cultivation of explants, acetosyringone (10-50 µ*M*) was added.

Agrobacterium infection and co-cultivation: The proximal end of the cotyledon was gently pricked for ten times to make wounds using sterile needle (Dispovan India Ltd., 0.63 X 25 mm). Then the cotyledon explants were immersed in the bacterial culture for 10 min. After that the explants were removed, blotted dry using sterile Whatman no.1 filter paper and inoculated (one explant/culture tube) on MS medium containing BA (1.0 mg L^{-1}) and ABA (0.5 mg L^{-1}). The co-cultivation was performed for 0, 1, 2, 3, 4 and 5 days under a 16h photoperiod with a light intensity of 30 µmol m^{-2} s^{-1} and kept at 25 ± 2 ºC.

Selection of transformants: After co-cultivation, the explants were washed three times with sterile distilled water containing filter sterilized cefotaxime (300 mg L^{-1}) (Hoechst Marion Roussel Ltd., Mumbai,

India), blotted dry and were subjected to selection. The cultures were maintained under 16h photoperiod (30 μmol m^{-2} s^{-1}) at 25 ± 2 ℃. Then the explants were transferred to MS medium supplemented with BA (1.0 mg L^{-1}), cefotaxime (300 mg L^{-1}) along with PPT (2.0 mg L^{-1}) for shoot bud induction. After 2-3 weeks, the explants with emerging shoots were transferred to MS medium containing BA (1.0 mg L^{-1}), cefotaxime (300 mg L^{-1}) and PPT (2.0 mg L^{-1}) for shoot proliferation. Two subcultures were done on the same medium at 25-d interval. The medium of the same composition was changed once in 10 days. The regenerated shoots were excised from the explants and transferred to MS medium supplemented with BA (1.0 mg L^{-1}), GA$_3$ (0.5 mg L^{-1}), cefotaxime (300 mg L^{-1}) and PPT (2.0 mg L^{-1}) for shoot elongation. The elongated shoots were then transferred to MS medium fortified with IBA (0.6 mg L^{-1}), cefotaxime (300 mg L^{-1}) and PPT (2.0 mg L^{-1}) for rooting. The rooted plants were transferred to pots containing sterilized sand, soil and vermiculite (2:1:1 v/v/v) mixture and were acclimatized in green-house for 30 days.

Leaf disc assay: Four week old putative transformed shoots derived from cotyledon explants were aseptically divided into small pieces using sterile blade. The leaves were transferred to petriplates containing MS medium with BA (1.0 mg L^{-1}) and PPT (2.0 mg L^{-1}) in order to find out the lethal effect of transformed and non-transformed shoots. The petriplates containing leaves from transformed and non-transformed shoots were incubated for 1 week under a 16h photoperiod with a light intensity of 30 μmol m^{-2} s^{-1} and kept at 25 ± 2 ℃.

GUS assay: Explants were assayed for the expression of *gus* A int gene following the histochemical procedure[19]. Cotyledon explants 8hr after co-cultivation and 3-week old young leaves from transformants were washed in distilled water three times and followed by incubation for 10 min in phosphate buffer (0.5 mM NaH$_2$PO$_4$ and 0.5 mM Na$_2$HPO$_4$), pH 7.0 containing 0.5 mM potassium ferri and ferro cyanide and 10 mM Na$_2$EDTA. The buffer was removed and fresh phosphate buffer containing 1% (v/v) Triton X-100 was added to the leaf tissues and incubated for 1h at 37 ℃ after draining the solution, again fresh phosphate buffer containing 1.0 mM X-gluc (5-bromo-4-chloro-3-indolyl β-D glucuronide) and 20% of 95% methanol was added. The reaction was placed under a mild vacuum for 5 minutes and incubated overnight at 37 ℃ and then the tissues were examined visually. Following the incubation the chlorophyll was removed and fixed in 95% (v/v) ethanol:1% (v/v) glacial acetic acid.

Statistical analysis: Each treatment consisted of a total of 110 explants and each experiment was repeated for three times. A completely randomized design was used in all experiments and analysis of variance and mean separations were carried out using Duncan's Multiple Range Test (DMRT). Significance was determined at $p < 0.05$ level[20].

