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Transformation of Insect and Herbicide Resistance Genes in Cotton (*Gossypium hirsutum* L.)

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ABSTRACT

Manipulation of different genes in crop plants to get desirable characters has become an important tool of plant biotechnology. In the current study, cotton variety MNH-786 was modified for its characteristics to show resistance against lepidopteran insects and herbicide by transformation of *Cry1Ac+Cry2A* and *GTGene* cloned in a different cassette under 35S Promoter. Mature embryos of cotton MNH-786 were injured by a sharp blade at the shoot apex and infected with the *Agrobacterim tumefaciens* harboring transgene constructs. Transformed cotton plants were successfully acclimatized in pots and later the green house. Gene specific PCR and ELISA confirmed the transgene presence and its protein expression which was considerably higher in transformed plants. Overall transformation efficiency was 1.05%. All larvae of *Helicoverpa armigera* feeding on transgenic cotton leaves of T_0 were found dead as compared to the control ones feeding on leaves from non-transgenic cotton. Transgenic plants also survived a glyphosate spray dose of 1,900 ml acre^{-1} as compared to herbs/weeds growing along with them, which burned completely five days post glyphosate application.

Keywords: Bt cotton, Glyphosate, Transgenic, Weedicide resistance.

INTRODUCTION

Cotton is the essential non-food cash crop and a major source of foreign exchange remunerations in Pakistan. It accounts for 8.2 percent of the value added in agriculture and about 2 percent to GDP (Khan and Khan, 2003; Batool *et al*., 2010). In Pakistan, cotton crop provides source of revenue to millions of people, but its production is gradually becoming expensive due to high water consumption, use of expensive pesticides, insecticides, and fertilizer and yield losses due to weeds. It has also been estimated that 14% losses in total agriculture occurs due to insect pests, of which 84% is in cotton (Shahid *et al*., 2012), 37% yield losses occur due to weeds while remaining 34% losses occur due to insects 11% due to micro-organisms and virus while 16-18% due to pathogens (Farooq et al., 2011; Oerke, 2006 and Orke *et al.* 2004). In the past, insects of cotton were controlled by spraying expensive pesticides, while weeds were controlled through manual hoeing. The heavy use of pesticide may result in import of pesticide of 81015498/- USD (Khooharo et.al 2008), and manual hoeing may result in labor intensive work along with yield losses up to 25% (Khan and Khan, 2003) due to delayed weed removal. Manual weeding is also expensive as it is time consuming and labour intensive (Cheema *et al*., 2005). Also, total weed seed numbers in the soil seed bank have been shown to increase significantly after changing from conventional chemical weed control to non-chemical means (Bond and Grundy, 2001). Hence, the levels of input required using non-chemical methods are

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not sustainable or economically viable. Combinations of mechanical weed control techniques fail to provide complete weed control (Bale *et al*., 2008), and also risk inflicting damage to the crop because the crop plants are usually small at the time when weeds are most vulnerable to these methods.

The advent of biotechnology offers an alternative to the weed control methods presently used in cotton. Glyphosate (N-phosphonomethylglycine) is a broad-spectrum systemic herbicide used to kill weeds, especially annual broadleaf weeds and grasses known to compete with commercial crops grown around the Globe. Glyphosate interferes in the shikimate metabolic pathway by inhibiting the synthesis of 5-enolpyrovyle 3-phosphoshikimate (EPSPS). It prevents the synthesis of three aromatic amino acids including tryptophan, phenylalanine, and tyrosine (Yamada *et al*., 2009). It has been reported that glyphosate causes the fruit (boll) abscission in cotton which ultimately reduces the yield of cotton (Monks *et al*., 2007). In these days, many ways have been devised to develop resistance in high yielding varieties of cotton against insect pests and herbicides. Conventional plant breeding has become an old discipline which takes plenty of time to produce resistant varieties. Genetic engineering is a breeding strategy that assures to avoid the problems associated with the transfer of large blocks of genetic materials between two parents (Bajaj, 1998) . The present study aimed to transform genes that had resistance against the lepidopteran insects' pests (Cry1Ac+Cry2A) and Glyphosate (GTG) in the cotton variety MNH-786 (due to its susceptibility to insect pests) through *A. tumifaciens.* A similar type of study was done by Nasir *et al*. (2014) by transformation of double *Bt* genes along with *GTGene* in sugarcane and they concluded that expression of these three exogenous genes result in development of insect- as well as weedicide-resistance in sugarcane.

