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An efficient and highly reproducible approach for the selection of upland transgenic cotton produced by pollen tube pathway method

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Abstract

Pollen tube pathway mediated transformation is most favorable genotype-independent approach and has been widely used for the production of transgenic crops all over the world. We used this technique to transform Zheda B, an upland cotton, by two different vectors i.e. pCAMBIA1301 with *hpt* gene for hygromycin resistance and pCAMBIA 2301 having *npt*II gene for kanamycin resistance. A preliminary experiment was conducted to find out suitable concentration of kanamycin (100-600 mg/L) and hygromycin (25-150 mg/L) for the screening of transgenic plants. Results revealed that both antibiotics caused a significant reduction in seed germination, seedling survival, plant height, root length, fresh and dry weight of root and shoot as compared to their respective controls. Furthermore, 500 mg/L kanamycin and 75 mg/L hygromycin were established as effective dozes to eliminate 80-85% of non-transgenic seedlings during seedling stage. The outcomes of the preliminary experiment were applied to select transgenic plants from T_1 population, which were later confirmed by PCR and GUS staining. The results were identification of positive transgenic plants with successful integration of GUS gene into recipient cotton genome. Comparing both antibiotics, upland cotton was found to be more sensitive to hygromycin application as compared to kanamycin. Therefore, designing gene constructs for pollen tube pathway mediated transformation; *hpt*II gene seems to be more appropriate choice than *npt*II gene for selection of transgenic plants. The protocols are robust and can be utilized for other crop species as well.

Keywords: Cotton transformation, leaf painting, growth inhibition, pollen tube pathway, seed germination, seedling survival.
Abbreviations: DPA_days post anthesis, GUS_β-glucuronidase, *hpt*_hygromycin phosphotransferase, *npt*II_neomycin phosphotransferase II, pNOS_nopaline synthase promoter.

Introduction

Production of transgenic crops with desirable traits is of paramount importance to meet the global needs of food, feed and fiber; and has much more advantages as compared to traditional breeding techniques. Transgenic plant development is mainly based upon *Agrobacterium tumifaciens* mediated transformation that accounts for production of 80% transgenic crops around the world (Wang and Fang, 1998). This method of genetic transformation is exclusively genotype dependent and needs extremely sterilized conditions during transformation and tissue regeneration. Due to this reason, this technique has been restricted to well develop laboratories only. In contrast, pollen tube pathway is a genotype-independent approach and provided first-hand choice for crop varieties lacking regeneration potential. This technique has been widely practiced for developing transgenic cotton (Zhou et al., 1983), rice (Luo and Wu, 1988) and soybean (Lei et al., 1991; Liu et al., 1992) and can easily produce next generation seeds (Song et al., 2007). The technique is very simple and follows injection of foreign DNA to stigma after one day of post anthesis (DPA) (Zhou et al., 1983).

During the process of transformation, some cells from nucellus tissue degenerate to make a channel for the entry of pollen tube towards embryo sac. This pathway is usually larger than pollen tube, so heterologous DNA can easily enter into embryo sac and gets incorporated into recipient plant genome (Song et al., 2007). The approach though did not require highly exclusive conditions for transformation of exogenous DNA but results in the production of thousands of seeds after transformation. Due to less availability of land, sometimes it becomes unattainable to directly sow all the seeds in the field and to follow commonly adopted methods of transgenic screening like leaf painting, PCR and southern blotting. Therefore, it is necessary to develop some cost effective and reproducible methods to eliminate most of non-transgenic plants at seedling stage. During gene transformation, a common phenomenon associated with exogenous genes is that they do not bestow a phenotype that can be used conveniently for the identification of transgenic cells. For this reason, phenotypically identifiable marker genes are co-transformed along with the gene of interest to selectively eliminate non-transformed tissues.

A selectable marker gene usually encodes a product that allows the transformed cell to survive and grow under conditions that can either kill or restrict the growth of non-transformed cells. Most commonly used marker genes are *npt*II (kanamycin resistant), *hpt* (hygromycin resistant) *Bar* (herbicide tolerant) and *pat* encoding phosphinothricin acetyl transferase; and have been extensively used for the selection of transgenic plants (Leelavathi, 2004; Zhang and Shangguan, 2006; Visarada et al. 2008; Daud et al., 2009; Zhang et al., 2010; Afolabi Balogun et al., 2011). Kanamycin is one kind of amino glycoside antibiotic, which inhibits the protein synthesis in green parts of the plants (Nap et al., 1992; Lu et al., 2001; Wang and Yi, 2003; Chen et al., 2005). The cells incorporated with *npt*II gene offer resistance to kanamycin application; while non-transformed cells either get died or show restrained growth. In this regard, appropriate concentration of antibiotics is one of the most critical factors for screening of transgenic plants. If the concentration of antibiotic is too low, it results in the selection of false positive plants while concentration exceeding the resistance level can cause mortality of transgenic plants as well. Previous report demonstrated the germination of cotton seeds on MS media supplemented with appropriate amount of antibiotics (Li et al., 2004). However, presence of sucrose/glucose in MS media escalates growth of microorganisms including bacteria and fungus; and therefore needs extremely sterilized conditions. Present study utilized water agar media as an alternative to MS media, which is highly cost effective, and has produced similar outcomes. Hence, the current study was planned to select terrific concentration of both kanamycin/hygromycin and growth indices which can be utilized to abolish non transgenic plants under field and lab conditions. Elimination of non-transgenic seeds and seedling will not only reduce the work for molecular studies but will also facilitate the timely selection of transgenic plants within a specific growing season.

