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Direct transformation through Particle gun using N_2 gas in *Capsicum annuum* L. cv. Mathania

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Abstract

A protocol for direct transformation through biolistic gun has been established for chilli (*Capsicum annuum* L. cv. Mathania). A disarmed strain of *Agrobacterium tumefaciens* EHA 105 carrying a binary vector plasmid p35SGUSINT has been used for transformation. This vector contains neomycin phosphotransferase gene (*npt* II), whose expression confirms Kanamycin resistance in transformants. In addition to *npt* II, plasmid encodes b -glucuronidase, reporter enzyme used for studying the expression of foreign genes in plants. We report for the first time, the use of Nitrogen gas for the biolistic transformation of chilli. We found physical parameter like 6 cm target distance with 900 psi rupture disk for partical gun experiment the most efficient for transient GUS expression. In case of nitrogen gas the frequency of transient GUS expression was better both in leaves (67.74%) and hypocotyl (69.18%) and frequency of conversion of transient to stable transformation was 3.4% in leaves and 4.0% in hypotocyls as against 63.91 % transient GUS expression showed by leaves and 63.77% showed by hypocotyls when helium gas was used in biolistic transformation and stable transformation frequency was 3.0% in leaves and 4.0% in hypocotyls. The transgenic nature of the regenerated plants was confirmed by the histochemical staining of GUS, and polymerase chain reaction (PCR) analysis of *npt* II gene.

Key words : *npt II* , Biolistic gun, Nitrogen gas, p35SGUSINT, BAP

Inroduction

Chilli (*Capsicum annuum*) is an important crop grown worldwide for its use as spices and vegetables [1]. However, chilli is highly susceptible to fungal and viral infection which causes considerable damage to the crop [2]. Although it is a Solanaceous member, the culture development in plant cell, tissue and organ culture as well as on plant genetic transformation, have lagged far behind compared to those achieved for other members of the same family, such as tobacco, tomato and potato. These latter species have been frequently used as model systems because of their ability to regenerate *in vitro,* and are able to be genetically engineered by currently available transformation methods. In contrast, members of the *Capsicum* family have been reported to be recalcitrant to differentiation and plant regeneration under *in vitro* conditions.

The mathania type chilli evolved in the mathania region of jodhpur district of Rajasthan (India)through decade of selection and cultivation is most preferred as vegetative ingredient.It is a long and wrinkled chilli with mild pungency but bright red color on maturity

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imparting desired color,flavor and taste. However, continued selection for uniformity and cultivation has rendered the crop vulnerable to various diseases and climate hazards. High incidence of pest and diseases, particularly,the leaf curl virus (LCV) and dieback disease, has resulted in high pest management costs accompanied with yield losses. This has led to gradual reduction in the cropping area of the admired land race; Mathania is now on the verge of extinction. Efforts to combat the dreaded diseases of chilli and to conserve the famous Mathania chilli cultivar have had limited impact.

The non-availability of resistance source in the germplasm necessitates development of suitable genotype using interventions of biotechnological tools. Thus genetic improvement efforts against pests and disease through recombinant DNA technology via genetic transformation is difficult [3,4] . The demand for chilli is expanding; therefore, there is widespread interest in incorporating novel genetic traits, which can often be introduced only via genetic transformation technology, in order to improve the quality and also quantity of the plants. Particle bombardment [6] is very effective for the delivery of DNA into intact cells and tissues. This method is especially beneficial for those plants which cannot be transformed by *Agrobacterium*-based gene transfer [5].

The biolistic process has found application in the transformation of diverse organisms including animal cells and tissues [7,8] monocot [9,10 and 11] and dicot [12,13 and 14] plants, yeasts, and other fungi [15], algae [16], and bacteria.

Present investigation has therefore been taken to develop an biolistic gun transformation protocol to enable development of agronomically superior transformants using various strategies to save the variety from extinction.

Materials and Methods

Bacterial strain and Plasmid used for transformation

Agrobacterium tumefaciens strain EHA 105 with plasmid p35SGUSINT [17] was used in present study. The plasmid harbored the *GUS* reporter gene driven by the CaMV35S promoter and *npt* II gene for Kanamycin resistance as selectable marker. The *Agrobacterium* strain was procured from National Research Centre on Plant Biotechnology, Indian Agriculture Research Institute, New Delhi.