MATERIALS AND METHODS

Plant Material

Cotton seeds (*G. hirsutum* var.MNH-786) were collected from the Cotton Research Institute (CCRI) Purana Shujabad Rd, Multan 60000. Seeds were delinted with 100% H_2SO_4, sterilized with 5% (W/V) $HgCl_2$ and 10% (w/v) SDS followed by 5-6 washings with autoclaved distilled water (Rao *et al*., 2013). Subsequently, seeds were allowed to germinate by incubating them at 30ºC for 48 hours. The germination index of cotton variety MNH-786 was calculated by counting germinated and un-germinated seeds of each experiment and dividing the number of germinated seed by total number of seeds and multiplied by hundred.

Agrobacterium-Mediated Transformation of Cotton with *Cry1Ac+Cry2A* and *CP4 EPSPSgene* Constructs

Glyphosate-tolerant lines of cotton have been produced using *Agrobacterium*-mediated transformation of mature embryos with *Cry1Ac+Cry2A* and *CP4 EPSPS* gene as done by Rao *et al.* (2009). Two constructs were used, all containing CaMV 35S constitutivepromoter NOS terminator as shown in Figure 1. After two months on MS medium with kanamycin selection, putative transgenic plants were shifted in shoot regeneration media (kinetin) and root regeneration media [1-Naphthaleneacetic acid (NAA) and Indole-3-butyric acid] without kanamycin, as described before (Rao *et al*., 2009).

Genomic DNA Isolation and Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from putative transgenic plants and purified from the immature leaves of putative transgenic

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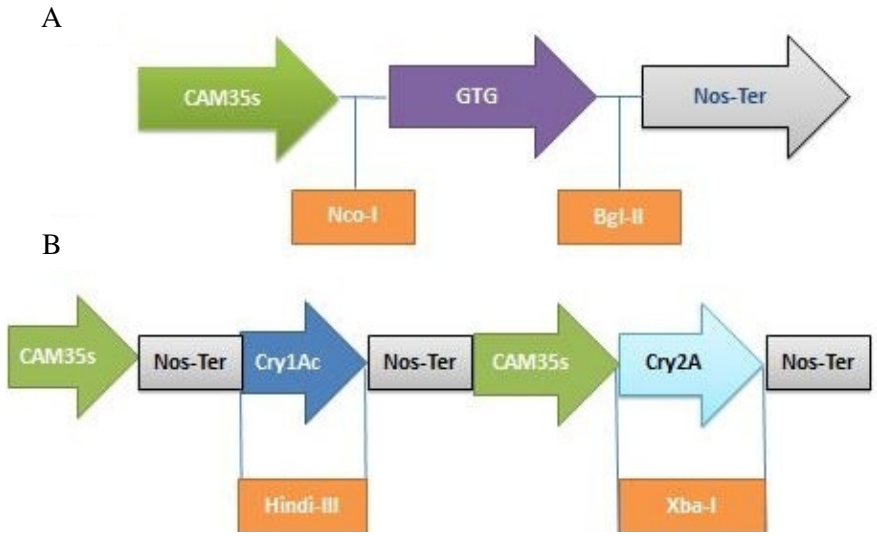

Figure 1. (A) Constructs map showing *GTGene* cloned by using NCoI and Bgl-II restriction sties while (B) *Cry1Ac* was cloned by using hindi-III and *Cry2A* gene was cloned by using XbaI restriction enzyme.

cotton plants using the protocol described by Lenin *et al*. (2001). PCR was done with the reaction mixture of 20 µL containing 1X PCR Buffer with 2.5 mM $MgCl_2$, 300 ng DNA template, 1 mM dNTPs,1 pico mole primers (forward and reverse; sequence given as Table 1), 2.5 U Taq DNA Polymerase. PCR was carried out in a thermo cycler according to following conditions, initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing at 51ºC for (Cry1Ac) for 1 minute followed by extension at 72ºC for 3 minutes. Final extension was done at 72ºC for 5 minutes. PCR amplified products of *Cry2A* and *GTGene* having sizes of 565 and 600 bp respectively were resolved onto 1% (w/v) agarose gel, stained with ethidium bromide and visualized by gel doc system.

