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Evaluation of two cotton varieties CRSP1 and CRSP2 for genetic transformation efficiency, expression of transgenes Cry1Ac + Cry2A, GT gene and insect mortality

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ABSTRACT

Expression of the transgene with a desirable character in crop plant is the ultimate goal of transgenic research. Transformation of two Bt genes namely Cry1Ac and Cry2A cloned as separate cassette under 35S promoter in pKHG4 plant expression vector was done by using shoot apex cut method of Agrobacterium. Molecular confirmation of putative transgenic cotton plants for Cry1Ac, Cry2A and GT gene was done through PCR and ELISA. Transformation efficiency of CRSP-1 and CRSP-2 was calculated to be 1.2 and 0.8% for Cry1Ac while 0.9 and 0.6% for Cry2A and 1.5 and 0.7% for GTG respectively. CRSP-1 was found to adopt natural environment (acclimatized) earlier than CRSP-2 when exposed to sunlight for one month. Expression of Cry1Ac, Cry2A and GTG was found to be 1.2, 1 and 1.3 ng/μl respectively for CRSP-1 as compared to CRSP-2 where expression was recorded to be 0.9, 0.5 and 0.9 ng/μl respectively. FISH analysis of the transgenic CRSP-1 and CRSP-2 demonstrated the presence of one and two copy numbers respectively. Similarly, the response of CRSP-1 against Glyphosate @1900 ml/acre was far better with almost negligible necrotic spot and efficient growth after spray as compared to CRSP-2 where some plants were found to have necrosis and negative control where the complete decay of plant was observed after seven days of spray assay. Similarly, almost 100% mortality of 2nd instar larvae of *Heliothis armigera* was recorded after three days in CRSP-1 as compared CRSP-2 where insect mortality was found to be less than 90%. Quantitatively speaking non transgenic plants were found with 23–90% leaf damage by insect, while CRSP-1 was with less than 5% and CRSP-2 with 17%. Taken together CRSP1 was found to have better insect control and weedicide resistance along with its natural ability of genetic modification and can be employed by the valuable farmers for better insect control and simultaneously for better production.

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1. Introduction

Cotton is the most important crop that contribute to feeding many people directly and indirectly [3]. Cotton act as a backbone of the economy and result in the generation of employment in the world especially in Pakistan [8]. Insect problem is the serious threat to world economy causing an estimated loss of $645 million dollars each year in the form of yield [10,1]. Among those insects losses caused by *Lepidopteron* insects estimated to be $216 million [11]. *Pectinophora gossypiella* which is pink bollworm is also a serious problem on a small percentage of the cotton acreage planted in USA where it accounts for an estimated loss of $71 million in the form of direct damage [15]. Control of these insects pests through manual techniques is quite expensive and problematic for today's farmers [21]). On an average 6–7 insect treatment in the form of pesticide/insecticide spray per season are applied to control these insects [22].

Chemical insecticide is hazardous/problem causing not only by the cost (expense) they cause, but also through their persistence in the environment and their escalating rate of application because of decreasing effectiveness [23]. Weeds are considered to be another limiting factor for reduction of plant yield. In past manual hoeing was the only reliable method for getting rid of these weeds but it was not beneficial due to time and labor it required and also because of the cost of labor [16]. Total seed numbers of weeds in

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the soil were found to rise significantly after shifting from chemical weed control method to non-chemical means [4].

Hence, non-chemical methods are not viable due to input it required. Biotechnology provides an alternative tool to control weeds which are more efficient as compared to conventional method. Glyphosate (*N*-phosphonomethylglycine) is known to kill annual broadleaf weeds and grasses, big competitor of crop plant all around the world owing to its persistent broad-based spectrum and its relation with herbicide group 'glycines'. The mode of action of Glyphosate is to interfere in the shikimate metabolic pathway through inhibition of synthesis of 5-enolpyrovyle 3-phosphoshikimate (EPSPS). It inhibits the synthesis of three aromatic amino acids including tryptophan, phenylalanine and trypsin [29].

