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*Research Article*

# Development of multigenic sugarcane to decrease the production cost in pakistan

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**Due to higher amount of sucrose, sugarcane is grown commercially. In order to save sucrose yields, various studies have been designed to develop resistance in sugarcane against weeds and stemborers. In this study, two problems had been addressed by genetic manipulation of sugarcane to make them resistant against both herbicides and insects by expressing glyphosate resistant gene (CEMB-GTGene) and borer resistant genes (CEMB-*Cry1Ac* and CEMB-*Cry2A*) under control of Nos terminator and maize ubiquitin promoter. Mortality percentage of shoot borers *Chilo infuscatellus* was determined by assessing the Cry proteins through insect Bio-toxicity assays. Results showed that in 80 days old transgenic plants, 100% mortality rates of *Chilo infuscatellus* have been found showing that there was high resistance in transgenic sugarcanes against shoot borers and sufficient gene expression to fully resist target pests. Weed management was done by glyphosate spray assays. 70%-76% of the transgenic plants were identified to be glyphosate resistant (3000 ml/Ha) in $V_1$ generation while 100% tolerant in $V_2$ generation. Thus, this transgenic sugarcane will help to boost sugarcane yield in the country as it now successfully provides resistance against both stemborers and glyphosate herbicides.**

**Key words:** Agrobacterium, Glyphosate herbicides, Plasmid, Sugarcane

## INTRODUCTION

Sugarcane is considered to be the world's significant cash crop as it is being cultivated around the globe in 58 countries (Yao et al., 2017). 26.9 million hectares of area is used for sugarcane cultivation worldwide (Mayavan et al., 2015). 80% of world's sugar need is fulfilled by sugarcane via chemically synthesized sweetener known as sucrose (Gao et al., 2016). A wide range of products are obtained from sugarcane like chemicals, biofuels, fibers, paper, beverages, detergents, insecticides, industrial enzymes, plastics, paints, pharmaceutical products, synthetics, chipboard and industrial chemicals like dextran, furfural and alcohol (Raghavi et al., 2016). Sugarcane contributes to 0.7% GDP and 3.4% of agriculture sector and is cultivated on ~1.3 million hectare area in Pakistan (Farooq, 2015). 37% of the agriculture production in Pakistan is lost out of which 13% is because of insects (Butt et al., 2016). Sugarcane crop is destroyed by ~1300 insect pests all over the world and by 61 insect species in Pakistan (Long et al., 1972). In Pakistan, 15%-36% of sugarcane yield is lost due to stemborers, 10%-20% by root-borers and 10%-15% by top-borers (Bhatti et al., 2008). Main objective of this study to prevent yield loss by making sugarcane resistant against stemborers and herbicides.

