INTRODUCTION

Cotton is the backbone of economy and employment in Pakistan. The sector employs about 40% of total force and earns approximately US $ 15 billion (Vogel, 2014). Nearly 45-60% of foreign exchange has been procured due to production and consumption of cotton (Batool et al., 2010; Khan et al., 2013; Khan and Khan, 2003). Pakistan being fourth largest producer of cotton suffered 14% of the losses in total agriculture due to insect pest of which 84% is in cotton (Shahid et al., 2012; Awan et al., 2015; Bale et al., 2008), 25% yield losses occur due to weeds (Khan and Khan, 2003) while remaining 30% losses occur due to CLCuV (Farooq et al., 2011) and 25% by abiotic factors. Initially, pre-sowing applications are applied to eliminate weeds already present and those that will appear before the crop (Farroq and Cheema, 2014).

Manual hoeing was the only reliable alternative for getting rid of weeds in the past, but it was time consuming and labour intensive (Cheema et al., 2005). Total weed seed numbers in the soil was found to rise significantly after shifting from conventional chemical weed control to non-chemical means (Bond and Grundy, 2001). Hence, total effort required using non-chemical methods are not viable or economically sustainable. Biotechnology provides a tool through which, this can happen easily compared with conventional method. Glyphosate (N'- phosphonomethylglycine) belongs to herbicide group ‘glycines’ which is a broad-spectrum universal herbicide used to kill annual broad leaf weeds and grasses which are known to compete with commercial crops grown around the World. Glyphosate affects in the shikimate metabolic pathway by preventing the synthesis of 5-enolpyruvyl-3-phosphoshikimate (EPSPS). It inhibits the synthesis of three aromatic amino acids including tryptophan, phenylalanine and trypsin (Yamada et al., 2009).

Resistance against insect pests and herbicide has been attained in high yield varieties by different ways now-a-days (Khan et al., 2014). Conventional breeding though necessary but takes much time to attain main objective. Genetic engineering is also a modern way of breeding that guarantees to avoid the problems related with the transfer of large blocks of genetic materials between two parents (Bajaj, 1998).

Genetic transformation of plants methodologies have been developed to upturn the efficiency of transformation and to
achieve stable expression of trans-genes in plants. In the present study, genes (Cry1Ac+Cry2A) and Glyphosate (GTG) that have resistance against the insect pests and weeds respectively were transformed in the cotton variety FBS-37 through Agrobacterium tumefaciens.

MATERIALS AND METHODS

Plant material: Cotton variety FBS-37 was selected for transformation of Cry1Ac+Cry2A genes along with cp4EPSPS genes because of its high germination. The seeds of cotton variety FBS-37 were collected from four brothers research station Multan. Delinting of cotton seeds was done by using concentrated H$_2$SO$_4$ while sterilization with 5% (W/V) HgCl$_2$ and 10% (w/v) SDS followed by 5-6 washings with autoclaved distilled water. Seeds were allowed to germinate by incubation at 30°C for 48 hours.

Transformation of cotton variety FBS-37 with Cry1Ac +Cry2A and CP4EPSPS gene: Transformation of Cry1Ac +Cry2A and cp4 EPSPS gene in cotton variety FBS-37 was done by following the method as done by (Rao et al., 2011). Two constructs containing CaMV 35S constitutive promoter and Nos terminator were used for transformation of cotton. Screening of putative transgenic plants was done on kanamycin at the rate (50mg/ml) medium for two months, putative transgenic plants were shifted to selection free medium for shoot and root formation, as determined by Rao et al. (2009).

Isolation of DNA from putative transgenic cotton plants and confirmation through PCR: Isolation of DNA from screened cotton plants was done by taking the fresh leaves and following the protocol described by (Lenin et al., 2001). Reaction mixture for PCR was prepared by using 10X PCR Buffer (2µl) with 2.5mM MgCl$_2$ (2µl), 300ng DNA template (1.5µl), 1mM dNTPs (2µl), 10 pmicrome primers (2µl) (forward and reverse) and 2.5U Taq DNA Polymerase for total volume of 20µl. The reaction was proceeded in ABI 9700 thermocycler having following conditions, initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 51°C for Cry2A and GTGene while 50°C for Cry1Ac for 1 min followed by extension at 72°C for 3 min. Final extension was done at 72°C for 5 minutes. PCR amplified products were resolved on 1 % (w/v) agarose gel and visualized by ethidium bromide staining.