Genetic engineering utilizing plant genes conferring resistance against diseases and controlling weeds offers an alternative to conventional breeding methods to control pathogens, insects, and weeds [19]. Genes encoding antifungal proteins, such as endochitinase, β-1,3-glucanases and glucose oxidase, or components of signaling pathways involved in the defense response, have already been used to generate transgenic plants resistant to various plant pathogens [25]. Sorting out best cotton and other crop varieties after genetic transformation are crucial step to generate highly efficient genetically modified plants [6]. Cotton varieties with high efficiency of genetic transformation, acclimatization and stable expression can help to save money and man power [31]. Methodologies have been developed to transform required genes through genetic engineering.

In this study transformation of three genes, CEMB double Bt (Cry1Ac and Cry2A) along with herbicide resistance gene (cp4EPSPS) was done to control insect pest and weeds. This study focuses on evaluating the potential of two cotton varieties for genetic transformation, acclimatization and stable expression of transgenes.

2. Materials and methods

2.1. Plant material

Two cotton varieties CRSP-1 and CRSP-2 were transformed with Cry1Ac + Cry2A along with cp4EPSPS gene. The seeds of cotton varieties were collected from cotton research station Multan (Pakistan). Concentrated H_2SO_4 was used for delinting while sterilization of seeds was done with 5% $HgCl_2$ and 10% SDS. Seeds were then allowed to germinate at 30 °C incubator overnight.

2.2. Genetic transformation of BT and herbicide gene in cotton

Cry1Ac + Cry2A and cp4EPSPS gene were transformed in CRSP-1 and CRSP-2 according to Rao et al. [26]. Two constructs having Bt and weedicide gene were used under CAMV35s promoter and NOS terminator for genetic transformation through Agrobacterium method of transformation. The genus of Agrobacterium has been divided into a number of species based on its disease symptomology and host range. *Agrobacterium tumefaciens* causes crown gall disease, *Agrobacterium rhizogenes* causes hairy root disease and a new species *Agrobacterium vitis* causes galls on grapes and a few other plant species [9]. The host range of Agrobacterium is extensive. As a genus, Agrobacterium can transfer DNA to a remarkably broad group of organisms including numerous dicot and monocot angiosperm species and gymnosperms [30]. The most widely used species in plant transformation is *A. tumefaciens*. *A. tumefaciens* is a naturally occurring soil borne pathogenic bacteria that causes grown gall. After transfer, T-DNA becomes integrated into the plant genome and its subsequent expression leads to the crown gall phenotype [12]. There are two bacterial genetic elements required for TDNA transfer to plants. The first element is the T-DNA border sequence that consists of 25 bp direct repeats flanking defining the T-DNA. The borders are the only 12 sequences required in cis for T-DNA transfer [28]. The second element consists of the virulence (vir) genes encoded by Ti Plasmid in a region outside of the T-DNA. The vir genes encode a set of proteins responsible for the excision, transfer and integration of the T-DNA into the plant genome [20]. Transgenic plants generated through Agrobacterium were screened on kanamycin antibiotic selection at the application rate of 50 mg/L of medium for 1.5 month, putative transgenic plants were shifted on selection free medium for shoot and root generation as done by Muzaffar [8].

2.3. Acclimatization

Two-month old putative transgenic plants were shifted to pot from tubes and were exposed to light for 15 min at the first day and then 15 min increases onward up to one month daily. During acclimatization plants were exposed from 10 a.m. onwards and were daily watered (Fig. 7).

2.4. Confirmation of transgenic plants through PCR

Genomic DNA from putative transgenic plants was isolated according to Lenin et al. [18]. Reaction mixture having 100 ng DNA (2 μl), 10X PCR buffer(2 μl), 2.5 mM $MgCl_2$ (2 μl), 1 mM dNTPs (2 μl) one picomole each primer(2 μl) and 2.5 U Taq DNA polymerase for a total volume of 20 μl was prepared with gene specific primers shown in Table 1. The reaction was proceeded in ABI 9700 thermocycler having following conditions, initial denaturation at 94 °C for 5 min then 35 cycles of denaturation at 94 °C for 1 min, annealing at 51 °C for Cry2A and GTG while 50 °C for Cry1Ac for 1 min followed by extension at 72 °C for 3 min. After amplification products were resolved on 1% agarose gel and visualized by ethidium bromide staining.

