PHYSICAL PROGRESS REPORT
OF THE PARB PROJECT NO. 235
(01-06-2010 TO 31-05-2017)

DEVELOPMENT & COMMERCIALIZATION OF INDIGENOUS TRANSGENIC BT AND HERBICIDE TOLERANT MAIZE HYBRIDS

By

Prof. Dr. Idrees Ahmad Nasir

Centre of Excellence in Molecular Biology, Lahore

DETAILED FINAL PHYSICAL PROGRESS REPORT FOR PARB CGS PROJECT

01 JUNE 2010 to 31 MAY 2017

A. Basic Information

1.	**Name of the project**	Development & commercialization of indigenous Bt and herbicide tolerant Maize hybrids
2.	**Project No.**	235
3.	**Total Project cost**	22.829 Million Rs
4.	**Total project duration**	60 Months
5.	**Funds released so far**	
6.	**Project commencement Date**	01-06-2010
7.	**Name of the Project Manager or Team Leader with designation**	Dr. Idrees Ahmad Nasir Professor CEMB, University of the Punjab Lahore

B. Physical Research Achievements

Output/Activity	**Description**	**Planned Starting Date**	**Planned Completion date**	**Achievement Indicator as planned**	**Achievements** (Please attach data in brief as annexure if activity completed)	**Annexure**
Output -1	**CEMB Bt and GTGene transformation in best selected Maize Inbred Lines.**	**1/6/2012**	**1/10/16**	**Transformed Maize Inbred lines will be available**	**Completed**	
Activity-1	Development of Construct with CEMB Bt and GTGenes harboring monocot promoter in plant expression vector.	1-6-2015	30-10-2015	Construct with CEMB Bt & GtGenes with monocot promoter in plant expression vector will be available.	**Completed**	**Annexure-I**
Activity -2	Transformation of best selected parents by Agrobacterium/ biolistic gun with CEMB Bt and GTGene (at least 10 insertion events)	1/11/2015	25/8/16	Putative transformed lines with different insertion events will be available	**Completed**	**Annexure-II**
Activity -3	Handing over seed of transformed lines to Agri Farm Services.	28/8/16	20/9/16	Seeds of transformed lines will be available to Agri Farm Services.	**Completed**	**Annexure-III**

Output -2	**Confirmation of transgenic maize lines via molecular analyses and bioassays.**	**20/4/16**	**15/05/17**	**Transgenic maize inbred lines will be available.**	**Completed**	**Annexure-IV**
Activity -1	ELISA and PCR analysis of putative transgenic plants	20/4/2016	10/06/2016	Confirmed transgenic plants will be available	**Completed**	**Annexure-IV**
Activity -2	Southern analyses of Confirmed transgenic plants	5/9/2016	10/10/2016	Copy number of genes in Confirmed transgenic plants will be Known	**Completed**	**Annexure-IV**
Output 3	**Bio-safety Assessments of new Bt inbred lines**	**1/10/2016**	**1/5/2017**	**Data for bio-safety study will be available**	**Completed**	
Activity 1	Study of Horizontal gene flow to check the crossover of target gene to the other plant species.	1/10/16	1/5/17	Data for Horizontal gene flow will be available	**Completed**	Annexure-V
Activity 2	Study of Vertical Gene flow to evaluate the presence of Bt protein in soil, its kinetics and effect on soil organisms.	1/10/16	1/5/17	Data for vertical gene flow will be available	**Completed**	Annexure-V
Activity 3	Study of Effect of Bt on non target insects	1/10/2016 1/3/2017	1/11/2016 1/5/2017	Data for effect of Bt on non target insects will be available.	**Completed** Chrysopa perla found in fields of transgenic maize.	Annexure-VI
Activity 4	Study of effect of Bt protein on Animals (Mice /Rabbit)	1/3/2017	20/5/2017	Data for effect of Bt on Animals (Mice and Rabbit) insects will be available.	**Completed** Experimental mice were fed on diet prepared by transgenic maize. Data is shown in Annexure-VII	Annexure-VII

C. Bottlenecks, if any:

Signature of Project Manager/Team Leader

Development of Constructs:

For expression of Cry2A, Cry1Ac, and GTG gene in maize inbred lines, the genes were cloned in pCAMBIA 2300. For expression of multiple genes in single plant two genes indivualy driven by ubiquitin promoter was cloned in multiple cloning sites of pCAMBIA 2300. Cry2A was combined with GTG, while Cry1Ac was combined with Cry2A genes. The cassette maps for constructs A+B, A+C were shown in figure 1.A and B. The vector and insert DNA fragments were amplified, ligated by seamless cloning, transformed in top10 competent cells. To confirm gene insertion and orientation was done by Restriction digestion (Figure 2).

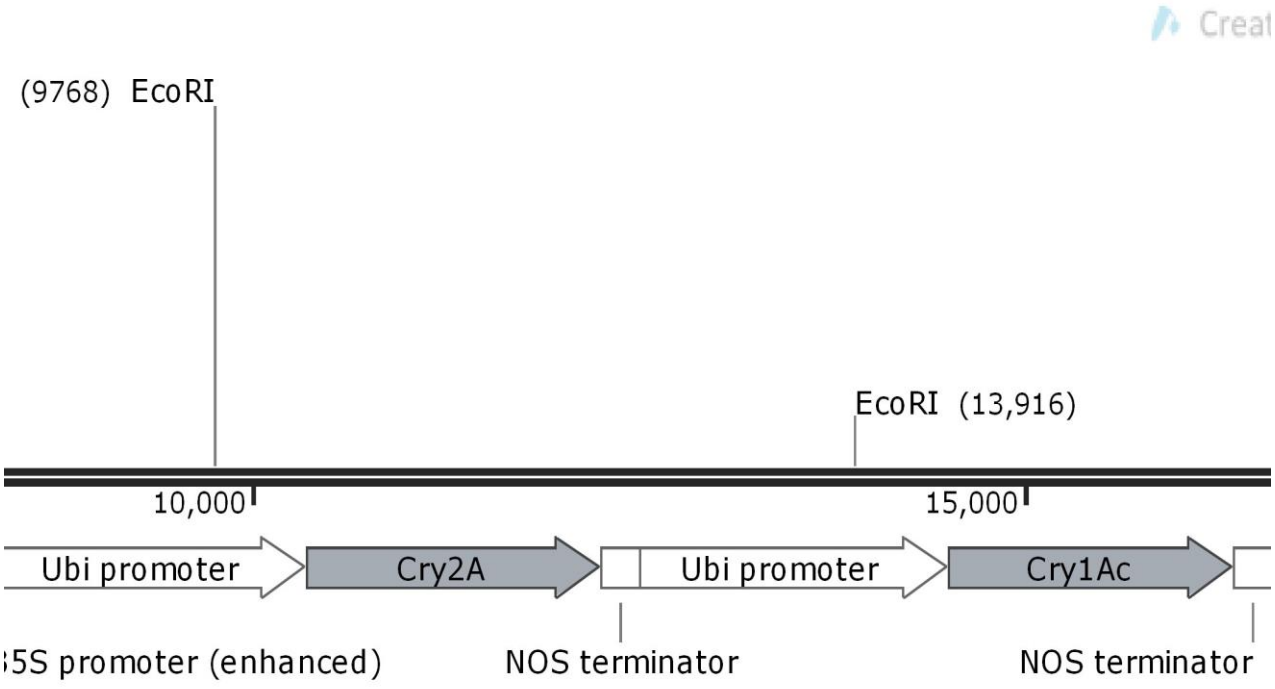


Figure 1.A Map of Construct A + C with Cry2A and Cry1Ac genes with EcoRI sites shown.

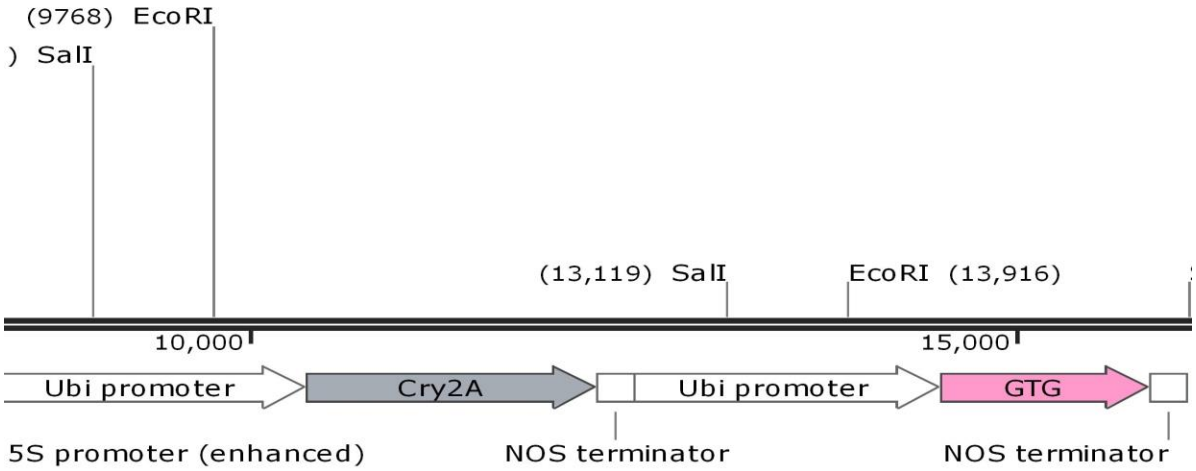


Figure 1.B Map of Construct A + B with Cry2A and GTG genes with EcoRI and SalI sites shown.

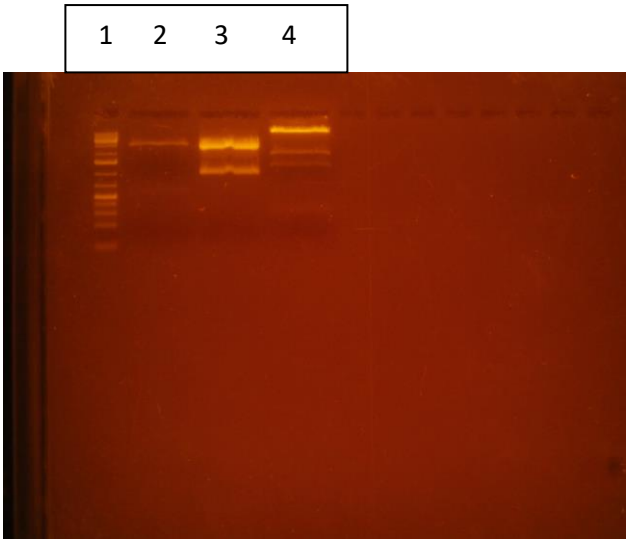


Figure 2. Lane 1: 1Kb plus DNA ladder, Lane 2: pCAMBIA 2300 digested with EcoRI, Lane 3: Construct A + B, Lane 4: Construct C + D

Annexure-II

Transformation in Maize Lines.

PCR **Confirmation of *Agrobacterium* transformation**

After the freeze thaw transformation of both constructs (Cry1Ac + Cry2A, Cry2A+GTG) in *Agrobacterium* strainLBA4404, Agrobacterium strain was confirmed through PCR by using the primer pairs mentioned in Table

Primer Name	Sequence	No of Bases	Tm C
Ach5FtsZ-F:	5′ -GAACTTACAGGCGGGCTGGGT-3′	21	72
Ach5FtsZ-R:	5′ -CGCCGTCTTCAGGGCACTTTCA-3′	22	72

Direct PCR of culture was performed using Phire Direct PCR Kit by Fermentas. Reaction mixture was made as per following

Name	Qunatity
10 Reaction buffer	10 ul
Ach5FtsZ F	1.5 ul
Ach5FtsZ R	1.5 ul
Taq	0.4 ul
water	5.8 ul
Agro culture	0.8 ul
Total	20 ul

PCR was done as per following profile and results were checked on 1% agarose gel (Figure 3).