Enzyme Linked Immuno Sorbent Assay (ELISA) of transgenic cotton plants: Cry1Ac, Cry2A and GTGene expression was evaluated through Enzyme Linked Immuno Sorbent Assay (ELISA) by using Envirologix Kit (Cat # 051). One gram leaves samples of transgenic cotton plants were taken in liquid nitrogen and ground to fine powder in pestle morator. 600 µl of protein extraction buffer (0.5M EDTA, Glycerol, 5M NaCl, 2M Tris-Cl, NH4Cl, PMSF, DTT) was added in the fine powder followed by incubation of 1hr on ice and centrifugation at 13000 rpm for 25 min.

Evaluation of Cry1Ac and Cry2A Toxicity through leaf bioassay: The transgenic cotton plants leaves were subjected to 2nd instar larvae of Helicoverpa armigera. Total five leaves from upper, middle and lower part of the transgenic cotton plants of 30, 60 and 90 day old (transformed and control, non-transgenic) were taken. The leaves were placed on moist filter paper present in petri plates. 2nd instar larvae of Heliothis were placed on the leaves for 2-3 days and their feeding was recorded. Insect mortality data was collected after 2-3 days feeding of insect on both transgenic and control plants.

Evaluation of herbicide tolerance of transgenic cotton plants through glyphosate spray assay: Total 1600ml/acre Glyphosate was applied to transgenic cotton plants in comparison to control. Herbicide Glyphosate is commercially available as Roundup™. Glyphosate was prepared up to final concentration of 1600ml/80L by dissolving it in water. Mixture was applied (1600ml/acre) to field grown transgenic cotton plants along with control non-transgenic cotton plants.

Table 1. Primer sequences used in the study:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry2A-F</td>
<td>AGATTACCCCAAGTCCAGAT</td>
<td>600bp</td>
</tr>
<tr>
<td>Cry2A-R</td>
<td>GTTCCCGAGCCGACTTCTAT</td>
<td>358bp</td>
</tr>
<tr>
<td>GTG-F</td>
<td>CCTGTTGACAAAGTCCATCT</td>
<td>565bp</td>
</tr>
<tr>
<td>GTG-R</td>
<td>CTGCACACCATCTCTCTGA</td>
<td></td>
</tr>
<tr>
<td>Cry1Ac-F</td>
<td>ACAGAAGACCTTCAATATC</td>
<td></td>
</tr>
<tr>
<td>Cry1Ac-R</td>
<td>GTTACCGAGTGATGTTAA</td>
<td></td>
</tr>
</tbody>
</table>

RESULTS

Genetic transformation of cotton variety FBS-37: Cotton variety FBS-37germination index was found to be 66.6%. Genetic transformation of cotton variety FBS-37 resulted in the development of 50 plantlets on selection medium after two month of screening through kanamycin. The overall transformation efficiency was found to be 1.05%.

Evaluation of putative transgenic cotton plants through molecular analysis: Putative transgenic cotton plants were evaluated for (cp4EPSPS) GTGene through PCR. 190bp fragment was amplified for GTGene by using gene specific primers Figure 1. Similarly, 1000bp fragment was amplified for each of Cry1Ac and Cry2A as shown in Figure 2 and 3, respectively. Transgenic cotton plants namely V4, V5, V7, V8, V9, V10 and V11 were confirmed positive not only for GTGene but also for CEMB- double Bt genes while no amplification was seen in negative control Figure 2, 3 and 4. The results of ELISA by using Envirologix kit clearly demonstrate successful expression of GTGene, Cry1Ac and Cry2A gene, respectively. Quantification of Cry1Ac, Cry2A
and GTG protein through ELISA determined maximum of 0.8µg/g, 1µg/g and 0.9µg/g of tissue, respectively (Fig. 4).

Figure 1. Amplification of cpEPSPS gene in transgenic cotton plants. Lane 1 1kb plus DNA Ladder, Lane 2-8 Transgenic cotton plants (V4, V5, V7, V8, V9, V10 and V11), Lane 9. Positive control plasmid, Lane 10. Negative control non transgenic cotton plant

Figure 2. Amplification of Cry1Ac gene in transgenic cotton plants. Lane 1 1kb plus DNA ladder, Lane 2-8 transgenic cotton plants (V4, V5, V7, V8, V9, V10 and V11). Lane 9. Positive control plasmid, Lane 10. Negative control non transgenic cotton plant

Figure 3. Amplification of Cry2A gene in transgenic cotton plants. Lane 1 1kb plus DNA Ladder, Lane 2. Negative control non transgenic cotton plant, Lane 3. Positive control plasmid, Lane 4-10

Transgenic cotton plants (V4, V5, V7, V8, V9, V10 and V11)

Figure 4. Graphical representation of quantification of GTG and Bt proteins.