2.5. Enzyme linked immune sorbent assay (ELISA)

Envirologix Kit (cat# 051) was used for the enzyme linked immune sorbent assay of Cry1Ac, Cry2A and GT gene expression. One gram leaves samples from transgenic cotton plants were subjected to grinding and total crude protein was isolated by using protein isolation buffer (0.5 M EDTA, Glycerol, 5 M NaCl, 2 M Tris–Cl, NH_4Cl, PMSF, DTT (Dithiothreitol).

2.6. Fluorescence in situ hybridisation (FISH)

The PCR-based best positive transgenic plants of CRSP-1 and CRSP-2 were subjected to Fluorescence in situ hybridization (FISH) for determination of copy number according to the procedure described by Rao et al. [10]. Mirus Label IT® FISH Cy3 Kit (cat# MIR6510, MJS Bio Lynx Inc., P.O. Bag 1150, 300 Laurier Blvd. Brockville, ON K6V 5W1, Canada) was used for labeling of probes. Chromosomes from growing root tips were prepared and were hybridized with the specific probe. The fluorescent microscope (Carl Zeiss AXIO100) was used for the detection of fluorescent signals using appropriate filter set. The CCD camera was employed

Table 1
Primers with sequences used in this study.

Primer name	Sequence (5′–3′)	Product size
Cry2A-F	AGATTACCCCAGTTCCAGAT	500 bp
Cry2A-R	GTTCCCGAAGGACTTTCTAT	
GTG-F	CCCTGGTGACAAGTCCATCT	800 bp
GTG-R	CTGCACACCCATCTCTCTGA	
Cry1Ac-F	ACAGAAGACCCTTCAATATC	1 Kb
Cry1Ac-R	GTTACCGAGTGAAGATGTAA	

for capturing fluorescent signals and analyzed by using Genus 3.7 software provided by Cytovision Applied Imaging System. The same software package was utilized for karyotyping.

2.7. Cry1Ac and Cry2A toxicity through leaf bioassay

Transgenic plants were subjected to 2nd instar larva of *Helicoverpa armigera* to check their toxic level. A total three leaves from upper, middle and lower part of transgenic cotton plants of 25, 55 and 85 day old were allowed to attack by *H. armigera*. After 2–3 days insect mortality picture was collected from transgenic and non-transgenic cotton plants.

2.8. Herbicide tolerance of transgenic cotton plants through Glyphosate spray assay

A total of 1900 ml/acre weedicide spray was done on both transgenic and non-transgenic cotton plants. Herbicide Glyphosate is commercially available as Roundup^{TM}. Glyphosate which was prepared a up-to final concentration of 1900 ml/acre by dissolving it in water.

3. Results

3.1. Embryo shooter generation efficiency of two cotton cultivars

One thousand embryos of two cotton cultivars subjected to transformation of double BT and herbicide resistant genes through Agrobacterium method of transformation were evaluated for embryo shoot regeneration on simple MS as well as on kanamycin selection medium. The overall embryo shoot regeneration efficiency of CRSP-1 was found to be 71% and 62% for CRSP-2. While the efficiency of embryos shoots regeneration was found to be reduced 20% for CRSP-1 and 14% for CRSP-2 after two months of continuous kanamycin selection (Table 2).

3.2. Shifting of putative transgenic cotton plants in soil pots and acclimatization

After one-month kanamycin selection putative transgenic cotton plants were made selection free by shifting on simple MS medium for new root formation followed by shifting in soil pots. The putative transgenic cotton plants were kept covered with plastic bags for three days.The acclimatization therapy was initially started by opening of plants for 15 min followed by a further increase of fifteen minutes up-to one month. During fist, five days plants showed a slight wilting due to dehydration which was recovered with the passage of time in both cultivars. However, the loss of 4% putative transgenic cotton plants was observed for CRSP-1 and 12% for CRSP-2 after10–20 days. The healthy survived putative transgenic cotton plants were shifted to the contained field of CEMB. CRSP-1 plants were found to be stabilized earlier and shown vigorous growth in contained field as compared to CRSP-2 (Fig. 1).

3.3. Confirmation of putative transgenic cotton plants through PCR

Genomic DNA was isolated from putative transgenic cotton plants of both cultivars and PCR was performed for detection of double Bt (Cry1Ac + Cry2A) and GT gene with gene specific primers. The amplification of 450 bp product size forCry1Ac and 500 bp for Cry2A was observed in putative transgenic cotton plants of both cultivars (Fig. 1(A and B) & Fig. 2). The amplification of 350 bp for cp4EPSPS (Glyphosate tolerant gene) was also observed with gene specific primers (Fig. 3).

