

Dissemination of Bt cotton in cotton growing belt of Pakistan

Saira Azam¹, Tahir Rehman Samiullah¹, Aneela Yasmeen¹, Salah ud Din¹, Adnan Iqbal¹, Abdul Qayyum Rao¹, Idrees Ahmad Nasir¹, Bushra Rashid¹, Ahmad Ali Shahid¹, Munir Ahmad², Tayyab Husnain¹

Key words: Double gene Bt cotton, Cry1Ac, Cry2A, ELISA, PCR, Pakistan,

Abstract

Background: Adoptability of biotech crops has climbed up dramatically in the world from 1.7 million hectares in its introductory period in 1996 to 170 million hectare in 2012. Area planted to biotech crop increased to over one-fourth of the world total in 2005-06. The data collected also demonstrates that developing countries like Pakistan are making tremendous development in production of Biotech crops especially cotton.

Methodology: Random samples have been collected and received from different cotton growing areas of Pakistan from 2007 to date for Bt gene confirmation and expression. Genomic DNA was isolated and analyzed through internal reference primers from SadI gene of cotton genome. Samples were analyzed by PCR for detection of Bt genes including CEMB Cry1Ac+2A double Bt gene. ELISA was done for confirmation of Bt protein by using Enviroligix Quantiplate ELISA kit Cry1Ab/Ac Cat # AP003 and Quantiplate ELISA kit Cry2A Cat # AP005 according to manufacturer instruction.

Results: Development of Bt Cotton in Pakistan is as old as in advanced countries of the world. Pakistan has become the fourth country of the world for production of indigenous Bt cotton by utilizing their local cotton varieties for genetic modifications. The support of various documentary proofs like research articles, patents, projects, Ph. D and M.Phil studies generated by Centre of Excellence in Molecular Biology (CEMB) greatly strengthen the data of Bt cotton development in Pakistan. Pakistan Central Cotton Committee (PCCC) declared locally developed Bt cotton as the best performing of all over Pakistan among other international product.

Conclusion: Progress made in development of indigenous Bt cotton variety by CEMB Pakistan and their excellent performance in field determined their increased adoptability ratio in farmers. The formal approval process which is going to be completed in near future will open the doors for farmers and breeders to utilize this material for better economy of Pakistan.

*Corresponding Author: Dr. Abdul Qayyum Rao (Email: qayyumabdul77@yahoo.com)

1Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore - Pakistan

2Ali Akbar Seed Company, 1 km Bhothian Chowk Defence Road off Raiwind Road, Lahore - Pakistan

Introduction

Cotton is the most important cash crop and backbone in developed as well as developing countries [1]. Cotton seed has the credit of being the oldest seed known so far. It is being cultivated since 3500 BC in Indus Valley. It was found near Bolan Pass in Baluchistan [2]. This crop is a good source of income for the farming community and textile industries as many countries are earning major GDP from cotton export. Cotton being source of income for more than 250 million people of world is the largest profit earning crop produced in the world [3]. Due to its economic importance Cotton has always received importance in Research and Development. Cotton is produced among 70 countries of the world but two third of it is cultivated in four major countries including China, US, India and Pakistan [4]. Figure 1 gives an insight regarding quantity of cotton produced by these countries from year 1980-81 to 2012-13.

Cotton is known as “White Gold”. It is one of the most important cash crops of Pakistan contributing major source of foreign exchange earnings. It accounts for 8.2 % of the value added in agriculture and about 2 % of the GDP. Pakistan has ranked as the 3rd largest exporter of Cotton as well as it also stands 2nd in Yarn export; 3rd in yarn production; 3rd in cloth export; and, 7th in Cloth production (Source: International Cotton Advisory Committee, Washington D.C., USA). Pakistan is the 4th largest consumer of cotton in the world. According to a rough estimate almost 26% of Pakistani farmers grow cotton. The economic survey

of Pakistan shows that 2689 thousand hectares of area was used for growing cotton crop during 2010-2011. Figure- 2 shows a comparison of cotton production in Pakistan.