Results and discussion

Effects of antibiotics treatment on seed germination and seedling survival

Both antibiotics significantly decreased the germination of cotton seeds in a dose-dependent manner as compared to control having no antibiotic (Fig. 1, 2). Likewise, the differences among various treatments of individual antibiotic were significant but the magnitude of reduction was more pronounced for hygromycin application than that of kanamycin. Highest concentration level (C6: in Fig. 1, 2) significantly decreased the seed germination which was 45% for kanamycin treatment and 30% for hygromycin as compared to 90% of control (Fig. 1). In present study, although the seeds were germinated but their roots were necrotic and could not either grow longer or develop any secondary root at higher concentration of antibiotics, as compared to the control neither having kanamycin nor hygromycin (Fig. 2, 3). After three weeks of transplantation, seedlings which developed true leaves were considered as survived while rests were scored as dead. According to our expectation, that high concentration of antibiotics might be lethal for root growth, all of the seedlings could not survive (Fig. 1, 4). The death of the seedling might be due to poor root growth (Visarada et al. 2008). Smiliarly, the seedling survival was highly dose-dependent for both kanamycin and hygromycin as compared to their respective controls (Fig. 1, 4). Kanamycin at level of 100 mg/L significantly reduced the seedling survival and this reduction was statistically similar to that of 200 mg/L (Fig. 1), but subsequent higher doses resulted gradual reduction in seedling survival with maximum reduction of 78% attained at highest concentration level (600 mg/L). On the other hand, the reduction in seedling survival was highly significant up to 75 mg/L of hygromycin relative to control, but none of the seedlings could survive at concentration higher than 75 mg/L (Fig. 3b, 4). Moreover, at C3 level, the seedling survival for kanamycin was 55% while for hygromycin it was only 15% as compared to 90% of control. Therefore, comparing both antibiotics, upland cotton was found to be more sensitive to application of hygromycin. Concomitant with our results, shoot development in case of grapevine was also found to be more sensitive to the application of hygromycin as compared to kanamycin (Torregrosa et al., 2000; Clevenger et al., 2004; Walters et al., 2010). But previous researchers (Guellec et al., 1990; Mullins et al., 1990; Mauro et al., 1995) have associated the failure of shoot development with the inefficiency of *npt*II gene, when it is controlled by pNOS, rather than assuming grapevine to be sensitive to kanamycin.

Morphological parameters of upland cotton seedlings

Plants were uprooted and various growth parameters were studied. Results revealed significant variations ($p < 0.05$) in seedling vigor. The plants raised from seeds initially germinated on media containing antibiotics were remained dwarf while their respective controls grew normally attaining plant height of about 35 cm (Fig. 5 A). Plant height was sharply declined up to 52 % of control at 200 mg/L of kanamycin and was statistically similar up to 400 mg/L followed by another sharp decrease at 500-600 mg/L. Whereas, in case of hygromycin plant height was highly dose dependent and continuously decreased with increase in applied concentration level (Fig. 5 A). At C2 level of both kanamycin (200 mg/L) and hygromycin (50 mg/L) plant height was about 18-20 cm as compared to 35 cm observed for control. Root is considered as the most important organ and determines the overall health of the plant. It not only anchors the plant in soil but also nourishes the plant through active absorption of water and nutrients from soil (Hoad et al., 2001). The seedling could not recover the inhibitory effects of antibiotics pretreatment even after growing under non stressed conditions for a period of three weeks. In present study, root growth was declined with both kanamycin and hygromycin. This decrease in root length was highly dose dependent and higher concentration of antibiotics escalated the reduction in root length. At C3 level of hygromycin (75 mg/L) the root length was about one third (7 cm) of control (21 cm) while similar reduction was observed at C6 level of kanamycin (600 mg/L). However, variations among treatments of both kanamycin and hygromycin were significant but magnitude of reduction was higher in case of hygromycin treatments (Fig. 5 B). From these results it seems that root of upland cotton is more sensitive to hygromycin application than kanamycin as low concentration of hygromycin resulted same growth inhibition as higher concentrations of kanamycin can do. The superior root and shoot mass have been proposed as reliable adaptation for the plants growing under stress environment in different plant species (Yang et al., 1991; Basal et al., 2005). Fresh and dry weights of the seedlings were also reduced in current investigation after germinating seeds on media supplemented with either kanamycin or hygromycin. In case of kanamycin, though fresh and dry weights were significantly decreased as compared to the control but differences among treatments were less pronounced. Whereas, in case of hygromycin these

Table 1. Primer sequences for PCR analysis.