98 5 min

98 8 sec

72 25 sec

72 5 min

4 infinity

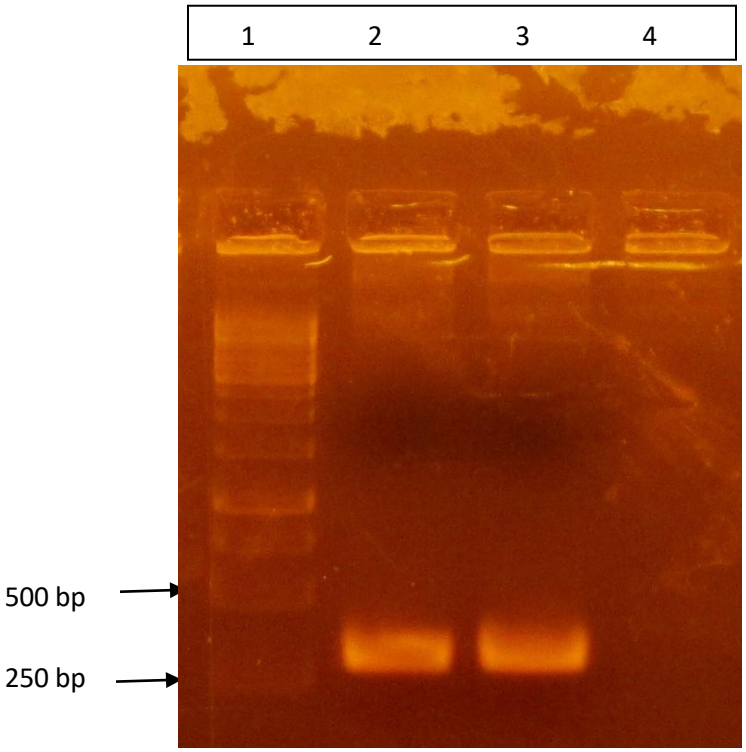


Figure 3. PCR for confirmation of Agrobacterium. Lane 1: 1 Kb DNA Ladder. Lane 2-3: Agrobacterium 380 bp band. Lane4: -ive control.

Immature Embryos:

Immature embryos of 2-3 mm of selected maize in bred lines were excised and treated for agrobacterium mediated transformation of Cry1Ac, Cry2A and GTG genes.

Plant tissue culture and regeneration

To identify the suitable lines of *Zea mays* for genetic transformation, the embryos of different sizes were cultured on callus inducing medium. Some of the embryos were sliced longitudinally into halves as a treatment, some were given a little cut at shoot side and on few embryos small holes were created by using the aucopancture needles to facilitate transformation. Embryos were sterilized by treating them with mercuric chloride solution.

Infection media contained 2g/l modified N6 basal salts mixture with vitamins, 1.5 mg/l 2,4-D, 700 mg/l L-proline, 500 mg/l MES, 30g/l sucrose in pH 5.8 and 3 g/l gelrite for solidification. The media were sterilized by autoclaving it at 121^{o}C for 20 minutes. Filter sterilized acetosyringone, 400mg/l Cystein and 0.85 mg/l Silver nitrate ($AgNO_3$) were added after autoclaving. The explants cultures were grown at 21^{o}C for 3 weeks in darkness. The embryogenic calli were further

transferred to the first regeneration medium, incubated at 25^{o}C in the 16/8 h (light/dark) photoperiod at 7000 luxlight intensity for 2 weeks.

The first regeneration medium contained 4.43 g/l MS salts and vitamins, 30 g/l sucrose adjusted pH at 5.8 and then added 3.0 g/l of phytagel in it. The cultures were maintained at 25±2^{o}C under high light intensity (18,000 lux) in the 16/8 h photoperiod for 4 weeks in the secondary regeneration medium until the roots of the plantlets reached to 10cm. **The secondary regeneration medium** components were same as the first regeneration medium except it contained 25 g/l of sucrose init. The number of regenerated plants were then determined for each line.

***Agrobacterium* strains and vectors**

Agrobacterium tummefacians strain LBA4404, harbouring the binary vector pCAMBIA1301 was used for transformation process. The plasmid pCAMBIA1301 contained ubiquitin promoter gene in the T-DNA, the broad host origin of replication (pVSI) and kanamycin resistant marker gene for bacterial selection.

A. tumefacians strain LBA4404, (harbouring a standard binary vector pCAMBIA1301) was streaked out from -80^{o}C glycerol stock onto an YEP agar plates containing appropriate antibiotics (for LBA4404 50 mg/l rifampicin and 50 mg/l kanamycin). The plates were placed in incubator set at 28^{o}C for 48 hours until single colonies were developed. That stock plates were usedon a weekly basis for upto a month. Single colony was further streaked on YEP medium plates containing the same antibiotics as stock plates. For all transformation experiments, *Agrobacterium* were grown at 28oC for 2 days.

Choice of antibiotics against bacterial overgrowth

At earlier, before performing the transformation experiments, different antibiotics were compared for their capability to control the growth of *Agrobacterium.* For this purpose, three combinations of antibiotics were tested. The immature embryos of one inbred line were infected with overnight cultures of three *Agrobacterium* strains. The infected embryos were blotted by using sterile filter paper and cultured on plates of callus inducing medium containing each of 250 mg/l Cefatoxime or Timentin, or 200 mg/l Vancomycin + 50 mg/l Jentamycin. After every week, embryos were transferred to a fresh medium. The efficiency of Agrobacterium elimination for each antibiotic was determined after three subcultures.

Embryo size and pretreatment

The role and effect of embryo size and pretreatment culture on transformation frequency was studied in one inbred line of *Zea mays* as an efficient regeneratable genotype. For the

transformation, the embryos of 0.5-5mm in length were used taken out of sterilized seeds. On the basis of length, embryos were divided in to three groups: 0.5-2 mm, 2-3 mm, 3-5 mm. These embryos were inoculated with A. *tumefacians* LBA4404 (harbouring pCAMBIA1301) suspension cultures. In all transformation experiments, the densities of bacterial cells were adjusted to OD550=0.4-0.5 in liquid infection (Inf) medium using a spectophotometer immediately before infection. The **Inf medium** consisted of 2g/l modified N6 basal salts with vitamins, 1.5 mg/l 2,4-D, 700 mg/l L-proline, 35g/l sucrose and 18 g/l glucose. The pH was adjusted at 5.2 and medium was filter sterilized before being autoclaved. In transformation experiments, the bacterial suspensions consisting of one full loop of large colony from 48 h old cultivation were used and added in the 50 ml falcon tube containing 5 ml of Inf medium supplemented with 100µM Acetosyringone (AS).

The tube was fixed in shaker incubator on low speed (75 rpm) at 28^{o}C for 5 hours. That pre-induction step was carried out in all experiments. For innoculation of bacterial cultures,15-25 immature zygotic embryos were washed twice in Inf medium containing acetosyringone in a 2ml tube and 1.5 ml of *A. tumefacians* suspension was added. Infection wasaccomplished by gently inverting the tube for 20 times before resting it upright for 20 minutes. For the better transformation of *Agrobacterium*, the embryos explants were dissected at tips with help of toothed dissecting forceps during the infection. The infected embryos were blotted dry on sterilized filter papers and were then placed on the plates of co-cultivation medium having scutellum side up. The cultures were incubated at 22±1^{o}C for 3 days in darkness.

The **co-cultivation medium** was consisted of modified N6 basal salts with vitamins, 1.5 mg/l 2,4-D, 700 mg/l L-proline, 30 g/l sucrose in pH 5.8 and 3 g/l of gelrite. After autoclaving the medium, 100µM AS, 400 mg/l Cystein and 0.85 mg/l Silvernitrate ($AgNO_3$) were added. The explants were then further transferred on to another resting medium for 7 days in darkness at 28^{o}C. The components of **resting medium** were same as in the co-cultivation medium, except that, AS was eliminated and 500 mg/l of MES and 250 mg/l Cefatoxime was added for elimination of *Agrobacterium*. After that, the explants were transferred to the next selection medium. The first, second and third selection mediums were supplemented with 1.5, 3 and 5 mg/l PPT (L-Phosphinothricin) respectively and pH was adjusted to 5.2. In each selection medium, the induced calli were maintained at 28^{o}C for 2 weeks at darkness.

Figure 4: Excitation of immature embryos, Sterlization and Transferring to Induction, co cultivation media after Agrobacterium treatment