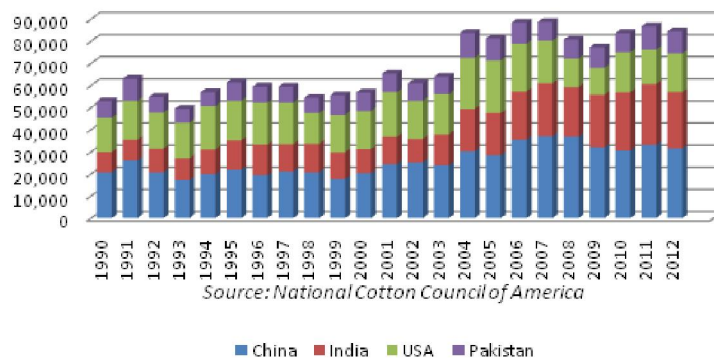


Figure 1: World Cotton production (in Million Tones) by Major Cottonproducers

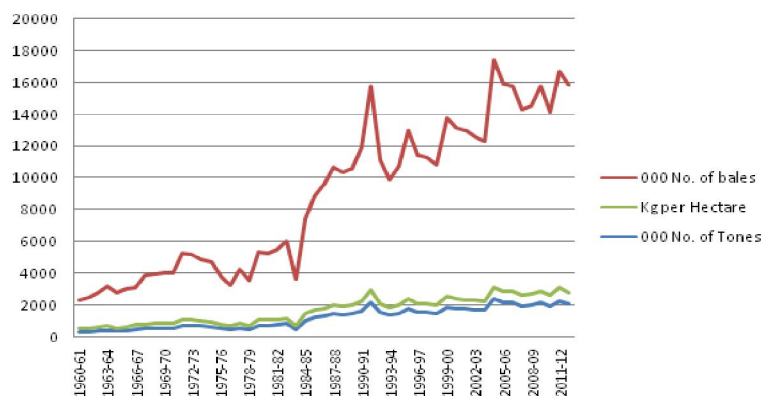


Figure 2: Yearly Cotton production trend in Pakistan (1960-2013), Source: Pakistan Cotton Ginners Association

The cotton belt of Pakistan ranges across Punjab and Sindh provinces. The production of cotton in these 2 provinces in past years is shown in figure 3 & 4.

In Pakistan, major insects including Pink Boll worm (*Pectinophora gossypiella*), American Boll worm (*Heliothis armigera*), Army worm (*Spodoptera lithura*), Spotted Boll worm (*Earias insulana/vitella*) cotton and Lepidopteron infect cotton crop drastically. In agricultural system, crop protection was exclusively depended upon

spectrum highly toxic agrochemicals and their broad spectrum use which has caused human health concerns and severe environmental problems. This situation provoked efforts to develop organic control measures for insects and their attack on crops [1]. Numerous distinctive opportunities are made possible by transgenic approaches to manage population of pest. However there are some prerequisites for above mentioned approach including proper inheritance, integration, and expression of transgenes to confer permanent resistance in crops against insects [5]. Defensive genes are present as constitutive part of the genome in different plant tissues or as defensive compounds produced when plant is attacked by pathogens and pests [6]. Many public and private sector institutes are working in developing new varieties and maintaining the cotton breeds. Each of such new varieties needs to get official approval before cultivation on large areas. The Pakistan Central cotton Committee (PCCC) is a national organization responsible for research on cotton and all its aspects. This committee is further co related to various bodies e.g. Federal Seed Certification and Registration Department (FSC&RD) and Expert Sub Committees which are performing many functions. Each new variety needs to go through the National Coordinated Varietal Trials (NCVT) conducted by PCCC. The varieties need to clear the DUS trails and bio-safety trials (in case of genetically modified crops) as well. Depending upon the trails results the variety is subjected to be approved or disapproved

for official permission of cultivation by the respective provincial seed council.

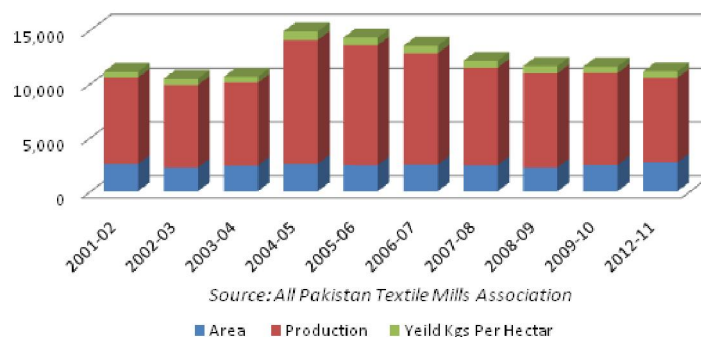


Figure 3: Area, Production and Yield (Kgs) per hectare of cotton in Punjab in last 10 Years