Primer name	Primer sequence (5′ - 3′)	Product size
Trnl-F	CGAAATCGGTAGACGCTACG	672 bp
Trnl-R	GGGGATAGAGGGACTTGAAC	
GUS-F	ACTTACGTGGCAAAGGATT	454 bp
GUS-R	GCGTCGCAGAACATTACA	

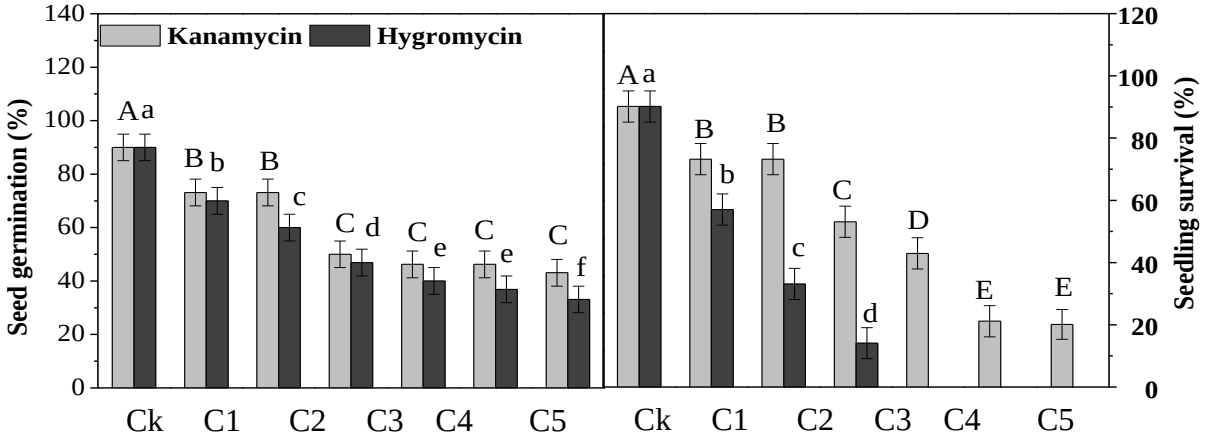

Fig 1. Effect of different levels of kanamycin and hygromycin on seed germination (after 3 days) and seedling survival (after 3 weeks) of upland cotton. Capital letters show differences among different treatments of kanamycin and control while small letters show differences among different concentrations of hygromycin as compared to control. CK; Control i.e no kanamycin, no hygromycin, C1 represents 100 mg/L kanamycin and 25 mg/L hygromycin, C2; 200 mg/L kanamycin and 50 mg/L hygromycin, C3; 300 mg/L kanamycin and 75 mg/L hygromycin, C4; 400 mg/L kanamycin and 100 mg/L, C5 stands for 500 mg/L kanamycin and 125 mg/L hygromycin while C6 shows 600 mg/L kanamycin and 150 mg/L hygromycin, respectively.

Table 2. Summary of transformation efficiency of pollen tube pathway method.

Construct	Antibiotic resistance	Flowers injected	Seeds harvested	Germination (% age)	Survival (% age)	No. of necrosis free plants	No. of Trnl PCR + plants	No. of GUS PCR + plants	GUS stained + plants
pCAMBIA 1301	Hygromycin	> 1200	3000	56	13	26	26	4	4
pCAMBIA 2301	Kanamycin	>1200	5000	62	33	310	310	6	6

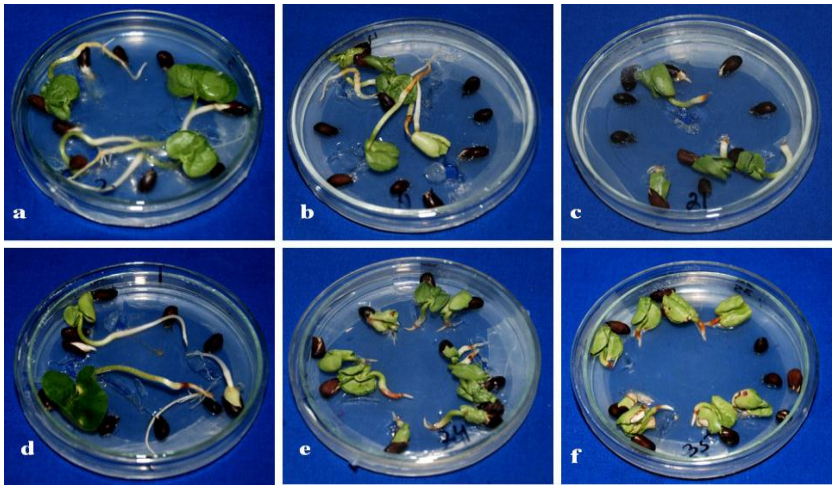

Fig 2. Effect of kanamycin and hygromycin on seed germination of non transgenic upland cotton. Control seeds, in the absence of antibiotics (a, d) developed long roots with white root tips while presence of 100 mg/L and 600 mg/L kanamycin (b, c); 25 mg/L and 150 mg/L hygromycin (e, f) respectively inhibited the growth of root and root tips showed necrosis.

differences were highly marked at all treatment levels and showed a gradual reduction in root length with increasing concentration of hygromycin (Fig. 5 C, D). Magnitude of reduction in dry weight was similar at highest concentration levels of both kanamycin (600 mg/L) and hygromycin (75 mg/L) again revealing high sensitivity of upland cotton to hygromycin application. Plant canopy is greatly dependent on the development of root which is the primary source for nutrients uptake. It has been reported previously (Zhao et al., 2007) that hygromycin at concentration level of 20 mg/L significantly suppressed shoot and root elongation of cotton seedlings. Likewise, in this experiment, a significant ($p < 0.5$) reduction in fresh and dry weight of root was observed due to kanamycin and hygromycin treatment at seed germination level, as compared to their respective controls. Moreover, magnitude of this reduction was directly proportional to the treatment level irrespective of the antibiotic type (Fig. 5 E, F). From the current results, seedling survival and seedling vigor were chosen as best indices for the elimination of non-transgenic plants. To eliminate almost 70-80% of the non- transgenic seedlings and to find out positive transgenic plants, 500 mg/L kanamycin and 75 mg/L hygromycin were found to be the optimal concentrations.

