THE ICAC RECORDER

International
Cotton
Advisory
Committee

Discover
Natural
Fibres
Initiative

Technical
Information Section

VOL. XXXV No. 3
SEPTEMBER 2017

Update on Cotton
Production Research




Content

Editorial

There are five articles in this issue; three are on traceability and two are on the cotton boll weevil (CBW). Both topics are important and have a strong bearing on the cotton sector. Traceability haunts textile credibility across the world, and boll weevils haunt productivity in America and Latin America.

Two important articles are related to the management of boll weevils in the American continents. These papers set the agenda for the XIV Meeting of the Latin American Association for Cotton Research and Development (ALIDA) held on 28th August at Maceió, Brazil. In his article, based on his enormous field experience, Mr. James Schoenholz recollects briefly the long history of planning, execution and lessons learnt in the war to eradicate boll weevils in USA and Mexico. Dr. Fatima Grossi and her colleagues describe their exciting results of successful genetic integration of the *Bt* gene *cry10Aa* into a native cotton cultivar BRS 372, and also the prospects of using gene silencing through Ribonucleic Acid Interference (RNAi) to control the dreaded cotton boll weevil. The newly-developed transgenic cotton technology immensely strengthens the arsenal for boll weevil eradication programs.

Three articles in this issue deal with Traceability. Mr. Kai Hughes gives an overview of the current state-of-art of cotton traceability technologies. Drs. Negm and Susan give an insight into the happenings on cotton traceability in Egypt. Mr. Joy and his team review the recent advances in DNA-based traceability techniques. The articles on DNA traceability concludes that the technology has promise but is not yet fully developed.

Traceability is defined by the International Organization for Standardization (IOS) as 'the ability to verify the history, location, or application of an item by means of documented recorded identification'. Traceability acts as the connecting link between all the elements and processes that are used for the creation of a product. The subject of 'cotton traceability' has acquired new dimensions in recent times due to controversies related to dubious claims and tarnished brand images. Textile companies are now concerned more than ever before about sustainability features in their business practices and traceability assurances in their products. Efforts are being made to develop techniques, tools and strategies to define product characteristics such as source and history of the raw material and other components used in their value chains.

There are several layers to the topic and several players in the field to whom the subject of traceability is of grave concern. The layers are related to sustainability of social aspects, economic factors and the environment; the players in the cotton value chain are consumers, farmers, traders, ginners, spinners, weavers, workers, input suppliers and industrialists, wholesalers and retailers. Then, there are other players who are in the Governments or in the private sector or in the non-government organizations who influence the cotton sector. The textile sector is characterized by complex supply chain networks comprising of independent suppliers of different kinds of raw materials such as lint, yarn, fabric and dyes. Is it possible to track and trace back the journey of a fiber from its origins to its destiny in a fabric? Is fiber traceability a practical possibility for cotton textiles? Does traceability lead towards sustainability? Is the consumer actually concerned about sustainability? Will traceability be transparent and reliable? Will it lend credibility to claims or will it be exploited commercially as yet another dubious tool to fool the industry and consumers? These questions are as difficult as is the subject of 'fiber traceability in cotton textiles'. An attempt is made in this issue of *THE ICAC RECORDER* to focus on the state-of-art of traceability technologies, which may partly address the questions.

Traceability technologies deal with documentation of

The ICAC RECORDER (ISSN 1022-6303) is published four times a year by the Secretariat of the International Cotton Advisory Committee, 1629 K Street, NW, Suite 702, Washington, DC 20006-1636, USA. Editor: Keshav Kranthi <keshav@icac.org>. Subscription rate: $220.00 hard copy, $180.00 electronic version.

material sources, production processes, certification and information to the consumer. Several management systems were introduced across the world to ensure environmentally responsible material sourcing and sustainable manufacturing practices in the value chain. These traceability systems deal with documentation of environmentally responsible supply chains. A few of them are bluesign®, CHEM-IQ, Global Traceable Down Standard (GTDS), HERproject, Oeko-Tex 100, Responsible Down Standard (RDS), The Higg Index, The Sustainable Apparel Coalition (SAC) etc., Bale tagging with labels and tracking the value chain, are being used for traceability in some countries.

However, there are other laboratory-oriented methods to track and detect the identity of fibers. One is to tag them first at the gin and detect them later in the fabric. The second method is to examine fibers for their innate genetic authenticity. Tagging fibers with extraneous labels of DNA nucleotides, or nanoparticles does not provide proof of genetic purity or authenticity. It only helps to identify a tagged fiber. Such methods can also be possibly misused to bring 'traceability' itself into disrepute. Further the DNA tags could be destroyed in the harsh process of bleaching, scouring, dyeing and washing. Whether DNA-tagging of fibers would be the method of choice is debatable indeed.

Among the fiber tagging methods, the use of cellulosic fibers with scanner-detectable nanoparticles, has several advantages of robustness, operational simplicity and cost-effectiveness over the DNA-tagging method. The second option of examining genetic identity of fibers can actually make scientific sense in identifying the true source and origins of fiber. But, the methodology requires strong technical skills in DNA isolation and assessment. DNA traceability is almost like forensic sciences; it needs detective prowess; it is prohibitively expensive and time consuming. In the textile value chain, cotton fibers undergo rigorous processes of heat and chemicals that degrade DNA.

Reliability and reproducibility of DNA-based traceability techniques are dependent on the quality of DNA extracted from fibers in a fabric. The isolation of good quality DNA from textiles will itself be a blend of art, science and skill. The next steps depend on the extent of genetic polymorphisms in the genome, the availability of polymorphic markers and how well they can be detected with the high-tech fingerprinting methods using polymerase chain reaction (PCR), electrophoresis, DNA sequencing, quantitative real-time PCR (qRT-PCR), loop-mediated amplification (LAMP), TaqMan probes, capillary electrophoresis, high-resolution melting (HRM), microarrays, micro-fluidic bead-based multiplex assays etc. However all these detection methods can be severely affected by nuclease activity and the presence of PCR inhibitors in the extracted DNA. Microsatellite markers and single nucleotide polymorphisms (SNPs) are commonly used in DNA fingerprinting. But a reference database is most crucial for a reliable fingerprint.

The article by Mr. Joy and his colleagues on 'cotton DNA traceability technologies' is of particular interest because it examines the feasibility of using DNA tags and DNA fingerprinting for fiber traceability. The authors take a closer look at the kind of native DNA present in cotton fibers, along with it's intactness in mature raw fibers and processed products, such as yarn and fabric. The article also describes a few commonly used molecular techniques for the extraction and detection of DNA, either native to the fibers or applied exogenously as tags. A brief description deals with the traceability methods used with raw fibers, yarn and fabric and the subsequent processes of polymerase chain reaction (PCR), electrophoresis, DNA sequencing, barcoding, DNA fingerprinting and phylogenetic analysis. Finally, the article concludes with the technical lacunae in the application of the methods, interpretation of results, and the associated pitfalls that are characteristic of the current state-of-art 'cotton traceability technology.'

As mentioned above, despite significant technological advances, none of the traceability techniques available today are scientifically robust enough to be considered precise and reliable to identify the genetic purity and genetic authenticity of fibers in a garment. But there is hope that sooner or later scientists will develop simple inexpensive tools to detect fibers of a specific variety in garments.

Similarly, there are no foolproof management solutions to the much dreaded cotton boll weevil. But recent scientific advances such as the results described by Dr. Fatima strengthen the hope for sustainable eradication of the CBW through a combination of strategies, including through farmer participation in implementation of area-wide management programs.

Traceability – An Overview

Kai Hughes, International Cotton Association, Liverpool. UK
(This article was written in August 2017; Mr. Hughes was still Managing Director of the ICA at the time)

Over the last 10 years or so, the world, and in particular the cotton world, has been concerned with sustainability. In those early years, the questions were around what do we mean by sustainability and how do we measure it, and I distinctly remember that the initial focus was on the environmental impact but subsequently evolved to a social and more latterly the business or economic impact. Then, the barriers to implementing sustainability were quite simple – cultural and perceived cost. What became clear as the debate developed was that those companies that invested in sustainability were themselves becoming more sustainable.

Last year, the 'Welspun incident' forced traceability into the spotlight and highlighted the need to fully understand our supply chains and in particular, where the cotton in our textiles comes from. That 'incident' cost Welspun and two major retailers not only a lot of money in compensation and lost contracts but more importantly, reputation. Today, we are once again hearing the same questions as in the sustainability debate; "What do we mean by traceability?" "How much will it cost?"

For some time now, it has been impossible to distinguish between cotton of different origins with typical laboratory technologies. DNA analysis methods have been developed to distinguish between *Gossypium hirsutum* and *Gossypium barbadense,* or between BT cotton and conventional cotton, but as DNA may be destroyed with bleaching, dyeing and washing, the test results cannot be assured for dyed textiles and garments. Attempts to measure the quantity of DNA after these processes have proved to be unreliable in all blind tests.

It is still early days in the debate, but on talking to retailers, it has become evident that they want traceability to provide four things:

- Identification – information about the cotton such as the origin, grower, etc.
- Authentication – the ability to prove that a branded product is authentic
- Quantification – proof that a product is 100% Pima, Organic, Egyptian, sustainable cotton, etc.
- Full supply chain knowledge – the ability to track where that cotton has been in a very complex supply chain.

Currently there are three main products in the market that can give some or all of these four strands of information. DNA marking developed by Applied DNA Science (ADNAS) mark the cotton at an early stage of processing (preferably at the gin) and then attempt to detect this marker material at later stages of processing or in the final garment. Concerns have been raised that this process is not 100% certain as the DNA marker may be destroyed in the process, especially when heavy metal or aggressive dyes are used, and it does not allow for the necessary quantification of the share of marked cotton blended with unmarked cotton. It is essentially just a marking system that can be used on fibres but washes off when used on synthetic fibres so cannot be used for identification or authentication on these fibres. In addition, it requires laboratory testing with the associated time and cost to do this, and there is no way to collect supply chain information unless the product is tested at frequent intervals.

Another method of tracing cotton is to use established blockchain technology which provides a very secure means of transferring documents via an open ledger system. This will provide full supply chain knowledge and allow identification and quantification of the cotton via an electronic 'paper trail'. However, it relies on trust to physically link the cotton at a gin with a blockchain document, and that trust continues through the supply chain. There needs to be verification along the chain that the ledger reflects exactly what it says it does and that the physical cotton has not been blended or switched.

The third method is the use of FibreTrace, produced by ICA Bremen in conjunction with an English company called Fibremark Solutions Ltd. FibreTrace uses cellulosic fibres that mimic cotton fibres and contain nano particles which have been engineered to give a specific signature. These fibres are added in minute quantities to the cotton at the gin if quantification is required or can be added at later stages if just identification or authentication is required. The signature is read by using a scanner and gives an instant reading of the cotton's identity and the quantification of marked cotton in a bale, year, cloth of finished garment. The information from that scanner is fed to a data base using cloud technology providing full supply chain knowledge. Where this technology has an advantage is that the cotton can be instantly verified at any point in the supply chain, and FibtreTrace is not affected by any of the dyeing or bleaching processes. It is also impossible to reverse engineer making it extremely secure.

It is this security and the fact that information is collected instantaneously and stored in a database makes it, in my view, an ideal partner with blockchain technology. By linking the two processes you negate the weakness of the blockchain technology by allowing continuous verification through the supply chain, and it allows additional documentation to be added to FibreTrace fibres within a totally secure environment.

The debate will no doubt continue as to what is required in a traceability product and what is the best technology to achieve this. That debate will also include the cost of the technology and whether retailers and customers are prepared to pay a premium in order to know where their cotton products have come from. But what is certain is that retailers and brands need traceability in their supply chain not only to reduce their risk exposure, but also to ensure that their products are ethically or sustainably sourced, pass through accredited suppliers and can be used to promote confidence in their brand and their products. Traceability is now appearing as a standalone item in big brand's and retailer's strategic plans. It is here to stay.