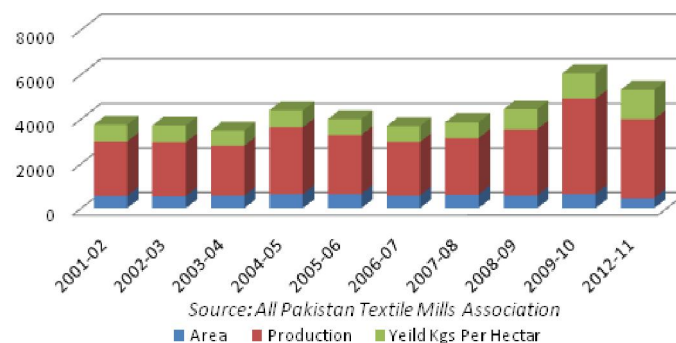


Figure 4: Area Production and Yield (Kgs) per hectare of cotton in Sindh in last 10 years

Sixteen Bt cotton varieties developed by various public and private institutes of Pakistan have been approved after following the set criteria (list given in table 1). A number of single gene Bt cotton varieties along with 10 varieties developed by CEMB double Bt gene (Cry1Ac+ Cry2A) namely; CEMB-02, CEMB-22, CEMB-33, CEMB-44, CEMB-55, CEMB 66, CA-12, CA-904, CA-919 and CR-333 are being evaluated by the PCCC, PSC, NBC etc. for varietal approval purposes.

Further thirteen single Bt gene cotton varieties namely VH-259, BH-178, CIM-

599, CIM-602, FH-118, FH-142, IR-NIAB-824, IUB-222, Sayban-201, Sitara-11M,

Sr. No	Variety Name	Developing Institute	Year of Approval
1	IR-3701	NIBGE, Faisalabad	2010
2	Ali Akbar 703	M/s. Ali Akbar Seeds, Multan	2010
3	MG-6	M/s. Nawab Gurmani Foundation, Kot Addu and M/s. Agri. Farm Services, Multan	2010
4	Sitara-008	M/s. Nawab Gurmani Foundation, Kot Addu and M/s. Agri. Farm Services, Multan	2010
5	IR-1524	NIBGE, Faisalabad	2010
6	FH-113	Cotton Research Institute, AARI, Faisalabad	2010
7	Ali Akbar-802	M/s. Ali Akbar Seeds, Multan	2010
8	Neelam-121	M/s. Neelam Seeds, Multan	2010
9	Tarzen-1	M/s Four Brothers Lahore.	2012
10	MNH-886	M/s. Ali Akbar Seeds, Multan	2012
11	NS-141	M/s. Neelam Seeds, Multan	2012
12	FH-114	Cotton Research Institute, AARI, Faisalabad	2012
13	IR-NIBGE_3	NIBGE, Faisalabad	2012
14	IR-NIBGE_901	NIBGE, Faisalabad	2012
15	CIM-598	Cotton Research Institute, Multan	2012
16	VH-259	Cotton Research Institute, Vehari	2012

Table: 01 List of Bt Varieties approved for General cultivation in Pakistan.

A-555, KZ-181 and Tarzan-2 including two varieties namely CA-12 and CEMB-33 containing CEMB double Bt genes have been recommended by Expert subcommittee Punjab, Pakistan in its 69th meeting held on 16-04-2013 for general cultivation in the Punjab Province. The Punjab Seed Council will consider these fifteen varieties for approval in its 44th meeting proposed to be held in the month of October-November 2013.

There are four labs which are responsible for testing of Bt contents of candidate varieties. These labs are part of following institutes:-

1. National Institute for Agricultural Biotechnology Research Institute Faisalabad (ABRI), Faisalabad
2. Centre of Excellence in Molecular Biology (CEMB), Lahore
3. National Institute of Biotechnology and Genetic Engineering (NIBGE), Faisalabad
4. Genomics and Advanced Biotechnology (NIGAB), Islamabad

During last 5 years various samples were received in CEMB from different cotton institutes of Punjab and Sind. These were analyzed for their Bt content. The results of all such experiments have been compiled in this paper to show the increasing content of Bt protein in cotton belt of Pakistan. Moreover, the spread of CEMB Cry1Ac+2A was also analyzed in received samples and the results are also reported here.

Methods

Plant Material

Random samples have been collected and received from different cotton growing areas of Pakistan from 2007 to date for Bt gene confirmation and expression.

Plant genomic DNA extraction and quality confirmation

Genomic DNA was isolated from apical leaves of cotton plants (growing in the field) by using Intron Scientific G Spin™ IIP kit cat# 17271 and analyzed through internal reference primers from SadI gene of cotton genome.