The percentage of GUS expression was calculated by the number of explants showing GUS positive divided by the number of explants subjected to co-cultivation and multiplied by 100[21].

Molecular confirmation of transformants

* Isolation of Genomic DNA: Genomic DNA was isolated from young leaves of control and transformed plants using previous method[22].
* PCR confirmation: For PCR analysis, DNA samples from putative transformants were amplified by *bar* specific primers. The *bar* gene fragment (0.46 kb) was amplified by using the forward primer - 5'-ATC GTC AAC TAC ATC GAG AC – 3' and reverse primer 5'-CCA GCT GCC AGA AAC CCA CGT C-3.'

All PCR reactions were performed using a Peltier effect thermal cycler (MJ Research Co., USA). Samples containing 50 ng genomic DNA were first heated at 94 ℃ for 5 min followed by 30 cycles at 94 ℃ for 30s, 55 ℃ and 72 ℃ for 30 s followed by 7 min final extension at 72 ℃. Fifty ng of plasmid DNA was used as positive control. The PCR reactions contained 10 pM of each primer, 10 mM dNTPs mix, 15 mM MgCl$_2$, 50 mM KCl, 10 mM Tris HCl (pH 9.0), 0.1% (v/v) Triton X-100, 2 U of Taq DNA polymerase and 50 ng of template DNA in 2X reaction buffer. The amplified DNA were analysed by using 1.5% agarose gel electrophoresis.

* Southern hybridization: The blotting procedures were followed by standardized method[23]. The *bar* gene probe was labeled by non radioactive labelling kit (ECL random labelling and detection system) (Amersham Biosciences, UK). The blot was prehybridized at 60 ℃ for one hour in hybridization buffer (5X SSC, blocking agent (0.5 % (w/v), SDS (0.1%) and dextran sulphate (5%). The denatured labeled probe was added to the hybridization oven (Amersham Biosciences, UK). Post hybridization washes were performed in high stringency conditions. The blot was washed in an excess of 1X SSC, 0.1% for 15 min, then in SSC (0.5%) and SSC (0.1% with SDS (0.1%).

After hybridization, the blot was rinsed briefly in antibody wash buffer (Tris-HCl 100 mM, NaCl 150 mM and pH 7.0). The blot was blocked with blocking agent (0.5%) (supplied by the manufacturer of ECL kit) at room temperature for one hour with continuous agitation. After a brief rinse in antibody wash buffer, the blot was incubated with antibody diluted 1000 fold in BSA (0.5%) (fraction V) (w/v) in antibody wash buffer and incubated for one hour at room temperature with continuous agitation. Membranes were washed with antibody wash buffer with Tween 20 (0.1%) (v/v) for 2 10 min followed by washing in 2 X 5 min at room temperature with continuous agitation.

After removal of excess antibody, the blot was wetted with the detection solutions 1 & 2 (Supplied by ECL manufactures). Excess of detection solution was drained from the membrane and the blot was covered by saran wrap. The covered blot was immediately placed in X-ray cassettes with X-ray film. After one hour the X-ray film was developed and the signal was detected in X-ray film.

RESULTS AND DISCUSSION

Plant material: Cotyledon explants (proximal half; 0.5 cm in length), derived from mature seeds of cucumber cultivar Poinsett 76 was used as target tissue for *Agrobacterium*-mediated gene transfer through direct regeneration. Mature cotyledon explants were used in the transformation study. *In vitro* regeneration of plants from cotyledons of mature seeds and young seedlings has received considerable attention in the recent years and this is most likely because of the easy accessibility, quick response and high ability for shoot organogenesis[24]. The use of mature cotyledon explant in the present study was justified with earlier transformation experiments in melon[25] and in cucumber cultivars[15,16,26].