Cotton DNA Traceability Technologies

Joy Das[1], K. P. Raghavendra[1], H. B. Santosh[1], M. Sabesh[1] and K. R. Kranthi[2]

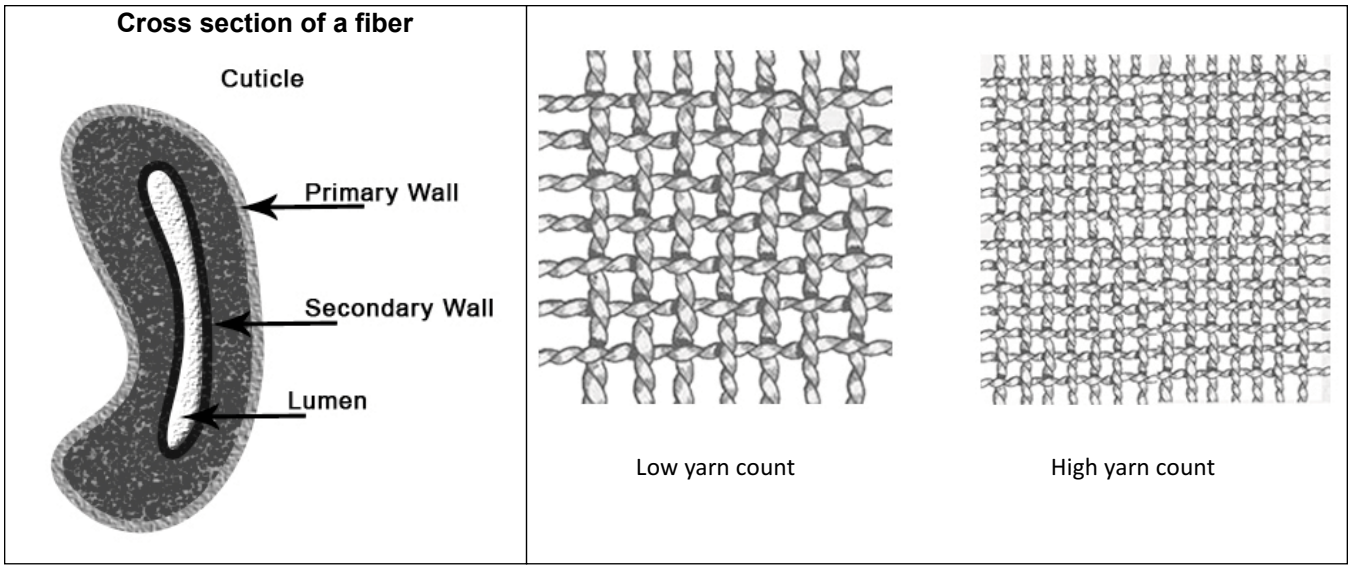
Introduction

'Cotton traceability' is becoming an important issue as retailers make certain product claims. Every cotton fabric and piece of apparel that we use today is constructed from millions of small fibers. The fabric may be labeled as 100% cotton, or 100% Egyptian cotton, or a cotton-blend, or a manufacturer might claim to have woven fabric from yarn of a specified count, or to have produced a product through a specific process, or in a particular country, or from a certain species or a specific cotton variety. Currently, it is very difficult to objectively verify such claims.

Several identity initiatives operate in the cotton sector, including Organic, Fairtrade, Cotton made in Africa (CmiA), the Better Cotton Initiative (BCI), and E[3] sustainable cotton program. There are also many national sustainability initiatives, including Cotton Leads and myBMP. Given the complexity of the cotton value chain, it is not easy to verify that a cotton fabric is correctly labeled as being of a particular quality or to have been produced in a certain manner in compliance with certain social norms, ethical guidelines, production systems or environmental standards, unless the entire production and processing chain is contained within a knowledge-intensive, trust-worthy system with high levels of accountability and integrity.

To understand the difficulty of verification of fiber, yarn or fabric content, quality or production claims, it is important to understand the complex sequence of processes that connect stake-holders in the cotton value-chain. The sequence starts with the farmer who produces seed cotton, which must be transported from farms to procurement centers to gins and is often co-mingled with seed cotton from other sources in the process. Seed cotton goes through several levels of processing, including ginning, spinning, dyeing, weaving and finally cutting and sewing into a finished product, with each stake-holder performing a role different from the other. In all likelihood, the farmer, ginner, spinner and the weaver do not know each other, though the fiber holds their occupations together.

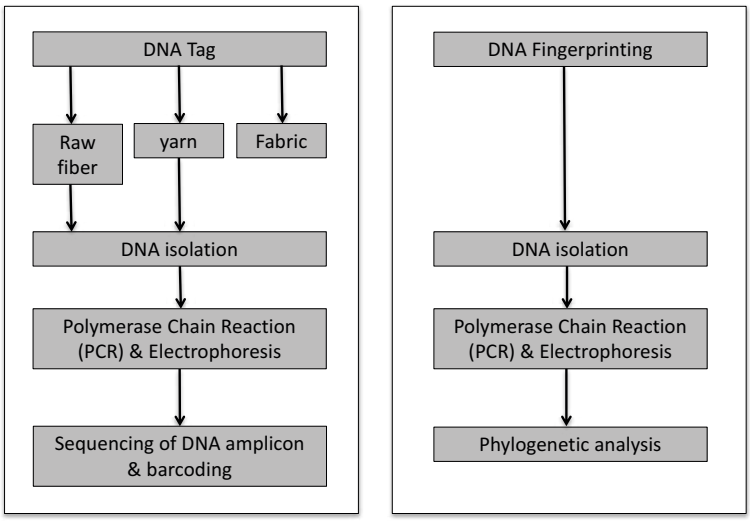
1) ICAR – Central Institute for Cotton Research, Nagpur, India
2) International Cotton Advisory Committee, Washington DC, USA



In a disaggregated value chain, labelling often depends on a claim backed by trust. For example, the farmer may claim, and may even produce a certificate to verify, that the cotton he/she produced is in compliance with strict organic-cotton guidelines. Based on this claim, the entire value chain could continue to label the product as 'organic-cotton'. The current identity programs are based at least partially on a 'belief-system' that all claims are trust-worthy. However, there have been recent cases of products labelled as 100% Egyptian cotton that were shown to be produced from upland varieties, and the organic cotton market is rife with rumors. Such incidents raise the need for a robust labelling/genotyping and tracking system to ensure labelling integrity. New Deoxyribo Nucleic Acid (DNA) based technologies provide avenues for a system that can finger-print and detect specific kinds of fiber through genotyping or exogenous DNA tags to fibers, yarn or fabric and detect them to provide traceability.

In an intense competitive environment, companies may fumble in their rigor of ascertaining standards of quality, or any specified kind of product characteristic. A few cases of dubious claims have been causing serious concerns in the market.

One of the most effective ways to identify fibers by origin is through a process called 'matured fiber genotyping'. There are several reports on this process where researchers have developed molecular marker-based tools which have the potential to identify the origin of matured and processed fibers, thus ensuring the genuineness of cotton fiber and textile materials. However, reports also suggest that chances of obtaining intact genetic material from matured fiber are narrow, thus contradicting the robustness of the claimed technological tools which are recommended for fiber genotyping. An alternative method is to tag cotton fibers with a DNA nucleotide-marker at the initial stage of processing. The tag remains always with the fiber in the fabric and can be detected at any point of time. Our review will relook into the scientific basis of several technological tools developed to analyze the genetic material of cotton fiber.



The Cultivated Cotton Species

The genus *Gossypium* comprises more than fifty species, of which only four are adopted for commercial cultivation, including *G. hirsutum* (American), *G. barbadense* (Egyptian), *G. arboreum* (Asiatic/ Indian) and *G. herbaceum* (sub-Saharan African and Arabian) (Wendel and Grover, 2015). All these four species produce unique types of fiber having distinguishable physical properties. For instance, fiber characteristics of the two diploid (two sets of 13 basic chromosomes of A genome) species, *G. arboreum* and *G. herbaceum* are inherently short and coarse compared to the relatively longer and finer fibers of the allotetraploid (two sets each of 13 basic chromosomes of A and D genomes) species, *G. hirsutum* and *G. barbadense*.

American and Egyptian cotton differ remarkably from each other, where the latter yields superior quality fiber typically termed as "extra long staple" (ELS) fiber (Liu *et al.,* 2015). (The ICAC Secretariat prefers the term extra fine to avoid confusion with national labels in Egypt and India that use the ELS designation.) As the name suggests, ELS fibers have naturally longer lint length as compared to that produced by *G. hirsutum* and hence, Egyptian cotton fiber qualifies as a favored raw material for the textile industry. Textile products manufactured out of genuine ELS fiber offer more value to the finished product and attract more vendors and consumers. The quality of ELS fiber from *G. barbadense* varies significantly depending upon the variety, geographical location and crop husbandry during cultivation. Many contentious cases relate to claims of 100% Egyptian ELS cotton that belongs to the species *G. barbadense.*

Endogenous DNA in Cotton Fibers

Cotton fibers are elongated desiccated single cells originating from individual cells of the external epidermal layer of cotton seed (Hernandez-Gomez *et al.,* 2017). Hence, they are also referred to as terminally differentiated unbranched seed trichromes (Tiwari and Wilkins, 1995; Kim and Triplett, 2001). Cotton fiber undergoes four developmental stages to reach maturity: *viz.* initiation, elongation, secondary wall deposition, and maturation. Each epidermal cell that gets transformed into a fiber strand possesses all the organelles similar to non-fiber cells. From the point of traceability, the fate of DNA in nucleus, chloroplast and mitochondria organelles, assumes significance. A sharp increase in the volume and number of nucleus, chloroplast and mitochondria was reported during the fiber initiation and elongation phases (Tatum, 1987). The molecular and physiological basis of initiation, elongation and secondary cell wall synthesis of fiber development is very well studied and documented (Haigler *et al.,* 2009 & 2012). Massive deposition of cellulose and micro-fibrillar rearrangement during secondary cell wall synthesis imparts strength and rigidity to the developing fiber. A difficulty in purification of biomolecules such as proteins and nucleic acids because of the strong cellulosic nature of fiber resulted in poor characterization of the maturation phase of fiber development (Kim and Triplett, 2001).

The fiber initiation and elongation phases are not mutually exclusive developmental events; they rather overlap in synchrony before the cotton fiber attains full maturity (Kim and Triplett, 2001; Haigler *et al.,* 2012). However, the concept of cotton fiber maturity is at times contested with varying opinions. For instance, one of the research groups headed by Hernandez-Gomez *et al.* (2017), referred to the notion of fiber maturity as "misleading" and instead renamed this as fiber desiccation phase. Earlier studies carried out by Berlin and co-workers (1986), had also highlighted desiccation and drying of cotton fiber during the final phase of development.

The cotton fiber development phase is also characterized by pure cellulose deposition during the secondary wall deposition phase, which eventually accounts for more than 95% of dry weight of matured or desiccated cotton fiber (Kim and Triplett, 2001; Stiff and Haigler, 2012). Fiber initiation commences from the day of anthesis arising from specific cells of the outer seed epidermis with a ratio of 1:3.7 among fiber initials and total ovular epidermal cells (Stewart, 1975). Fiber elongates for the next 21-26 days which gradually coincides with secondary cell wall synthesis, which commences at about 16 days post anthesis (DPA), and lasts until approximately 32 to 40 DPA (Meinert and Delmer, 1977; John and Crow, 1992), followed by the desiccation or maturation phase until 45 to 60 DPA (Kim and Triplett, 2001).

Meanwhile, during the course of the fiber development phase over a period of about two months, fiber cells had been hypothesized to undergo programmed cell death (PCD) and die, roughly after 40 DPA (Potikha *et al.,* 1999). PCD is the process of organized and regulated destruction of cells for survival and maintenance of organisms. It is a well documented process in both animals and plants. The process of programmed cell death in tracheary elements differentiation has been extensively studied in plants (Fukuda *et al.,* 2000; Kacprzyk *et al*., 2016; Dauphinee *et al*., 2017). Similarity in the process of tracheary elements (xylem vessels) differentiation and later phases of cotton fiber development led researchers to hypothesize the theory of PCD in cotton fiber development. Coincidence of production of reactive oxygen species such as H_2O_2 in response to initiation of secondary cell wall synthesis convinced Pothika *et al.* (1999) to postulate the theory of PCD in cotton fiber. However, in a subsequent report, Kim and Triplett (2001) contested this theory due to the lack of concrete evidence showing typical disintegration of cellular organelles or any detectable biochemical markers to support PCD in cotton fiber. In the same context, a study was carried out by Roche (2007) exclusively to decipher PCD in cotton fiber.