Protein Expression Analysis

Expression analysis of *Cry1Ac, Cry2A* and *GTGene* was done by ELISA assay using Envirologix Kit (Cat# 051) 500 Riverside Industrial Parkway Portland, Maine, 04103-1486 USA. Plant samples were ground to fine powder in liquid nitrogen and 600 µL of protein extraction buffer (0.5M EDTA, 10% Glycerol, 5M NaCl, 2M Tris-Cl, 150 mM NH_4Cl, 1.0 mM PMSF, 0.05% DTT) was added followed by 1 hour incubation on ice, centrifuged at 13,000 rpm for 25 minutes. Supernatant was used for further analysis. ELISA was performed according to the instruction provided in kit manual as done by Kiani *et al*. (2013) and .

Table1. Primer sequences used in the study.

Primer Name	Sequence (5'-3')	Product Size
Cry2A-F	*AGATTACCCCAGTTCCAGAT*	600 bp
Cry2A-R	*GTTCCCGAAGGACTTTCTAT*	
GTG-F	*CCCTGGTGACAAGTCCATCT*	358 bp
GTG-R	*CTGCACACCCATCTCTCTGA*	
Cry1Ac-F	*ACAGAAGACCCTTCAATATC*	565 bp
Cry1Ac-R	*GTTACCGAGTGAAGATGTAA*	

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Transgenic Cotton Plants Resistance against Insect Resistance/Bio-toxicity Assay

To check the efficacy of *Cry1Ac* and *Cry2A* endotoxins against targeted insect pests, laboratory bio-toxicity assays of cotton leaves with *H. armigera* larvae (2nd instar) were conducted. Five leaves from upper, middle, and lower portion of the cotton plants (transformed and control, non-transgenic) were detached in three times i.e. after 30, 60, and 90 days of crop age. The leaves were placed in petriplates having moist filter paper pieces. Subsequently, the second instars larvae of *H. armigera* were fed on them. After 3 days, leaves were observed for leaf damage and insect mortality.

Glyphosate Spray Assay

To check the effectiveness of GTG transgene in cotton, Glyphosate spray was applied on acclimatized transgenic cotton plants (in three repeats) in a greenhouse at 35±5°C. Herbicide Glyphosate is commercially available as Roundup™®. It was prepared in water at a final concentration of 1,900 ml 80 L^{-1}. Prepared solution was applied (1,900 ml ha^{-1}) to field grown transgenic cotton plants along with the control non-transgenic cotton plants. The plants with production of aromatic amino acid would survive while others which failed to produce aromatic amino acid would die.

RESULTS

Transformation of *G. hirsutum* MNH-786 and Confirmation of Putative Transgenic Plants

Germination index of MNH-786 cotton variety was found to be 66.6% as shown in Figure 2.Total of 50-plantlets were obtained after two months selection of putative transgenic plants on kanamycin and the transformation efficiency was calculated to be 1.05% (Table 2). Amplification of *CEMB-GTGene* at 358 bp as shown in Figure 3 in transformed cotton plants confirmed the presence of Glyphosate gene in them. Amplification results of *Cry1Ac*

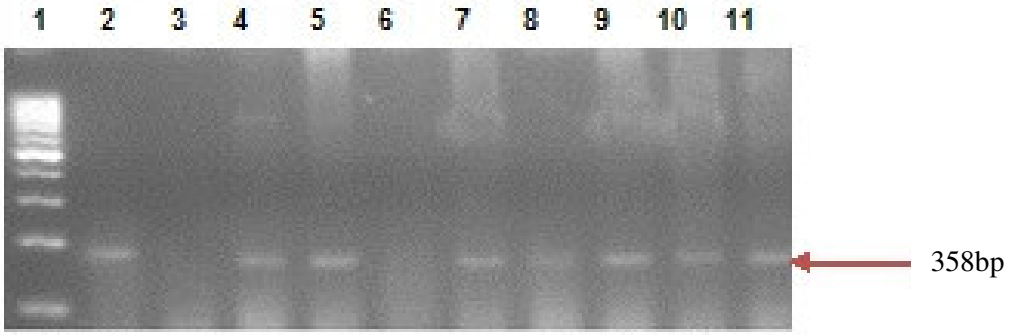

Figure 2. PCR Amplification of *Cry1Ac* genes in putative transformed plants. (Lane 1) 1 kb molecular weight marker; (Lane 2) Plasmid was used as positive control; (Lane 3) Non-transgenic plant was used as negative control, and (Lane 4-11) Putative transformed plants.