3.4. Transformation Efficiency

Total twelve and seven putative transgenic cotton plants were amplified for Cry1Ac in CRSP-1 and CRSP-2 respectively out of one thousand embryos utilized for transformation. Similarly, amplification of nine and six plants was observed for Cry2A in CRSP-1 and CRSP-2 respectively.While fifteen plants of CRSP-1 and CRSP-2 were amplified for cp4EPSPS (Glyphosate tolerant gene)

Table 2
Transformation efficiency of cultivar embryos transformed through Agrobacterium method of transformation.

Cultivar	Total Transformed embryos	Cry1Ac positive	Cry2Apositive	GTG positive
CRSP-1	1000	12	9	15
%		1.2%	0.0%	1.5%
CRSP-2	1000	8	6	7
%		0.8%	0.6%	0.7%

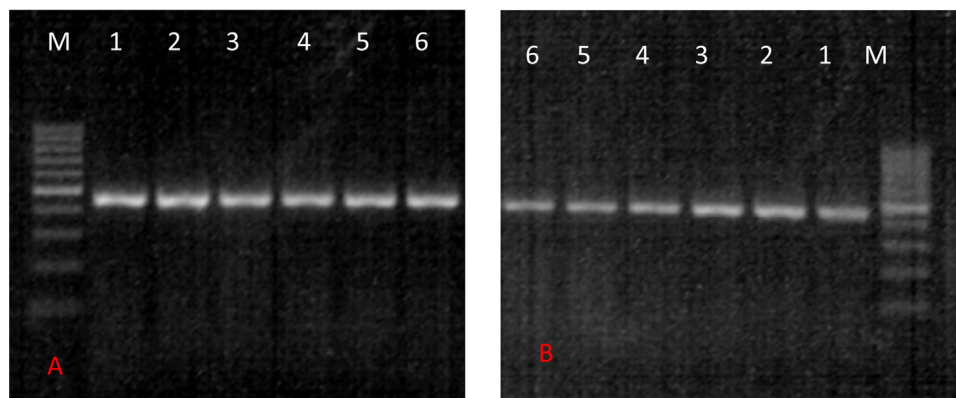

Fig. 1. Confirmation of cry1Ac through PCR amplification. Gene Specific primers were used for PCR amplification (CRSP-1 A): M-1 kb ladder, 1–6 positive plants for Cry1Ac (CRSP-2 B): M-1 kb ladder, 1–6 positive plants for Cry1Ac.

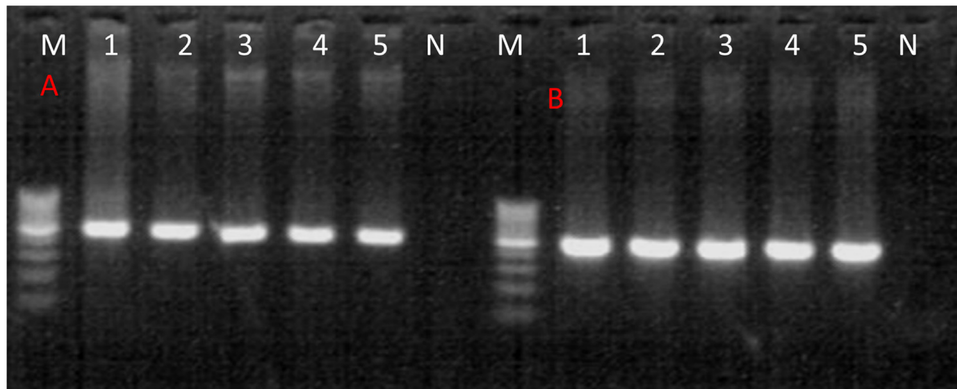

Fig. 2. A: Cry2A 500 bp amplification with gene specific primers. M-100 bp ladder, 1–5 CRSP-1Cry2A positive, N—negative control, B: M-100 bp ladder, 1–5 positive plants of CRSP-2, N—Negative control.