Screening of samples through PCR

Genomic DNA of collected and received samples were analyzed by PCR for detection of Bt genes by amplification of internal fragments of Bt genes. These samples were analyzed for Cry1Ac and Cry 2A genes by using PCR as done in other reported studies [7,8]. Presence of CEMB Cry1Ac+2A double Bt gene was also done in these samples by using CEMB gene specific primers.

Expression analysis of cotton samples for Bt protein

The total proteins of all plants samples were isolated from apical leaves, Bradford assay was performed for estimation of total protein as done by Bradford [9]. ELISA was used to screen the plants for expression of Bt protein. Confirmation of Bt protein in samples was done by using Enviroligix Quantiplate ELISA kit Cry1Ab/Ac Cat# AP003 and Quantiplate ELISA kit Cry2A Cat# AP005 according to manufacturer instruction.

Results

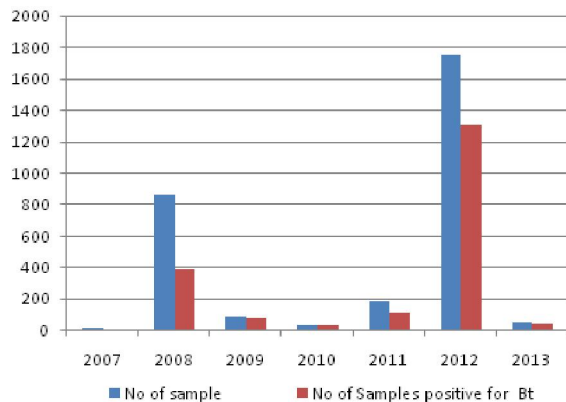
Samples were collected from different research institutes and breeding center since 2007 to 2012. The samples were tested for their Bt content via PCR and ELISA tests. The number of cotton samples received in various dates and the percentage of positive Bt cotton samples is given in table 02. From table 2 it is clear that Bt was not detected in received samples until 2007 but from year 2008 to date insect resistant gene was detected in various samples. A comparison of Bt cotton samples in various years is shown in graph 1 & 2. Graph 2 shows the increase of Bt cotton in cotton belt of Pakistan with the passage of time. CEMB GMO detection lab has also analyzed the presence of CEMB double Bt gene (Cry1Ac/2A) in various samples. The results of those tests are compiled in table 3.

Year	No. of sample Analyzed	No. of Bt +ve Samples
2007	19	0
2008	860	393
2009	92	77
2010	35	33
2011	190	112
2012	1750	1310
2013	49	46

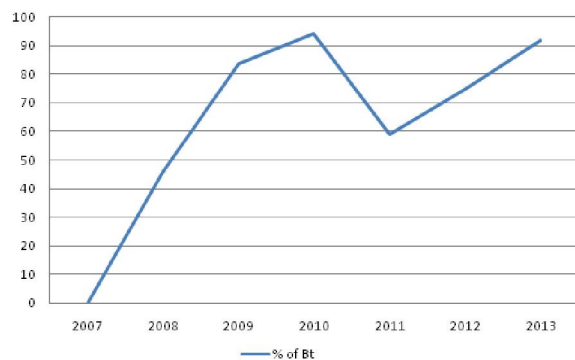
Table 2: Number of cotton samples received in various dates and the percentage of positive Bt cotton Samples

Graph 1 determines the comparison of Bt cotton samples received in different years starting from 2007 to date. From the graph it is also cleared that maximum samples has been received in year 2012 which shows increase dissemination of Bt cotton with the passage of time. However the small bar in

2013 indicates that there are samples still to be analyzed in future years.



Graph 1: A comparison of total cotton seeds samples with the Bt positive samples

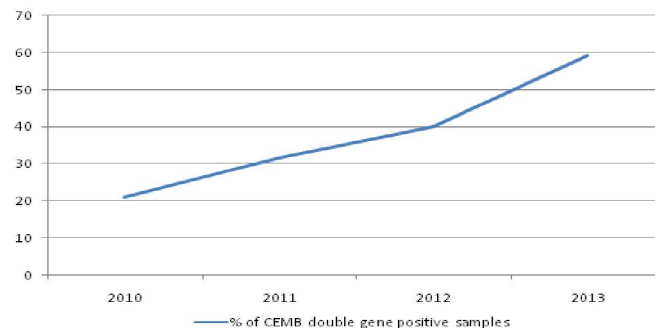


Graph 2: Graph 02: Dissemination of BT seeds in cotton belt of Pakistan from 2007 to 2012 % of Bt positive samples during last seven years

Graph 2 represents the %age of cotton samples positive for Bt protein in different years. The continuous increase over last two years is also obvious from the graph. The discontinuity in graph line in years 2010 and 2011 was observed which reflects less percentage of Bt samples in cotton areas in these years.