Sterilization of culture media

For sterilization, the culture medium was poured in flasks and pluged with cotton and wrapped with aluminium foil. Autoclaving was done at 15-16 psi (1.06 kg/cm^2) for 18 minutes. After autoclaving media was poured in pre-autoclaved 100 ml flasks in laminar air flow chamber and stored at 25 ± 2^{0}C.

Establishment of aseptic chilli cultures

To obtain aseptic seedling, seeds were surface sterilized for 4 min with 0.1% (w/v) Mercuric Chloride ($HgCl_2$) followed by 3-4 rinses with sterile double distilled water. The aseptic seeds were then transferred aseptically into sterilized culture tubes containing filter paper bridges whose lower half is dipped in 1/4th MS salt [18]. Jam bottles with the basal MS medium (BM) containing 3% (m/v) sucrose and 0.8% (m/v) agar was adjusted to pH 5.8 were used as germination vessels. All media were autoclaved at 121^{0}C (1.4 Kg cm $^{-2)}$ for 18 min. Basal medium was supplemented with different concentration of BAP for shoot bud induction. All cultures were incubated in growth chamber at temperature of 25±2^{o}C, 16- h photoperiod with an irradiance of 50µmol m^{-2} s^{-1} provided by cool white fluorescent lamps.Cotyledonary leaves and hypocotyls were excised and inoculated on MS medium supplemented with various concentrations of BAP (4mgL^{-1} , 5mgL^{-1} , 6mgL-1 , 7mgL^{-1} , 8mgL^{-1} , 9mgL^{-1} , 10mgL^{-1}) to evaluate its effect on shoot bud induction. The shoot buds were transferred to shoot elongation and rooting medium supplemented with various concentration of auxins (IAA,IBA,NAA) for further elongation and rooting. The

rooted shoots were gently removed from the jam bottles and the roots were washed with autoclaved distilled water to remove the traces of agar. The plantlets were then transplanted in magenta box (PAC) filled with germination sand containing ¼ M.S. medium and kept covered with cap of magenta box (PAC) having a few holes on it. The plantlets were watered alternately with autoclaved distilled water to maintain high humidity. After 12-15 days, humidity was gradually decreased by open the holes of magenta box (PAC). The caps of magenta box (PAC) were finally removed. Four to six week hardened plants were then transplanted to bigger pot or to the field.

Determination of tolerance level of chilli leaf and hypocotyls and explant to Kanamycin

Before attempting transformation experiments, the concentration of Kanamycin to be used for the selection of transformed cells, were determined in preliminary experiments. Cotyledonary leaves and hypocotyl explants were inoculated on MS medium supplemented with different concentration of BAP and IAA along with different levels of Kanamycin (25, 50, 100, 200, 300, 400, 500, 600, and 700 mgL^{-1}. The concentration of Kanamycin at which explants turned brown or bleachy spotted was considered appropriate for selective medium.

Cultivation of *Agrobacterium tumefaciens*

A single colony of *Agrobacterium tumefaciens* was inoculated in 50 ml of YEP liquid medium containing Kanamycin (50 mgL^{-1}) and rifampicin (10mgL^{-1}) to get pure culture. Culture was incubated for 10-18 hours at 27^0C on incubator shaker @ 190 rpm to an A_{600} 0.6-0.8. The bacterial culture was centrifuged at 5000 rpm and then resuspened at the ratio of 1:20 in YEP liquid medium.

Isolation of p35SGUSINT Plasmid of *A.tumefaciens*

Single bacterial colony was transferred into 5 ml of YEP medium containing appropriate antibiotics (Kanamycin 50 mgL^{-1} and refampicin 10 mgL^{-1}) in a loosely capped 30 ml tube and was incubated at 28°C 190 rpm for overnight. The method used is a modified method of Birnboim and Doly [19] and Ish-Howowicz and Burke [20].