Leaf assays for antibiotic resistance

Leaf is considered as food factory of the plant and is the main centre for photosynthesis, responsible for the production of carbohydrates, fats and proteins. This food is not only utilized by leaf but also transported to other plant parts (Zhang and Shangguan, 2006; Zeeman et al., 2007). In present study, survived seedlings were further screened by leaf painting using 750 mg/L kanamycin solution (already optimized). The treated leaves exhibited chlorosis after 5-7 days and then necrotic patches after 10 days (Fig. 7 A, B). The reason for this toxicity is that kanamycin can combine with 30 S sub-unit of ribosome of chloroplast and mitochondrion, hampering the normal process of translation and hence affects synthesis of different proteins and their translocation (Chen et al., 2005). This peculiar characteristic of kanamycin and hygromycin has been employed by various researchers for the screening of kanamycin resistant plants in various crops (Finer and Michael, 1990; Noor et al., 2000; Li et al., 2004; Kumar et al., 2013). Selection conditions were optimized for hygromycin resistance in many crops including rice and cotton using leaf tip assay method (Wang and Waterhouse, 1997; Noor et al., 2000). But little attention has been given to use hygromycin for leaf painting method, despite being most convenient under field conditions. Present study tested a wide range of hygromycin (25-200 mg/L) on leaves at different growth stages. Cotton leaves exhibited differential sensitivity to hygromycin application under field conditions. Leaves from top of the plant canopy (node 12) were more sensitive as compared to middle (node 8) and lower canopy (node 4, data not shown). The possible reason for this differential sensitivity might be leaf age, young leaves from top canopy being more sensitive to hygromycin application. After 48 hrs of hygromycin application, no necrotic symptoms were observed for low concentrations (25-50 mg/L); while higher concentrations produced marked symptoms (Fig 6). However after 96 hrs, necrotic symptoms were uniformly observed with all concentrations including 25-50 mg/L as well. However, differences among treatments higher than C3 level (75 mg/L) were non-significant ($p > 0.05$) at both time intervals. This shows that low concentration of hygromycin (25 mg/L) is effective too but need more time (96 hrs) for the appearance of necrotic symptoms (Fig 6). Similarly, concentration of 75 mg/L hygromycin can differentiate three types of leaves with highest score in top leaves followed by middle and lower leaves (data not shown). Compared with our selection levels, several studies used much higher hygromycin levels (50-100 mg/L) for cotton callus selection and seed germination (Li et al., 2004). The reason for these necrotic patches might be due to absence of innate resistance against hygromycin in plants (Christou and Ford, 1995), thus can be useful for providing strong discrimination between transformed and non-transformed cells. Leaves of susceptible seedlings changed color from green to yellow after 3-5 days of hygromycin application (Tian et al., 2010). Similarly, to avoid the impact of direct climatic conditions, a parallel experiment was conducted under laboratory conditions on detached leaf. Hygromycin application (10 mg/L) on detached leaves resulted necrotic spots similar to that observed under field conditions (Fig 7 C, D). Combining together field and lab observations, it is concluded that hygromycin is effective even at very low concentrations (10 mg/L). However, in field due to fluctuation in climatic conditions, sometimes low concentration is not affective. So, our result suggested 50-75 mg/L hygromycin concentration is appropriate in field condition whereas under controlled condition (water agar plate) 10 mg/L is an affective concentration to remove 80-90% non-transgenic plants and is very simple in application.

Analysis of putative transformants

The outcomes of the preliminary experiment were used to screen the transgenic plants. More than 1200 flowers were injected with each plasmid i.e. pCAMBIA 1301 and pCAMBIA 2301. However, the number of T_1 seeds harvested was different for both constructs (Table 2). Germination of T_1 seeds on media supplemented with appropriate antibiotic offer rapid selection of transgenic plants (Weymann et al., 1993). In current study, T_1 seeds from pCAMBIA 1301 (hygromycin resistance) population were germinated on water agar plates supplemented with 75 mg/L of hygromycin; while seeds from pCAMBIA 2301 (kanamycin resistance) were germinated on water agar plates containing 500 mg/L kanamycin. Only 56% seeds from pCAMBIA1301, while 62% seeds of pCAMBIA 2301 were germinated, however most of the seedlings had necrotic roots. Three days old seedlings were then transferred to small pots containing peat moss and maintained well in glass house under optimum conditions of light and humidity. After 3 weeks of transplantation, seedling survival for pCAMBIA 1301 was only 13%, while for pCAMBIA 2301 it was as high as 33%. This might be due to sample destruction during germination test; presumably the seedlings could not survive high concentrations (100-150 mg/L) selection which might have been exceeded than expression levels of the resistant gene (Visarada et al.. 2008). Previous studies have associated death of non-transgenic seedlings with poor root growth (Li et al., 2004; Meng et al., 2007) resulted from inhibitory effects of antibiotics in seed germination media. Low efficiency of kanamycin in current study contradicts the previous justifications (Guellec et al., 1990; Mullins et al., 1990; Mauro et al., 1995) as both pCAMBIA 1301 and pCAMBIA 2301 have same promoter (35 S) regulating the expression of *hpt*II and *npt*II gene for hygromycin and kanamycin, respectively.