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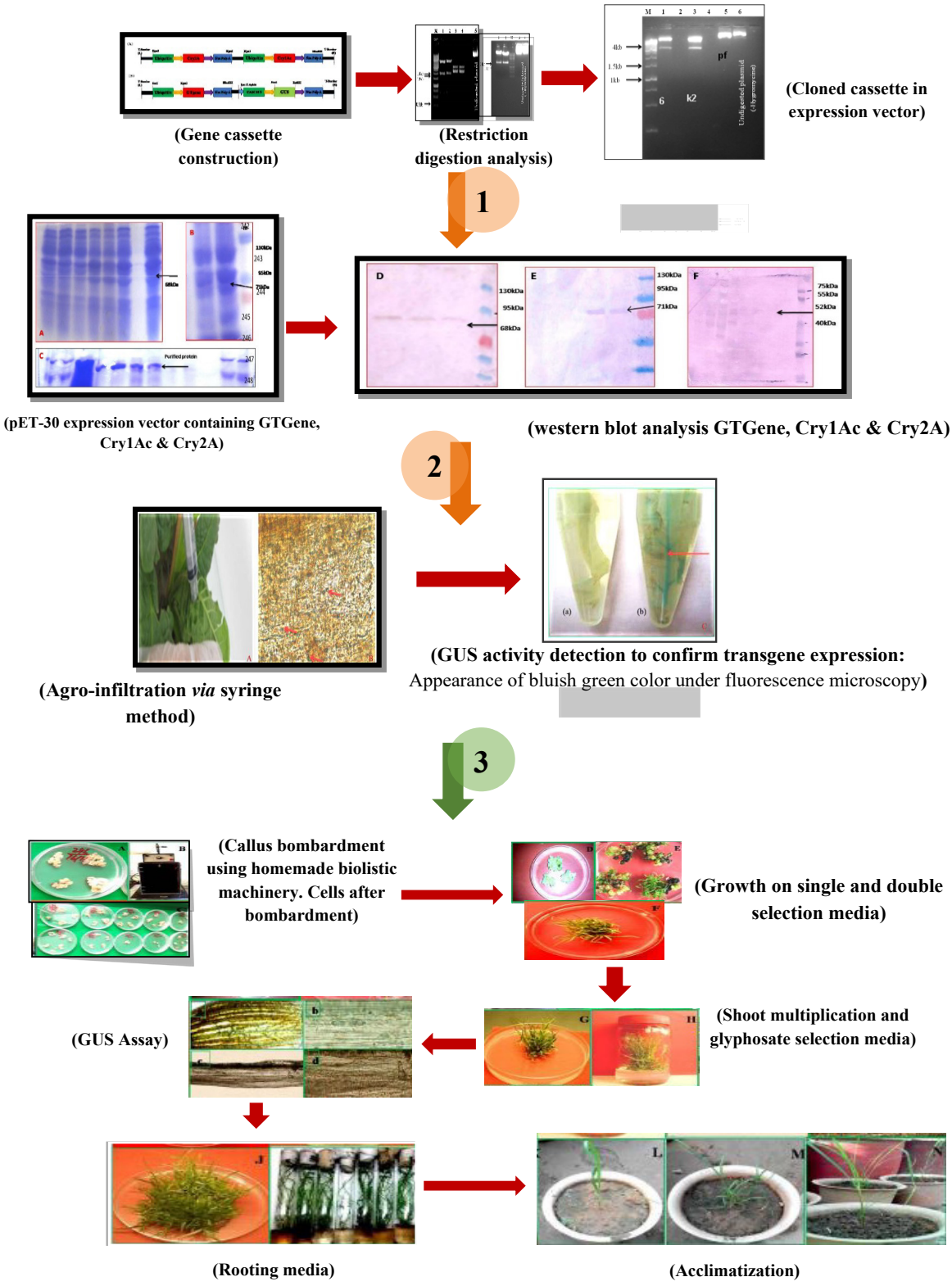
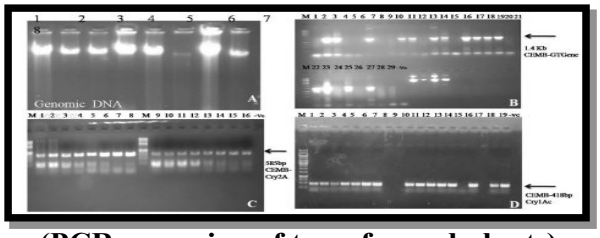
## MATERIALS AND METHODS

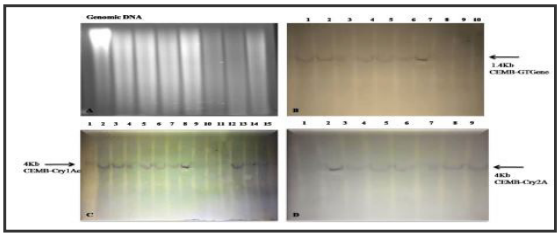
A gene cassette was designed that contains herbicides and stemborers resistant genes i.e. CEMB-GTGene, CEMB-*Cry1Ac* and CEMB-*Cry2A* under control of Nos terminator and maize ubiquitin promoter. These constructs pCEMB-SGTG and pCEMB-SC12 were introduced via electroporation into the Agrobacterium cells (Qamar et al., 2015). Colony PCR was performed to confirmed gene transformation via gene specific primers. 8-10 weeks old leaves of tobacco plants were co-cultured with agrobacterium to induce agrobacterium mediated transformation (Bhaskar et al., 2009). Expression of transgenes was indicated by histochemical detection of the GUS activity that was used as a reporter using agro infiltrated leaves.