Preparation of competent cells of E.coli DH5á-strain and transformation of E.coli DH5á-strain with p35SGUSINT plasmid

Competent cells were prepared according to the method of Mendel and Higa [21]. For transformation, 2 ml plasmid was added to 100 ml DH5á-competent cell kept on ice and stored for 30 min. A brief heat shock at 42^0C for 90 second was given followed by chilling on ice for 10 min. Luria Broth was added to each tube and the tube was incubated for 60 min. at 37^0C in thermo shaker. The Luria agar plate containing Kanamycin (50 mgL^{-1}) was prepared, and allowed to dry. Then the transformed cells were given a brief spin to pellet the cells and pellet was resuspended in 100 ml Luria broth and spreaded on each agar plate. The plates were incubated for 16-18 h at 37^0C.

Isolation of p35SGUSINT plasmid from transformed E.coli DH5á-strain cell

The plasmid DNA was isolated from the white colonies and the white colony was streaked on a fresh YEP plates and then colony was inoculated in 5 ml YEP containing Kanamycin (50 mgL^{-1}) and refampicin (10 mgL^{-1}) and followed the plasmid isolation method as described above.

Preparation of biolistic gun system components for bombardments

(A)Instrument Preparation

Verify that helium and nitrogen tank has 200 psi in excess of desired rupture disk pressure for bombardment. Set gap distance between rupture disk retaining cap and microcarrier launch assembly. Prepare the Rupture Disk Retaining Cap. After setting the gap between cap and microcarrier launch assembly, wrap the Rupture Disk Retaining Cap in

aluminum foil and sterilize by autoclaving. Prepare the Microcarrier Launch Assembly. Sterilize the target shelf by wiping with 70% ethanol, followed by drying in a sterile environment just prior to use. This part may not be autoclaved.

(B) Preparation of sample and other accessories

Place the macrocarrier into the macrocarrier holder using the seating tool. Transfer selected rupture disks to individual Petri dishes for easier handling. Sterilize rupture disks by briefly dipping them in 70% isopropanol just prior to insertion in the Retaining Cap. All disks, with the exception of those rated at 450, 650, and 1,100 psi are laminated. Transfer selected stopping screens to individual Petri dishes for easier handling. Sterilization by autoclaving is recommended.Alternatively, these parts can be sterilized by soaking in 70% ethanol, followed by drying in a sterile environment.The particle suspension was obtained out by the sequential addition of 10 ìl of 50 mg/ml gold particles, 10 ìl of 1ìg/ìl plasmid DNA, 50 ìl of 2.5 M $CaCl_2$ and 20 ìl of 0.1M spermidine.

Plant tissue preparation for bombardment.

Place 4 explants in the centre of a petri dish on top of a filter paper moistened with distilled water and keep covered untill needed.

Performing a Bombardment

Before the Bombardment, Select 6 cm. gap distance between rupture disk retaining cap and microcarrier assembly. Placement of stopping screen support in proper position inside fixed nest of microcarier launch assembly.Check helium and nitrogen supply (200 psi in excess of desired rupture pressure).Clean/sterilize Equipment: rupture disk retaining cap, microcarrier launch assembly Consumables: macrocarriers/ macrocarrier holders.Wash microcarriers and resuspend in 50% glycerol.Coat microcarriers with DNA and load onto sterile macrocarrier/ macrocarrier holder then fire the biolistic gun and remove the explants from the gene gun.

Regeneration and Selection of transformants The explants were kept on selective medium containing appropriate levels of BAP, and Kanamycin. Only transformed explants were expected to show regeneration since non-transformed cells were likely to be killed due to be the presence of Kanamycin in the medium.

Histochemical Gus assay in transformed tissues

The transient *GUS* expression was assayed with 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as substrate after 3 days of co-cultivation The GUS assay was carried out as per the method described by Jefferson [23] in following steps:

The leaf /hypocotyl explants and callus/shoot developed from leaf/hypocotyl were incubated in phosphate buffer (50 mM pH 7.0) that contained 0.3% (w/v) x-Gluc. & potassium ferricyanide. The plant material was left for 6 to 20 h at 37^0C in an incubator. After staining, the plant material was rinsed in 70% ethanol for at least 25- 30 min to remove the chlorophyll. GUS expression units (Blue spots and regions) were seen under microscope to ascertain GUS activity.