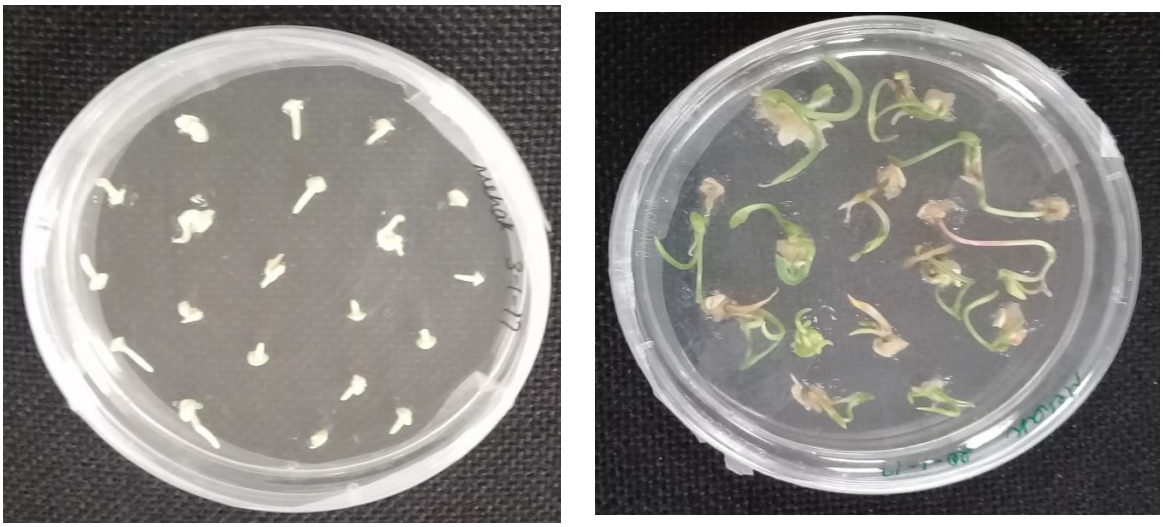


Figure 5: Regeneration of embryos on regeneration l and ll media

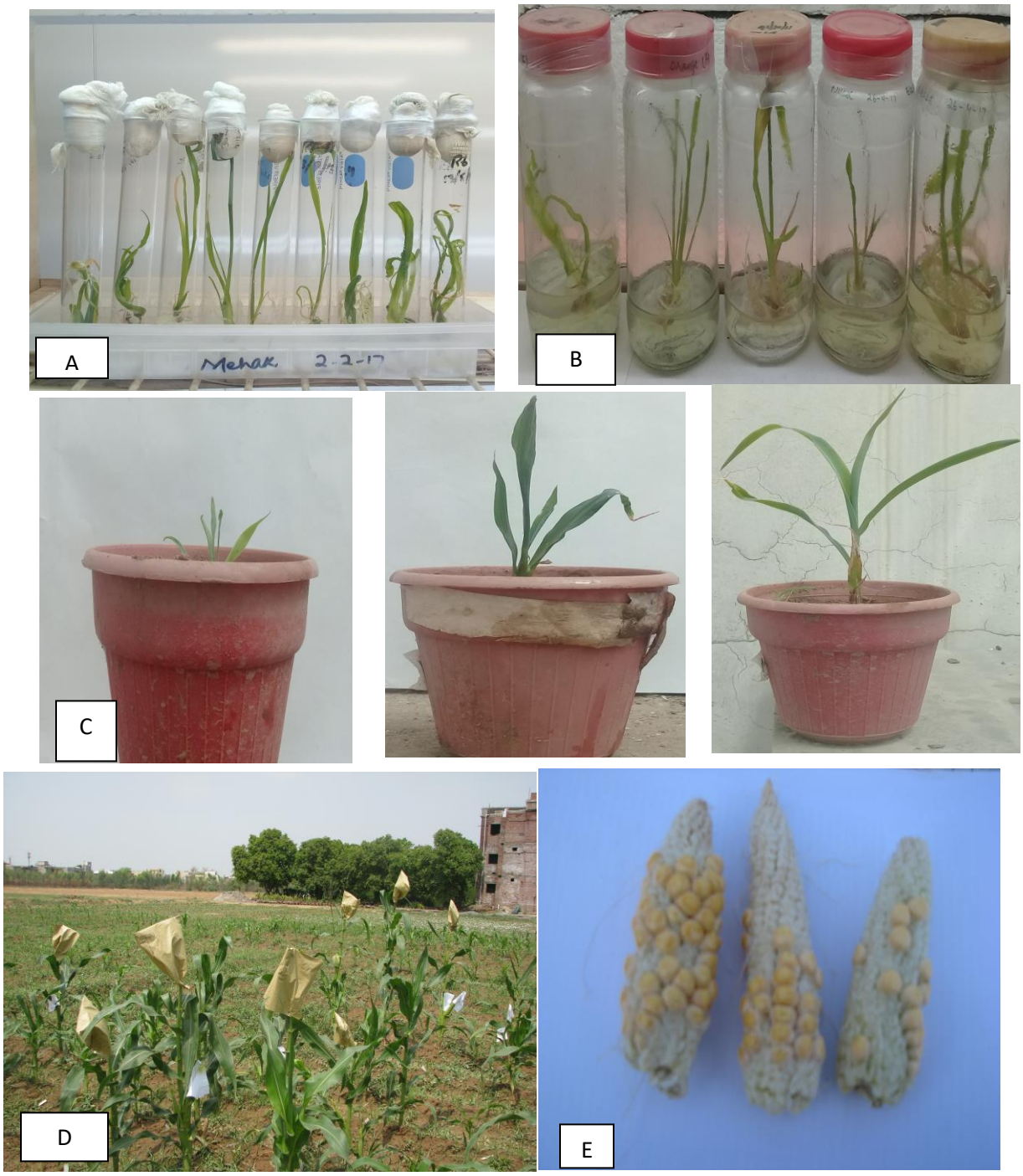


Figure 6: A. Regenerated transgenic plants in tubes. (**B)**. Regenerated transgenic plants in glass jars. **(C)** Putative transgenic plants shifted to pots for acclimatization. **(D)** Putative transgenic plants at stage of selfing. **(E)** Putative transgenic Cobs harvested.

Annexure-IV

Histochemical detection of GUS activity

GUS activity was histochemically detected in plant tissues by overnight incubation at 37°C in staining solution composed of 0.1% w/v 5-bromo-4-chloro-3-inodolyl-b-D-glucuronic acid (X-Gluc sigma) in 100mM Na_2HPO_4/ KH_2PO_4 pH 7.0, 0.01% w/v Chloramphenicol, 20% Triton X-100, 20% w/v Methanol. Chlorophyll was extracted from photosynthetic tissues with 70% w/v ethanol. The GUS expression was detected microscopically by the distinct blue color which results from the enzymatic cleavage of X-Gluc. Samples were stored at 4°C (in 70% w/v ethanol).

Figure 7: Gus expression in seeds of transgenic maize lines at different stages.

DNA extraction

For DNA extraction, Thermo Scientific GeneJet Plant Genomic DNA Purification Mini Kit #K0792 was used with little modifications.

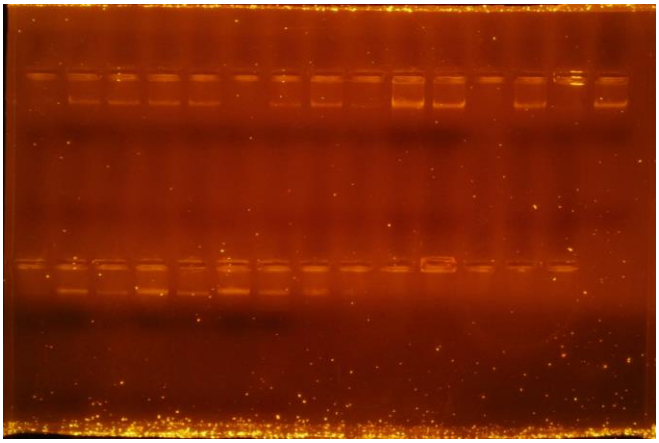


Figure 8: DNA Extracted from putative transgenic plants of maize inbred lines.

Dot Blot Hybridization

Dot Blot analysis:

Dot blot analysis was performed on genomic DNA extracted from leaves of both transformed plants and non-transformed control plants using DIG High Prime DNA Labeling and Detection Starter Kit-II (catalogue No 11 585 614 910, Roche). According to the instruction given in the manual, genomic DNA (3µg) of transformed plants, genomic DNA (3µg) of non-transformed plant as a negative control and pCAMBIA- construct (3µg) as a positive control were denatured by incubation in boiling water for 10 minutes, followed by rapid chilling on ice. The denatured DNA was spotted on positively charged nylon membrane: Hybond-N (Amersham Biosciences). The nylon membrane was air dried and placed on whatman 3MM-paper soaked with 10x SSC (**Appendix**) and subjected to UV-crosslinking for 3-5 minutes. The membrane was put into DIG easy hyb buffer (10ml/100 cm^2 membrane) followed by pre hybridization at 42 ºC for 30 minutes with gentle agitation in hybridization tube. DIG-labelled DNA probe was denatured by boiling it for 5 minutes and rapid cooling it on ice. Added denatured DIG-labelled DNA probe to pre-heated DIG easy hyb buffer (3.5ml/100 cm^2 membrane) and mixed. The membrane was left in DIG easy hyb buffer at 42 ºC for overnight. After hybridization, detection procedure was performed as follows:

- Nylon membrane was washed two times in ample 2x SSC, 0.1 % SDS solution at 25 ºC for 5 minutes under constant agitation.
- Membrane was washed two times in 0.5x SSC, 0.1 % SDS solution at 68 ºC for 15 minutes under constant agitation.
- After hybridization and stringency washes, membrane was rinsed for 1-5 minute(s) in washing buffer.
- After washing, membrane was incubated for 30 minutes in 100 ml blocking solution.
- Incubated for 30 minutes in 20 ml antibody solution.
- Washed 2 times for 15 minutes in 100 ml washing buffer.
- Equilibrated for 2-5 minutes in 20 ml Detection buffer.
- Finally, enzymatic reaction was performed by incubating the membrane in 10ml of freshly prepared 1X NBT/BCIP dissolved in distilled water.

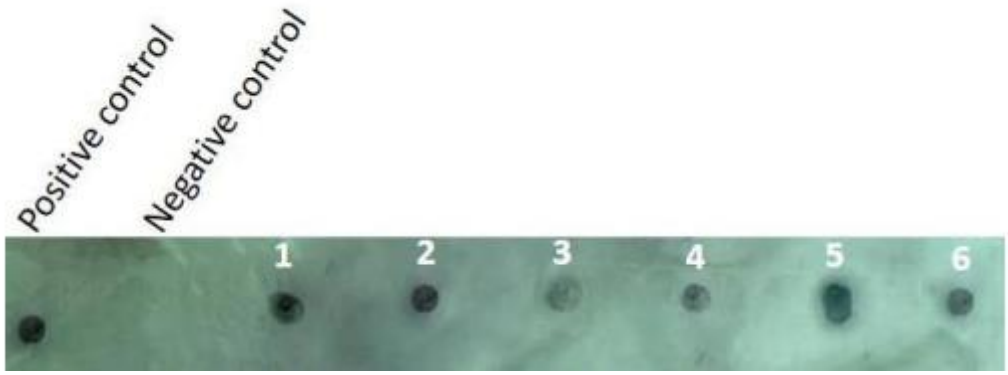


Figure 9: Dot blot of putative Transgenic Plants.

PCR Identification of Transgenic Plants.

Putative Transgenic Maize lines were identified through PCR of Cry1Ac, Cry2A and GTGenes among lines of Agri Farm Services and CEMB.

PCR of Cry1AC:

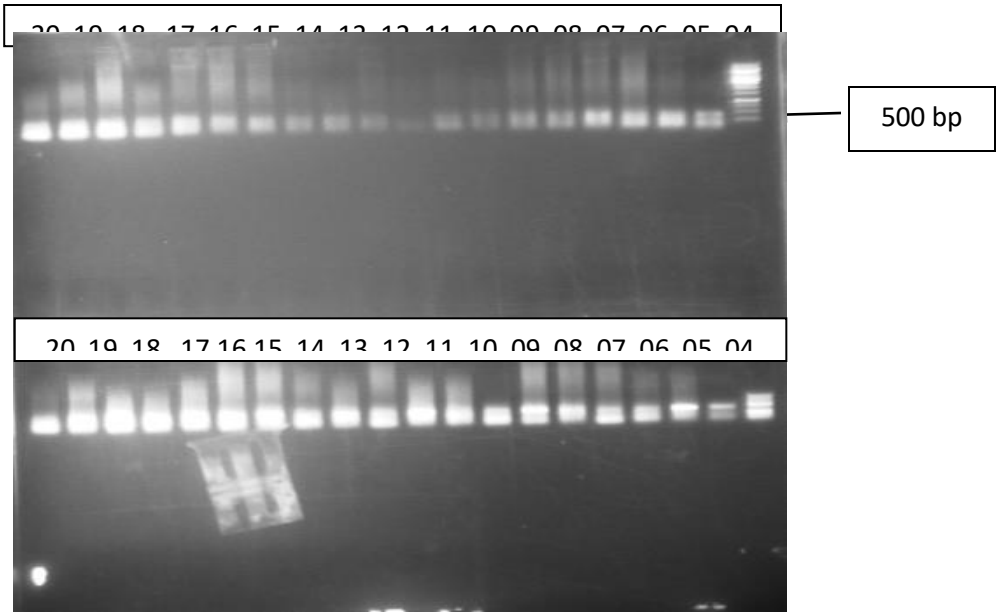


Figure 10: PCR of Cry1Ac of transgenic maize inbred lines. The plant were from T0 generation.

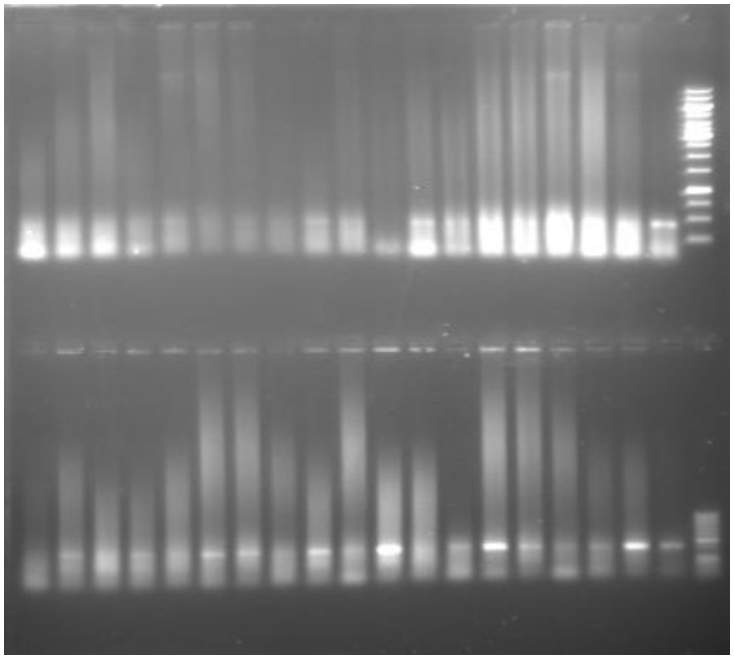


Figure 11: PCR of Maize Transgenic plants for gene Cry1Ac from T1 generation.