Determination of efficacy of Cry1Ac and Cry2A through leaf bioassay of insects: Mortality percentage of Heliothis armigera 2nd larvae was found variable after 30, 60 and 90 days due to variation in level of expression at different time of growth of plants. Both dead and alive larvae were counted in each petri plate. Mortality 90-100% of larvae was found in larvae fed on transgenic plant leaves while no mortality was found in larvae fed on leaves of non-transgenic control plants and insects remain healthy and also advances their cycle to next instar (Fig. 5).

Figure 5. Bioassay of transgenic and control plant leaves. 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.

Determination of herbicide resistance of transgenic cotton plants through glyphosate spray assay: Transgenic cotton plants were sown in field containment and no application of weed removal was applied until 3 months. After three months when cotton field was full of different kinds of weeds glyphosate spray at the rate of 1600ml/acre was applied. The necrotic effect of glyphosate became visible after 3-5 days post herbicide application (Fig. 6A, B). Symptoms which appear comprised of wilting, drooping and slight burning spot appearance and ultimate death of all
transgenic cotton plants (Fig. 6B).

**Figure 6A. Field spray assay: Presence of weeds in field along with control and transgenic plants. Glyphosate was being sprayed.**

**Figure 6B. After five days weeds and control plants were dead while transgenic plants were healthy. It shows the presence of GTGene in cotton plants.**

**DISCUSSION**

Cotton plays very significant role in economy of Pakistan by generating 30% of foreign exchange and contributing 80% of raw material to textile industry. Despite of its importance cotton is being harmed by attack of different insect pests and weeds. Total 20% yield losses in cotton occur due to insect pests (Gruere et al., 2011) while 25% of yield losses in cotton occur due to weeds (Khan et al., 2003; Monks et al., 2007;). To overcome such losses an attempt was made in current study to develop insect and herbicide resistant cotton plants by introduction of Cry1Ac +Cry2A as done by Muzaffar et al. (2015) and cp4EPSPS gene as done by Awan et al. (2015).

Transformation of double Bt and GTGene was done by shoot apex cut method as done by Rao et al. (2011). Transformation efficiency of experiments was calculated to be 1.05% which is almost similar to the results obtained by Bakhsh et al. (2012); Rao et al. (2011). Amplification of 190bp fragment for GTGene and 1000bp for each of Cry1Ac +Cry2A confirmed successful transformation of three gene in cotton variety FBS-37 conferring resistance to the broad-spectrum glyphosate and insects, respectively (Ali et al., 2016; Rao et al., 2013). The results of ELISA of Bt and GTGene in transgenic cotton plants confirmed their successful expression. Quantification of Cry1Ac, Cry2A and GTG protein through ELISA determined maximum of 0.8µg/g, 1µg/g and 0.9µg/g of tissue, respectively (Fig. 4). The efficacy of transgenes in transgenic plants were further confirmed by insect bioassay and glyphosate spray assay as shown in Figure 5 and 6, respectively. Mortality of insects (100%) on transgenic cotton plant leaves while complete survival on non-transgenic control plant leaves determined successful expression of Cry1Ac +Cry2A genes as was obtained by Kiani et al. (2013). Similarly, complete survival of transgenic cotton plants against 1600ml/acre glyphosate spray in comparison to control and weeds where 100% death was obtained and determines efficacy of transgene cpEPSPS against glyphosate herbicide. The results are in accordance as obtained by Zhao et al. (2011) and Awan et al. (2015).

**Conclusion:** FBS-37 harboring CEMB Cry1Ac + Cry2A genes along with cp4EPSPS gene holds good potential to combat with serious problems of insects and weeds. It may be proved to be a good asset for national breeders to utilize this material for development of high yielding insect and weedicide resistant cotton varieties which can pave their role in boosting up of national economy.

**REFERENCES**


Bakhsh, A., S. Siddique and T. Husnain. 2012. A molecular approach to combat spatio-temporal variation in
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