Table 2. Efficiency of transformation. Total no. of 4,745 embryos were isolated for transformation out of which 50 developed into positive plants transformation efficiency was calculated by dividing positive plants to total no of isolated embryos and multiplied by 100.

No. of embryos isolated		Plants obtained after 8 weeks		Transformation efficiency	
Control	experimental	Control	experimental	Control	Experimental
100	4745	86	50	86%	1.05%

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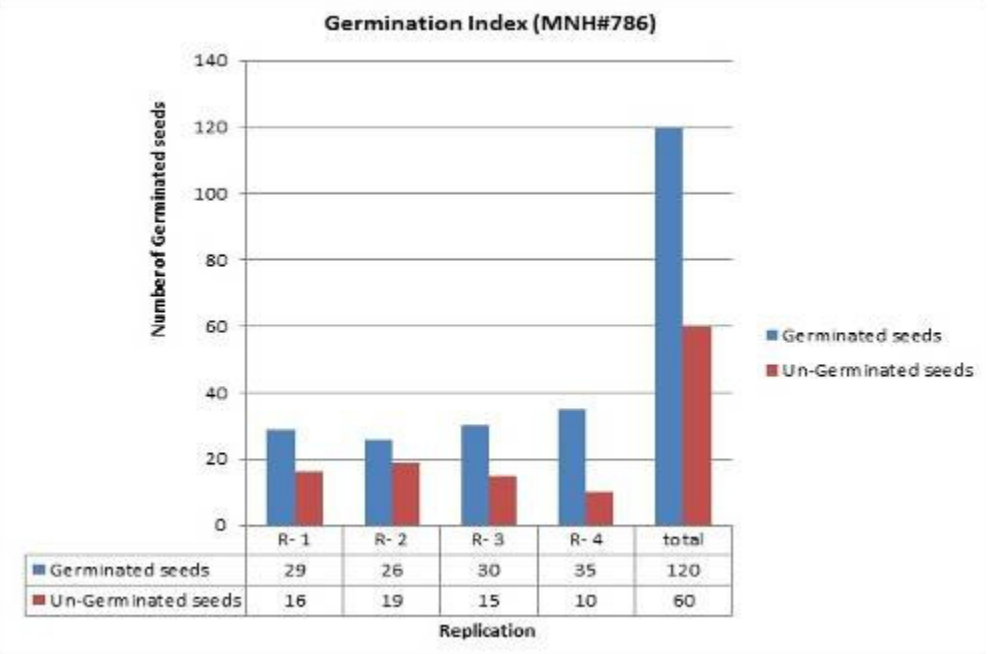

Figure 3: Germination Index of MNH-786 was calculated in three replicates in each replicate total germinated seeds were divided by total no of seeds and multiplied with 100.

and *Cry2A* at 565 and 600 bp for *Cry1Ac* and *Cry2A* genes, respectively, was revealed as shown in Figures 4 and 5. It was also found that some transformed plants (V4, V5, V7, V8, V9, V10 and V11) were PCR positive not only for *GTGene* but also for *CEMB-Bt* genes, as both constructs were separately electroporated in Agrobacterium as shown in Table 3. No amplification was detected in negative control. PCR positive transformed cotton plants were further confirmed for transgene specific protein expression. ELISA with GTG and Bt proteins with specific antibodies was done whose results demonstrated and confirmed PCR results. Quantification of GTGene and Bt proteins was done and represented Figure 6. These results counter confirmed PCR data, where similar transformed cotton plantlets showed PCR amplification for all three transgenes. One cotton plant (V6) was observed to be non-transgenic while all other experimental plants were transgenic and were successfully acclimatized in the field.

Insect Bioassays

Larvae of American cotton bollworm (*H. armigera*) were fed on transgenic and non-transgenic cotton leaves. All *H. armigera* larvae feeding transgenic cotton leaves that have Bt/Cry toxin proteins expressed in them were found dead, whereas larvae feeding on non-transgenic control cotton leaves were alive and remained healthy as shown in Figure 7. Mortality rate of feeding larvae was 100%, concluding that all tested transgenic cotton plants in T_0 generation exhibited strong anti-pest activity.