Year	No. of sample Analyzed	Cry1/2A Positive
2010	91	19
2011	35	11
2012	1750	700
2013	49	29

Table 3: Number of cotton samples with positive CEMB double Bt Gene



Graph 03: Comparison of % of CEMB Double Bt Gene Positive samples during last 4 years

Discussion:

Cotton is the major export material earning sizable foreign exchange for Pakistan but its annual yield is decreasing due to many factors including H. Armigera attack as a major pathogen [6]. Using conventional plant breeding techniques, cotton breeders have continuously sought to improve the cotton which, in past century, has added number of different improved traits in crop yield. But in existing germplasm resistance against insect pests is not present; this has led transfer of new transgenes into plants to create plant varieties with novel characters through plant breeding techniques that's why transgenic cotton expressing insecticidal proteins from B. Thuringiensis (Bt) has been one of the most rapidly adopted GM crops in the world [5]. Plants transformed with single Bt gene are liable to develop insect resistance and this has already been reported in a number of studies carried out around the world where Bt cotton was cultivated on commercial scale. Later, it was envisaged to transform plants with more than one Bt genes in order to combat with resistant larvae. This approach seems valid as various Bt genes possess different binding domains which could

delay the likely hazards of insect resistance against a particular Bt toxin [10]. Many public and private institutes are working on development of Bt cotton. In transformation of virus resistant genotype of *Gossypium hirsutum* L., with pesticidal gene CEMB has developed protocol for cotton genetic modification as reported by different studies [11,12]. CEMB claims to have developed Bt cotton with both single and double gene and citation of many PhD studies right from 1996 to time, with nearly fifty research articles and five patents in support of this claim along with approval of their more than ten lines from National Bio-safety Committee hence strengthening their claim [2,5,13-19]. There is a set procedure for Bt variety to get approval. The variety has to pass through NBC approval process, two years DUS studies by the FSC&RD, two years field trial at all Pakistan level by the PCCC and one year trial at PSC Khanewal for spot examination by the expert team of scientists has also to be done before the case can be submitted to Punjab Seed Council for approval by expert subcommittee. Sindh Seed Council also follows same procedure for giving approval. So far, sixteen Bt Cotton varieties have been approved by Punjab Seed council; eight of them were approved in 39th meeting of Punjab Seed Council while the other eight are recently approved in 42nd Meeting of Punjab Seed Council (Punjab Seed Council, 2012). The details are given in table 02.

Single gene varieties have been approved for cultivation in 2012. But the record of CEMB shows that Bt cotton was there in cotton belt of Pakistan since 2008. In Pakistan, insect Resistant cotton was grown

on 2.4 million hectares out of 2.8 million hectares allocated land. In addition, the CEMB double Bt gene variety which got NBC approval/commercialization license in 2009 has been spread all over the cotton belt as shown in table 3 and graph 3 and also reported in different studies [1,3]. PhD dissertations along with number of M.Phil research dissertations have highlighted insect resistant cotton development in CEMB during these years [2,5,10,18].

In conclusion from above discussion it is clear that development of Bt cotton in Pakistan is as old as from 1996 as the outcome comes in form of publication from these dates. Bt cotton introduction in Pakistan in the form of Single and double gene is not new but its local development and multiplication has introduced it very early and now the data reflects its limited spread all over Pakistan.

Moreover from CEMB data it is also clear that bio-safety trial of insect resistant plants having Bt genes singly and double Bt genes (CEMB 02 Events) has been started from early 2000 on different organisms including Mice, Fish, Earthworm, Chicks and Rabbits along with gene flow studies including horizontal and vertical gene flow which results in delaying their efforts to be brought in front of public. Thirteen years studies of risk assessment clearly demonstrate that CEMB-02 event is safe from risk point of view for all organisms.

The adoptability ratio of CEMB double Bt gene by farmers demonstrate the excellent performance of CEMB-02 event in farmers field as it is being transformed in

local cotton varieties and it will prove to be milestone for farmers and breeders to develop new cultivars after its approval from Punjab seed council in near future.

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