To examine whether cotton fiber cells succumb to the usual process of PCD, Roche (2007) made keen observations on the fate of the genetic material (i.e. DNA) within cotton fiber cells. DNA content and DNA disintegration were particularly monitored in the study as typical markers for tracing the occurrence and timing of PCD in cotton fiber. DNA was extracted from nuclei isolated from cotton fibers at specific time intervals *viz.* 5, 20, 25, 30, 35, 40, 45, 50, 55, 60 and 65 DPA. It was observed that DNA could be extracted and visualized from fiber cells until 40 DPA, after which it remained undetected. Notably, there was no DNA laddering, a typical hallmark indicator for PCD linked apoptotic DNA fragmentation (Kressel and Groscurth, 1994; Ryerson and Heath, 1996; Orzaez and Granell, 1997) observed at any point of time. The author therefore, said that her study remained rather inconclusive and was unable to uncover any firm evidence to mark the process of PCD in cotton fiber.

However, the fact that DNA could be traced from cotton fiber

during the course of fiber development has been documented in earlier studies as well (Van't Hof, 1999; Taliercio *et al.,* 2005). Nuclear degradation, vacuolar rupture, organelle destruction are typical hallmark events, along with other important features of PCD that could not be established in cotton fiber. Hence, the fate of DNA after cotton fiber gets fully matured or desiccated and subsequently harvested, is still an enigma to be resolved.

Extraneous DNA Tag on Cotton Fiber

Studies to assess the stability of DNA extraneously introduced or adhered onto various types of fabrics, show that cellulosic fibers such as cotton are good substrates. Such studies are of vital importance in forensic science. For instance, one study included six different fabrics stained with dried blood, including cotton, nylon, rayon, polyester, acrylic and wool. The study revealed that a superior DNA profiling could be derived from the samples of dried blood stained on cotton and nylon as compared to other fabrics tested, even on 14-day old bloodstains (Seah *et al.,* 2004). Thus, cotton fiber acts as a rather good binding substrate for extraneous DNA. Hence, the cotton swab has long been used as a favorable forensic tool for collection and analysis of DNA as a part of evidence in crime scenes (Hansson *et al.,* 2009; Brownlow *et al.,* 2012; Adamowicz *et al.,* 2014).

Apart from forensic applications, the use of extraneous DNA as a marker to tag and authenticate cotton fabrics to combat counterfeiting of branded clothing has been in the news for quite some time. Crypton® and Applied DNA Sciences, Inc. (both are private companies) have developed a unique DNA-marker-based, anti-forging technology which enabled tagging and forensic authentication of textile materials, including cotton fibers in finished products. The technology has been named 'SigNature T DNA platform' which the companies claim provides legitimate proof of the identity and purity of textile goods.

(http://adnas.com/signature_dna/;https://crypton.com/crpt-content/uploads/2017/03/crypton-companies-initiate-forensic-dna-program-with-applied-dna-sciences.pdf).

Stability of DNA During Fiber Development

Several reports suggest that there is considerable evidence for the presence of DNA in matured or harvested cotton fiber. Patent documents claim successful detection and extraction of PCR amplifiable DNA from matured harvested cotton fiber (Liang *et al*., 2014, 2015). As discussed above, the phenomenon of PCD in cotton fiber cells could has not been proven conclusively, due to a lack of conformity with typical PCD associated symptoms. In this case, there arises an obvious question regarding the DNA content and its stability in cotton fiber. Unfortunately, there is no report available that depicts the time line for the decline in integrity of DNA inside fiber cells after 40 DPA. However, based upon earlier studies which relate to storability of genomic DNA of plant samples, some logical conclusions can be drawn in favor of the stability of cotton fiber DNA.

There are reports of the recovery of measurable amount of DNA extracted from herbarium specimens as old as 118 years, and from mummified seeds and embryos ranging, astonishingly, from 500 years to greater than 44,600 years old (Rogers and Bendich, 1985). It is well documented that the nuclear material of biological samples can be preserved for a fairly long duration simply by drying the samples (Doyle and Dickson, 1987). Good quality genomic DNA could be extracted from desiccated plant tissue samples stored for weeks, and even months, at room temperature (Liston *et al.,* 1990; Chase and Hills, 1991; Till *et al.,* 2015). In fact, the DNA extracted from the samples was good enough to carry out restriction site analysis and polymerase chain reaction (PCR) amplification (Chase and Hills, 1991). The final phase of cotton fiber development is marked by a natural process of desiccation of fiber cells (Berlin, 1986; Hernandez-Gomez *et al.,* 2017). In relation to the aforementioned studies, it can be postulated that the genetic material of cotton fiber holds a fair chance of survival for a long period of time. However, a thorough chronological study is needed to define the precise time length for which DNA in cotton fiber may remain intact.

DNA Stability During Fiber Processing

Extraction of DNA from cotton fiber cells from a processed product such as cloth or fabric is a challenging task. The need for traceability has accentuated the demand for methods to extract DNA of a reliable quality that can act as the basic material for detection using DNA-tagging or genotyping to establish the identity of the test cotton fiber. Recovery of good quality DNA from matured cotton fiber, finished fabrics and apparel is the basic prerequisite step to carry out DNA bar-coding for fiber typing and fabric authentication. There is a common belief that, amongst all the currently available methods, technologies based on fiber DNA could provide the most reliable tools for the textile industry as an anti-counterfeiting tool.

Cotton fiber usually undergoes extensive processing before being converted to finished textile products. Moreover, the degree and number of steps of fiber processing vary immensely depending upon the desired end product. Thus, a full cycle of fiber processing in textile mills may impart severe mechanical, thermal and chemical stresses on fiber. For instance, machine harvesting and ginning causes thermal and mechanical damage to cotton fiber, and thermal stress is applied when drying excess moisture from fiber. Chemical treatments during the final phases of spinning and knitting are also common in fiber processing.

In addition, cotton fiber is a unique kind of plant cell with a thick secondary cell wall and is composed of more than 95% pure cellulose. Therefore, there are substantial challenges

in isolating intact and high quality DNA from matured, harvested fiber. Nevertheless, despite the thermal, chemical and mechanical stresses, it has been shown that DNA typing, using either extraneously introduced or naturally occurring DNA, can be carried out reliably from cotton fiber or its processed products which could be further utilized for PCR-based fiber genotyping assays. (http://adnas.com/signature_dna/; Patent EP2318676B1, 2011; Patent US8669079, 2014; Patent US8940485, 2015).

Methods to Isolate DNA Reliably from Raw Fibers, Yarn, Fabric and Apparel

There are a few patent documents available which described protocols for good quality DNA extraction from raw cotton fibers and processed fabrics (Patent EP2318676B1, 2011; Patent US8669079, 2014; Patent US8940485, 2015). The DNA extraction protocols mentioned in these patents are more or less similar to the standard molecular biology protocols (Sambrook *et al.,* 1989) with minor modifications in a few instances. Surprisingly, by following the standard Cetyl-trimethyl-ammonium-bromide (CTAB) method with minor modifications (like enhanced incubation period), it was possible to extract adequate amounts of genomic DNA from matured cotton fibers (Patent EP2318541B1, 2011). Not surprisingly, some earlier reports also suggest that good quality genomic DNA can be isolated from tough (Moncada *et al.,* 2013), mummified (Rogers and Bendich, 1985), dried and old archaeo-botanical plant tissues (Schlumbaum *et al.,* 2008). For example, protocols for DNA extraction from bark cloth, a fabric made from beating fibrous tree barks into sheets, have been elaborated in recent studies (Moncada *et al.,* 2013; Seelenfreund *et al.,* 2016). The DNA of bark cloth was successfully utilized in genetic analysis and characterization of archaeological samples as well. Therefore, these protocols may be explored for the extraction of cotton fiber genomic DNA to ensure its quality and authenticity.

Applied DNA Sciences (ADNAS) Company claims to have discovered that chloroplast DNA has better stability and survival chances compared to nuclear DNA in the fibers. A meticulous study carried out by Roche in 2007 (as discussed earlier), could not comprehend the exact fate of nuclear DNA of cotton fiber due to certain practically translatable experimental limitations. Notably, the author (Roche, 2007) clearly stated in her results that visually detectable nuclear DNA could be isolated from cotton fiber only until 40 DPA, after which (55, 60 and 65 DPA), DNA could not be visualized. It was further found that there was no DNA laddering observed at any point of time. However, there is no clear evidence to ascertain that nuclear genome is more prone to degradation as compared to chloroplast genome. Thus, if ADNAS has validated the survival of chloroplast DNA in mature cotton fibers, there may be an equal possibility of survival of mitochondrial and nuclear DNA as well.

A few patents show that it is very much possible to recover DNA irrespective of nuclear, chloroplast or mitochondrial origin, from matured harvested cotton fibers. A group of inventors, Ming-Hwa Liang and co-workers (2014, 2015) published a patent revealing successful extraction of genomic DNA from mature cotton fibers which can be further utilized for identification of specific cotton cultivar/species and detect genetic variations among cotton species. They have even claimed to have isolated DNA from processed and finished textile materials including anthropological textiles, garments, artwork canvases etc. They have used specific sets of primers targeting sequence polymorphism between distinct cultivars of *Gossypium barbadense*. In addition, they were also able to distinguish between different cotton varieties cultivated at different geographical locations. They could also genotype 25 different ELS cultivars of *G. barbadense* using a combination of merely 5 sets of SSR primers. Using such SSR primers, thousands of different cultivars can be discretely identified, and a genotype profile database can be created to identify specific cultivars of cotton from matured harvested cotton fiber (Liang *et al*., 2015). The patents claim to distinguish between *G. barbadense* and *G. hirsutum*, the two main cultivated cotton species worldwide. In yet another patent Arioli *et al*. (2016) claimed successful extraction of biological macromolecules of DNA, RNA, proteins, peptides etc. from matured or processed cotton fiber. The document has also revealed robust protocols for isolating DNA by incubating lint and processed fibers in specific buffers for a specific time period. Mohamed Negm & Suzan Sanad of Cotton Research Institute, Giza (Egypt) developed specific protocols and fiber-DNA based methods based on DNA melting curves to identify Egyptian varieties and authenticate the purity and presence of Egyptian cotton fibers in textile products (Negm and Sanad., 2015).

Experiments conducted at ICAR-Central Institute for Cotton Research, Nagpur (India) have shown that with a slight modification of standard protocols, good quality PCR amplifiable DNA could be extracted from mature cotton fibers of *G. hirsutum*, *G. barbadense* and *G. arboreum* (Raghavendra et al, unpublished).

Finally, it must be said that so far, research papers have not yet affirmed whether intact nuclear DNA can be isolated from mature fibers, either raw or processed. Interestingly, all such claims of extracting nuclear DNA from mature fibers of fabric or apparel have been made only in patents, which could possibly have been done from a commercial perspective and need to be test-verified for scientific correctness.

Traceability of *Gossypium* Species Using DNA Fingerprinting

DNA fingerprinting is a powerful tool to ascertain the identity of individuals across the animal and plant kingdoms. The DNA fingerprint of a genotype reveals the pattern of allelic variation present in the genome as detected by the molecular

markers. Since its discovery by Jeffreys and co-workers in 1985, many techniques have been developed, optimized, utilized and eventually abandoned when novel and more efficient and/or more reliable methods became available. Of the various molecular marker systems, locus-specific microsatellite analysis is the most popular method for DNA fingerprinting in plants (Nybom *et al.,* 2014) owing to their reproducibility, co-dominant inheritance, genome-wide presence, robustness, higher polymorphism and analytical simplicity. DNA fingerprinting is a relative assessment that is subject to the sample size of the individuals under study and the number of markers employed. It would be practically impossible to genotype an entire population, including the related genotypes within the species and individuals of related species. The credibility of a DNA fingerprint is assessed by using a statistical parameter called 'probability of identical match by chance.' The parameter can be calculated using the formula $(\overline{X}_D)^n$ as described by Ramakishana *et al.* (1994), where '$\overline{X}_D$' is the average similarity index and 'n' is the average number of amplified products per cultivar. The smaller the probability of an identical match, the greater the reliability of the DNA fingerprint.