Pre-culture of explants: In the present study, a 5-day-old pre-cultivation period was found suitable for cotyledon explants of Poinsett 76 in shoot bud induction medium [MS + BA (1.0 mg L^{-1}) + L-Glutamine (20 mg L^{-1})] to induce shoot bud at the proximal region and more GUS expression (Fig. 2). If explants were co-cultivated immediately without pre-culture, they were not able to withstand the infection and eventually died (data not shown). Similarly, more escapes were observed from cotyledon explants pre-cultured beyond five days (data not shown). Pre-culture of explants is a critical factor to achieve high frequency of transformation. It makes the explant tissue competent
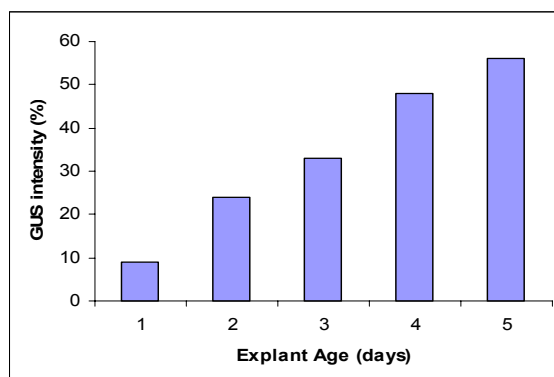

Fig. 2: Effect of age of the cotyledon explants of GUS expression in cucumber cultivars co-cultivated with EHA 105

enough to withstand the bacterial infection and the related stress that followed it. Pre-culturing of explants prior to inoculation and co-cultivation with *Agrobacterium* has been shown to improve genetic transformation frequencies in some woody fruit plants such as plum[27] and almond[28]. In this study, 5-day-old pre-cultured cotyledons were used for transformation. Five-day-old cotyledon explants did produce higher number of transformed shoots than the explants of other age. Beyond 5th day, cotyledon explants produced shoots but most of them were escapes due to pre-emergence of shoot primordia from the explants before agro infection. Our study was in conformity with earlier reports on cucumber[15,26]. For cucumber transformation studies, three to seven-day-old *in vitro* grown seedlings were used as donors of cotyledon explants[9,10,15,16,27,29]. On the other hand, 1-day-old cultured cotyledon explants of cucumber used for transformation procedure[14].

Sensitivity of cotyledon explants to PPT: In order to find out the appropriate concentration of selection agent, to effectively screen transformed shoots in the cultivar Poinsett 76, we employed different concentrations of PPT during shoot bud production. Control shoots developed normally in the selective agent free medium. Maximum number of shoot buds was obtained at 0.5 mg L^{-1} PPT. At 1.0 mg L^{-1} PPT fifty percentage of explants showed necrosis. Further increase in the level of PPT led to a corresponding decrease in the shoot bud production. PPT at 2.0 mg L^{-1} caused almost total inhibition of bud production and regeneration from cotyledon explants (Fig. 3A). Therefore, this concentration was used for the selection of shoots with minimal escapes. In the earlier studies, PPT selection of transformed shoots of cucumber has been adopted with successful results[15].

Table 1: Transformation efficiency of *Agrobacterium* strain EHA 105 with 20 µ*M* acetosyringone for cv. Poinsett 76 in PPT (2.0 mg l⁻¹)

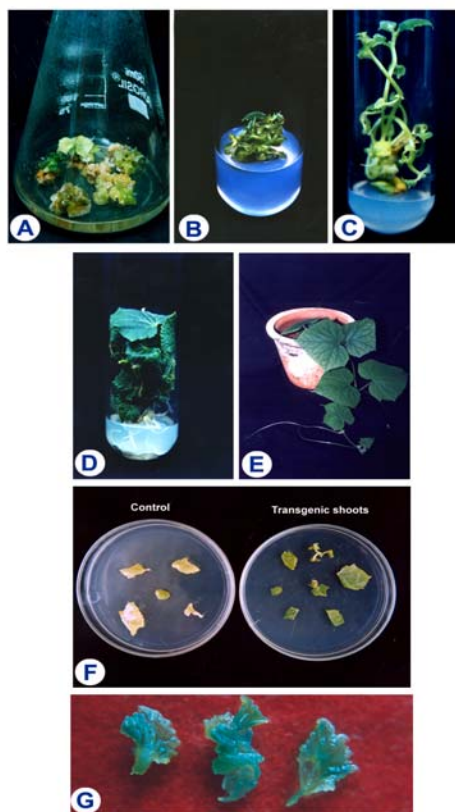| Agrobacterium strain	Exp. No.	No of cotyledon explants infected	No of GUS positive shoots (without acetosyringone)	No of GUS positive shoots (with acetosyringone)	Efficiency of transformation (%)	
					Without acetosyringone	With acetosyringone
EHA105	1	110	1	8	0.9	7.2
	2	110	1	7	0.9	6.0
	3	110	3	3	2.7	6.4
	4	110	2	5	1.8	6.8
	5	110	1	15	0.9	6.8
	Total	550	8	36	1.5	6.6