Isolation of DNA from transformed plantlets

Five gram of leaf material was homogenized in liquid nitrogen (Liq. N_2). The homogenized material was handled as per the method described by Doyle and Doyle

Quantification of DNA

The quantification of DNA/ Plasmid was done by observing it at 260 nm and 280 nm wavelengths by using a UV visible spectrophotometer (UNICAM)

PCR amplification of *npt*II gen specific sequence from genomic DNA of GUS positive transformed plantlets

For PCR analysis, DNA was isolated from leaves according Doyle and Doyle (1990). Two set of primers of the *npt* II genes were used: 5'-GAG GCT ATT CGG CTA TGA CTG-3' and 5'-ATC GGG AGG GGC GAT ACC GTA-3'. Expected size of the fragment was 700

bp. PCR Reaction were performed in final volume of 25ml containing dNTPs (200µm) 2 µl ØTaq Buffer 2.5 µl Primers 2 µl (1µl forward+1µl reverse) Taq DNA Polymerase 0.33 µl DNA 2µl Water18 µl.

Following the amplification, the PCR products were loaded on 1.2% Agarose gel which was prepared in 1X TBE buffer containing 0.5 mg/ml of the ethidium bromide. The amplified products were electrophoresed for 3-3.5 h at 100 V with cooling. After seperation the gel was viewed under UV Transilluminater and photographed by digital camera.

Results and Discussion

Regeneration

Prior to attempting transformation experiments, Protocol for regeneration in Chilli cv. Mathania using cotyledonary leaf and hypocotyls explant was established [24].

Determination of suitable concentration of Kanamycin

Cotyledonary leaves and hypocotyls were taken from 12-13 d old *in vitro* raised seedlings and inoculated on regeneration medium containing BAP 10 mgL^{-1} and IAA 1 mgL^{-1}along with different concentrations of Kanamycin. Effect of different concentrations of Kanamycin was examined after 15 d of inoculation (Table 1). At a concentration of 25 mgL^{-1}nearly all the leaf were green and show callusing were as 3 out of 4 hypocotyls were healthy and showed callusing. All the explants showed brown spots with no callusing at concentration 50 mgL^{-1}. However, higher concentrations of Kanamycin (above 50 mgL^{-1}) showed more chlorosis and browning. Therefore, it was concluded that concentration of 50 mgL^{-1}would be used for the selection of putative transformants in transformation experiment using vector p35SGUSINT, which contain *npt* II gene for both types of explants**.**

Transformation via Gene gun

Gene delivery by particle bombardment is regarded as the most efficient and consistent genotype-independent method for transfer of foreign DNA. Unlike *Agrobacterium*-mediated method, which is a biological process, particle bombardment is a physical process of gene delivery and theoretically any plant tissue can be transformed by this method [25, 26]. However, to date there is no report available for the use of this technology in chilli except one by Nianiou *et al.,* [27] reporting limited success.

Nitrogen gas was also considered as an alternative of the helium gas for pressure firing of the micro carriers in biolistic gun. This was included the option for the use of nitrogen gas was considered because of non-availability of helium in less developed area's like our's at the same time more expensive.

Bombarded by Nitrogen

Table 2 shows the frequency of callus development and shoot formation from callus while using Gene gun with Nitrogen gas. The transformation was tried using three different rupture disks 650, 900 and 1100 psi and associated gas pressure keeping the target distance with explant fixed at 6 cm. The explants (Cotyledonary leaf and hypocotyl) were monitored for transient expression of GUS activity after seven d of transformation and subsequent incubation on selective regeneration media containing Kanamycin. When we used rupture disk of 650 (psi) 3 (13.63%) leaves out of 22 leaf explants and only 1 (12.5%) hypocotyl out of 8 hypocotyl explants were found GUS positive. Subsequent culturing resulted in callus induction in 2 (66.66%) leaves and none from hypocotyl explant. While using rupture disk of 900 (Psi) 16 (64%) leaves and 7 (70%) hypocotyls were found GUS positive out of 25 leaf and 10 hypocotyl explants respectively. Most of these GUS positive leaves (14, 87.5%) and hypocotyl (6, 85.71%) formed callus in selection media. At 1100 (Psi) rupture disk 16 (61.53%) leaves were GUS positive out of 26 leaf explants and 7(58.33%) hypocotyls were GUS positive from 12 hypocotyls explants. Callus was formed in 13 (81.25%) leaves and 5 (71.42%) hypocotyl explants.