Figs. 1–3).Transformation efficiency of 1.2%, 0.7% for Cry1Ac and 0.9%, 0.6% for Cry2A was calculated for CRSP-1 and CRSP-2 respectively. Similarly, transformation efficiency of GTG was found to be 1.5 and 0.8% respectively (Table 2)

3.5. Quantification of transgenes protein through ELISA

The best three positive plants for each gene were subjected to crude protein isolation. Isolation of protein was done from total three phenotypically best plants. Gene-specific antibodies were used for ELISA detection and quantification by using Envirologix kit (cat# AP010) On an average quantification of Cry1Ac, Cry2A and GTG were calculated to be 1.2, 1 and 1.3 units respectively for CRSP-1 and 0.9, 0.5 and 0.9 units respectively for CRSP-2 (Fig. 4).

3.6. Determination of copy number of transgenic cultivars

Fish is more reliable technique than Southern hybridisation in determining the copy number [27]. The best transgenic plant with better protein expression of Cry1Ac were subjected to Fish for

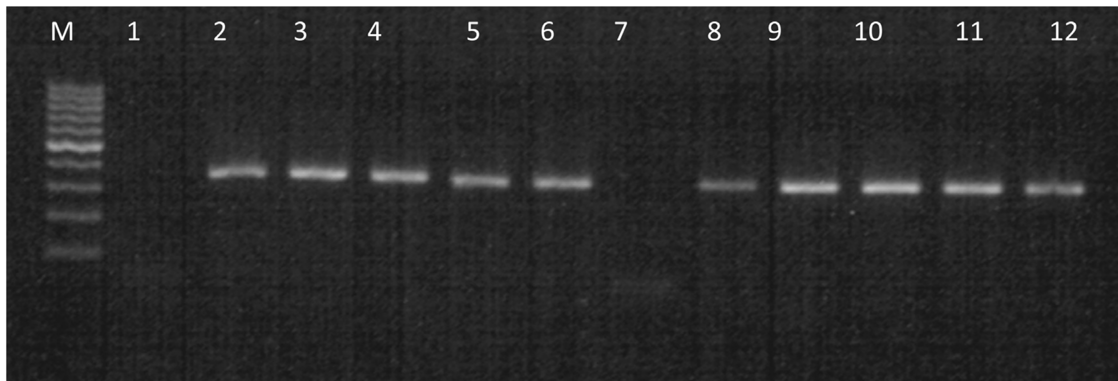

Fig. 3. Glyphosate gene (350 bp) amplification with gene specific primers. M-1 kb plus DNA ladder, 1,7 negative control, 2–7 positive GTG transgenic CRSP-1 plants, 8–12 CRSP-2 GTG positive plants.

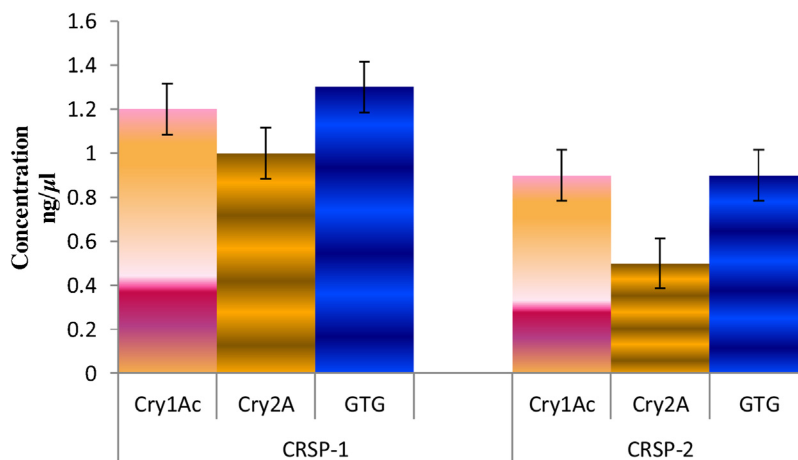

Fig. 4. Graphical representation of quantification of Cry1Ac, Cry2A and GTG for both cultivars each representation is the average of three plants.