Fig. 3: Transformation of *Cucumis sativus* L. cotyledon explants by *Agrobacterium tumefaciens* strain EHA 105
A. Sensitivity of cotyledon explants [MS + BA (1.0 mg L⁻¹) _ L-Glutamine (20 mg L⁻¹) + PPT (2.0 mg L⁻¹)]
B. Proliferation of transformed shoots [MS + BA (1.0 mg L⁻¹) + Cefotaxime 9300 mg L⁻¹) + PPT (2.0 mg L⁻¹)]
C. Elongation of transformed shoots [MS + BA (1.0 mg L⁻¹) + GA3 (0.5 mg L⁻¹) + Cefotaxime (300 mg L⁻¹) + PPT (2.0 mg L⁻¹)]
D. Rooting of elongated transformed shoots [MS + BA (1.0 mg L⁻¹) + IBA (0.6 mg L⁻¹) + Cefotaxime (300 mg L⁻¹) + PPT (2.0 mg L⁻¹)]
E. Hardening of transformed plant in pot (Sand:Soil:vermiculite 1:1:1 v/v/v)
F. Leaf disc assay of transformed shoots [(MS + BA (1.0 mg L⁻¹) + PPT (2.0 mg L⁻¹)]
G. GUS expression in 2-week-old transformed shoots

***Agrobacterium* strain and plasmid vector:** *Agrobacterium* strain EHA 105 containing the plasmid pGA492GL-*Bar* (+) was used in this study. The total number of explants infected was 550 for each strain and the experiment was repeated 3 times. EHA 105 showed greater infectivity in transformation. The transformation frequency was 6.6% for EHA 105 in Poinsett 76 (Table 1). A large number of *Agrobacterium tumefaciens* strains were isolated and several of them have been modified for use in transformation studies. Virulence of *Agrobacterium tumefaciens* strains varies widely among plant hosts[30-32]. In the present study, EHA 105 strain was employed for transformation into cucumber. This strain have already been deployed in earlier study in cucumber[16]. This is in agreement with previous study[16] where the transformation efficiency was 6.4 in EHA 105. *Agrobacterium* strain EHA 105 was more effective because its derived from super virulent wild type strain A281[18].

***Agrobacterium* infection and co-cultivation:** Transformation experiments were performed to optimize the effective *Agrobacterium* density, acetosyringone concentration and co-cultivation period. In the present study, an inoculum density of 1.0 OD (600 nm) was optimal for co-cultivation of cotyledon explants (data not shown). Co-cultivation period was assessed for each day from 0 day to 5th day. A 2-day co-cultivation was found optimal for Poinsett 76 in MS medium containing BA (1.0 mg L⁻¹) and ABA (0.5 mg L⁻¹) which led to the production of significantly higher rate of GUS expression as compared to other days of co-cultivation (Table 2). In this cultivar the optical density greater than 1.0 OD and co-cultivation beyond 3 days led to bacterial over growth and leaching of bacteria from explants (data not shown).

Among the different concentrations of acetosyringone tested, 20 µ*M* was optimum for Poinsett 76, beyond which shoot recovery was not possible due to bacterial overgrowth from explants (Table 1). The transformation efficiency in the cultivar Poinsett 76 was

Table 2: Effect of co-cultivation period on GUS expression in cotyledon explants derived from 1-5 – day - old seedlings of cucumber cultivars co-cultivated with EHA 105

Cultivar	Percentage of GUS expression Days of co-cultivation				
	1	2	3	4	5
Poinsett 76	20c, 16bc	31c, 27bc	45c, 39bc	64bc, 57bc	78bc, 61c

Each value represents the treatment means of 110 explants and repeated three times.
Values with the same letter within columns are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