These plants were transferred into field and after one weak adaptation; healthy plants were further screened using respective antibiotics by leaf painting using 750 mg/L

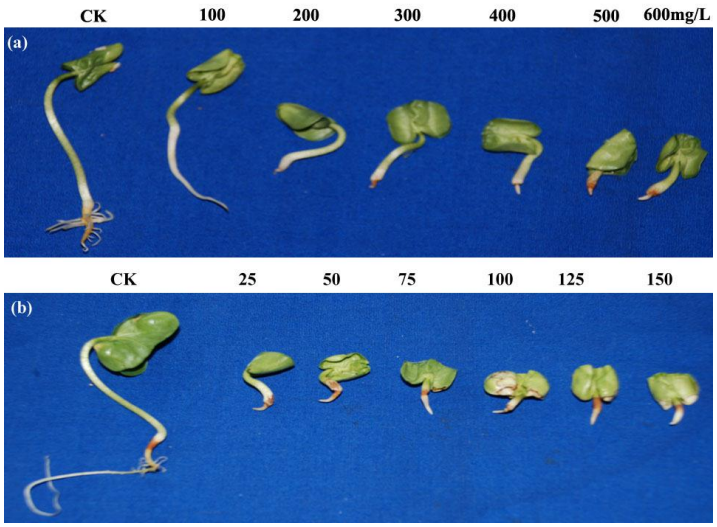

Fig 3. Effect of different concentrations of kanamycin and hygromycin on root development of non-transgenic upland cotton. CK; control seeds in absence of kanamycin and hygromycin developed long roots with root hairs while presence of kanamycin (a) or hygromycin (b) inhibited the root growth and have no root hair.

Fig 4. Effect of kanamycin (a) and hygromycin (b) treatment during seed germination on seedling survival. Number of survived seedling was reduced at higher concentration of both kanamycin and hygromycin.

kanamycin and 75 mg/L hygromycin in afternoon of sunny day on respective plants. After one weak, plants were observed for the appearance or absence of leaf necrosis. About 26 plants (11%) for hygromycin resistance while 310 plants (30%) for kanamycin resistance were selected due to the absence of any necrotic symptom (Table 2) on treated leaves while other plants exhibiting leaf necrosis were removed from the field. For further confirmation of the integration of exogenous gene into cotton genome, DNA was extracted from plants found to be resistant for both antibiotics after leaf painting assay. Primers from housekeeping cotton gene actin were synthesized and PCR was performed with a wide range of DNA concentrations (10-100 ng/µL). The concentration levels 30-50 ng/µL were found to be best as they could uniformly amplify the targeted product of 680 bp in all samples (Fig. 8 A). In initial screening, kanamycin and hygromycin were used as products of *npt*II and *hpt* genes. Therefore, PCR was performed with primers designed from GUS gene, a common gene between pCAMBIA 1301 and pCAMBIA 2301, and can be visualized as well via histochemical staining. Data revealed that 4 plants out of 26 hygromycin resistant plants and 6 plants out of 310 kanamycin resistant plants were found to be positive for the presence of GUS gene. PCR was repeated 3 times with new DNA extraction from these ten plants to ensure that whether they were true transgenic plants or not. PCR product of 455 bp was amplified in these ten plants every time with no such amplification in non-transgenic cotton genomic DNA (Fig. 8). Histochemical staining revealed that recombinant plasmid was successfully integrated into cotton genome. The young leaves from PCR positive plants were stained dark blue while no color change was observed in non-transgenic control (Fig. 8 C). Results of the GUS staining proved these ten plants as positive transgenic plants. However, the expression of GUS gene was different among plants some were darkly stained and some were stained as light blue. This might be because of different copy numbers of GUS gene in individual plant which will be further confirmed through southern blotting. Thus, selection during germination successfully eliminated progeny lacking the gene of interest and resulted in identification of positive transgenic plants.

Materials and methods

Cotton seeds of Zheda B, an upland cotton (*Gossypium hirsutum*) were sown in polythene bags (40 mm diameter × 100 mm high) filled with nutrient-rich soil and placed on an already watered seed bed. This soil was taken directly from the field and placed in the polyethene bags one week before manual sowing. Each polythene bag contained 2-3 seeds at a depth of 15 mm and covered by a thin layer of fine soil. After sowing, the bags were covered with transparent plastic sheet to keep the soil moist. Three to five days after germination, the bags were uncovered during the day time but re-covered at night to protect the young seedlings from low temperatures. The surface of the soil was kept moist by watering the seed bed at interval of every 2 days. After 7-10 days of seed sowing, only one seedling was allowed to grow in each polythene bag to ensure healthy seedlings before transplanting into the field at 20 days old. Field was characterized by having clay-loam soil, pH 6·5 and organic matter content of 25·4 g/kg. Nitrogen fertilizer (0·12 t/ha) was applied to the experimental plots before transplanting for healthy crop.