Evaluation of Herbicide Resistance in Transgenic Cotton Plants

Field grown transgenic cotton plants in T_0 generation were evaluated for their resistance against glyphosate, a commonly used weedicide in cotton field. Three-month-old transgenic cotton plants were

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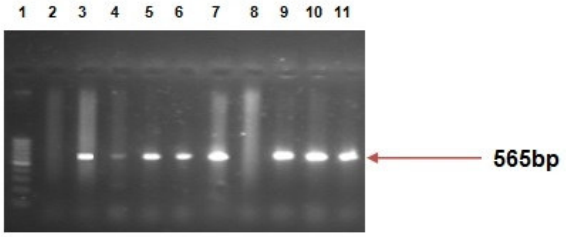

Figure 4. PCR Amplification of *Cry1Ac* genes in putative transformed plants. (Lane 1) 100 bp molecular weight marker; (Lane 2) Non-transgenic plant was used as negative control; (Lane 3) Plasmid was used as positive control, and (Lane 4-11) Putative transformed plants.

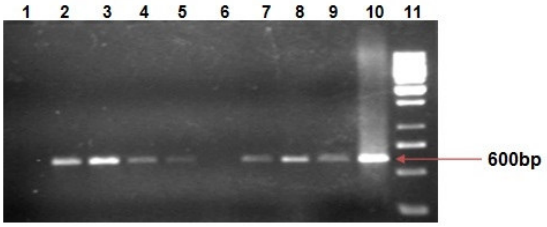

Figure 5. Amplification of *Cry2A* gene by PCR in putative transformed plants. (Lane1) Non transgenic plant as negative control; (Lane 2) Plasmid as positive control; (Lane 3-10) Putative transformed plants, and (Lane 11) 1 kb molecular weight marker.

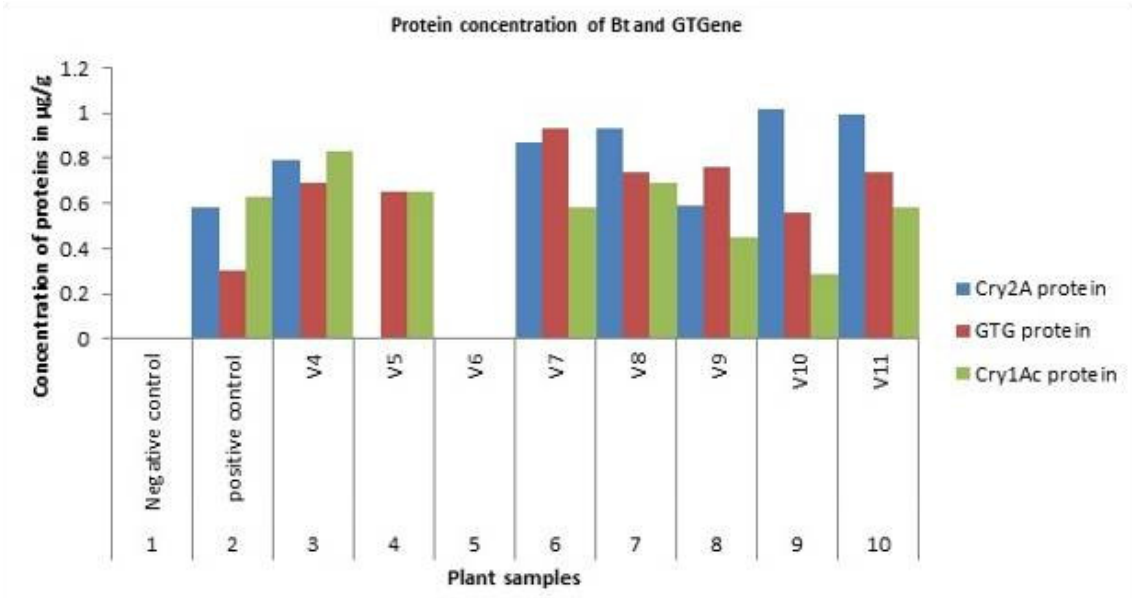

Figure 6. Graphical representation of Quantification of GTG and Bt Proteins. Blue bars shows the concentration of *Cry1Ac*, Red shows the concentration of *GTGene* and Green bars shows the concentration of Cry2A protein in µg g^{-1} of the leaves.

Table 3. Summary of PCR results. Putative transgenic plants were confirmed by using PCR amplification plants named as P_{786} (V4), P_{786} (V5), P_{786} (V7), P_{786} (V8), P_{786} (V9), P_{786}(V10) and P_{786} (V11) were found positive for *GTGene*, *Cry1Ac* and *Cry2A* while plant P_{786} (V6) was PCR negative.