11 10 09 08 07 06 05 04 03 02 01

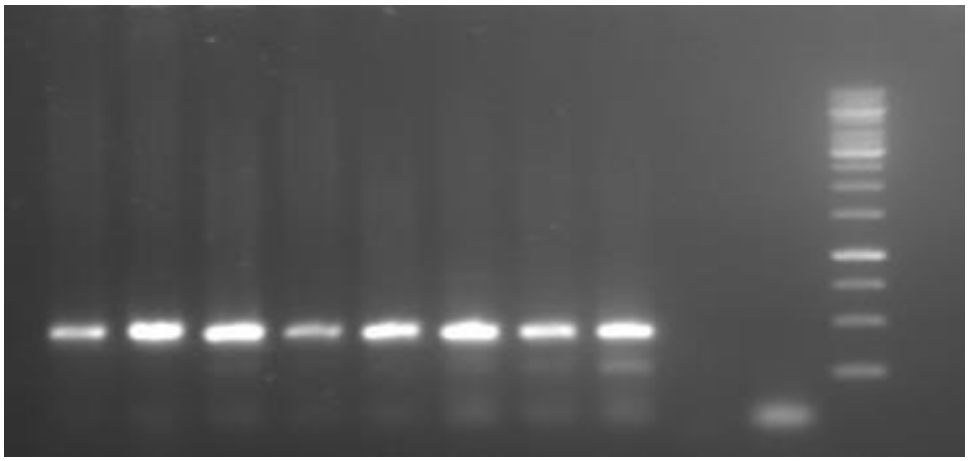


Figure 12: PCR of transgenic maize plants of CEMB Lines from Line 4-7: T3 generation. Line 8-11: T4 generation. For gene Cry1Ac.

GTG and Cry2A PCR

Putative transgenic plants of maize inbred lines from T0, T1 and T3 generations were checked for GTG and Cry2A genes with multiple set of primers. Multiplex PCR for Cry2A and GTGene was also performed to confirm construct A + B.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

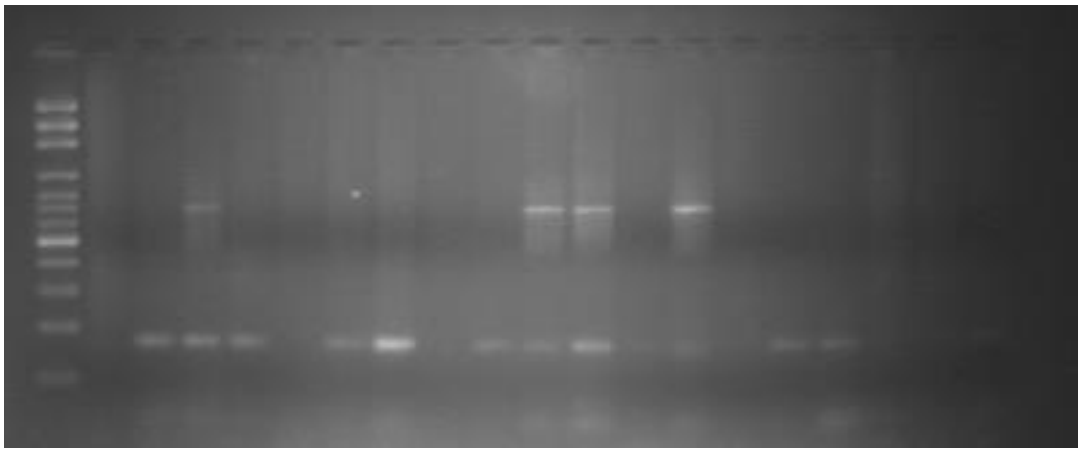


Figure 13: Multiplex PCR of Cry2A and GTGenes in T0 transgenic inbred lines. Lane 1: DNA Ladder, Lane 2: Control inbred maize line. Lane 4,11,12,14: +ive for GTG and Cry2A Lane 3,5,7,8,10,16,17: +ive for Cry2A only.

1 2 3 4 5 6 7 8 9 10 11 12 13 14

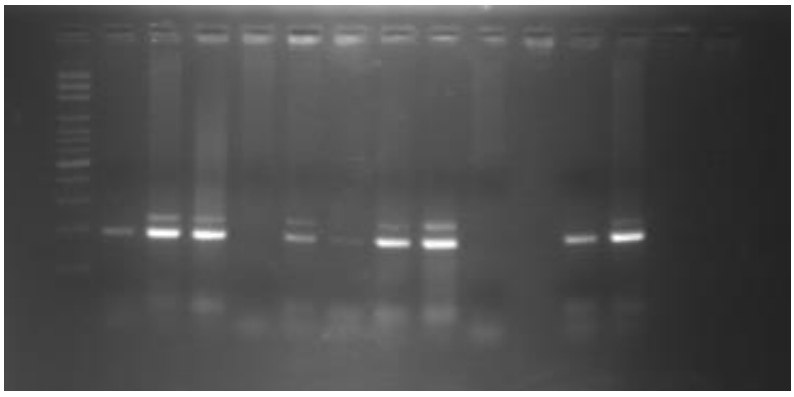


Figure 14: Multiplex PCR of Cry2A and GTGenes in T1 transgenic inbred lines. Lane 1: DNA Ladder, Lane 2: Cry2A + inbred maize line. Lane3,4,6,8,9,13: +ive for GTG and Cry2A. Lane 7,12: +ive for Cry2A only.

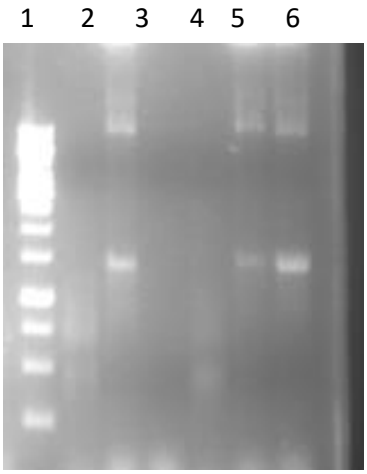


Figure 15: Multiplex PCR of GTGenes in T2 transgenic inbred lines. Lane 1: DNA Ladder, Lane 2: Control inbred maize line. Lane3,,6,7:

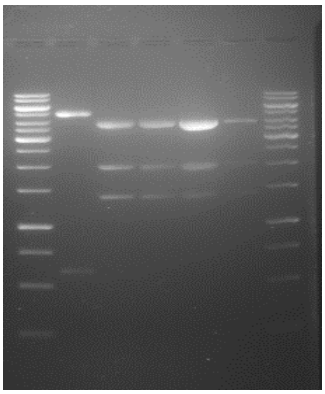


Figure 15: Multiplex PCR of Cry1Ac, Cry2A and GTGenes in T2 transgenic inbred lines. Lane 1: DNA Ladder,

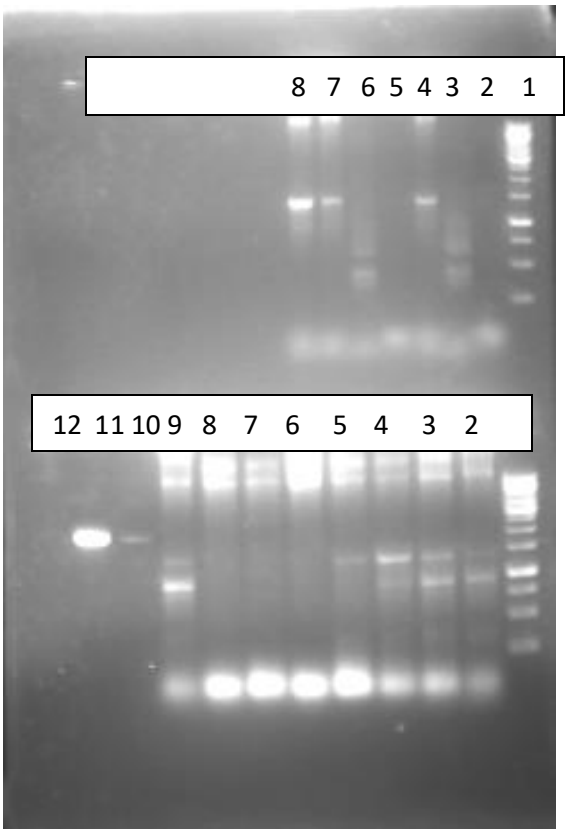
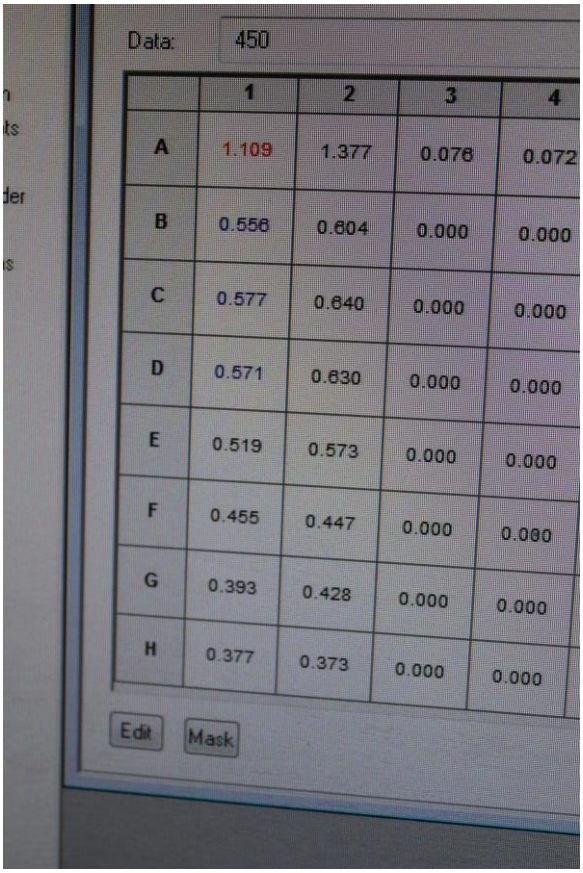


Figure 15: Multiplex PCR of GTGene and Cry2A in lower lanes in T3 transgenic inbred lines. Lane 1: DNA Ladder, Lane2,3,,4,9: +ive for GTGene and Cry2A.

ELISA of Cry1Ac, Cry2A and GTGenes.



Data: 450

	1	2	3	4
A	1.109	1.377	0.076	0.072
B	0.556	0.604	0.000	0.000
C	0.577	0.640	0.000	0.000
D	0.571	0.630	0.000	0.000
E	0.519	0.573	0.000	0.000
F	0.455	0.447	0.000	0.000
G	0.393	0.428	0.000	0.000
H	0.377	0.373	0.000	0.000

Edit Mask

Figure 16: Reading taken at 450 nm for Cry2A gene.