Plasmid isolation

Plasmid pCAMBIA1301 and pCAMBIA 2301, having *hpt* and *nptII* as marker gene respectively in addition to GUS reporter gene in both, were used in this study. *E. coli* cells harboring these plasmids were separately grown on LB plates supplemented with 50 mg/L of kanamycin. Single colonies were then picked; sub cultured in 1 mL of liquid LB media at 37 ^{o}C with continuous agitation of about 200 rpm. The presence of targeted constructs was confirmed through PCR using GUS based primer. Positive clones were finally grown in large volume of LB media and plasmid was isolated according to the alkaline lysis method (Sambrook and Russell, 2001). Plasmid concentration was measured using

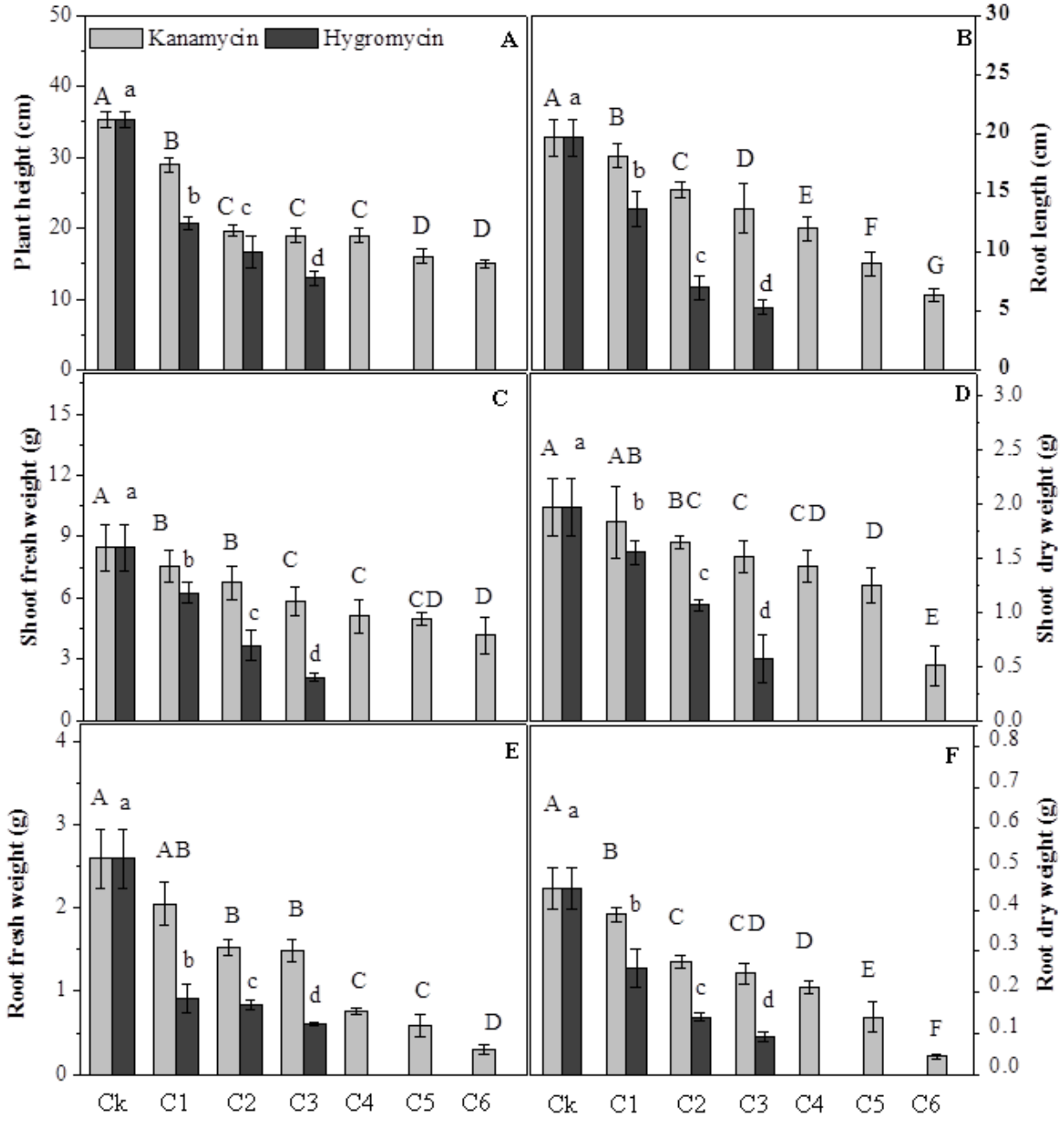

Fig 5. Effect of kanamycin and hygromycin treatment on different growth parameters of survived cotton seedlings. Capital letters show differences among different treatments of kanamycin and control while small letters show differences among different concentrations of hygromycin as compared to control. CK; Control, C1; 100 mg/L kanamycin and 25 mg/L hygromycin, C2; 200 mg/L kanamycin and 50 mg/L hygromycin, C3; 300 mg/L kanamycin and 75 mg/L hygromycin, C4; 400 mg/L kanamycin and 100 mg/L hygromycin, C5; 500 mg/L kanamycin and 125 mg/L hygromycin, C6; 600 mg/L kanamycin and 150 mg/L hygromycin

nano drop and 30 µg/mL dilutions were prepared using double distilled autoclaved water.