		PCR results		
Plant no.	Plant name	*GTG*	*Cry1Ac*	*Cry2A*
1	P_{786} (V4)	Positive	Positive	Positive
2	P_{786} (V5)	Positive	Positive	Positive
3	P_{786} (V6)	Negative	Negative	Negative
4	P_{786} (V7)	Positive	Positive	Positive
5	P_{786} (V8)	Positive	Positive	Positive
6	P_{786} (V9)	Positive	Positive	Positive
7	P_{786} (V10)	Positive	Positive	Positive
8	P_{786} (V11)	Positive	Positive	Positive

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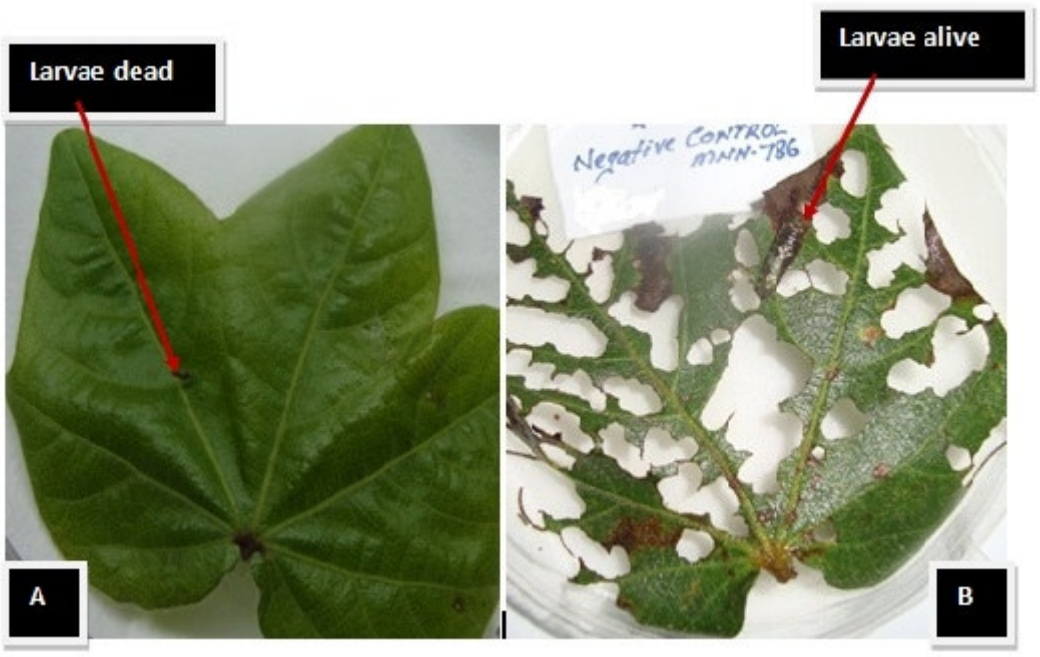

Figure 7. Bioassay of transgenic and control plant leaves. *Helicoverpa* was fed on both the control and non -transgenic cotton leaves and were observed after 3 days. A. In case of transgenic plant leaf and larva died after eating a small portion of leaf, while in non-transgenic control larva remained alive. (A) A transgenic plant leaf and larva is killed after eating a small portion of leaf, (B) A control non-transgenic plant. Larva ate it and is alive.

sprayed with Glyphosate at a final concentration of 1,900 ml acre^{-1}. At the time of glyphosate assay, transgenic cotton field was shown to be full of weeds/herbs as shown in Figure 8-A. Toxic effects of glyphosate became visible on weeds three days post herbicide application, as can be clearly seen in Figure 8-B. These symptoms include plant wilting, drooping and slight burning spot appearance. Also, weeds were dead completely five days post glyphosate application as shown in Figure 8-C as compared to transgenic cotton plants which remained healthy and no visible symptoms of any kind of toxicity were observed. Conclusively, developed transgenic cotton plants were weedicide/herbicide and insect resistant while control plants along with weeds could not withstand glyphosate and were dead five days after spray.

DISCUSSION

Cotton is not only an important cash crop of Pakistan but also a significant segment of agricultural profile. Cotton generates 30% of foreign exchange earnings for Pakistan by contributing approximately 80% raw material to textile industry. In this particular study, an effort was made to improve the agronomic characteristics of this high input crop through the development of insect and herbicide resistant cotton plants.