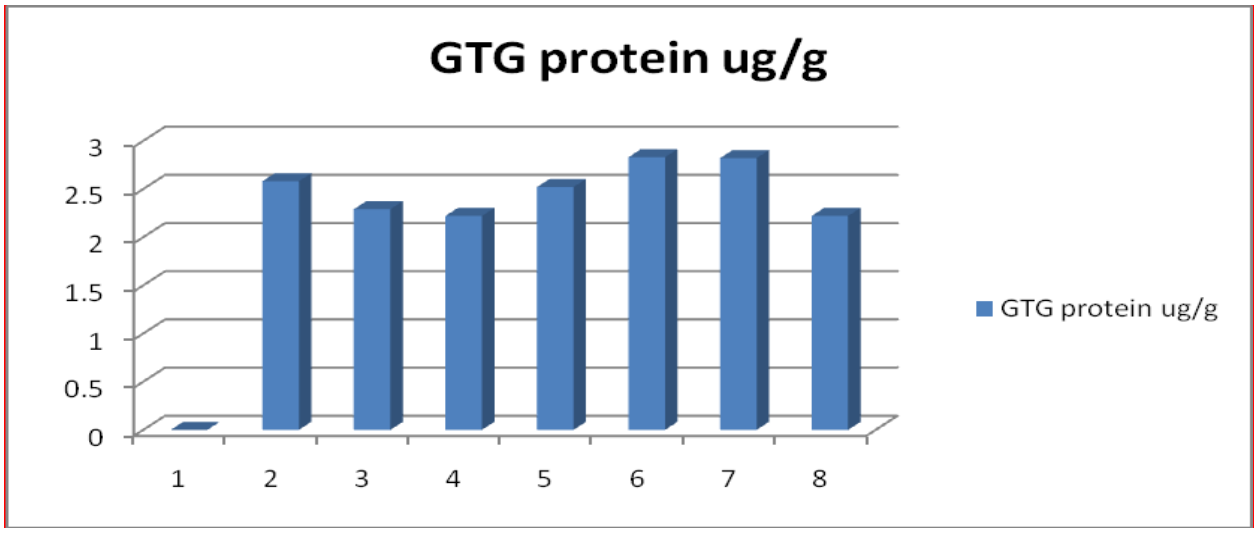


Fig: 17 Quantification of CEMB GTG protein in advance generation through ELISA

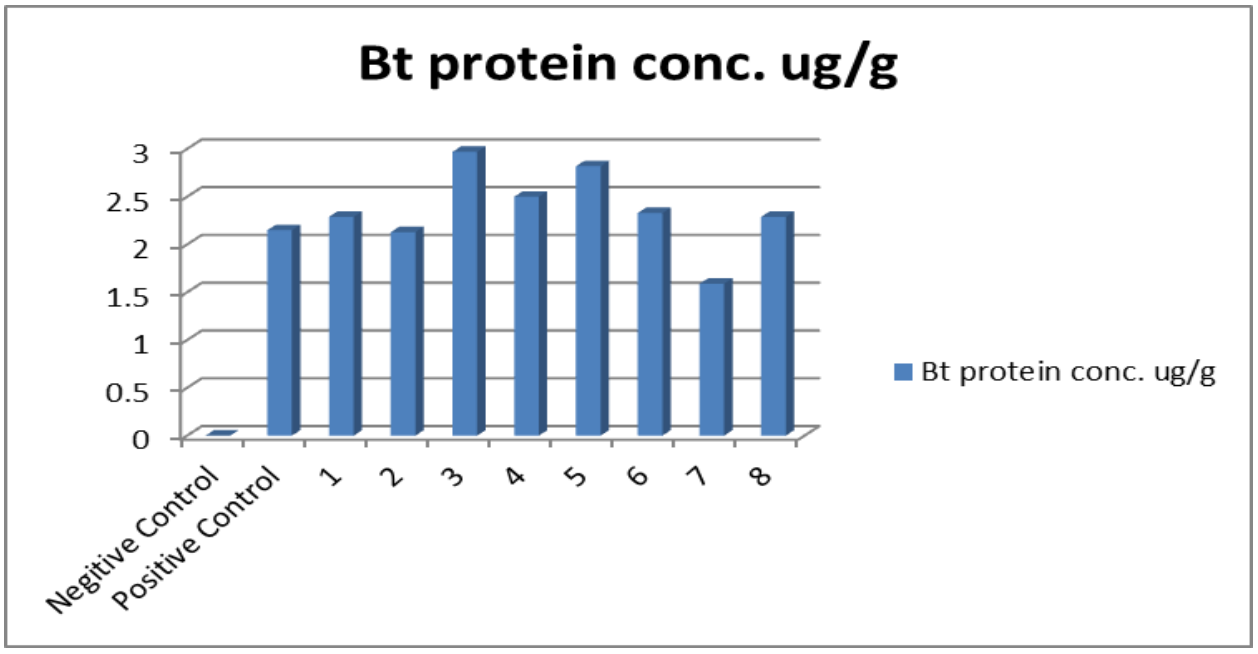


Fig: 18 Quantification of CEMB BT protein in advance generation through ELISA

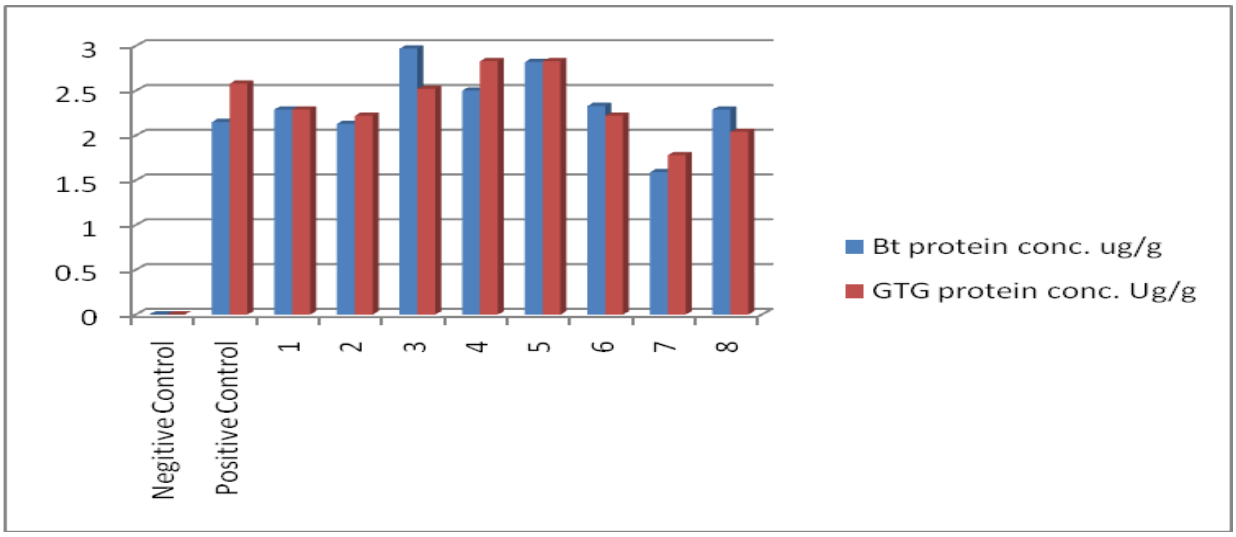


Figure 19: Comparison of Bt and GTG Protein quantity in selected advance lines of transgenic Corn varieties.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.104	2.491	0.604	3.096	3.018	1.998	3.109	1.083	3.064	1.459	0.701	0.041
B	0.388	2.562	1.671	1.019	3.404	1.917	1.706	3.281	3.072	2.731	3.283	0.040
C	1.741	2.217	3.086	2.870	3.249	1.835	2.299	1.398	3.294	2.839	2.584	0.042
D	1.233	0.863	0.774	1.104	1.595	1.332	1.729	2.692	1.905	1.676	2.969	0.043
E	1.165	0.796	3.066	1.519	2.425	1.225	1.581	2.412	2.870	2.139	1.348	0.039
F	1.021	1.039	1.277	1.247	2.824	1.158	3.383	0.907	3.513	1.603	3.445	0.039
G	1.365	2.136	1.986	3.495	3.482	3.484	3.297	2.041	3.493	2.490	3.342	0.064
H	1.536	1.647	1.127	2.112	2.533	1.649	2.322	2.815	3.143	2.759	1.813	0.047

Table: Showing readings of GTG protein concentration read at 450 nm.

Southern Blot Analysis Maize transgenic Inbred Lines

Southern blotting was used for checking the stable integration of transformants in the genome of the potato. The digested fragment was transferred on a nitro cellulose membrane; after the hybridisation of the probe, its detection was carried out using an NBT/BCIP substrate.

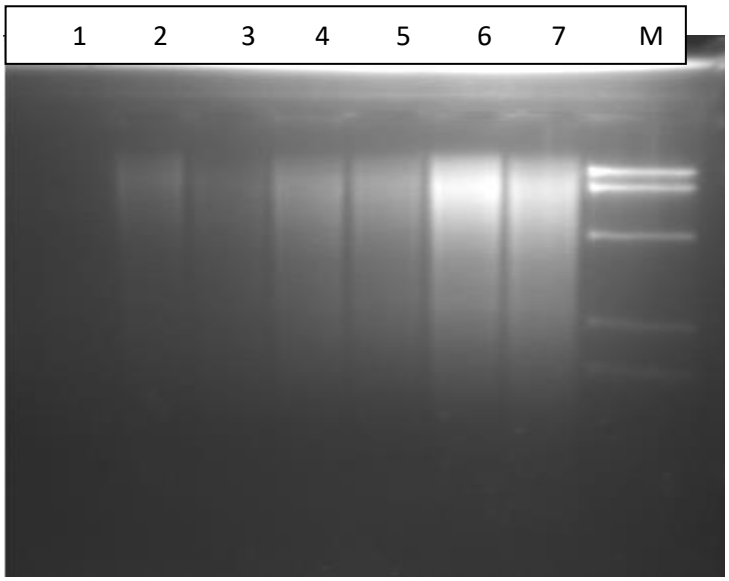


Figure 20: Digestion with *EcoR1* restriction enzyme. **M**: Marker 1kb, **1**: L3/1, **2**: L10/1, **3**: L7/2, **4**: L14/4, **5**: L16/2, **6**: L15/3, **7**: L17/2

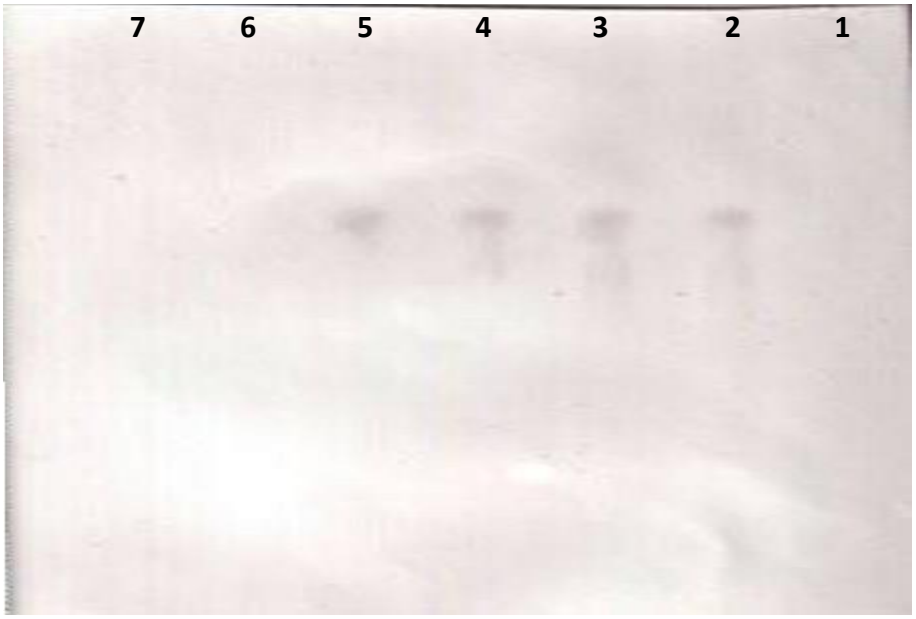


Figure 21: Southern Blot Analysis of positive plants.**1**: L3/1, **2**: L10/1, **3**: L7/2, **4**: L14/4, **5**: L16/2, **6**: L15/3, **7**: L17/2

Southern Blot for GTG

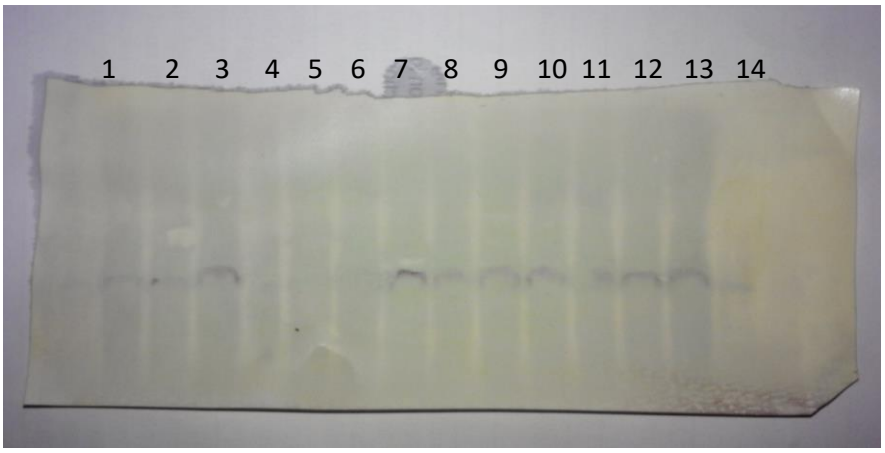


Figure: 1: L1/4, 2: L1/5, 3: L2/1, 4: L2/4, 5: L3/3, 6: L4/3, 7: L11/2, 8: L14/4, 9: L15/1, 10: L15/2, 11: L20/2, 12: l16/4, 13: L16/3, 14: L16/4