Ovarian injection method

Unopened flower buds (2 cm in length) were tied in afternoon with a thread to prevent the entry of foreign pollen and next morning after pollination (identified from pink and withered petals), petals were removed and about 5-7 µL of 30 µg/mL of each plasmid (afore mentioned) was taken in Hamilton syringe. Meanwhile, style was pierced in the center with needle and needle was taken out little to create some space and finally DNA solution taken in the syringe was injected. The treated flowers were labeled and gibberlic acid (GA_3 20 ppm) was sprayed over the injured part of the ovary to prevent flower shedding. More than 800 flowers were injected with each construct i.e. pCAMBIA 1301 and pCAMBIA 2301 and plants were allowed to grow to produce seeds.

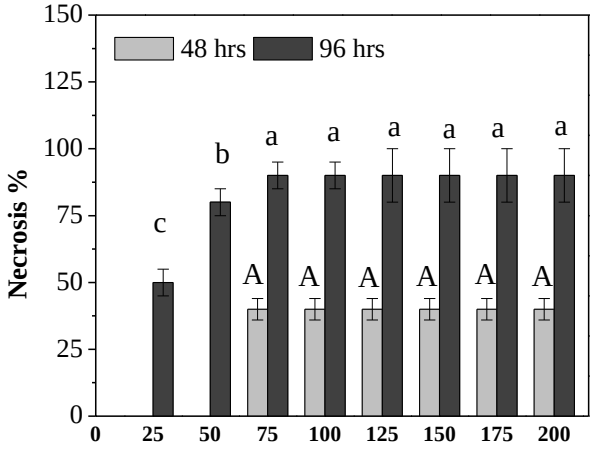

Fig 6. Necrosis % age by different concentration of hygromycin during different time intervals

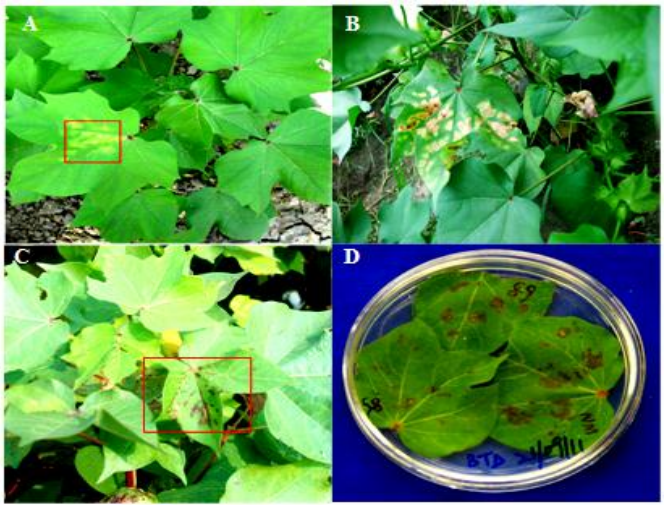

Fig 7. Necrotic patches produced after application of kanamycin (A, B) and hygromycin (C, D) on leaves using leaf painting method. Outlined area show the treated portion, only treated leaves show symptoms while others are green.

Optimization of antibiotics concentration for selection of transgenic plants

To select transgenic cotton plants, a preliminary experiment was conducted using non transgenic cotton to find out the appropriate concentration of antibiotics that can eliminate 70-80% of non transgenic plants. Seeds were delinted with concentrated H_2SO_4, washed well with distilled water 5-6 times and finally blot dried. Water agar (0.7 %) was prepared and autoclaved at 121 ^oC for about 30 min. After autoclaving when temperature fell down up to 60 ^oC, antibiotics were added, mixed well and poured into autoclaved petri plates. A wide range of concentrations labeled as C1-C6 were tested for both kanamycin (100-600 mg/L) and hygromycin (25-150 mg/L) with five replications for individual treatment. About ten delinted seeds were germinated per plate supplemented with different concentration of antibiotic along with control. Seeds which developed root length $\geq$ 1 cm were scored as germinated seeds. After 3-5 days, young seedlings (distinct radical and plumule) were transferred into pots containing peat moss and allowed to grow under natural conditions of light and temperature. Plants were nurtured well with Hoagland solution and harvested after three weeks. Data for seed germination, seedling survival, root length, plant height, seedling fresh/dry weight and root fresh/dry weight were recorded. For the leaf painting screening in field, kanamycin was used at concentration of 500 mg/L while hygromycin concentration was optimized in this study. Leaves from different positions of the plant like top (12 node), middle (8 node), and lower canopy (4 node) were utilized to select best leaf *vs* concentration combination. Different concentrations of hygromycin solution (25-200 mg/L) were prepared and applied on leaf surface using cotton swab in afternoon. Necrotic symptoms were observed after 48 hrs and 96 hrs. For laboratory screening 0.7% water agar was prepared and poured into petri plates and solidified. Leaf from node 12 below apex was detached, labeled similar to plant number in field and kept on these plates (3-4 leaf/ plate). Then 10% of hygromycin solution was applied on these leaves with the help of pippettman in the form of 5-10 drops (each drop 100 µL) on every leaf. Plates were sealed using parafilm and kept in growth camber under 16 h light/ 8 h dark at 26 ^oC and observed after 3 days.