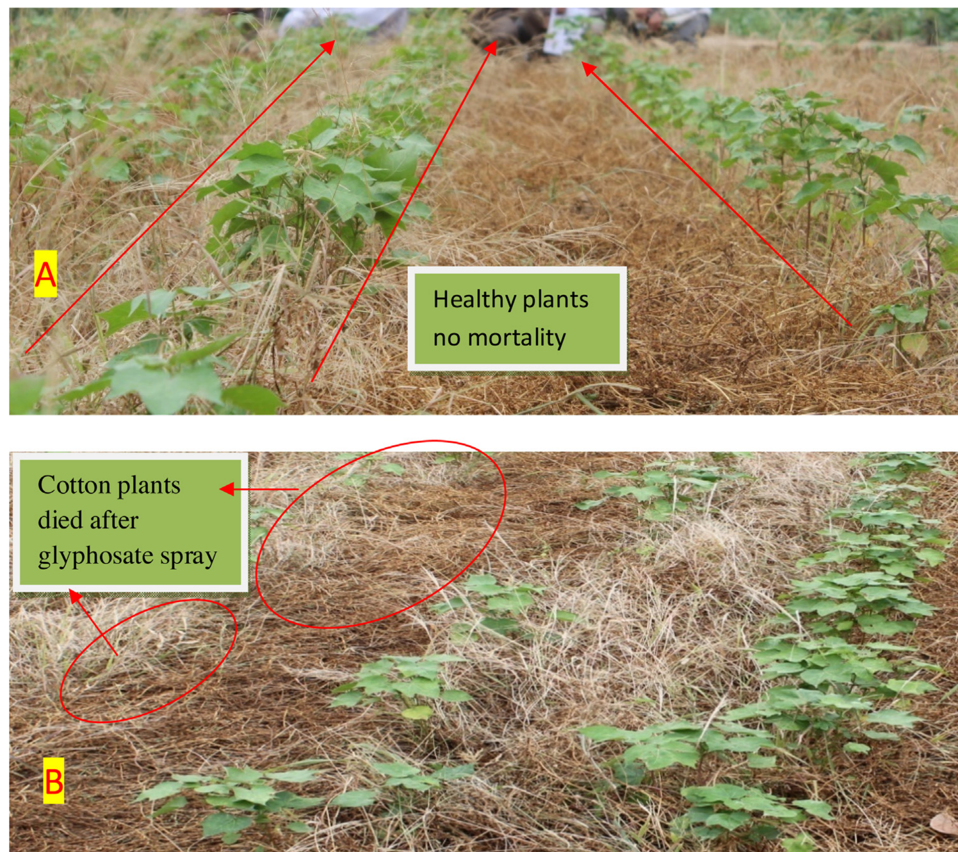

Fig. 5. After six days weeds were died in both fied of CRSP-1 and CRSP-2. In the B field (CRSP-2) mortality of cotton plants was observed along with weeds shown as red but in field A (CRSP-1) plants were healthier no mortality was observed.

determining the copy number of the gene as well as a location of the gene on a chromosome. This analysis is very much important because copy number and the position of a gene on chromosomes are directly related with better expression and ultimately better control against insects [24]. CRSP-1 was found to have one copy number at chromosome 1 as compared to CRSP-2 which was found to have 2 copy number at chromosome number 6 &10 (Fig. 8)

3.7. Evaluation of transgenic cotton plants for resistance against insects and weedicide through insect leaf bioassay and Glyphosate spray assay

Variation in mortality % age of *H. armigera* 2nd instar larvae was observed after 30, 60 and 90 days depending upon expression of Cry proteins. Damage percentage of CRSP-2 transgenic cotton plants was higher as compared CRSP-1 (Fig. 6). Transgenic cotton plants were subjected to a full pressure of weeds in containment without manual hoeing until 3 month. After the three months when the cotton field was full of different kinds of weeds glyphosate spray at the rate of 1900 ml/acre (300 ml of 99% Glyphosate of Galaxy brand FMC mixed with 1900 L and 700 ml of water in the tank) was applied. The necrotic effect was seen on weeds along with nontransgenic cotton plants which ultimately lead to death. However, no effect of spray was observed on CRSP-1 plants which remained healthy and showed the full potential of growth while CRSP-2 transgenic plants survived spray assay but showed stunted growth (Fig. 5). In order to measure insect damage quantitatively leaf area of leaves was measured by Easy Leaf Area software [13].