Southern Blot for Cry2a

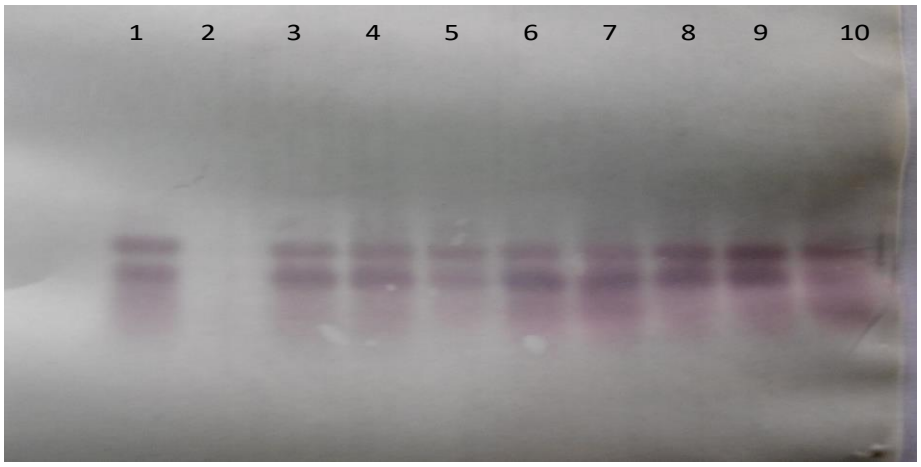


Figure: L1/4, 2: L1/5, 3: L2/1, 4: L2/4, 5: L3/3, 6: L4/3, 7: L11/2, 8: L14/4, 9: L15/1, 10: L15/2

Glyphosate Spray Bioassay of Transgenic Maize Lines.

Figure 24: Survived Maize transgenic plant after spray of 1400 ml/acre

Greenhouse and field trail for Glyphosate application

Insect Bioassay:

Chilo Partillus, American worm were also collected and feed on control as well as transgenic maize lines in field.

Dead insects found on leaves of transgenic maize lines.

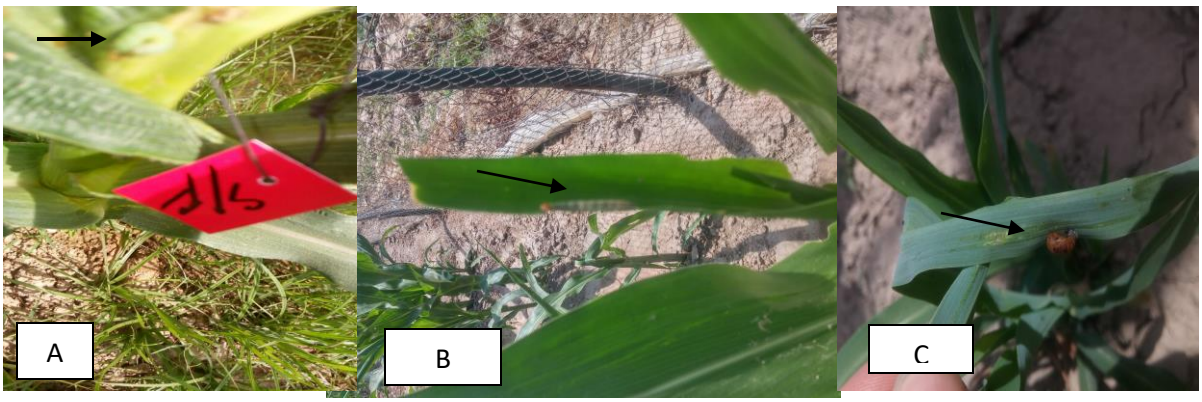
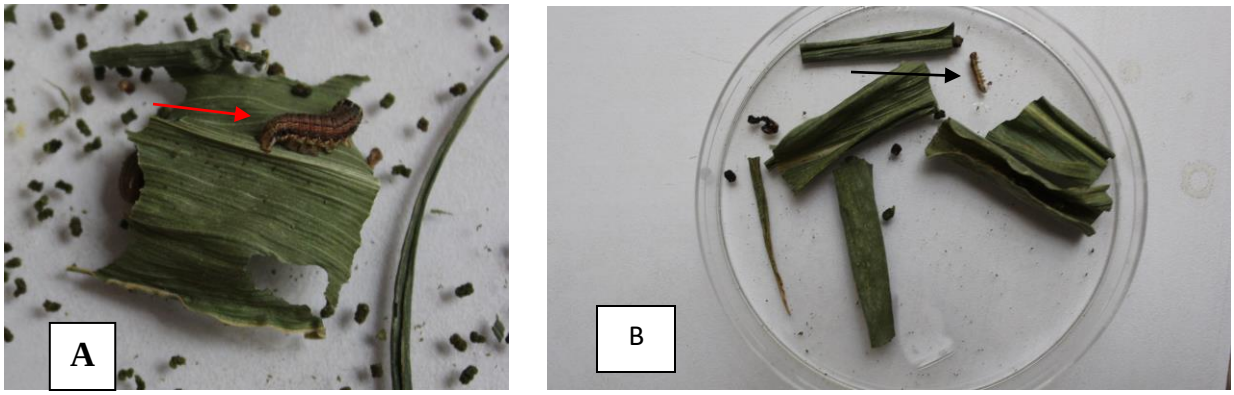


Figure **A**. Living insect feeding on maize leaves. **B**. Control maize line**. C**. African Pink worm dead on transgenic maize plant.



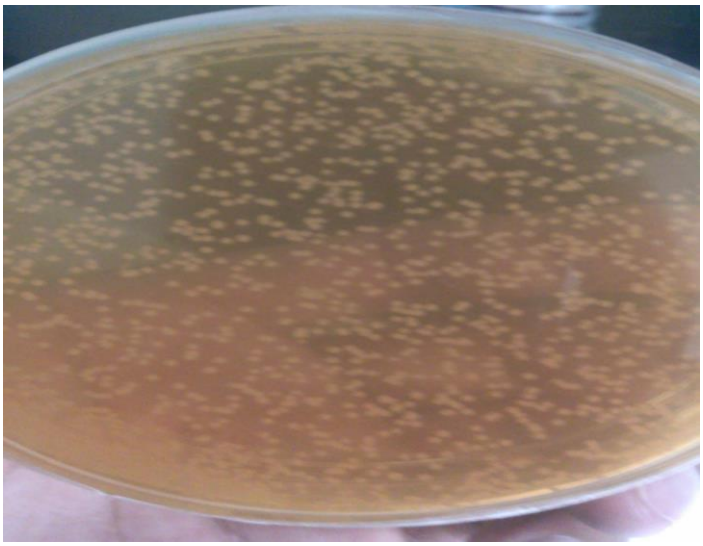
A. Army Worm Feeding on leaves of control plants in lab.
B. Dead army worm on transgenic maize leaves.

Annexure V

Vertical and Horizontal Gene Flow

For vertical and horizontal gene flow DNA was isolated from soil, soil bacteria grown in lab, root exaduates, weeds, etc. the isolated DNA was checked by PCR for presence and absence of Ubiquitin promoter region.

DNA will be isolated from roots. PCR will be carried out to confirm horizontal gene transfer of Bt and GTGene to plant rooting medium. PCR will be carried out in reaction volume of 20 µl containing the genomic DNA template 100 ng, forward and reverse primers 50 pM each, dNTPs 200 µM, 1X PCR Buffer (50 mM KCl, 1.5mM MgCl2 and 10mM Tris-HCl) and Taq Polymerase 1 unit. Amplification will be detected by gel electrophoresis (1% Agarose).



Soil Micro Organisms grown on culture media plate.

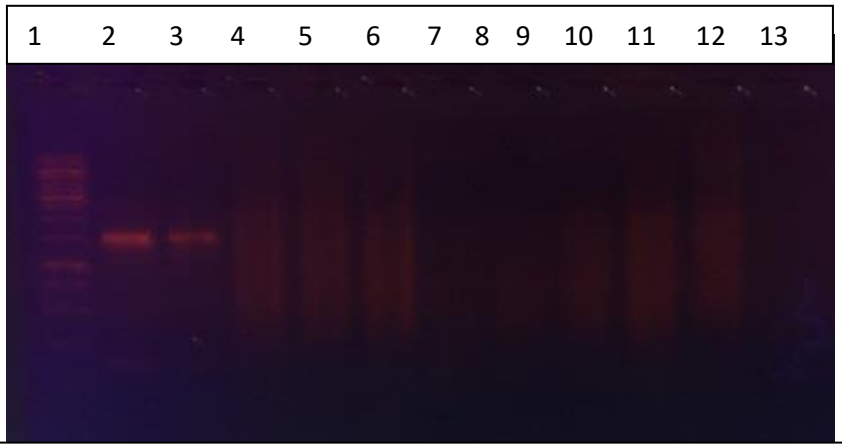


Figure: Lane 1 (from left to right): Ladder (1kb DNA ladder), lane 2: +ive Clone, lane 3: +ive Maize plant, Lane 4-6: DNA from tunnel soil samples, Lane 7-12: DNA of Micro Organisms extracted from soil samples, 13: Negative Control.

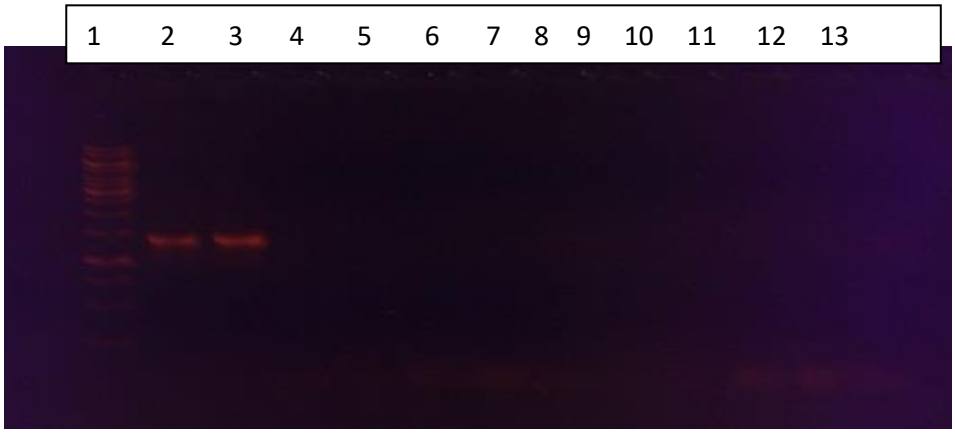


Figure: Lane 1 (from left to right): Ladder (1kb DNA ladder), lane 2: +ive Maize plant, lane 3: +ive Clone, Lane 4-12: DNA from tunnel plants other than maize like weeds etc, Lane 13: Negative Control.

Annexure VI

Effect on Non-Target Insects:

As maize transgenic fields were not sprayed with any pesticide, therefore *Chrysopa perla* were found on maize plants in Transgenic fields.

Figure 30: *Chrysopa perla* in CEMB Bt transformed maize fields.

Annexure-VII

Biosafety Studies of Transgenic Maize Lines

Biosafety studies of five transgenic maize lines were performed at CEMB. Line 1, belong to Agri farm services, whereas CEMB 4 and CEMB 2 belongs to CEMB Lahore. ALL lines were confirmed for presence of CEMB Cry1Ac, Cry2A and GTGenes in them. The cobs were collected from CEMB tunnels after self-pollination.

White American mice were selected for biosafety assessment of transgenic maize lines. Mice were divided into 4 groups. One is control while rest belong to each transgenic line.