In the present study, transformation of double *Bt* genes and *GTGene* was done by shoot apex cut method as done by Rao *et al*. (2011). Total of 4745 embryos were inoculated with the *Agrobacterium* strain LBA4404 containing gene of interest. Out of these 4,745 inoculated embryos, 560 transformed plants were obtained, but only 50 plants survived in the field after

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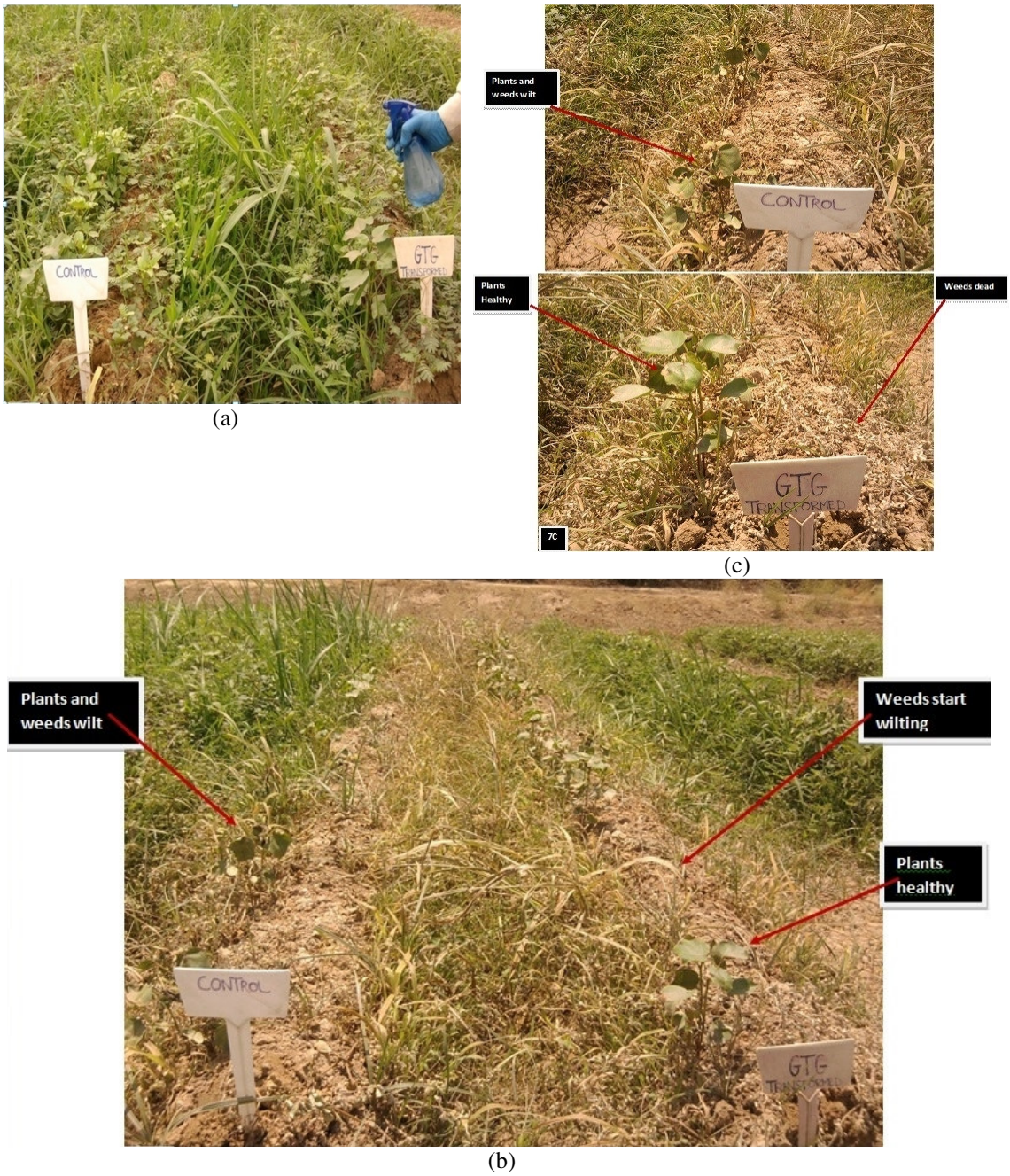