Table 1: Some example markers for genotyping *Gossypium* sp.

Simple Sequence Repeats (SSR) / Microsatellites	**Reference**
Gossypium hirsutum, G. barbadense, G. darwinii and G. tomentosum.	(Lacape *et al.,* 2007)
378 accessions of *G. hirsutum* and 3 from *G. barbadense*	(Tyagi *et al.,* 2014)
47 upland cotton genotypes	(Rakshit *et al.,* 2010)
157 elite *G. hirsutum* cultivar accessions	(Zhao *et al.,* 2015)
410 *G. barbadense* and 1,523 *G. hirsutum* accessions	(Hinze *et al.,* 2016)
193 *G. hirsutum*	(Fang *et al.,* 2013)
24 *G. hirsutum* accessions with varying degree of drought tolerance	(Abd El-Moghny *et al.,* 2017)
Single nucleotide polymorphism (SNP)	**Reference**
G. hirsutum Texas Marker-1	(Ashrafi *et al.,* 2015)
18 *G. hirsutum* varieties	(Zhu *et al.,* 2014)
363 *G. hirsutum*: 292 cultivated and 71 non-cultivated relatives, 27 from 10 diploid and tetraploid *Gossypium* species which included 6 diploid species (*G. arboreum*, *G. amourianum*, *G. longicalyx, G. raimondii, G. thurberi*, and *G. trilobum*) and four tetraploid species (*G. barbadense, G. ekmanianum, G. mustelinum, and G. tomentosum*).	(Hinze *et al.,* 2017)
Amplified fragment length polymorphism (AFLP) & internal transcribed Spacer (ITS)	**Reference**
41 cultivars of *Gossypium hirsutum, G. barbadense, G. herbaceum and G. arboreum*	(Jena *et al.,* 2011)

DNA fingerprinting in cotton has its own challenges. Cotton is often a self-pollinated, complex, allotetraploid species with a huge genome (2400Mb) and having a high proportion of repetitive sequences. Outcrossing, mediated by insects of varying proportions (5-30%), is reported to happen in cotton. With every outcrossing, the purity of a cotton variety is at risk, and must be maintained through timely selfing and proper roguing. Outcrossing and amplifications emanating from the repetitive sequences of the genome infuses spurious heterozygosity and can reduce the credibility of a DNA fingerprint. However, the number of SSR markers available for research has increased over the years, and their polymorphism in cotton is reported to be low. Nevertheless, techniques used for the separation of PCR amplicons should have a higher resolution and the scoring of gel profiles should be automated to achieve precise estimation of allele size. Studies employing Agarose or Metaphor for amplicon separation and manual scoring of gel profiles can lead to imprecise results. Compared to genomic SSRs, the use of EST-derived SSRs can provide more robust information, as they represent the true variation in the expressed part of a genome connected to trait variation. A common set of markers are to be employed in DNA fingerprinting to compare the genotype profiles across countries and laboratories.

Researchers across the globe have reported several DNA markers that have been developed specifically for genotyping several *Gossypium* species and cultivars. The availability of the genome sequences of diploid progenitors and tetraploid cultivated species of cotton can be explored for the development of robust polymorphic markers for the identification of *Gossypium spp* (Paterson *et al.,* 2012; Wang *et al.,* 2012; Li *et al.,* 2014a; Li *et al.,* 2015; Liu *et al.,* 2015). For instance, utilizing the diversity of chloroplast genome of *G. hirsutum* and *G. barbadense*, Li and co-workers (2014b) identified 50 polymorphic chloroplast simple sequence repeat (cpSSR) markers for diversity analysis and the identification of *Gossypium* species. Similar kinds of markers can be extended to DNA-based tagging and authentication of cotton fiber and textile fabrics. Likewise, several other studies have also revealed many other molecular markers for cotton diversity analysis as depicted in Table 1.

Rakshit *et al.* (2010) developed the DNA fingerprints of 47 upland cotton genotypes using ten identified SSR markers with a moderate probability of an identical match by chance (0.01). Forty-eight of the most popular tetraploid cotton varieties of India were profiled using 68 identified polymorphic SSR markers at the ICAR-Central Institute for Cotton Research,

Nagpur. A robust DNA fingerprint having a low probability of identical match by chance was developed using a selected set of 14 markers (Santhy *et al.,* unpublished). If the probability of identical match by chance is 3.55×10^{-12}, it means one pair in 3.55×10^{12} combinations can have an identical DNA profile by chance. Therefore, to develop a robust DNA fingerprint having a very low probability of identical match by chance, a set of highly polymorphic markers should be utilized for molecular profiling, in combination with a fragment separation system having high resolution power and being amenable to automation. DNA fingerprint profiles can be maintained as databases in the public domain to achieve effective cultivar identification and differentiation across laboratories and countries.

The cultivated tetraploid species, *viz., G. hirsutum* and *G. barbadense,* differ significantly for most fiber quality traits, and many SSR markers tightly linked to these fiber quality characters have been identified through meta-QTL analysis of *G. hirsutum* × *G. barbadense* populations (Said *et al.,* 2015). A dedicated and updatable cotton QTL database (http://www2.cottonqtldb.org:8081/) is being maintained to assist cotton molecular breeding. In order to differentiate lint samples of *G. hirsutum* and *G. barbadense* through DNA fingerprinting, SSR markers, either genic or tightly linked for fiber quality traits sourced from the cotton QTL database can be utilized for greater success.

For over a hundred years, plant breeders have exchanged cotton germplasm lines across continents and used them in varietal improvement programs. For example, varieties from Egypt may have been used by breeders to improve varieties of *Gossypium barbadense* and *Gossypium hirsutum* in other countries. Therefore, a fair amount of the genome from Egyptian cotton varieties would be present in the improved extra-long staple varieties that are commercially cultivated in the major cotton growing countries. Similarly, extra-long staple fibers are produced from inter-specific hybrids of *G. barbadense* × *G. hirsutum* (H×B), which are commonly cultivated in India. It is probable that small-scale farmers may not even have the technical knowledge to differentiate between *Gossypium barbadense* varieties and interspecific hybrids of *G. barbadense* × *G. hirsutum.* Thus, the extra-long staple fibers harvested from H×B hybrids may be traded as *Gossypium barbadense* (Egyptian cotton) fibers mostly out of ignorance and not necessarily with dubious intentions. The quality of extra-long staple fibers of interspecific H×B hybrids may be as good as several *G. barbadense* varieties, but almost half the DNA in the genome of these fibers will come from *G. hirsutum.*

Conclusion & Future Prospects

DNA based 'traceability' methods are being developed as commercial products. With technological advancement, it is possible that very soon techniques will be available that can provide consumers with an absolute assurance of the specified identity, origin or species of fibers within a fabric. However, based on current science, there are still a few challenges that remain to be addressed before a foolproof technology is developed.

The key questions are:

1) Are the DNA methods cost effective and easy to use?

2) Is the DNA tag foolproof?

3) Is DNA fingerprinting / genotyping foolproof for traceability?

The simple answer to the above questions is 'No, not yet'.

Neither the DNA tags nor the DNA fingerprinting and genotyping techniques available today for traceability are foolproof. In addition, whether tagging or fingerprinting techniques are used, DNA methods are very expensive, and the techniques require special labs for DNA isolation, PCR, electrophoresis and interpretation. The DNA testing method itself takes 2-3 days. Tagging fibers with 50 to 100-mer oligos (short single stranded molecules of DNA/RNA oligonucleotides) or small double stranded DNA fragments may not be very expensive, but the detection-testing process can be tedious, time consuming and expensive. For example, each random DNA test to verify a claim could cost about US$50 or more, which could be equal to the cost of the apparel itself.

DNA tags can be misused by dubious operators, and DNA finger printing technology is yet to evolve to the stage where it can provide a credible specific profile of each genotype without any chance of overlap with other genotypes. DNA fingerprinting and the genotyping technology are based on a set of molecular markers that can be used to distinguish genotypes from one another within a population. Single DNA markers that are genotype-specific are very rare. It is possible to develop a multiplex PCR technique using a few reliable markers to obtain robust genotype-specific profiles, but this requires considerable expertise in highly specialized laboratories for testing unknown samples to verify the veracity of claims.

DNA nucleotide fragments are extraneous labels. Molecular tags in the form of small DNA fragments of defined nucleotide sequences are absorbed into fibers during any stage of the textile value chain. The extraneous or exogenous DNA can be incorporated into the fibers at the ginning unit, or in spinning mills or during treatment of the fabric, and they can be tracked at any stage of production. DNA is isolated from the fiber, yarn or fabric and used as a template for PCR amplification of the DNA-tag, separated through electrophoresis to obtain nucleotide amplicons of an expected size, and sequenced to be used as a bar-code for final identification. Some private companies have sensed a commercial opportunity, and they have quickly made claims of having developing reliable technologies of tagging and detection.

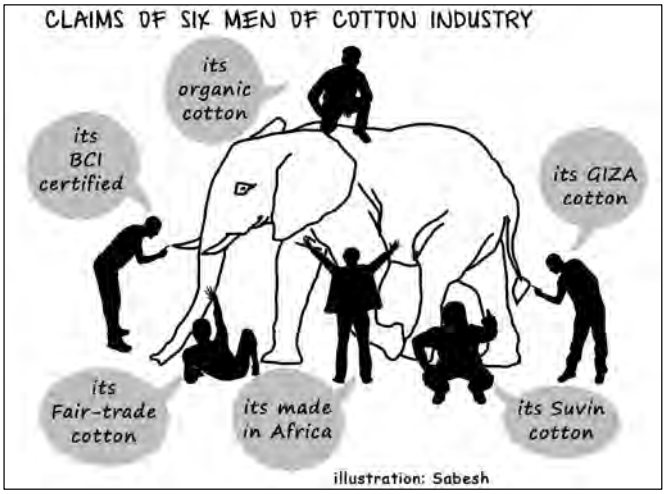
However, one of the main issues with the technology is that, with dubious intensions, any DNA can be tagged with any kind of fiber at the initial stage of processing, and any kind of

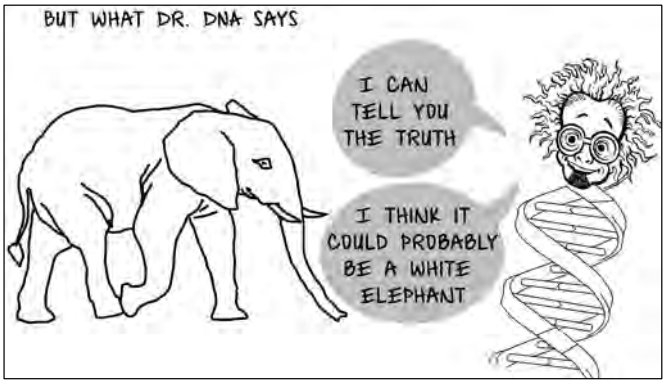
a final claim can be made for the product. In crude terms, a cat's fur can be exogenously tagged with a tiger's DNA and passed off as tiger's fur. It may even pass the legal test, depending on the lawyer's talent! The question once again is of 'integrity'. Therefore, it is possible that DNA tags could be misused by dubious companies as a marketing ploy, or the tags could be used mistakenly by genuine companies to label a product based on their trust in the source of the raw fibers rather than relying on robust scientific methods that confirm the veracity of the claim.

In the absence of traceability technologies, 'trust' plays a major role all through the value chain. Ironically, even if traceability technologies are implemented , products may be labeled with DNA tags based on disclosures made by raw fiber providers, rather than being based on DNA genotyping to confirm the authenticity of a claim of species, variety or geographical area. Thus, the problem of providing an authentic, foolproof traceability system is still a few steps away from being solved through DNA tagging technology. DNA genotyping technology may partly provide traceability information, but this technology is currently not used by any ginners, traders or spinners or any raw material suppliers.