Diet Preparation:

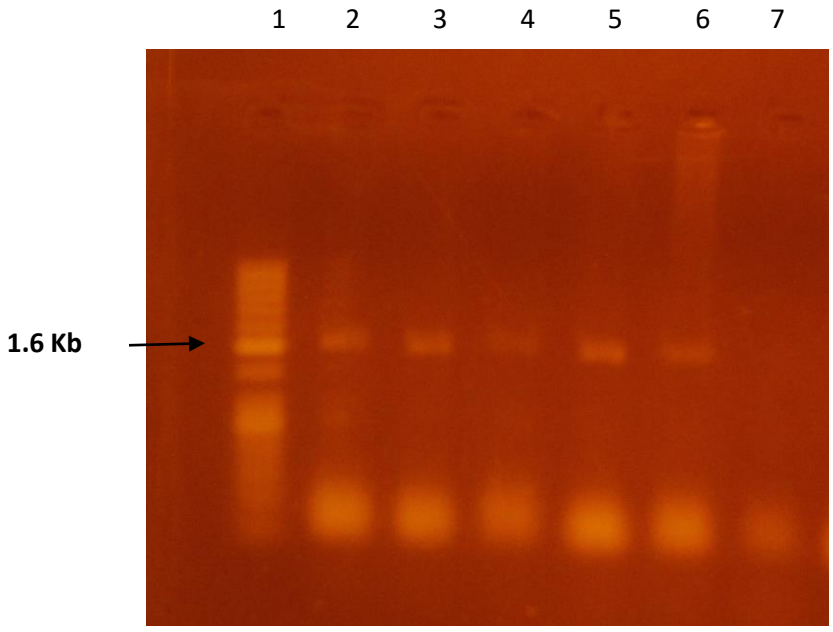
There are four group of diets were prepared. The group one contain maize flour from non transgenic lines. Group two contain maize flour from line 1, group 3 CEMB 2 and group 4 from CEMB 4 respectively. The recipe for mice diet is Baking powder 50g, White Bean 25g, Maize Flour 25g, Oat Bean 12.5g, Wheat flour 75g, Milk powder 25g, Barley Flour 25g, Fine Wheat Flour 50g, Red Bean 25g, Black gram 25g, Oil 25ml.

Detection of Feed for presence of genes:

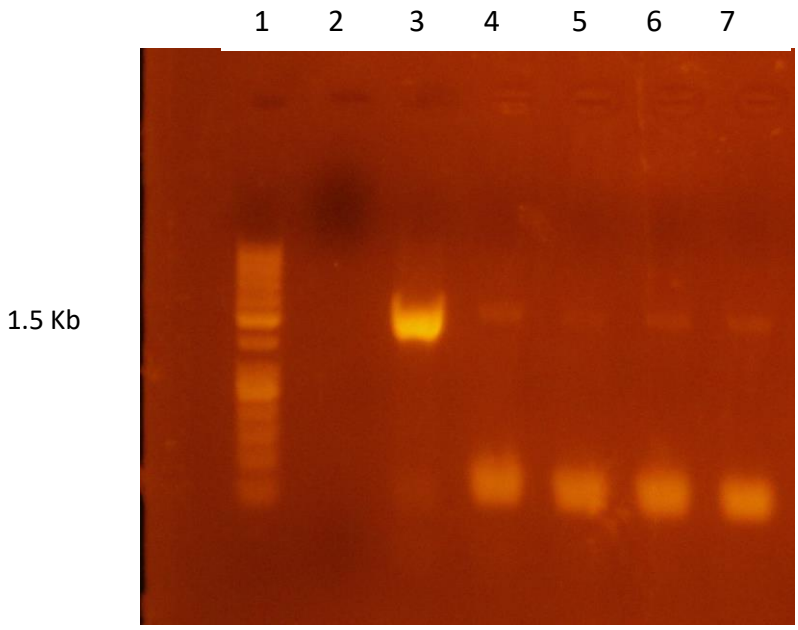
The feed was prepared as per standard instructions and presence of genes were checked through PCR and Dip stick assays. The results are here as under.

PCR for Cry1Ac, Cry2A and GTGene:

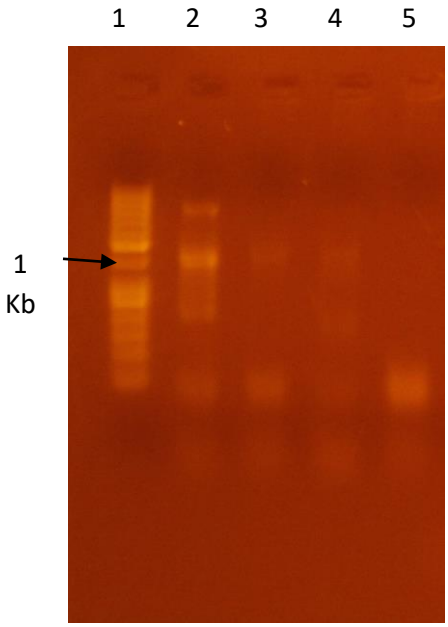
Feeds were checked for presence of Cry1Ac, Cry2A and GTGene in them.



Cry 1Ac: Line 1: DNA Ladder, Line 2: CEMB2, Line 3,4,5: CEMB 4, Line 6 Agrifarm line1. Line7 control Feed



Cry2A: Lane 1: DNA Ladder, Lane2: Control Feed, Lane 3: plasmid, Lane 4 to 7: Transgenic Feed.



GTG: Lane 1: DNA Ladder, Lane2-4: Transgenic Feed, Lane 5: control Feed.

DipStick Assay:

Biochemical Analysis of blood and urine of rat after feeding transgenic Corn

	ALT	AST	AL.phos	Creatine	U-Acid	Urea	Cholesterol	LDH
Control	17.5	15.3	170.3	2.7	3.05	0.9	176.3	1234
CEMB-2	17.6	14.3	181.3	3.2	2.7	1	176.6	1259.6
CEMB-4	19.1	15.8		3.35	3.15	0.9	180	1344.5
Agri Farm Line1	18	14.1	178	2.9	2.51	1	177.8	1264
N values	10-30µ/l	10-30µ/l	167-305µ/l	2.6-7.5g/dl	2.1-5.4mg/dl	0.8-1.5mg/dl	114-244µ/l	1130-1438µ/l

	Hb	TLC	PCV	RBC	DLC:Neut	Lymph	Mono	Eos
Control	12.11	13.4	42.1	4	9.6	44.1	0	3.5
CEMB-2	12	13.8	41.91	4.1	9.6	43.6	0	3.8
CEMB-4	12.15	13.66	42.16	4	10	44.16	0	3.5
Agri Farm Line1	11.96	13.4	41.6	4	9.8	43.1	0	2.6
N. values	11.04-12.56g/dl	13.65-14.34*10^9/l	40.95-43.05 %	3.99-4.49*10^6/µl	10+1	43+3	0	4.2± 0.8

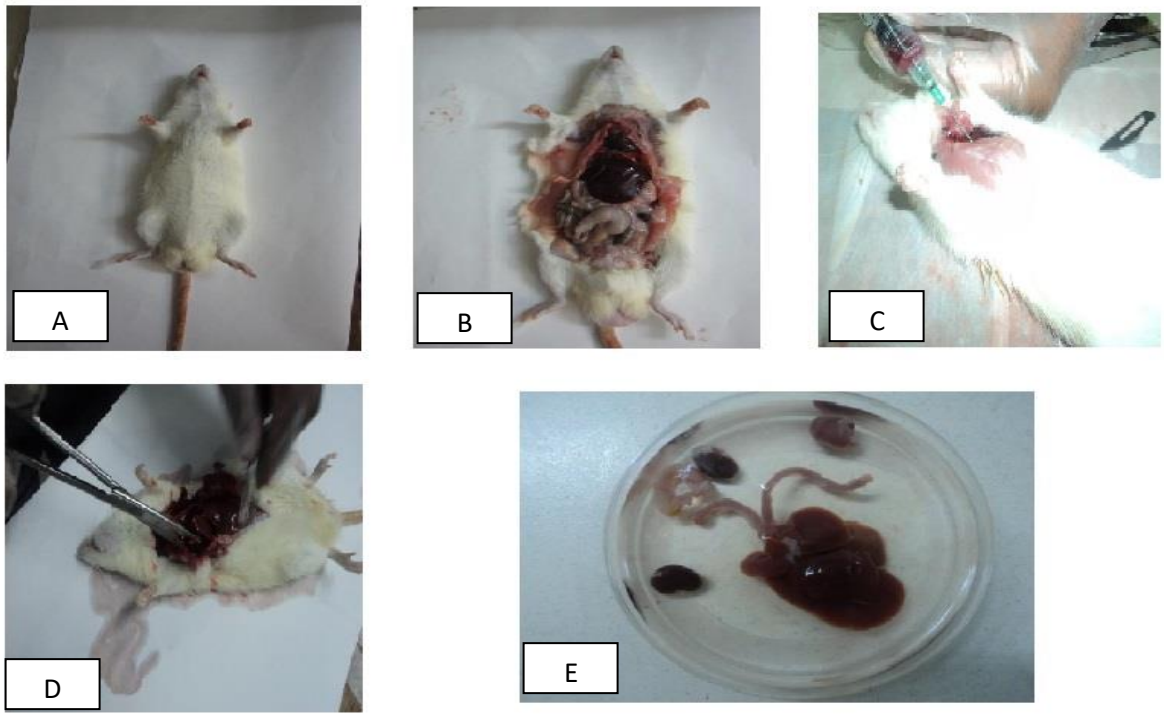
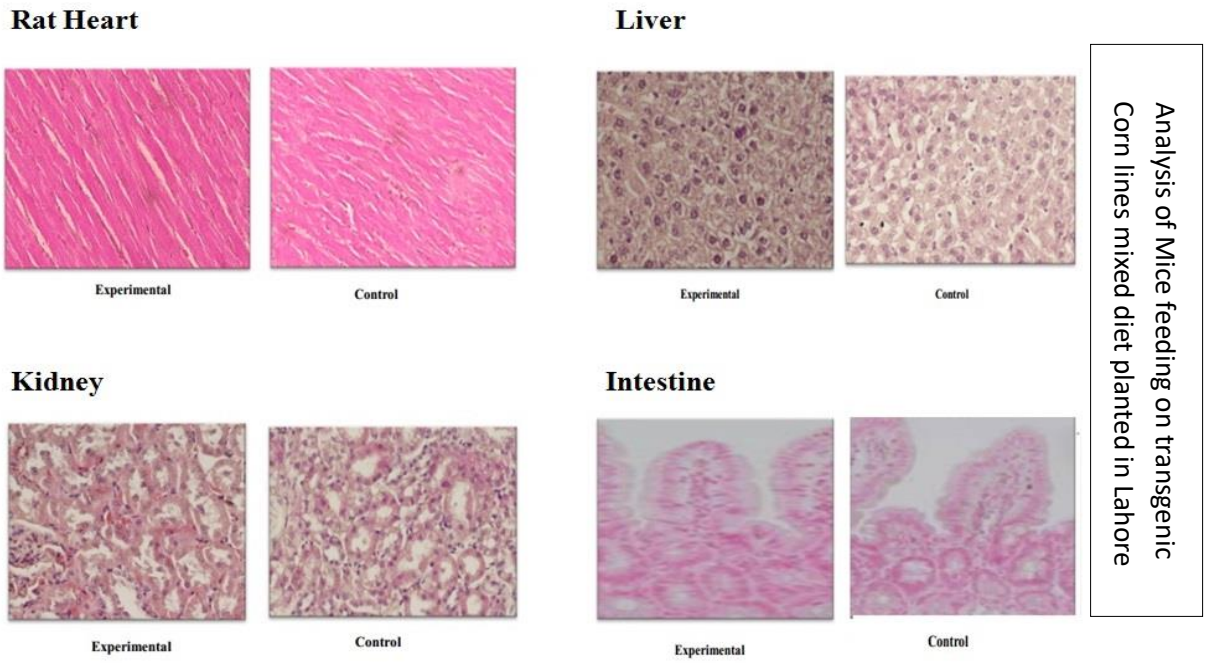