Figure 8. Glyphosate spray on transgenic plants (a) Glyphosate (1,900 ml acer^{-1}) spray in the field of GTG positive transgenic plant (b) Field 3 days after spray (c) After five days weeds and control plants were dead while transgenic plants were healthy. It shows the presence of *GTGene* in cotton plants. Presence of weeds in the field along with the control and transgenic plants. Glyphosate was being sprayed.

acclimatization. The transformation efficiency was recorded to be 1.05% which was similar as obtained by Rao *et al*. (2011). Insects including bollworms are major pests of cotton causing significant yield losses. Insects not only decrease crop productivity but also their extensive uses have deleterious consequences/impacts towards human

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health, environmental quality, and biodiversity. For the sake of sustainable agriculture, environmentally friendly approaches to manage insects have to be developed through the use of biotechnology. The most effective and the cheapest method is to develop 'insect resistance' in transgenic crops. CEMB has isolated genes that code potential Cry toxin proteins from a soil bacterium '*Bacillus thuringenesis*' (Bt) that proved to be lethal for targeting insects. Transformation of double *Bt* genes, namely, *Cry1Ac* and *Cry2A* in *G.hirsutum* was done as done by Bakhsh *et al*. (2012) and developed insect resistant transgenic crop of cotton. The transgenic plants were confirmed by using PCR analysis (Khan *et al*., 2013b, Akhtar *et al.* 2014) and ELISA as done by Khan *et al*. (2013a). Both transformed genes differ in their sequence of amino acid and insecticidal activity.

Modern agriculture is supported by the use of herbicide treatment that enhances the crop production cost effectively. Weeds compete with cultivated crop for water, light and available nutrients (Tauseef *et al*., 2012). Although herbicide applications improve crop yield by breaking the competition, their extensive use destabilizes the soil ecosystem and nitrification cycles. A transgenic cotton resistant to herbicide Glyphosate, which inhibited the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSPS) synthase, a key catalyst in the production of aromatic amino acids, was developed. Glyphosate gene was expressed in transgenic cotton plants under the control of CaMV 35S, a strong constitutive promoter as done by Nasir *et al*. (2014) The results of ELISA of *Bt* and *GTGene* in transgenic cotton plants confirmed their successful expression. The principal motivation in developing glyphosate resistance in cotton is related to the broad spectrum activity of the herbicide and it is relatively low level of environmental contamination. In the present study, the transgenic cotton plants harboring *CEMB GTGene* have shown survival after the spray of Glyphosate at the rate of 1,900 ml $acre^{-1}$, which was more than the previously reported rate of 1,600 ml $acre^{-1}$ as recommended by Monsanto, 2011.

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تاریختی ژن مقاومت به حشرات و علف کش ها در پنبه (*Gossypium hirsutum* L.)

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چکیده

برای به دست آوردن صفات مناسب در گیاهان دستکاری ژن ها ابزاری مهم در فناوری گیاهان به شمار می رود. در این پژوهش رقم پنبه MNH-786 با ترا ریختیCry1Ac+Cry2A و GTGene همسانه شده در یک کاست متفاوت تحت راه انداز (پروموتر) 35S برای ویژگی های مقاومت در برابر حشرات بال پولکدار(لپیدوپترا) و

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علف کش ها تغییر داده شد. به این منظور، جنین های بالغ پنبه MNH786 با تیغه تیزی در نوک ساقه بریده شد و با اگروباکتریوم *Agrobacterim tumefacien* حامل ساختار ژن تراریخت تلقیح شد. گیاهان پنبه تراریخت شده با موفقیت در گلدان ها و سپس در محیط گلخانه خو گرفتند. پی.سی.آر. و الایزای ژن ویژه حضور ژن تراریخت و بیان پروتئین آن را که در گیاهان تراریخت بسیار بیشتر بود تایید کرد. کارایی کلی فرایند تراریختی برابر ۱/۰۵٪ بود. بر خلاف لاروهای شاهد که از برگ پنبه بدون تراریختی تغذیه می کردند، لاروهای *Heliothis armigera* که روی برگ پنبه تراریخت شده T_0 تغذیه کرده بودند ، مردند. همچنین، بعد از پاشش glyphosate به مقدار $1,900\ ml\ acre^{-1}$، بوته های تراریختی همچنان به رشد ادامه دادند در حالی که علف ها و دیگر گیاهانی که کنار آنها بودند پنج روز بعد از پاشش این ماده به کلی نابود شدند.

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