Comparative performance of regeneration and transient transformation with 900 (Psi) rupture disk was considered better for obtaining transformed

plants. overall 336 (67.74%) leaves out of 496 and 220 (69.18%) hypocotyls out of 318 were GUS positive. About, 86.01% leaves and 78.63% hypocotyls formed callus. A number of elongated plants from transformed explant were rooted: 17 (3.4%) from leaf and 13 (4.0%) plants from hypocotyl explants.

Bombarded by Helium

Though our experiment has clearly demonstrated efficiency of nitrogen gas for inducing transformation it was felt that its efficiency should be compared with commonly recommended and used helium gas. Similar to the above experiment with nitrogen gas, the transformation was tried using three different rupture disks 650, 900 and 1100 psi and associated gas pressure keeping the target distance with explant fixed at 6 cm. The bombarded explants (Cotyledonary leaf and hypocotyl) were monitored for transient expression of GUS activity by taking half of the treated leaf, after seven d of transformation and subsequent incubation on selective regeneration media containing Kanamycin and Cefotaxime. Table 3 shows the frequency of callus development and shoot formation from callus while using Gene gun with helium gas. When we used rupture disk of 650 psi 5 (20.83%) leaves out of 24 leaf explants and only 3 (30%) hypocotyl out of 10 hypocotyl explants were found GUS positive. Subsequent culturing resulted in callus induction in 2 (40%) leaves and 2 (66.66%) from hypocotyl explants. While using rupture disk of 900 (Psi) 16 (64%) leaves and 7 (70%) hypocotyls were found GUS positive out of 25 leaf and 10 hypocotyl explants respectively. Most of these GUS positive leaves (14, 87.5%) and hypocotyls (6, 85.7%) formed callus. At 1100 (Psi) rupture disk 14 (53.84%) leaves were GUS positive out of 26 leaf explants and 7 (58.33%) hypocotyls were GUS positive from 12 hypocotyls explants. Callus was formed in12 (85.71%) bombarded leaves and 5 bombarded (71.42%) hypocotyl explants.

The most effective 900 (Psi) rupture disk was taken for our further experiment and development of transformants. At which overall 310 (63.91%) leaves were GUS positive out of 485 leaves explants and 206 (63.77%) hypocotyls were GUS positive out of 323 hypocotyls explants. 241 (77.74%) leaves formed callus out of 310 GUS positive leaves and 158 (76.69%) hypocotyls formed callus from 206 GUS positive hypocotyl explants.15 (3.0%) plants with roots were obtained from leaf explants where as 13 (4.0%) plants with roots obtained from hypocotyls explants.

Conformation of transformation

Through GUS expression

Explants transformed directly using biolistic gun were examined for transient Gus expression after 7 d of firing (Plate 1&2) However, this GUS activity could be because of transient expression of the gene. Thus, in order to confirm the stable integration of T-DNA into host genome callus developed from infected and bombardad explants grown on Kanamycin containing medium was used for GUS assay (Plate 3). Gus expression was monitored on whole leaf of kanamycin resistant callus derived plantlets to conform transformation. (Plat 4).

The results for transient expression for various transformation methods have presented earlier. In order to make the system more efficient all the explants were tested for transient expression taking half of its part. A good frequency of plants showing transient expression was considered for further culturing. The explants infected with *Agrobacterium* showed GUS expression near the cut edges, where as in case of gene gun GUS expression was scattered all over the explant in dotted pattern (Plate 1&2).

The explants were incubated on selective medium showed callusing after 20-25 d of incubation. The entire callus samples developed on selective media containing 50 mgL^{-1} Kanamycin, showed GUS activity in all the cases taken (24, 4 for each method-explant combination) for test in both the explants. However, certain regions devoid of GUS activity were also seen i.e. showing no blue coloration on GUS test. The GUS activity was also tested on callus developed from untreated explants (control) on MS media devoid of Kanamycin as control. No activity

was detected in such callus tissues (Plate3). The GUS activity was visible after 4-5 hours of x-gluc treatment in transformed callus or tissue. The blue color developed in tissues after 4-5 hours got diffused to treatment buffer after 10 hrs. Though no blue color developed in callus generated from control explants, during the period of 10-12 hrs. Confirmation of transformation through PCR analysis: In direct biolistic gun mediated transformation experiments using either nitrogen gas or helium gas, 12 plants each were put to PCR analysis (6 from leaf and 6 from hypocotyl explants each). All the 24 plants amplified a single fragment of expected 700 bp fragment of npt-II (Plate 5&6).