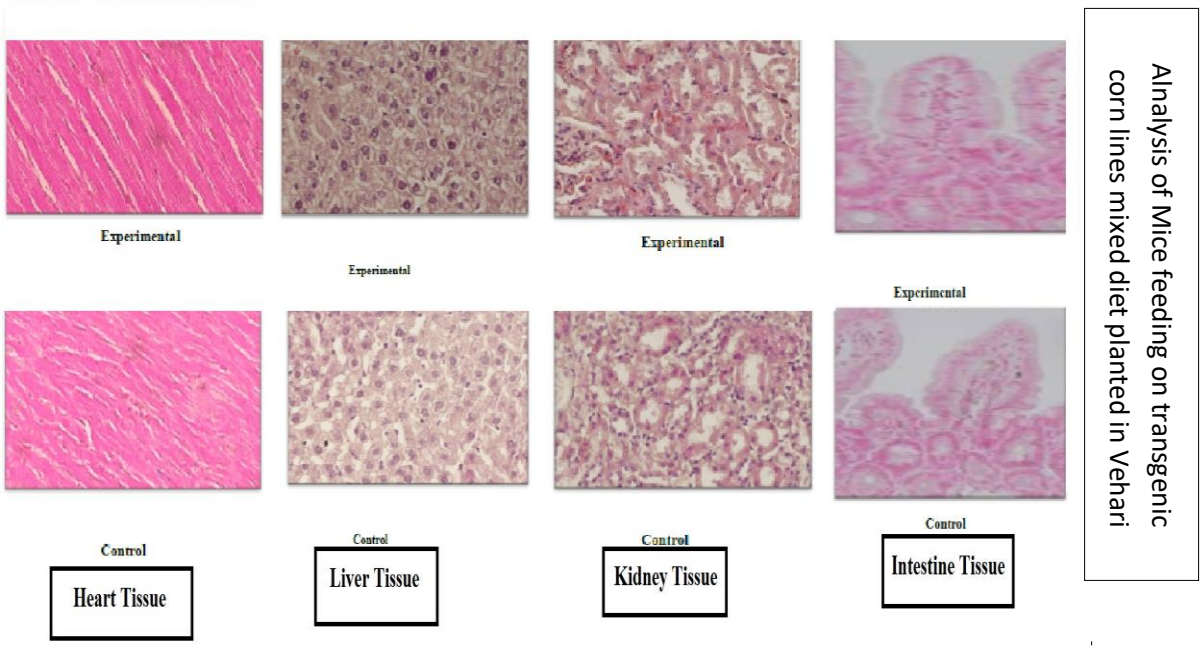
Figure 5: Morphology of Rats after feeding transgenic corn plants with Cry1Ac+Cry2A and GTGene

Fig A .Fainted mice with chloroform **B**. Dissection of mice **C**. Profusion of mice organs, **D**. Cutting of Mice organs **E**. Preservation of Mice organs in 4% Formalin.

Morphology of mice organ

Two mice from transgenic group and two mice from control group are used for the analysis of morphological studies with four vital organs e.g. liver, heart, kidney and intestine.





feeding transgenic diet at different stages

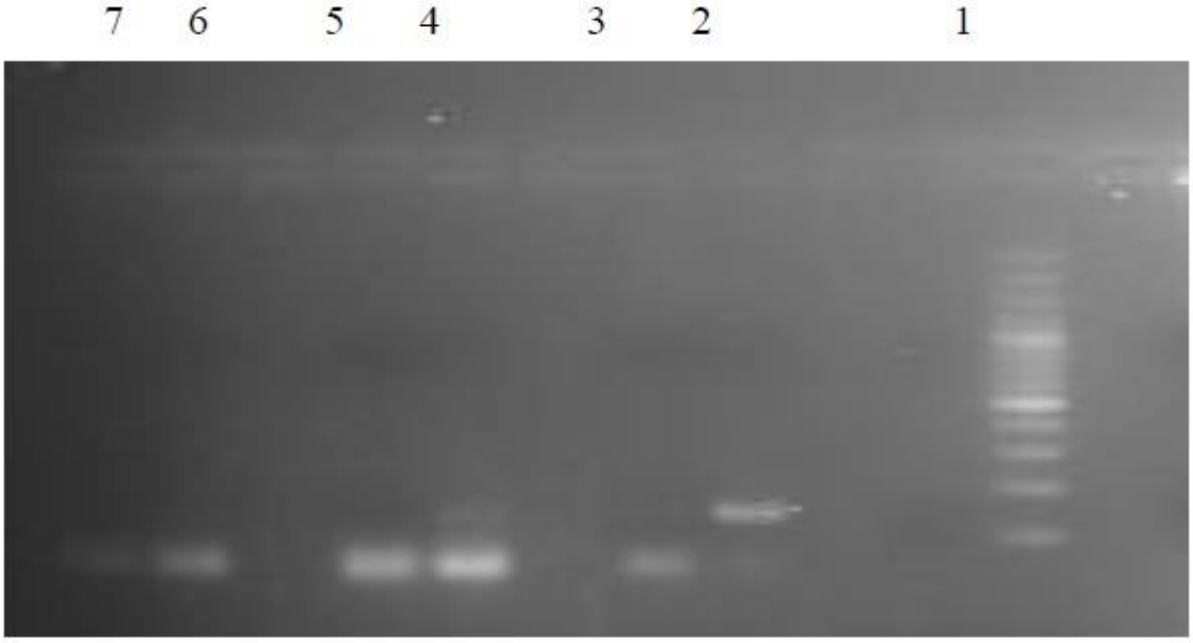


Figure 7: PCR analysis of blood of mice fed on transgenic corn plants along with control diet mice; Lane1: 1kb plus ladder Lane 2: +ve control Lane 3: -ve control Lane 4: Experimental Male Lane 5: Experimental Female Lane 6: Control Male Lane 7: Control Female.

Samples	Protein µg/g
Negative	0.004
Positive	0.256
Transgenic diet	0.243
Transgenic diet	0.245
Transgenic diet	0.233
Control diet	ND
Control diet	ND
Control diet	ND

Figure 8: ELISA for Transgenic and Control diet

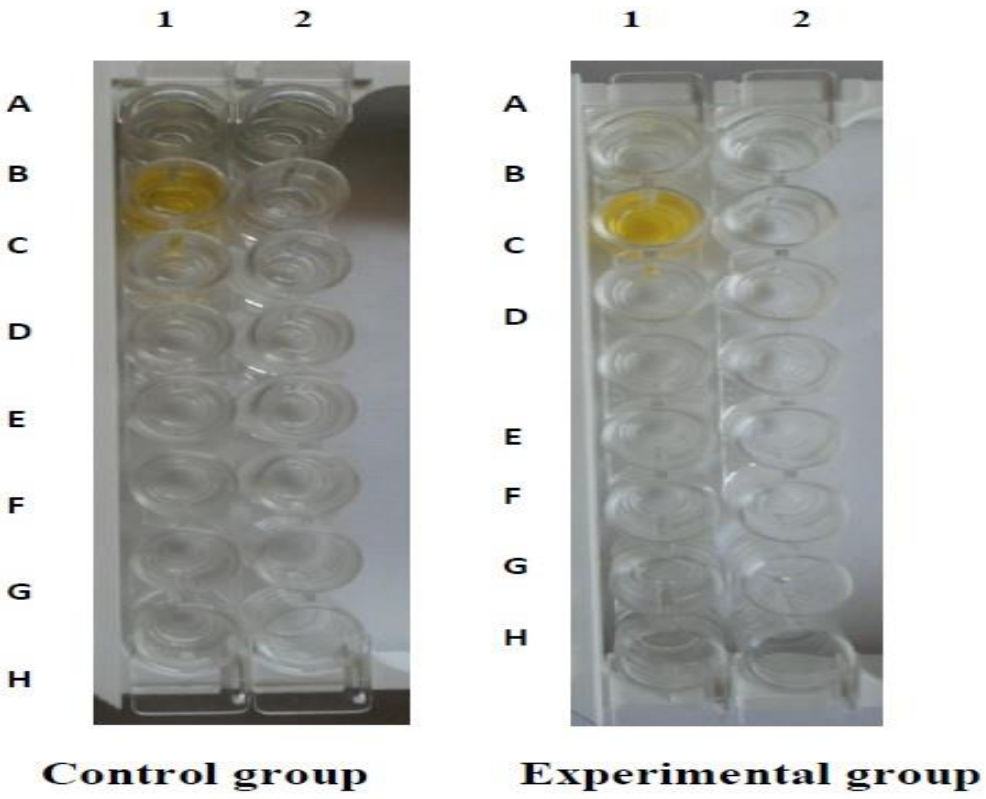


Figure 9: Enzyme linked immune-sorbent assay was performed from blood serum of mice after feeding.

Outcomes of Project:

Development of Product

Five Corn inbred lines resistant to insects and weedicide were developed; handed over to Agri-Farm Services Multan for multiplication and to develop high yielding F1 Hybrids with insect resistance, Weedicide resistant traits. It will add up in national economy which was major expectation of this project.

Published 2 articles in world reputed journals

1. Qamar, Z, Aaliya, k, **Nasir**, I. A, Farooq, A. M, Tabassum, B, Ali,Q. Husnain, T. (2015): An overview of genetic transformation of glyphosate resistant gene in Zea mays. Nature and Science 2015;13
2. Aaliya, k., Qamar, Z., **Nasir**, I. A., ALI, Q., Farooq, A. M. and Husnain, T. (2016). Transformation, Evaluation of GTGene and Multivariate Genetic Analysis for Morpho-Physiological and Yield Attributing Traits in Zea mays. GENETIKA, 48(1):423-443.

PHYSICAL PROGRESS REPORT
OF THE PARB PROJECT NO. 235
(01-06-2010 TO 31-05-2017)

DEVELOPMENT & COMMERCIALIZATION OF INDIGENOUS TRANSGENIC BT AND HERBICIDE TOLERANT MAIZE HYBRIDS

By

Muhammad Imran

AGRI FARM SERVICES
Multan

PHYSICAL PROGRESS REPORT FOR PARB CGS PROJECT

Basic Information

1.	Name of the project	Development and commercialization of indigenous Bt and herbicide maize hybrids.
2.	Project No.	235
3.	Total Project cost	22.89 million
4.	Total project duration	84-months
5.	Funds released so far	4.891494 (fund release to Agriri Farm from parb)
6.	Project commencement Date	01-06-2010
7.	Name of the Project Manager	Dr Adrees Ahmad Nasir

PHYSICAL RESEARCH ACHIEVEMENTS

Item	Description	Planned Completion date	Achievement indicators as planned	Achievements	Reason for deviation if any
Overall Project Objective	Development & commercialization of indigenous transgenic Bt and herbicide tolerant maize hybrid	1-6-10 to 31-5-17	Five single cross best high yielding hybrids were selected		
Output-3	Development of single cross hybrids from transformed inbred lines and their evaluation.	1-1-15 to 31-5-17	Five nigh yielding transgenic F1 corn hybrid will be available for varietal approval	Transgenic parental line seed received from CEMB Were selfed event wise (plant wise)	-
Activity-1	Crossing of transformed inbred line	1-12-16 to 31-05-17	Seed of five hybrid will be available for trials in sufficient quantity	1. Transformed inbred line seed is sown in field dated 6/2/2017 Event wise plant wise plot size 15 feet length Two rows of each plant of each event, RR distance : 2.5 feet PP distance: 8 inches 2.Round up is spray @ 1600 ml/acre 1/3 of total plot of each event of each plant on31/3.2017 3.After 20 days of spray plant of event -1 of p5 survive normally and 3&4 plant of event 7& 8 were abnormally survive respectively 3. shoot borer and shoot fly resistant were found in E1, E5&E6.	Roundup is spray against weeds to check RR gene reistan

Team Leader
PARB Project
No 235
Multan