DNA typing or fingerprinting depends on several factors. The technology deploys a set of markers and cannot rely on one or a few markers to distinguish a unique genotype from a population of genotypes. The uniqueness of the DNA fingerprint of a specific genotype is a relative term with reference to the number and type of polymorphic markers used and the relative density of related individual genotypes subjected to the test. The fingerprint of a particular genotype can be considered to be absolutely authentic only if the entire population of its related genotypes / species has been genotyped with a set of markers having high polymorphic resolution power to provide a clearly distinguishable phylogeny profile for all the genotypes within the population. Recent advances in next generation high throughput DNA sequencing techniques such as massively parallel signature sequencing, 454 pyrosequencing, Illumina Sequencing, Polony sequencing, SOLiD sequencing and Single molecule sequencing have not only reduced the cost of DNA sequencing but have also resulted in rapid identification of microsatellites and SNPs in the cotton genome.

The SSR and SNP markers are being either complemented or replaced by new methods of 'genotyping-by-sequencing'. Multiplex-SNP genotyping is also becoming more common. Cotton genome sequence data of *hirsutum* $(AD)_1$, *G. barbadense* $(AD)_2$, *Gossypium arboreum* (AA) and *Gossypium raimondii* (DD) are now available and will provide tremendous help for the identification of novel, highly polymorphic molecular markers that would accelerate future work on DNA fingerprinting. With diligent efforts, it may also be possible to develop species-specific and genotype-specific sequences that can be of immense value in traceability. However, as mentioned above, the development of markers





unique to a specific genotype will depend on sampling density and sequence coverage.

Finally, for the 'traceability-technology' to be reliable and robust, at this point of time, with the given state of the art, it may become necessary to combine an exogenous DNA-tag with at least two or more endogenous DNA markers that represent the species or variety/genotype or geographical indication, etc. Further, it would be important to develop simple and reliable detection methods. Such a set of markers could consist of a combination-tag in consonance for traceability. For example, fibers of Giza-86 could have an endogenous tag of a few SSR markers, combined with an exogenous DNA-tag, to confer high reliability to the technology of traceability. Nevertheless, there is hope that very soon simple and handy 'traceability' tools will be developed and put in place so that consumers get a trustworthy certificate-claim.

While the 'traceability' technology is progressing, some issues still remain. With the given 'state-of-the-art DNA techniques,' fabrics made out of cotton blends either with natural fibers or synthetics will need the more expensive 'Real-time-PCR' to figure out the blending proportion of cotton. Real-time PCR would only reveal the proportion of cotton used in the blend but will not resolve the identity of the components, especially the synthetic fibers used in the blend.

In summary, it would be appropriate to conclude that 'DNA based traceability technology' has evolved commendably in

recent years, but is not foolproof as yet. The major issues with it are as follows:

- DNA or chemical tags are useful but can be easily misused.
- DNA degradation during fiber processing is a serious concern and can affect the isolation and detection of the DNA. The degree of degradation depends on the processing methods employed and the level of stress to which fibers are subjected.
- Varietal-fingerprinting technology is available, but is complicated and difficult to be used for traceability as of now.
- DNA fingerprinting can be very difficult with samples containing physical mixtures of fibers from different varieties.
- DNA fingerprinting tests may suffer false-negatives due to cross-pollinating crops.
- DNA testing cannot reveal proportions and components of a synthetic blend.
- DNA testing can indicate genetic identity, but can be unreliable for geographic identity.
- DNA testing cannot identify or differentiate fibers derived from sustainability initiatives or identity initiatives.
- DNA testing requires considerable infrastructure and technical expertise, apart from being tedious, time consuming, cumbersome and expensive.

References

Adamowicz, M.S., Stasulli, D.M., Sobestanovich, E.M., Bille, T.W. and Budowle, B., 2014. Evaluation of methods to improve the extraction and recovery of DNA from cotton swabs for forensic analysis. *PLoS One,* 9(12), e116351.

Abd El-Moghny, A.M., Santosh, H.B., Raghavendra, K.P., Sheeba, J.A., Singh, S.B. and Kranthi K.R., 2017. Microsatellite marker based genetic diversity analysis among cotton (*Gossypium hirsutum*) accessions differing for their response to drought stress. *J. Plant Biochem. Biotechnol.,* doi:10.1007/s13562-016-0395-1.

Arioli, A. and Engelen, S., Bayer Bioscience NV, 2016. *Methods for plant fiber characterization and identification*. U.S. Patent 9,371,564.

Ashrafi, H., Hulse-Kemp, A.M., Wang, F. et al., 2015. A long-read transcriptome assembly of cotton (*Gossypium hirsutum* L.) and intraspecific single nucleotide polymorphism discovery. *Plant Genome,* 8(2), pp.1-14.

Berlin, J.D., 1986. The outer epidermis of the cottonseed. *In:* Cotton Physiology, The Cotton Foundation, Memphis. pp. 375-414.

Brownlow, R.J., Dagnall, K.E. and Ames, C.E., 2012. A comparison of DNA collection and retrieval from two swab types (cotton and nylon flocked swab) when processed using three QIAGEN extraction methods. *Journal of Forensic Sciences*, 57(3), pp. 713–717.

Chase, M.W. and Hills, H.H., 1991. Silica Gel: an ideal material for field preservation of leaf samples for DNA studies. *Taxon,* 40(2), pp. 215-220.

Dauphinee, A.N., Fletcher, J.I., Denbigh, G.L., Lacroix, C.R. and Gunawardena, A.H., 2017. Remodelling of lace plant leaves: antioxidants and ROS are key regulators of programmed cell death. *Planta*, pp.1-15.

Doyle, J.J. and Dickson, E.E., 1987. Preservation of plant samples for DNA restriction endonuclease analysis. *Taxon,* 36(4), pp. 715-722.

Fang, D.D., Hinze, L.L., Percy, R.G., Li, P., Deng, D. and Thyssen, G., 2013. A microsatellite-based genome-wide analysis of genetic diversity and linkage disequilibrium in Upland cotton (*Gossypium hirsutum* L.) cultivars from major cotton-growing countries. *Euphytica,* 191(3), pp. 391–401.

Haigler, C.H., Betancur, L., Stiff, M.R. and Tuttle, J.R., 2012. Cotton fiber: a powerful single-cell model for cell wall and cellulose research. *Frontiers in Plant Science,* 3:104. doi: 10.3389/fpls.2012.00104.

Haigler, C.H., Singh, B., Wang, G. and Zhang, D. 2009. Genomics of cotton fiber secondary wall deposition and cellulose biogenesis. *In:* Genetics and Genomics of Cotton, Volume 3, Paterson, A.H. (Ed.) Springer US, New York, NY, pp. 385–417.

Hansson, O., Finnebraaten, M., Heitmann, I.K., Ramse, M. and Bouzga, M., 2009. Trace DNA collection - Performance of minitape and three different swabs. *Forensic Science International: Genetics Supplement Series*, 2(1), pp. 189–190.

Hernandez-Gomez, M.C., Runavot, J.L., Meulewaeter, F. and Knox, J.P., 2017. Developmental features of cotton fiber middle lamellae in relation to cell adhesion and cell detachment in cultivars with distinct fiber qualities. *BMC Plant Biology,* 17,69. doi: 10.1186/s12870-017-1017-3.

Hinze, L.L., Gazave, E., Gore, M.A., Fang, D.D., Scheffler, B.E., Yu, J.Z., Jones, D.C., Frelichowski, J. and Percy, R.G., 2016. Genetic diversity of the two commercial tetraploid cotton species in the *Gossypium* diversity reference set. *Journal of Heredity,* 107(3), pp. 274–286.

Hinze, L.L., Hulse-Kemp, A.M., Wilson, I.W., et al., 2017. Diversity analysis of cotton (*Gossypium hirsutum* L.) germplasm using the cotton SNP63K array. *BMC Plant Biology,* 17, 37. doi: 10.1186/s12870-017-0981-y.

Jeffreys, A.J., Wilson, V. and Thein, S.L., 1985. Hypervariable'minisatellite' regions in human DNA. *Nature*, *314*(6006), pp.67-73.

Jena, S.N., Srivastava, A., Singh, U.M., et al., 2011. Analysis of genetic diversity, population structure and linkage disequilibrium in elite cotton (*Gossypium* L.) germplasm in India. *Crop and Pasture Science,* 62(10), pp. 859–875.

John, M.E. and Crow, L.J., 1992. Gene expression in cotton (*Gossypium hirsutum* L.) fiber: Cloning of the mRNAs. *Proceedings of the National Academy of Sciences (USA),* 89(13), pp. 5769–5773.

Kacprzyk, J., Dauphinee, A.N., Gallois, P., Gunawardena, A.H. and McCabe, P.F., 2016. Methods to Study Plant Programmed Cell Death. *Programmed Cell Death: Methods and Protocols*, pp.145-160.

Kim, H.J. and Triplett, B.A., 2001. Cotton fiber growth in planta and in vitro. Models for plant cell elongation and cell wall biogenesis. *Plant Physiology,* 127(4), pp. 1361–1366.

Kressel, M. and Groscurth, P., 1994. Distinction of apoptotic and necrotic cell death by in situ labelling of fragmented DNA. *Cell and Tissue Research,* 278(3), pp. 549–556.

Lacape, J.M., Dessauw, D., Rajab, M., Noyer, J.L. and Hau, B., 2007. Microsatellite diversity in tetraploid *Gossypium* germplasm: Assembling a highly informative genotyping set of cotton SSRs. *Molecular Breeding,* 19(1), pp. 45–58.

Li, F., Fan, G., Lu, C., et al., 2015. Genome sequence of cultivated Upland cotton (*Gossypium hirsutum* TM-1) provides insights into genome evolution. *Nature Biotechnology,* 33, pp. 524–530.

Li, F., Fan, G., Wang, K., et al., 2014. Genome sequence of the cultivated cotton *Gossypium arboreum*. *Nature Genetics,* 46, pp. 567–572.

Li, P., Li, Z., Liu, H. and Hua, J., 2014. Cytoplasmic diversity of the cotton genus as revealed by chloroplast microsatellite markers. *Genetic Resources and Crop Evolution,* 61(1), pp. 107–119.

Liang, M.H. and So, S.S.K., Apdn (B.V.I), Inc., 2015. *Methods for genotyping mature cotton fibers and textiles*. U.S. Patent US8940485.

Liang, M.H. and So, S.S.K., Cara Therapeutics, Inc., 2014. *Methods for genetic analysis of textiles made of Gossypium barbadense and Gossypium hirsutum cotton*. U.S. Patent 8,669,079.

Liston, A., Rieseberg, L.H., Adams, R.P., Do, N., Ge-lin, Z., 1990. A method for collecting dried plant specimens for DNA and isozyme analyses, and the results of a field test in Xinjiang, china. *Annals of the Missouri Botanical Garden,* 77(4), pp. 859-863.

Liu, X., Zhao, B., Zheng, H.J., et al., 2015. *Gossypium barbadense* genome sequence provides insight into the evolution of extra-long staple fiber and specialized metabolites. *Scientific Reports,* 5, 14139. doi:10.1038/srep14139.

Meinert, M.C. and Delmer, D.P., 1977. Changes in biochemical composition of the cell wall of the cotton fiber during development. *Plant Physiology,* 59(6), pp. 1088–1097.

Moncada, X., Payacán, C., Arriaza, F., Lobos, S., Seelenfreund, D. and Seelenfreund, A., 2013. DNA Extraction and Amplification from Contemporary Polynesian Bark-Cloth. *PLoS One*. doi: 10.1371/journal.pone.0056549.

Negm, M. and Sanad, S., 2015. Developing a DNA -based Technology for Identifying the presence and percentage of Egyptian cotton fibers in various textile products, https://www.icac.org/getattachment/tech/Regional-Networks/Inter-Regional-Cooperative-Research-Network-on-Cot/Twelfth-Regional-Meeting-Documents/DNA-base-technology.pdf.