4. Discussion

Cotton is cash crop that enables farmers and farm workers to earn their living. Cotton provides 80% raw material to industries and generates 30% of foreign exchange [8]. But there are so many factors which hinder or cause the reduction in its production [5]. One of the serious concerns of cotton is the competition of nutrition with weeds along with insect pest attack [24]. Insects are responsible for 20% loss while 25% losses occur due to weeds [1]. It has been well documented that full control of insect and weeds can be possible by over expression of the responsible genes [7]. In the present study, an effort was made to transform two local cotton cultivars namely CRSP-1 and CRSP-2 with two Bt(Cry1Ac + Cry2A) and one herbicide resistant gene (cp4EPSPS). The purpose was not only to transform cotton but also to compare two cotton cultivars for transformation efficiency, gene expression, weedicide tolerance and insect mortality.

Transformation of the Bt(Cry1Ac + Cry2A) and weedicide codon optimized cp4EPSPS gene was done by using shoot apex method as done by Rao et al. [26]. Transformation efficiency of both cotton cultivars was found different under similar condition depending upon genotype. Transformation efficiency of CRSP-1 and CRSP-2 was 1.2%, 0.7% for Cry1Ac while it was 0.9, 0.6% and 1.5%, 0.8% for Cry2A and GT gene respectively (Table 2). Plant genotype, healthy seed embryo with integration site of the gene to show its dominant effect can be the reasons to withstand harsh environmental conditions [17]. Similar transformation efficiency of cotton was observed by Rao et al. [26] in cotton variety CIM-496 which was different from Bakhsh et al. [32] in cotton variety NIAB 846.

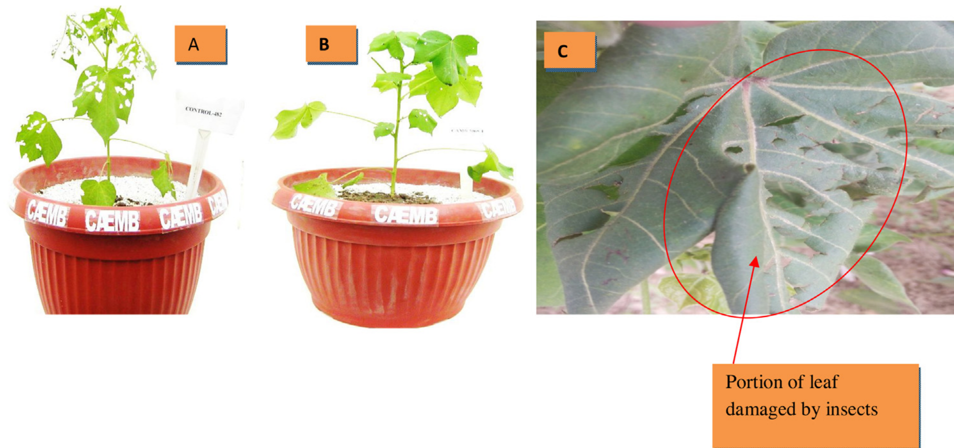

Fig. 6. Bioassay of Transgenic and non-Transgenic plants. A: Non-transgenic plant almost fully damaged, B: Transgenic CRSP-1 variety stayed healthy, no insect attack, C: CRSP-2 transgenic variety, a portion damaged by insects.

Fig. 7. Transgenic plants during acclimatization and in filed phase. A-CRSP_1 plants in pot being acclimatized more healthy and fresh B-CRSP-1transgenic plants in the field in very good condition C&D CRSP-2 transgenic plants during acclimatization and filed phase were weak and unhealthy as compared with CRSP-1 transgenic plants.