Statistical analysis

Three replicates for each treatment were maintained. The analysis of variance was conducted between different treatment levels for each antibiotic. The significant differences within hygromycin (small letters) and kanamycin (capital letters) concentrations under control and treatments were evaluated by LSD multiple range tests ($p < 0.05$) using the SAS 9.2 statistical software designed by SAS institute, North Carolina. All graphic presentations were generated using Orgin pro 7.5 version.

Screening of putatively transformed plants

About 4000 seeds for each construct were harvested and named as T_1 seeds. Seeds were delinted, sterilized and grown on water agar supplemented with 75 mg/L hygromycin (pCAMBIA 1301) and 500 mg/L of kanamycin (pCAMBIA 2301) for about 3 days. Seedlings were then transferred to pots containing peat moss and grown under glass house conditions. To further eliminate non transgenic seedlings, leaf painting was done using 500 mg/L kanamycin and 50 mg/L hygromycin on 3-5 leaf stage plants. After 5-7 days seedling with necrotic symptoms were removed while remaining seedlings were transferred to field and grown under agronomic conditions mentioned earlier. Total genomic DNA was isolated from young leaves, using the already devised methods (Paterson et al., 1993; Li et al., 2002). To check the amplification of cotton genomic DNA, chloroplast primers given in the Table 1 were used while to identify positive transgenic plants primers against GUS gene were designed using primer 5 software. A total of 20 µL reaction mixture was prepared containing 10 µL Taq mix (Takara), 7 µL PCR water, 0.5 µL forward/reverse primer (20 µM/µL) and 2 µL DNA (30 ng/µL). The PCR profile was adjusted with initial denaturation of 94^oC for 90 sec, 30 cycles of 30 sec at 94 ^oC, followed by annealing at 57 ^oC for 30 sec and at 72 ^oC for 1 min with final extension of about 5 min. The primer sequences are given in the Table 1. Amplified product was eluted from agarose gel and sequenced. Results were blast in NCBI to confirm that the amplified product is part of GUS gene.

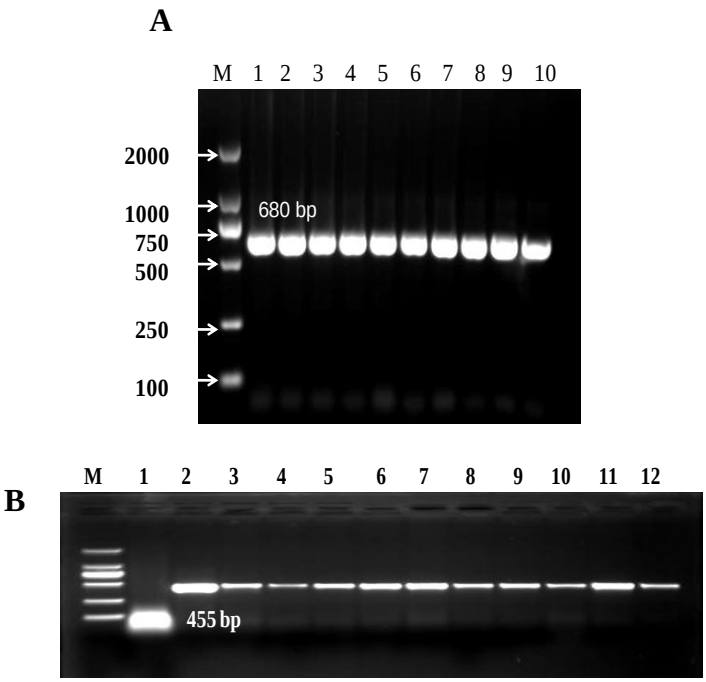

Fig 8. PCR amplification of genomic DNA using chloroplast primers (A) and GUS gene (B) of the recombinant plasmid. M; Marker- DL 2000, Lane 1; Non transgenic DNA, Lane 2; Transgenic DNA, Lane 3-12 (transgenic plants). Histochemical staining (C) showing activity of glucuronidase in transgenic leaves.

GUS Histochemical assay

Young leaves were collected from PCR positive plants of T_1 generation in 2 mL microfuge tubes containing pre chilled 90 % acetone and kept at room temperature for about 1h. This treatment not only pre fixed the leaf tissue but also removed chlorophyll. Leaves were then rinsed with distilled water, completely dipped in GUS staining solution (Jefferson et al., 1987), and incubated at 37 ^{o}C in dark. After 8-10 hours leaves were washed with a series of ethanol (25%, 50%, 70% and 95%) at continuous low agitation until the blue color become visible.

Conclusion

This paper presented various approaches using a wide range kanamycin (100-600mg/L) and hygromycin (25-150 mg/L) for the selection of transgenic plants at different developmental stages and revealed that upland cotton is more sensitive to the application of hygromycin. The hygromycin application exhibited higher magnitude of reduction for seed germination, seedling survival and other growth parameters as compared to kanamycin application Therefore, in designing vector constructs for pollen tube mediated pathway transformation, *hpt*II gene (hygromycin resistance) can be a preferred choice as a marker gene than *npt*II (kanamycin resistance). Similarly utilization of 0.7 % water agar media is a best substitution of MS media and can be utilized in less developed laboratories. The current methodology will deepen the knowledge for cotton transformation and accelerate the screening of the transgenic plants.

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