Nybom, H., Weising, K. and Rotter, B., 2014. DNA fingerprinting in botany: past, present, future, *Investigative Genetics,* 5,1. doi: 10.1186/2041-2223-5-1.

Orzaez, D. and Granell, A. 1997. DNA fragmentation is regulated by ethylene during carpel senescence in *Pisum sativum*. *Plant Journal,* 11(1), pp. 137–144.

Paterson, A.H., Wendel, J.F., Gundlach, H., et al., 2012. Repeated polyploidization of Gossypium genomes and the evolution of spinnable cotton fibers. *Nature,* 492, pp. 423–427.

Potikha, T.S., Collins, C.C., Johnson, D.I., Delmer, D.P., Levine, A., 1999. The involvement of hydrogen peroxide in the differentiation of secondary walls in cotton fibers. *Plant Physiology,* 119(3), pp. 849–858.

Rakshit, A., Rakshit, S., Santhy, V., Gotmare, V.P., Mohan, P., Singh, V.V., Singh, S., Singh, J., Balyan, H.S., Gupta, P.K. and Bhat, S.R., 2010. Evaluation of SSR markers for the assessment of genetic diversity and fingerprinting of *Gossypium hirsutum* accessions. *Journal of Plant Biochemistry and Biotechnology,* 19(2), pp. 153-160.

Ramakishana, W., Lagu, M.D., Gupta, V.S. and Ranjekar, P.K., 1994. DNA fingerprinting in rice using oligonucleotide probes specific for simple repetitive DNA sequence. *Theoretical and Applied Genetics,* 88(3), pp. 402–406.

Roche, M.C., 2007. A study of programmed cell death in cotton (*Gossypium hirsutum*). M.Sc. thesis submitted to Texas A&M University, USA; available at http://oaktrust.library.tamu.edu/bitstream/handle/1969.1/ETD-TAMU-1599/ROCHE-THESIS.pdf?sequence=1.

Rogers, S.O. and Bendich, A.J., 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Molecular Biology,* 5(2), pp. 69–76.

Ryerson, D.E. and Heath, M.C., 1996. Cleavage of Nuclear DNA into Oligonucleosomal Fragments during Cell Death Induced by Fungal Infection or by Abiotic Treatments. *Plant Cell,* 8(3), pp. 393–402.

Said, J.I., Song, M., Wang, H., Lin, Z., Zhang, X., Fang, D.D. and Zhang, J., 2015. A comparative Meta- analysis of QTL between intraspecific Gossypium hirsutum and interspecific *G. hirsutum* × *G. barbadense* populations. *Molecular Genetics and Genomics*, 290(3), pp. 1003-25.

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989. Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press.

Schlumbaum, A., Tensen, M. and Jaenicke-Després, V. 2008. Ancient plant DNA in archaeobotany. *Vegetation History and Archaeobotany,* 17(2), pp. 233–244.

Seah, L.H., Othman, M.I. Jaya, P. and Jeevan, N.H., 2004. DNA profiling on fabrics: an *in-situ* method. *International Congress Series*, 1261, pp. 565-567. doi: 10.1016/S0531-5131(03)01832-6.

Seelenfreund, A., Sepúlveda, M., Petchey, F., et al., 2016. Characterization of an archaeological decorated bark cloth from Agakauitai Island, Gambier archipelago, French Polynesia. *Journal of Archaeological Science,* 76, pp. 56–69.

Stewart, J.M., 1975. Fiber Initiation on the cotton ovule (*Gossypium hirsutum*). *American Journal of Botany,* 62(7), 723.

Stiff, M.R. and Haigler, C.H., 2012. Recent advances in cotton fiber development, The Cotton Foundation, pp. 163-192; available at https://www.cotton.org/foundation/upload/F-F-Chapter-10.pdf.

Taliercio, E., Hendrix, B. and Stewart, J.M., 2005. DNA content and expression of genes related to cell cycling in developing *Gossypium hirsutum* (Malvaceae) fibers. *American Journal of Botany,* 92(12), pp. 1942–1947

Tatum, J.A.W., 1987. A stereological study of the development of cotton fibers. An M. Sc. Thesis submitted to Texas Tech University; available at https://ttu-ir.tdl.org/ttu-ir/bitstream/handle/2346/11092/31295005227573.pdf?sequence=1&isAllowed=y.

Till, B.J., Jankowicz-Cieslak, J., Huynh, O.A., Beshir, M.M., Laport, R.G. and Hofinger, B.J., 2015. Sample Collection and Storage. Low-Cost Methods Mol. Charact. Mutant Plants. Springer International Publishing, Cham, pp 9–11.

Tiwari, S.C. and Wilkins, T.A., 1995. Cotton (*Gossypium hirsutum*) seed trichomes expand via diffuse growing mechanism. *Canadian Journal of Botany,* 73(5), pp. 746–757.

Tyagi, P., Gore, M.A., Bowman, D.T., Campbell, B.T., Udall, J.A. and Kuraparthy, V., 2014. Genetic diversity and population structure in the US upland cotton (*Gossypium hirsutum* L.). *Theoretical and Applied Genetics,* 127(2), pp. 283–295.

Van't Hof, J., 1999. Increased nuclear DNA content in developing cotton fiber cells. *American Journal of Botany,* 86(6), pp. 776–779.

Wang, K., Wang, Z., Li, F., et al., 2012. The draft genome of a diploid cotton *Gossypium raimondii*. *Nature Genetics,* 44, pp. 1098–1103.

Wendel, J.F. and Grover, C.E., 2015. Taxonomy and Evolution of the Cotton Genus, *Gossypium*. *In:* Cotton, 2nd ed., Agron. Monogr. 57. ASA, CSSA, and SSSA, Madison, WI, USA. doi:10.2134/agronmonogr57.2013.0020.

Zhao, Y., Wang, H., Chen, W., Li, Y., Gong, H., Sang, X., Huo, F. and Zeng, F. 2015. Genetic diversity and population structure of elite cotton (*Gossypium hirsutum* L.) germplasm revealed by SSR markers. *Plant Systematics and Evolution,* 301(1), pp. 327–336.

Zhu, Q.H., Spriggs, A., Taylor, J.M., Llewellyn, D. and Wilson, I. 2014. Transcriptome and complexity-reduced, DNA-based identification of intraspecies single-nucleotide polymorphisms in the polyploid *Gossypium hirsutum* L. *G3: Genes, Genomes, Genetics,* 4(10), pp. *1893–1905.*

Egyptian Cotton Traceability

Dr. Mohamed Negm & Dr. Suzan Sanad, Spinning Research Dept. Cotton Research Institute, Giza Egypt

Introduction

"Egyptian Cotton" is characterized by its superior quality. It gives Egypt a competitive advantage on which a comprehensive industry could be developed to make Egypt the main producer of extra fine count yarns which could be processed and exported as the finest and highest quality cottons in the world. Such products need an identity that can be reliably traced and detected. It is now up to Egypt to develop a comprehensive program to establish 'competitive advantage' by developing a distinct brand, increased demand for new products, creating systems to ensure varietal purity associated with robust traceability technologies.

Unlike many other cotton-producing countries, Egypt exclusively produces *Gossypium barbadense*, a type of extra fine cotton endowed with a longer and finer staple than upland cotton. In Egypt, seed for planting has been strictly controlled by the government, which for many years operated as the sole supplier and distributor of cotton planting seed.

This article describes all the traceability initiatives being undertaken in Egypt to ensure seed purity, varietal purity, bale purity and finally to track the fibers from gin to fabric. The DNA testing method developed by us is simple, yet robust enough to detect and distinguish Egyptian varieties. We briefly describe all traceability initiatives, including the DNA techniques used to identify and verify the authenticity of Egyptian cotton.

Traceability of Seed Purity

The Cotton Research Institute, (CRI), of the Ministry of Agriculture and Land Reclamation (MALR), continues to breed high quality cotton varieties. New varieties are developed each year, and the most promising are submitted to a ten to fifteen year path towards the seed's progenies, through Foundation Seeds, Certified Seeds, and Registered Commercial Seeds, grown by 'seed multipliers'. After a few years (generally 2-3 at the Registered Commercial level), new seeds replace the old seeds, and new varieties are promulgated.

Lastly, a revamped cotton extension and marketing system is updated. A new traceability system is being designed in collaboration with the MALR and the cooperatives, including the Central Department for Seed Administration. The structure of the system is briefly described here.

There are three major components of the new traceability strategy that will depend on implementing information technology throughout the process from distribution of seeds to the farmer, follow up on extension programs, agriculture practices, designated cotton production area by variety and yield, Cotton Arbitration & Testing General Organization "CATGO", inspection, and market place bidding.

First, as indicated by the MALR, the cooperatives will play a major role. The MALR initiated measures to prevent genetic deterioration, which was considered one of the principal reasons for a decline in yields. The following strategies were defined: The CRI produces breeder seeds (generation one) and foundation seeds (generation two). The cooperatives will receive the foundation Seeds and distribute them to a set of 'Elite' farmers, who follow the production recommendations developed by the 'extension service', to obtain the highest possible yields of certified seeds (generation three). All seed cotton bags will be delivered to the cooperative warehouse upon harvesting in colored and signed bags. The seed cotton will then proceed to the cotton gins for ginning, and the seeds will be separated into their respective bags, for seed testing, germination, and fiber quality evaluation.

Seeds are selected, graded, labeled as certified seeds and returned to the MALR General Administration. After testing for germination and other characteristics, the MALR will return the seeds back to the cooperatives for the next season's distribution to master lead farmers to act as 'seed multipliers' to produce registered commercial seeds for general distribution. These seeds will have a different colored bag, different markings and labeled as 'registered commercial' seeds.

The traders with the highest bids will purchase the seed cotton bags, and pay the farmers 80% of the value upon CATGO's initial inspection, and the remaining 20% will be paid based upon final CATGO ginning out-turn (GOT) results. At this point, the traders who purchased the seed cotton from the farmer will gin the seed cotton according to MALR approved ginneries and seed cotton transportation licenses. Foundation and certified seeds are packed in different colored bags for testing and returned to MALR's Central Administration, while leaving the commercial seed for the trader. The trading company will sell or dispose of the gin co-products, sometimes referred to as gin trash.

Traceability of Varietal Purity

The cultivated area devoted to cotton used to extend from Alexandria in the north to Aswan in the south, about 600 Km; the two southern-most governorates of Quena and Aswan were excluded from growing cotton.

The marked climatic difference from north to south created difficulties in finding a single variety that would be suitable for the whole range of conditions prevailing in Egypt. Therefore, a few varieties, up to ten, are usually grown in any season. Under such conditions, contamination by natural crossing is almost inevitable. Zoning and ginning control are the two main measures that have been taken to achieve the objective of varietal purity.

Zoning

Variety zoning has been adopted on two levels; the first category is of varieties zoning, i.e. Extra-Long Staple (ELS) and Long Staple (LS) zoning in the Delta and Upper Egypt regions, and the second level is single-variety zoning within each region. The country is divided into as many varietal zones as the number of varieties under cultivation. The area of each zone, i.e. the area to be cultivated by a variety, is determined based on the expected total demand of the local industry and exports of the variety and the expected yield per Feddan (1 Hectare= 2.38 Feddan).

Ginning Control

In accordance with single-variety zoning, the one-gin one-variety system is also applied. In this system, each gin is assigned one variety for the whole season; in this way, any possibility of seed contamination with different varieties in ginneries is strictly avoided. Also, within each ginnery, precautions are taken to prevent mixing of strains of the same variety. Consequently, strains are ginned starting with the newest one, and thoroughly cleaning the gin-stands from seeds in between each of two consequent strains.

Traceability of Bales

Currently, the Egyptian Government has a policy objective to restore Egypt's position as the world's leading producer of fine cottons. Accordingly, the government has designed an ambitious programme of redevelopment of fine cotton output with realistic objectives.

The Holding Company (HC) of cotton and textile Industries has an important part to play in achieving the policy objective. First, it is the HC's responsibility to provide the sector with ginning capacities that separate lint from the annual seed cotton output to the highest quality standards.