Variation in expression of Cry1Ac, Cry2A, and GT gene in two cotton varieties was also evident as 1.2, 1 and 1.3 units respectively for CRSP-1 and 0.9, 0.5 and 0.9 ng/ml respectively for CRSP-2 (Fig. 4). These differences in expression may due to T-DNA transfer rate, insertion points of genes, genetic make-up of plants and Vir genes activity during transformation [10]. Similar types of results were obtained by Rao et al. [24] for PhyB gene expression. Amplification of 450 bp product for Cry1Ac, 500 bp for Cry2A and 350 bp for GTG confirmed the successful transformation in cotton plants of CRSP-1 and CRSP-2 [14]. From the results, it was clear that expression of Cry1Ac, Cry2A, as well as GT gene, was lower in CRSP-2 than CRSP-1. This may be due to the reason that different germ plasm may have different expression capacity of foreign genes [24]. Fluorescent in situ hybridization was used for determining the copy number. The plant with best protein expression of each cultivar was used for FISH analysis. The results clearly demonstrated that CRSP-1 having one copy no has better adopted to show resistance against insects as compared to CRSP-2 with two copy no at chromosome no. 6 and 10. The results are in accordance with Rao et al. [24] who determined the best expression of phytochrome B in transgenic *cotton* plants having single a copy no [24]. The transgenes of both cultivars were further subjected to insect bioassay and Glyphosate spray assay. Toxicity level of transgenic cotton variety CRSP-1 was found to be higher than CRSP-2 as evident from insect damage (Fig. 6B and C) and negative controls (Fig. 6A). CRSP-2 was found a little bit susceptible to insect attack has showed stunted growth after spray of 1900 ml/acre Roundup™ Glyphosate. The total leaf damage as for as control was concerned it was 23–90% while CRSP-1 was found with less than 5% and CRSP-2 was with 17%. These results are consistent with [13]. Almost 100%

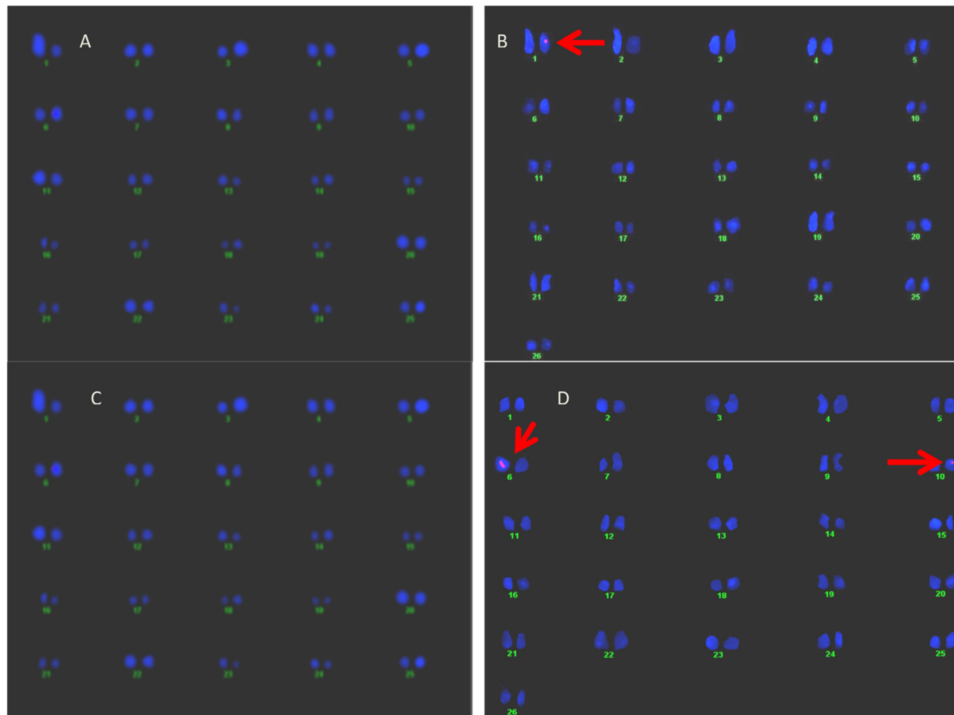

Fig. 8. Copy no and location of transgene of Cotton variety CRSP1 and CRSP-2. A and C are negative control, while B is CRSP-1 and D is CRSP-2. Arrow determined the fluorescent signal.

mortality of insect and healthy growth of CRSP-1 as compared to CRSP-2 with complete removal of weeds confirmed its successful utilization in variety developmental program. The difference in expression capacities of both cultivars may be due to the fact that the ancestors of both cultivars may be descendent from germplasm which is more dominant in one cultivar and have the resistance capacity to insects and weeds as compared to other. The results are comparable with results of Refs. [16,1,2]. Based on results CRSP-1 was found better than CRSP-2 for resistance against insects and herbicide expression and action.

5. Conclusions

CRSP-1 harboring double Bt and GTG gene holds good potential to combat with serious problems of insects and weeds and may be good assets for farmers and National breeders to develop further good varieties using this material against insects and weeds which can pave their role in boosting up economy of Pakistan as compared with CRSP-2

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