6.6% at 20 μM acetosyringone The inoculum density at 1.0 OD was used in the present experiment. Our results were in conformity with previous studies[9,10,16,26] in cucumber that 1.0 OD was essential for effective transformation. Different co-cultivation period was found optimal for the effective infection and regeneration of transgenic shoots[10,12,14,26]. The co-cultivation period beyond 4 days the calluses with free of *Agrobacterium* contamination were more difficult to obtain for cucumber[13]. In the present study, a 2-day-old co-cultivation for Poinsett 76 was found suitable for effective transformation. The use of acetosyringone (20-50 μM) enhanced the infection frequency during co-cultivation. *Agrobacterium* is presumably attracted to a wounded plant in response to signal molecules released by the plant cells to which it then becomes attracted[33,34]. The results of the present study are in agreement with previous observations[12,13,27,35].

Selection of transformants: Inclusion of selection agent in the regeneration and rooting media was responsible for the production of putative transformed shoots that stably expressed transgenes. In the present study, PPT was employed for selection of transformants. The explants inoculated in MS medium containing selection agent PPT (2.0 mg L^{-1}) produced transformed shoots with a transformation frequency of 6.3% for cv. Poinsett 76 (Fig. 3B). Among the 110 explants infected with *Agrobacterium* strain EHA 105, 7 explants produced transgenic shoots in Poinsett 76. Other explants did not respond to shoot regeneration and ultimately died. After 3 weeks of culture the selected shoots were elongated, rooted and hardened (Figs. 2.3C, D & E). *Bar* gene in conjunction with phosphinothricin has been shown to be an effective selectable marker in obtaining transgenic plants in cucumber[15], *Arabidopsis*[36], Carrot[37], Lotus[38], Medicago[39], Pea[40], Soybean[41] and Sugarcane[42].

Leaf disc assay: The leaf disc (5 cm long) taken from 2-month-old putative transformed shoots in the medium

containing BA (1.0 mg L^{-1}) and PPT (2.0 mg L^{-1}) survived without any necrotic symptoms, while non-transformed leaf discs showed necrotic symptoms (Fig. 2.3F). The percentage of survival of transgenic shoots was 90%, while non-transformed shoots showed 100% necrosis within a week in the same medium (data not shown). Chimeras were not observed in the transgenic shoots. Leaf disc assay is one of the preliminary tests to identify and compare the transgenic shoots from non-transgenic shoots. Because of high toxicity of PPT, the non-transformed shoots (without *bar* gene in its genome) were not able to withstand. Phosphinothricin is an analogue of glutamic acid and two L-alanine residues. It is a powerful inhibitor of glutamine synthetase[43]. It is believed that inhibition of glutamine synthetase leads to ammonia accumulation resulting in symptoms of chlorophyll bleaching in non-transformed shoots while transformed shoots remained alive without necrotic symptoms or bleaching. The advantage of using PPT for the screening procedure is that it has a localized effect, i.e only those plant cells that uses the active *bar* gene have the ability to detoxify the herbicide. Leaf disc assay and colour viability test for the plants transformed with *Agrobacterium* strain EHA 105 under the selection of PPT revealed more positive results compared to the wild type control plants[44]. In tobacco primary mode of action for glyphosate and PPT herbicides on non-transformed shoots leads to lethal effect[45]. Transgenic lines arising after the herbicide selection phase were subjected to leaf disc assay showed non lethal effect. Hence in the present treatment, leaves of non-infected explants showed chlorophyll bleaching whereas those of transformed plants remained alive without chlorophyll bleaching.

GUS assay: The present study was investigated for the GUS expression in cucumber cultivar Poinsett 76. The strong GUS expression was observed in 3-week-old young leaves (Fig. 3G). Our result was collaborative with previous study[16].