Second, the HC also has a role to play in contributing with other responsible government agencies in the policy program to promote cotton agriculture and the enabling mechanisms to assist and incentivize farmers to grow more cotton.

The new ginning capacity will be set up to operate to efficient lint conversion rates and ginning outturn (GOT) to the highest quality standards.

Those standards are critically important and refer to:

- Elimination of contamination
- Minimizing residual trash,
- HVI bale certification and traceability systems from field to bale

The field to bale cotton traceable system will have identifying barcode and/or RFID "Radio Frequency Identification" with the following fiber quality and bale data:

- Cotton variety
- Name of cotton ginnery and location
- Lot number
- Numbers of bales
- Bale number
- CATGO lot number
- Cotton grade "CATGO"
- Date of gin
- HVI data results; UHML, UI, Strength, Elongation, Micronaire, SFI, and Color attribute

Cotton Egypt Association and the Egyptian Cotton Logo Trade Mark and Traceability

The Cotton Egypt Association is a non-profit association established in 2005 with support from the Ministry of Industry and Foreign Trade. The association works closely with local and international companies involved in the Egyptian cotton supply chain. The association's mission, is to protect Egyptian cotton's legacy of luxury and help promote all Egyptian cotton licensees and their products. Cotton Egypt's mission is to manage, market, promote, license and monitor the Egyptian Cotton logo and its licensees, as well as guarantee the authenticity of products licensed to use the logo. This ensures that products carrying the official Egyptian Cotton logo are softer, finer and more resilient than products made from other fibers. The licensing system covers the entire supply chain of Egyptian Cotton users to monitor quantities purchased and

sold by each licensee and draw a map of its usage and establish a traceability system around the world. This will enable monitoring of location, quantities, brands, manufacturers and retailers to track the journey from bale to stores.

Complete Traceability of Product in System as well as on Production Floor

- Supplier should have a robust system in place for identification and traceability of product in the system as well physically on the production floor
- Products should have unique and independent material codification systems for raw material, material in-process, and the final products at each stage of production which are well integrated as per the process flow chart of each product.

Spinning

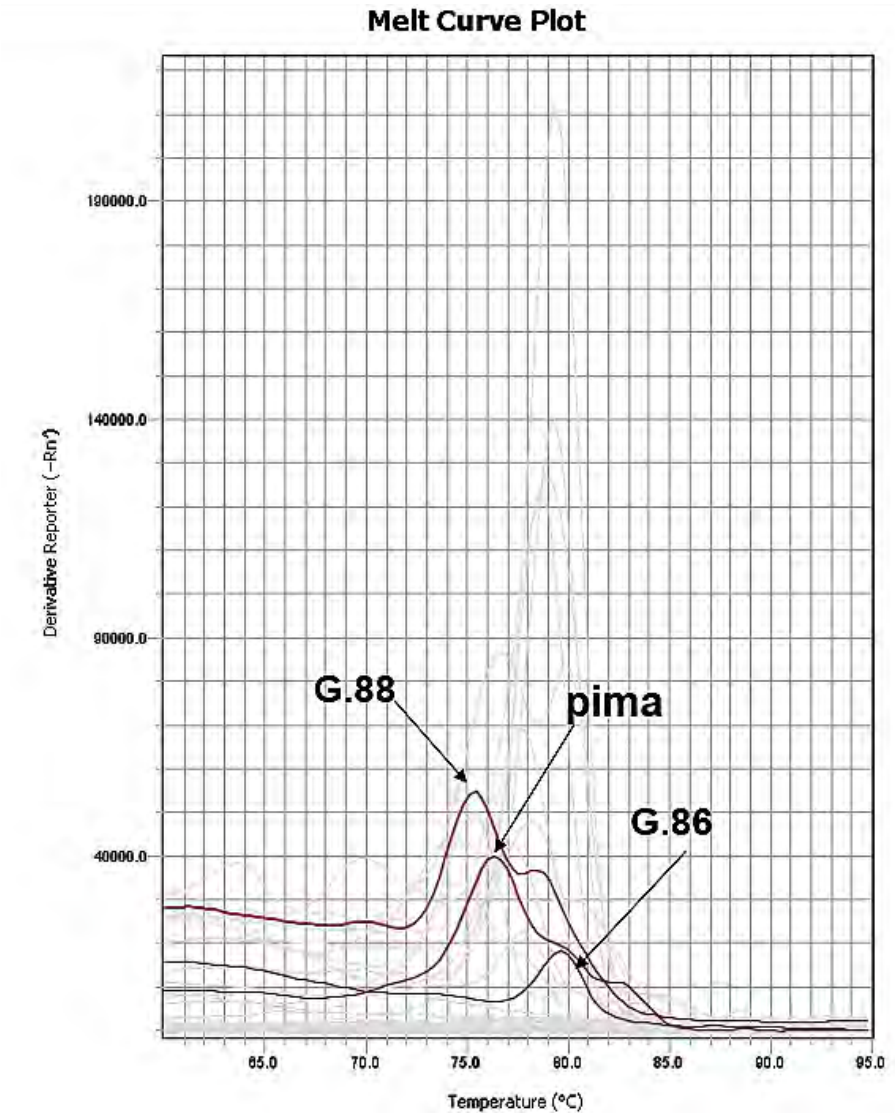
- Dedicated spinning line for special fiber mixing to prevent contamination and mixing of other fibers.
- Complete traceability system must be in place connecting cotton lot with yarn lot number.
- Every spun yarn lot must be coded with a unique number connected to the cotton lot number.

Weaving

- Unique specification sheet for warp/ weft and fabric doffs should be made which has the details of yarn lot number.
- Unique sort number should be provided to every doff which will be connected to yarn lot number.

DNA Based Traceability Method

The authors developed a novel process of DNA analysis for Egyptian cotton. The testing relied on the innate genetic differences between different species of cotton, such as *G. barbadense* (i.e., Egyptian cotton) and *G. hirsutum* (Upland cotton), to determine the species from which the fibers are derived. The test can also differentiate between the Egyptian cotton Varieties, all commercial Giza Varieties and Pima cotton. The authors developed the CTAB extraction method to extract DNA from Egyptian cotton fibers throughout the supply chain, up to the finished product. The laboratory is already accredited with ISO 17025 from EGAC (Egyptian Accreditation Council), Accreditation Certificate No. 216031A, and approved from ILAC (International Laboratory Accreditation Cooperation).



Development of Transgenic Cotton Plants – Effectiveness and Advances in Boll Weevil Control

Thuanne P. Ribeiro[3], Fabrício B. M. Arraes[3], Maria E. Lisei-de-Sá[3], Rayssa A. Garcia[3], Leonardo L. P. Macedo[3], Ana G.B. Leite[3], Dagna M. L. Silva[3], Maria C. M. Silva[3], Isabela T. Lourenço-Tessutti[3], Wagner A. Lucena[3,4], Maria F. Grossi-de-Sa[3,*]

Agriculture aims to achieve a sustainable economy while maintaining and improving crop yields worldwide. Several constraints limit crop production and, in this context, insect pests represent one of the most critical threats with adverse and damaging impacts on agricultural production and market access. In Brazil, the losses to 35 major crops caused by insect pest attacks generate an annual loss of approximately US$ 17.7 billion to the Brazilian economy, despite the adoption of several control measures (Oliveira *et al.,* 2014).

Insect-pest control categories can be classified into cultural, mechanical/physical, biological, and genetic/biotechnological practices. Several of these practices have been implemented in Brazilian agriculture, including the increased adoption of the no-tillage system, the reduction in the row-spacing of several crops, changes in plant architecture, variations in the harvest cycle, and the widespread adoption of transgenic crops (Oliveira *et al.,* 2014). Currently, several options of transgenic cotton are available for insect resistance and herbicide tolerance; these have contributed to improvement in crop management and reductions in pesticide applications.

Cotton (*Gossypium hirsutum*) is an economically important crop in Brazil and the leading source of natural fiber used globally. Genetically modified (GM) cotton provides effective crop protection, thereby increasing production levels and improving fiber quality. GM cotton has become the third most cultivated GM crop worldwide and currently corresponds to 11.0% of the total area planted to GM crops in the world. However, cotton plants are still strongly affected by a great number of insect pests (Gallo *et al.,* 2002). The coleopteran *Curculionidae,* the cotton boll weevil (CBW - *Anthonomus grandis*) represents a major cotton insect pest in Brazil and Latin America. Since its life cycle occurs inside the cotton flower buds and fruits (Gallo *et al.,* 2002; Azambuja & Degrande, 2014; Gullan & Cranston, 2014), the damage caused by the insect to the floral structures may lead to abortion of the flower bud and/or reduction in the fiber quality (Silvie *et al.,* 2013). Several factors contribute to CBW prevalence in Brazilian fields, including the large cotton cultivated areas (around 1.0 million ha), an extremely favorable climate for the insect's development and the absence of effective natural enemies to regulate such populations. Nowadays, considerable efforts have been made by the cotton production sector to reduce the impact caused by CBW on Brazilian cultivars. The use of biotechnological strategies is regarded as an important advancement in the control of insect pests, such as the incorporation and expression of insecticidal-proteins to obtain resistant GM cotton plants.

Biotech insect-resistant (IR) cotton was first commercialized in Brazil in 2006; in 2016 the cultivated area under biotech-cotton represented around 78% of the total cotton crop area, indicating a significant rate of adoption by farmers. In this context, Cry toxins from *Bacillus thuringiensis (Bt)* have become the major biotechnological tools for insect pest control. There are currently more than 790 characterized *Bt*-encoded entomotoxic crystal proteins (Crickmore *et al.,* 2016), which are highly specific to targets and safe to human health. Furthermore, the expression of Bt toxins in transgenic crops has significantly reduced the use of chemical pesticides in agriculture (James, 2017). When used alongside with proper agricultural practices, Cry-based technology can offer many benefits to crops, farmers, and consumers alike. Accordingly, numerous commercial Cry-GM events have been released for crop protection. In cotton, several events have become particularly successful, such as Bollgard II® (Monsanto), WideStrike™ (Dow AgroSciences) and TwinLink® (Bayer CropScience), which express Cry1Ac-Cry2Ab, Cry1F-Cry1Ac, and Cry1Ab-Cry2Ae toxins, respectively. Nonetheless, these events are only effective against lepidopteran pests and cannot control CBW. As an alternative, researchers from the Brazilian Agricultural Research Corporation (EMBRAPA) are working on different approaches to control CBW, especially using Cry toxins genes that have shown to be effective against specific coleopterans. Data reported on Cry8Ka5 (Oliveira *et al.,* 2011), Cry1Ia12 (Silva *et al.,* 2015; Oliveira *et al.,* 2016), and Cry10Aa toxins (Aguiar *et al.,* 2012) have demonstrated the potential of these Cry toxins for controlling CBW in cotton cultivars; more recently Ribeiro et al (2017) reported the first GM cotton plants able to control the CBW.