Discussion

Gene delivery by particle bombardment is regarded as the most efficient and consistent genotype-independent method for transfer of foreign DNA. Unlike *Agrobacterium*-mediated method, which is a biological process, particle bombardment is a physical process of gene delivery and theoretically any plant tissue can be transformed by this method [25, 26]. However, to date there is no report available for the use of this technology in chilli except one by Nianiou *et al.,* [27] reporting limited success.In particle bombardment experiments, the different physical and biological parameters such as the mechanism of delivery, the velocity of particle delivery, DNA particle concentration and precipitation procedures, amenability of target tissues for transformation need to be carefully considered. The target distances (3, 6, 9, 12 cm.) traveled by the micro projectile from the micro carrier launch assembly to the target tissues significantly affected the rate of transient GUS expression [28]. It was opined that reduced frequency of transient GUS expression with increased target distance could be due to the deceleration of the microprojectiles caused by the air resistance that increase as distance is increased. On the basis of earlier reports viz. in sugarcane [29], in broccoli [30], in cauliflower [31] and in tomato [28] 6-cm target distance giving best results was finalized for present experiments. Keeping the target distance fixed at 6 cm. Protocol for direct transformation using biolistic gun was standardized taking 3 rupture disc of various pressures (650,900,1100 psi) on two types of explants (Cotyledonary leaf and hypocotyl).

Nitrogen gas was also considered as an alternative of the helium gas for pressure firing of the micro carriers in biolistic gun. This was included the option for the use of nitrogen gas was considered because of non-availability of helium in less developed area's like our's at the same time more expensive.

The frequency of direct transformation was better than co-cultivation with both nitrogen and helium gas for both the explants leaf and hypocotyls used in the present study. Moreover, use of nitrogen for bombardment increased the frequency of transient GUS expression in leaf (67.74%) and hypocotyls (69.18%) with a higher frequency of its conversion to stable transformation (3.4% in leaves and 4.0% in hypocotyls) as compared to helium gas, the frequency of transient GUS expression being 63.91% in leaf and 63.77% in hypocotyls and stable transformation frequency was 3.0% and 4.0% in leaf and hypocotyls respectively. Low conversion rates of transient-to-stable expression has been suggested to be due to several factors such as, low efficiency of integration of the introduced DNA, low survival of the bombardment cells, toxicity of the heavy metals used for bombardment, mechanical damage due to particle penetration in the cell wall [32]. However, higher frequency of transformants in direct method than co-cultivation rules out negative effect of gold used in the present study and particle penetration. Biolistic transformation in chilli has not been previously reported.

But in other solanaceae plants, transient GUS expression frequency ranged from 22.20% to 36.56% in three different explants of tomato [28] and 85% frequency of transient GUS expression in datura plants [33]. The frequency of transformants reported in our experiment was better than repoted for tomato and poor compared to datura.

Table 1. Effect of Kanamycin on explants of *Capsicum annuum* L. var. Mathania.

Conc. of Kanamycin (mgL^{-1})	% Survival of leaf explants	% Survival of hypocotyl
0	100 [a]	100 [a]
25	100 [a]	75 [a]
50	50 [b]	50 [b]
75	25 [b]	00 [c]
100	00 [c]	00 [c]
200	00 [c]	00 [c]
300	00 [c]	00 [c]
400	00 [c]	00 [c]
500	00 [c]	00 [c]

A= All explants green, healthy, callusing/shoot regeneration.

B=Green, No callusing/Shoot regeneration.