Molecular confirmation of transformants
* PCR confirmation: Molecular analysis through PCR amplification confirmed the presence of *bar* gene in the putative transformants, co-cultivated either with EHA 105 strain. The DNA isolated from putative transformed shoots, control plants and plasmid pGA492GL were used as template DNA. The suitable primers for *bar* gene was used for PCR amplification. The presence of amplified band at 0.46 kb in transformed shoots confirmed the presence of *bar* gene (Fig. 4; lanes 3-8).
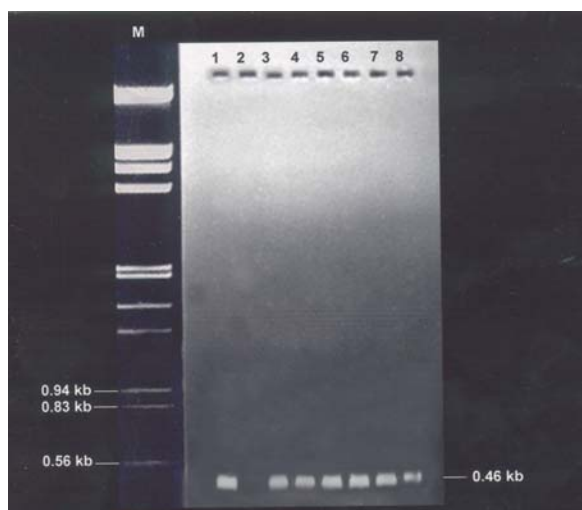
Fig. 4: PCR confirmation of transformed shoots. Arrows indicates the amplification of bar gene (0.46 kb)
M- Lambda Hind III Marker (1.0 kb)
Lane 1: Plasmid DNA (pGA492GL) (positive control)
Lane 2: DNA samples from non-transformed shoots (negative control)
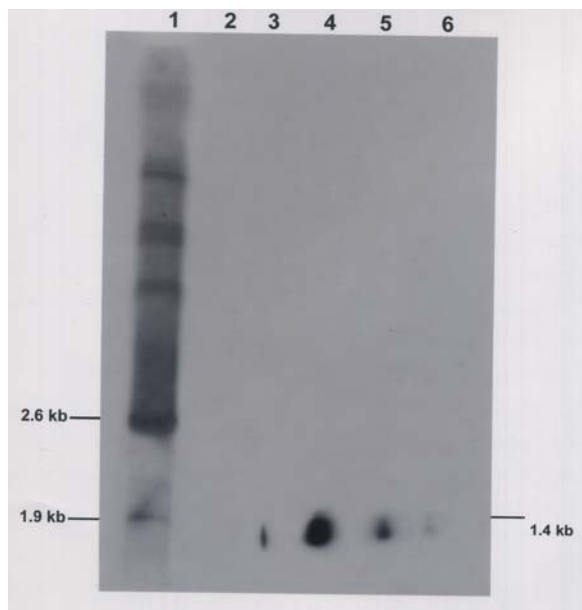Lane 3 – 8: DNA samples from transformed shoots

Fig.5: Southern Hybridization. Arrow indicates the presence of bar gene (1.4 kb)
Lane 1: Plasmid DNA (pGA492GL) digested with *Bam HI* and *Hind III*
Lane 2: DNA from non-transformed shoots
Lane 3-6: DNA samples from transformed shoots

The amplification was not seen in non-transformed control shoots (Fig. 4; lane 2). The amplification of the same (0.46 kb) was observed from the lanes loaded with plasmid DNA pGA492GL (Fig. 4; lane 1).

* Southern hybridization: Southern hybridization was carried out on genomic DNA from GUS positive, shoots developed on cotyledon explants. The *bar* gene was detected as a *Bam* HI and *Hind* III digested *bar* fragment of expected size (1.4 kb) in the shoots transformed with EHA 105. As the *bar* fragment probe hybridized to digested DNA from transgenic shoots (Fig. 5; lanes 3-6) but not hybridized to non-transformed control leaves (Fig. 5; lane 2). Fig. 5; lane 1 indicated plasmid DNA (pGA492GL) carried *bar* fragment which cleaved under digestion with restriction enzymes (*Bam* HI and *Hind* III).

CONCLUSION

The success in production of stably transformed shoots from cotyledon explants reported here appears to be due to at least three key factors; first was the choice of the explant (cotyledon), second was the use of efficient reporter gene, the GUS and the third was the use of PPT as the selective agent. This method offers several advantages: i) the cotyledons are available throughout the year from mature dry seeds; the protocol is rapid and ii) as the regeneration occurs through direct organogenesis, genetic variation/abnormalities often associated with regeneration of plants from callus are expectedly avoided.

In conclusion, a reliable transformation protocol was standardized using *Agrobacterium* strain EHA 105 with reference to cucumber cultivar Poinsett 76. This protocol may be adopted for transferring any character genes of agronomic interest.

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