Biolistic transformation method was used for transformation of transgene in 4 sugarcane varieties i.e. CPF-246, HSF-240, SPF-234 and SPF-213 (Nasir et al., 2014). During early transgenesis, transgene expression was determined by performing GUS assays on young shoots. Presence of transgenes was confirmed through PCR screening using CEMB-GTGene, CEMB-*Cry1Ac* and CEMB-*Cry2A* genes specific primers. Stable transgene integration was determined by performing southern blotting (Edwards et al., 1991) on PCR positive transformants. CEMB-GTGene, CEMB-*Cry1Ac* and CEMB-*Cry2A* genes expression were determined through dipstick assays that were coated with the IgG monoclonal antibodies for each gene. These sticks were dipped in total proteins that were isolated from the fresh transgenic plants leaves (Qamar et al., 2017). ELISA was performed to quantify the transgene expressions. Toxicity effects of CEMB-*Cry1Ac* and CEMB-*Cry2A* endotoxins were determined by performing leaf biotoxicity assay on the leaves. CEMB-GTGene expression and activity was confirmed by spraying glyphosate on the transgenic plants. Comparison between different lines (control and transgenic) was done through statistical analysis (Dunnett's tests, LSD and ANOVA) (Figure 1).






(PCR screening of transformed plants)



(Western blot analysis for determining transgene expressions)

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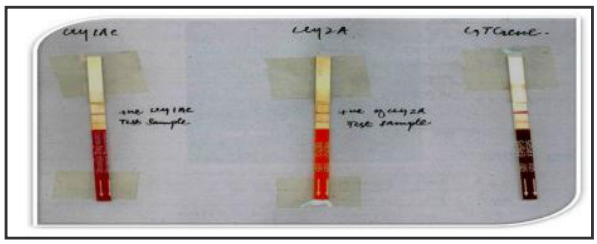

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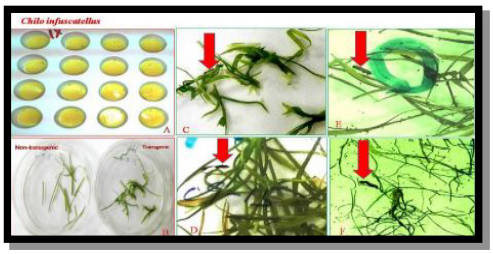


(Leaf bioassay of transgenic sugarcane plantlets)

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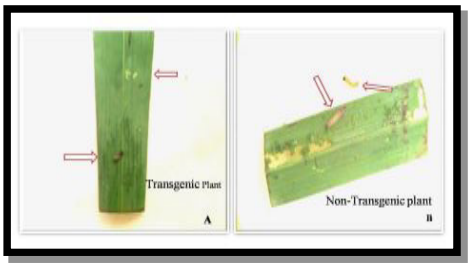
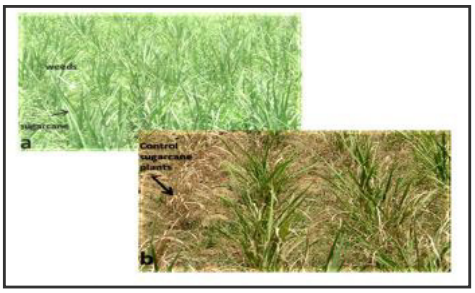



(80 days old transgenic and control plants leaf bioassay)



(Herbicide glyphosate spray assay)

6

($V_1$ **generation:** Resistance against cane-borers and glyphosate)

($V_2$ **generation:** 15 $V_1$ generation transgenic lines completely resistant to any insect attack and glyphosate)