C=Bleached

Table 2. Frequency of callus development and shoot formation from callus (Bombarded with nitrogen)

Rupture disk	No. of explants		GUS positive		No. of calli formed		No. of shoots with roots	
	Leaf	Hypocotyl	Leaf	Hypocotyl	Leaf	Hypocotyl	Leaf	Hypocotyl
650 (Psi)	22	8	3	1	2	0	-	-
900 (Psi)	25	10	16	7	14	6		
1100 (Psi)	26	12	16	7	13	5		
900 (Psi)	496	318	336	220	289	173	17	13

Table 3. Frequency of callus development and shoot formation from callus (Bombarded with Helium)

Rupture disk	No. of explants		GUS positive		No. of calli formed		No. of shoots with roots	
	Leaf	Hypocotyl	Leaf	Hypocotyl	Leaf	Hypocotyl	Leaf	Hypocotyl
650 (Psi)	24	10	5	3	2	2	-	-
900 (Psi)	25	10	16	7	14	6		
1100 (Psi)	26	12	14	7	12	5	-	-
900 (Psi)	485	323	310	206	241	158	15	13

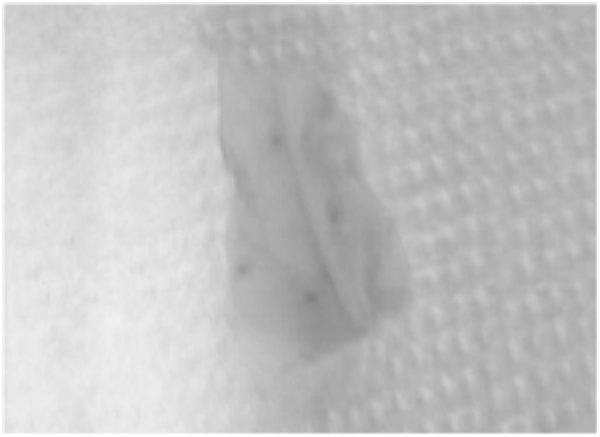

Fig:1 Cotyledonary leaf explants showing GUS expression after 7 days of gun shoot

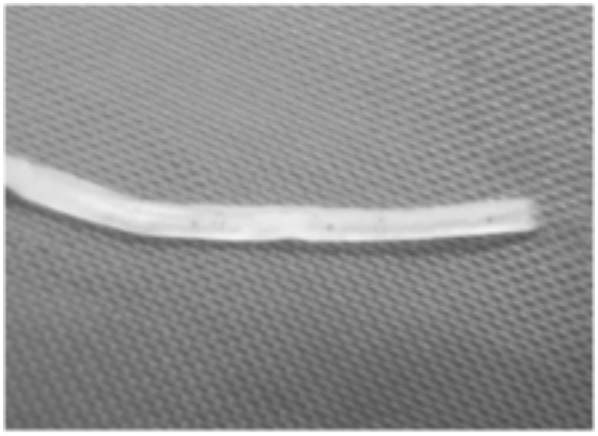

Fig:2 Hypocotyl leaf explants showing GUS expression after 7 days of gun shoot

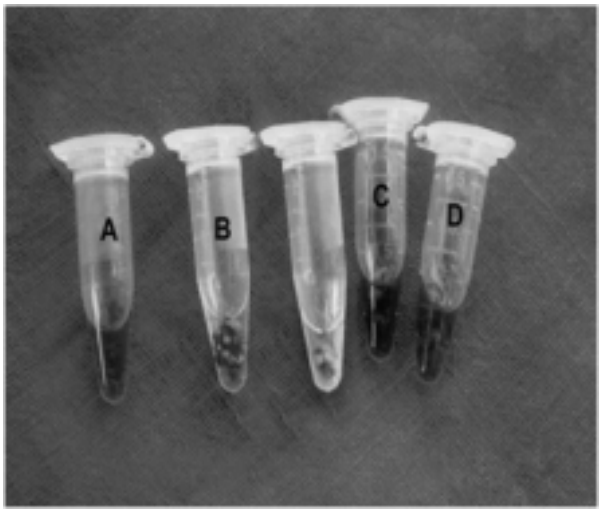

Fig:3 Showing GUS activity in transformed and transformed

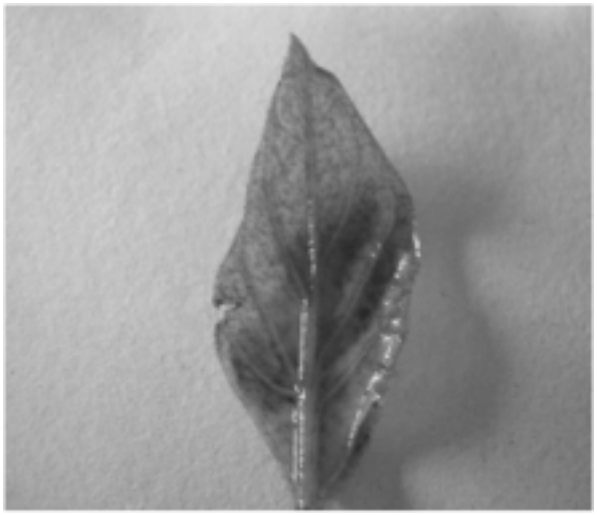

Fig:4 Leaf taken from fully developed

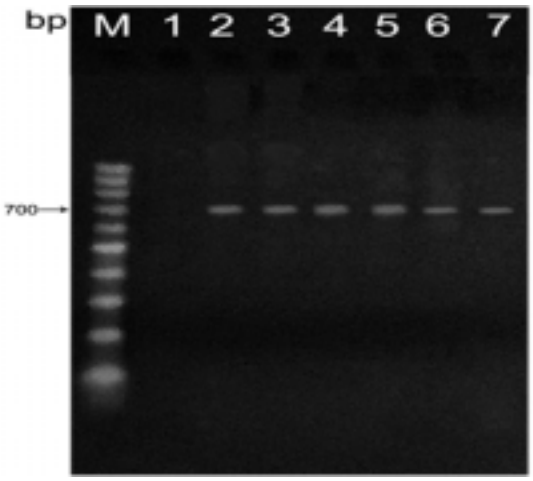

Fig:5 *npt II* gene specific amplicons (700 bp)generated in putative transgenic shot with gun using nitrogen gas.M: 100 bp ladder,Lane 1: Control (Non transformed),Lane 2-7: Transgenic Plants.

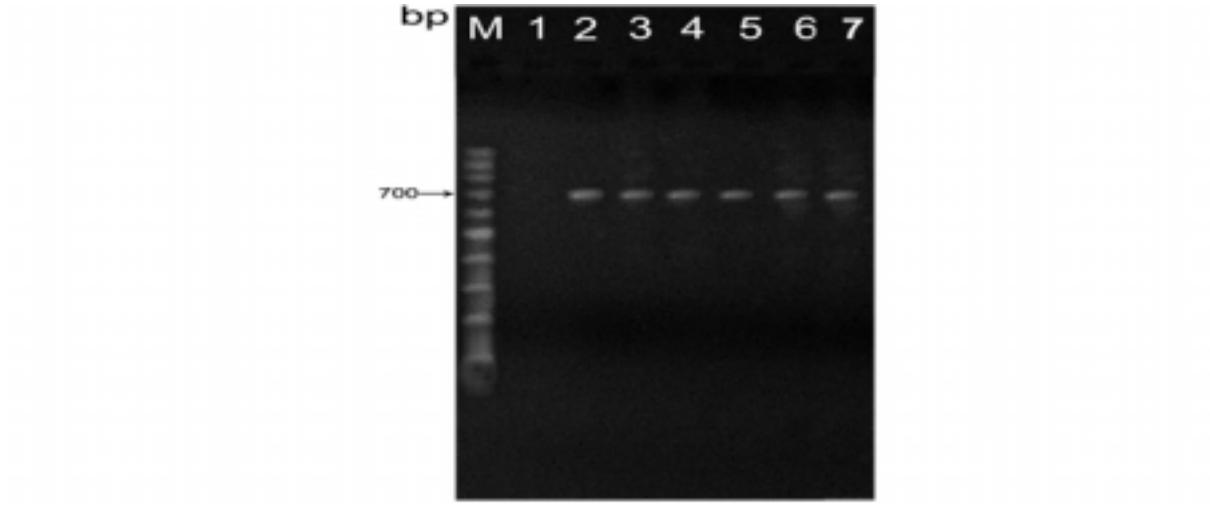

Fig:6 *npt II* gene specific amplicons (700 bp)generated in putative transgenic shot with gun using Helium gas. M: 100 bp ladder, Lane 1: Control (Non transformed),Lane 2-7: Transgenic Plants.

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