**Figure 1.** The expression of GUS was confirmed by bluish green color under fluorescent microscope.

## RESULTS

Restriction of the CEMB-GTGene, CEMB-*Cry1Ac* and CEMB-*Cry2A* genes generated 1.4 kb, 1.8 kb and 1.9 kb fragments respectively which were then integrated into expression vector (pCAMBIA-1301). These constructs were introduced via electroporation into agrobacterium. PCR analysis confirmed presence of transgenes. PCR positive transformants were subjected to agro-filtration using tobacco leaves in presence of GUS receptor. The expression of GUS was confirmed by bluish green color under fluorescent microscope. For sugarcane transformation and tissue culturing, CPF-246, HSF-240, SPF-234 and SPF-213 varieties of sugarcane were selected. To obtain maximum embryogenic calli from the selected varieties, 4 different combinations were used for callus induction media. Maximum embryogenic calli was observed in CPF-246 (100%) followed by SPF-213 (90%), SPF-234 (90%) and HSF-240 (81%). Plasmid constructs were then transferred to these varieties via biolistic methods. Total of 400 explants were used for transformation. On single selection media (kanamycin), 91% of CPF-246, 74% of SPF-234, 70% of SPF-213 and 45% of HSF-240 survived while on double selection media (glyphosate and kanamycin), 81% of CPF-246, 40% of SPF-234, 34% of SPF-213 and 29% of HSF-240 transformed calli survived. Then after it, GUS assay was performed to screen the transgenic putative plants. PCR, southern blotting, dipstick assay and ELISA was performed for transgenic plants at Vo, V1 and V2 generation. Leaf bio-assay was performed to determine the efficiency of CEMB-GTGene, CEMB-*Cry1Ac* and CEMB-*Cry2A* genes.60%-100% mortality rate Chilo infuscattellus was determined in transgenic leaves. Weed management was done by glyphosate spray assays. 70%-76% of the transgenic plants were identified to be glyphosate resistant (3000 ml/Ha) in V1 generation while 100% tolerant in V2 generation.

## DISCUSSION

Main objective of crop production is to obtain high yields even for sugarcane (Ali et al., 2014). Different viruses, drought stress, weeds and insects are the major constrains for sugarcane (Thiebaut et al., 2012). Present study aimed to control insects and weeds through genetic manipulation of sugarcanes. In this study, for maximum callus regeneration, an efficient procedure was developed to instill tolerance against glyphosate and cane borers. For embryogenic callus formation, immature leaves were found to be excellent explants (Snyman et al., 2006). It basically strengthens the procedure for gene transformation in sugarcane (Fitch et al., 2001). From a callus inducing media with 2,4-D, embryogenic calli were obtained for all 4 varieties (Nawaz et al., 2013). To enhance potential of embryogenic calli of sugarcane, it was supplemented with casein (Joyce et al., 2014). Tissue culture response was observed to critically screen all 4 varieties (Ali et al., 2015). For genetic modification, varieties were selected on basis of regeneration response (Bakhsh et al., 2012). Studies have also disclosed that resistant against lepidopteran insects were best provided by Cry proteins (Riaz et al., 2006). Most commonly used herbicide for weed control is glyphosate which is a broad spectrum herbicide. One of the main drawbacks of using glyphosate is that along with weeds and herbs, it also stunts the plant growth thus affecting its yield (Nawaz et al., 2020). It inhibits formation of EPSPS enzyme in shikimate pathway which leads to shikimate pathway being shut down. This inhibits the formation of 3 essential amino acids i.e. phenylalanine, tyrosine and tryptophan that humans can't synthesize and is required from plant source (Castle et al., 2004). In this study, resistant against glyphosate and stemborers are provided by introducing CEMB-GTGene, CEMB-*Cry1Ac* and CEMB-*Cry2A* genes into the sugarcane varieties. Measuring small amounts of bismuth in

## CONCLUSION

In this study, 100% mortality rates of *Chilo infuscatellus* have been found in CPF-246 variety showing that there was high resistant in transgenic sugarcanes against shoot borers and sufficient gene expression to fully resist target pests. Weed management was done by glyphosate spray assays. 70%-76% of the transgenic plants were identified to be glyphosate resistant (3000 ml/Ha) in V1 generation while 100% tolerant in V2 generation. This study reported that after approval from biosafety committee, farmers can use this sugarcane variety as starting material for cost effective weeds and insect's control. More studies should be done to enhance stable Bt. toxin expression. Glyphosate resistant crops against 5000 ml/Ha were recommended to be successful in controlling all sugarcane weeds.

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