Successful Generation of Cry10Aa GM Cotton Plants

Due to the *in vitro* entomotoxic potential presented by Cry10Aa against the CBW (Aguiar *et al.,* 2012), a Brazilian cotton cultivar (BRS 372) was transformed with the cry10Aa gene under control of the uceA 1.7 cotton promoter (Grossi-de-Sa *et*

3) Embrapa Genetic Resources and Biotechnology, Brasília-DF, Brazil

4) Embrapa Cotton, Campina Grande-PB, Brazil

* Corresponding author: fatima.grossi@embrapa.br, phone +55 61 3448 4705

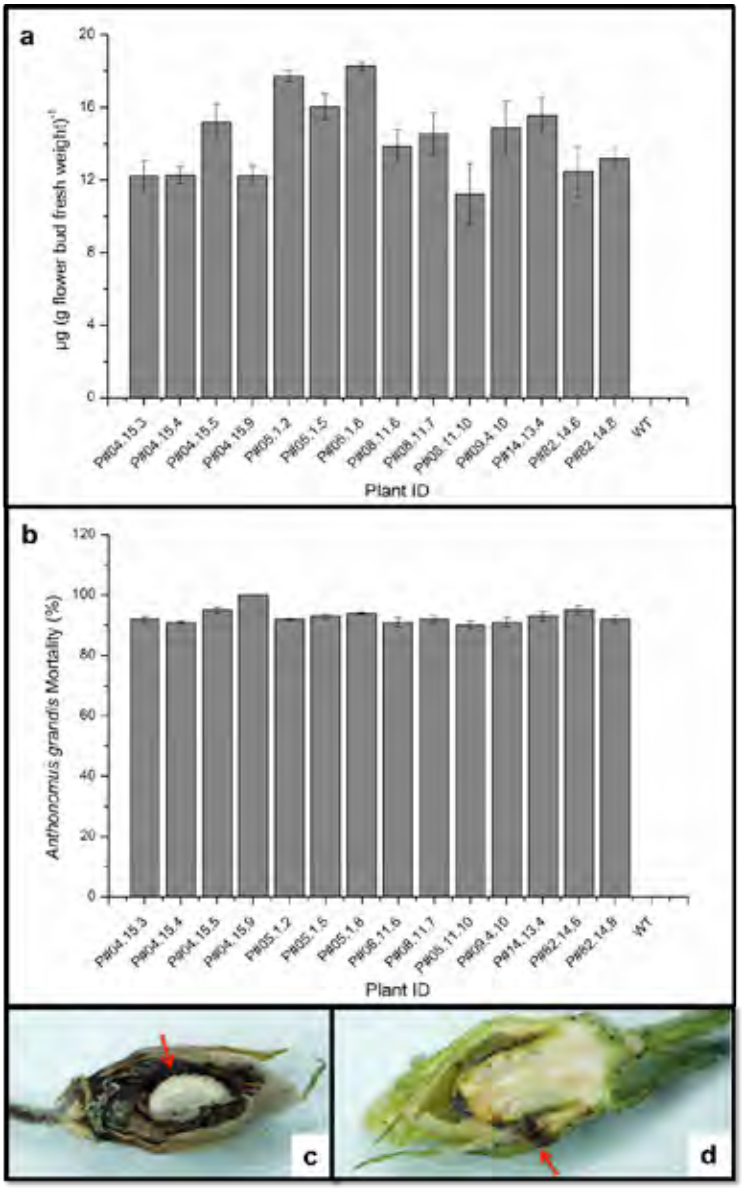


Figure 1. Cry10Aa GM Cotton Resistant to CBW. (a) Cry10Aa expression levels in T_2 GM cotton flower buds; **(b)** Mortality rate of CBW larvae in T_2 GM cotton flower buds expressing the Cry10Aa toxin; **(c)** CBW larval development in WT cotton floral buds **(d)** CBW larval development in Cry10Aa T_2 GM cotton flower buds. Red arrows indicate the location of CBW oviposition. WT – wild type non-transformed plants.

al., 2007 – Patent US 20090320153 A1), which yielded high transgene expression levels of the toxin in the floral tissues (Ribeiro *et al.,* 2017). Complete molecular characterization, including qPCR, southern blot, immunoassays, and *in vivo* bioassays in the presence of CBWs was carried out for eleven T_0 transgenic events. Quantitative and qualitative analyses using both T_0 flower buds and leaf tissues showed protein expression levels ranging from 3.0 to 14.0 µg per gram of fresh tissue (Ribeiro *et al.,* 2017). The CBW susceptibility bioassays, performed by feeding larvae and adults with leaves and flower buds from T_0 plants, resulted in high levels of CBW mortality (up to 100%). Subsequently, molecular analysis revealed that the transgene stability and the entomotoxic effect on the CBW were retained in T_2 plants, which presented high Cry10Aa toxin expression levels in the cotton flower buds (11.05 to 18.57 µg g^{-1} of fresh tissue) and a CBW mortality rate around 100% (Figure 1). The results represent a significant advance for the control of CBW in Brazil and can positively impact the Brazilian cotton agribusiness.

Cotton Production and Impacts of GM Cotton Plants on CBW Control

The rapid adoption of biotech-cotton over the past two decades has resulted in reduced chemical pesticide usage and increased crop yields. Among more than 480 transgenic events commercially approved worldwide, 57 are biotech cotton, and 75% of them have been developed for the insect resistance traits (James, 2017). By 2023-24 it is estimated that the area of planted cotton will increase from 0.9 million hectares (2014/15) to 1.5 million hectares in Brazil; during the same period, the adoption of biotech cotton is expected to increase from 0.6 million hectares to 1.2 million hectares (Céleres, 2016).

Continuous cotton production accelerates the build-up of pests, which can cause substantial production losses. The persistent occurrence of CBW during the last 33 years (1983- 2016) in the Brazilian cotton fields indicates that this insect is one of the most damaging pests of the Brazilian agriculture (Céleres, 2016). After the growing season, the CBW leaves the crop and disperses to refugee areas, where it finds alternative plant species that serve as food (pollen grains), shelter and hiding places during the off-season (Cuadrado, 2002; Macêdo *et al.,* 2015; Ribeiro *et al.,* 2010). Besides, the volunteer transgenic cotton plants resistant to lepdopterans (*Bt*) are also potential hosts for the CBW and other important cotton crop pests (Silvie *et al.*, 2015).

Due to the multiple factors involving CBW infestation and reproduction in cotton fields, many efforts have been undertaken by the cotton sector to reduce its impact on Brazilian cotton production. Despite consistent emphasis to reduce the use of insecticides to control this pest, chemical control is still a critical component in the CBW management. The number of insecticide applications to control CBW in the Brazilian Cerrado region varied from 17 to 23 per harvest, in particular in the transgenic cotton areas (Céleres, 2016). The cost of CBW control in the 2013/2014 harvest was 35% of the total pest control expenses. In Mato Grosso State, for the major Brazilian cotton producer, the average production cost average is estimated to be US$2,176.00 per

hectare. Of this total, 9% (US$192.25) is spent only for CBW control, with 15 to 20 insecticidal applications. In 2015/2016, the losses exceeded US $360 million per year (Céleres, 2016).

Despite the measures adopted to control CBW, there is an urgent need to rescue the profitability of cotton for the production sector, considering the vast importance of the Brazilian cotton sector in the domestic and international economies. The adoption of Cry10-cotton plants will have a positive impact in the producing regions, since it represents an improved practice of CBW management that strengthens attempts to eradicate this pest. One of the most important factors for achieving the desired expression levels of a transgene is the choice of the promoter that regulates its transcription. In the Cry10-cotton plants generated by the Brazilian researchers, the reported gene expression driven by *uce*A1.7 revealed that this cotton promoter is particularly more active in flowers and fruits, the tissues targeted by the CBW in cotton (Ribeiro *et al.,* 2017). Besides, the significant toxicity of Cry10Aa makes it a promising biotechnological tool for the development of transgenic cotton resistant to CBW. Additionally, biosafety studies revealed that Cry10Aa did not cause any genotoxic and hepatotoxic effects in Swiss mice, which confirm that the Cry toxin is not harmful to animal and human health (Freire *et al.,* 2014). Finally, the deployment of *cry10Aa* gene based biotech cotton to control the CBW can lead to significant reductions in the use of insecticides to improve insect management and, consequently, pave the way towards an eco-friendly agriculture.

Perspectives – RNA Interference as an Alternative for CBW Control

Cry proteins are widely used to control insect pests of cotton; and, several cases of insect populations resistant to these toxins have been reported (Wu, 2014; Tay *et al.,* 2015). It is believed that in the future, a similar situation could arise with CBW populations against the Cry10Aa proteins. An alternative approach to obtain GM cotton resistant to CBW is the RNA interference (RNAi) technology. RNAi is a promising strategy for controlling crop insect pests that show the advantage of using the insect's systemic gene-silencing machinery to suppress essential gene expression (Baum *et al.,* 2007; Burand & Hunter, 2013). Double-stranded RNA (dsRNA) is the RNAi trigger molecule that primes the post-transcriptional down-regulation of a target gene (Fire *et al.*, 1998). Studies using the RNAi technology showed efficient silencing of several target genes (i.e., genes related to CBW development) through different methods of dsRNA administration into larvae and adults of CBW insects (Baum *et al.,* 2007; Firmino *et al.,* 2013; Coelho *et al.,* 2016; Macedo *et al.,* 2017) (Figure 2).

Efficient RNAi-induced gene silencing in insects requires certain essential factors, including dsRNA processing by RNAi enzymes (Christiaens *et al.,* 2014), intracellular transport (Bolognesi *et al.,* 2012), expression of the core RNAi machinery (Garbutt & Reynolds, 2012), a delivery method (Luo *et al.,* 2013), and uptake from the hemolymph

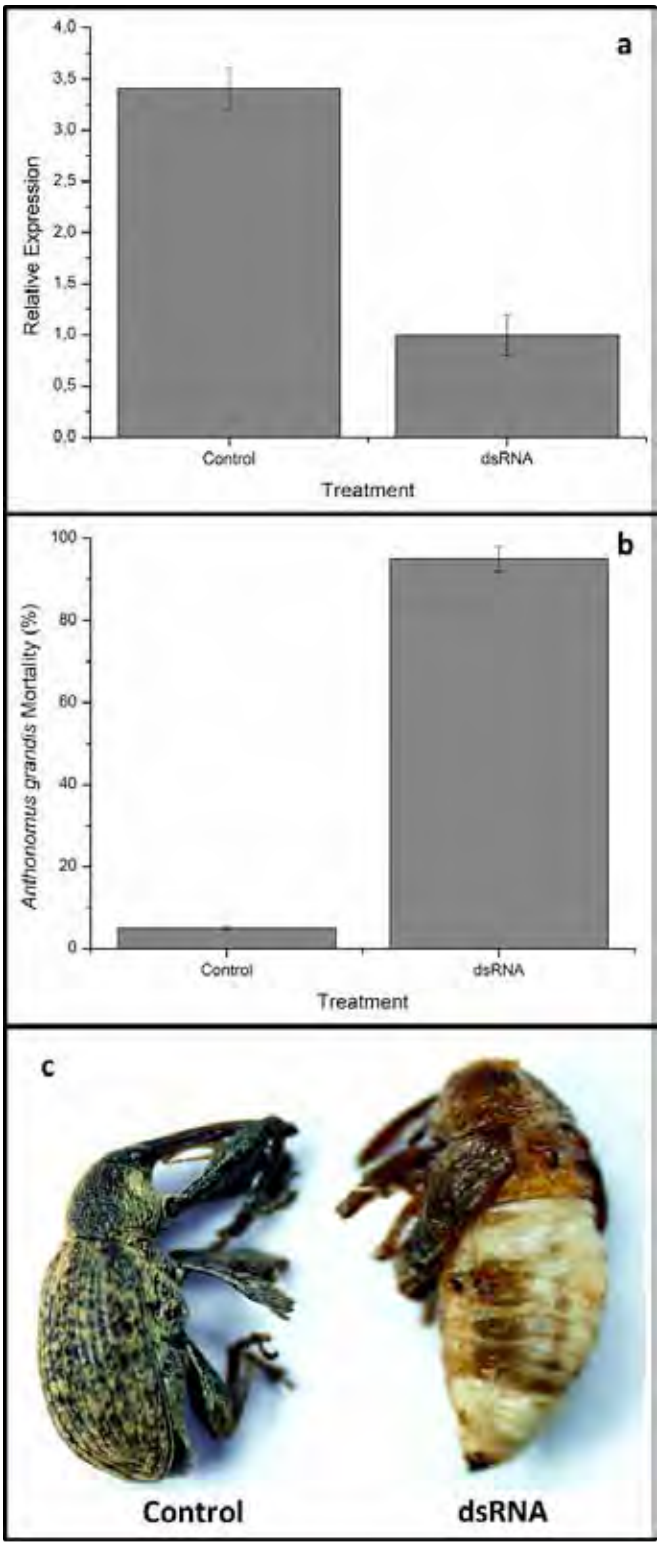


Figure 2. Effects of RNAi-mediated silencing on *Anthonomus grandis* insects. (a) Relative expression of a gene related to CBW development (*dev* – developmental gene). Adult females 48 hours after microinjection of 500 ng of *dev* dsRNA; **(b)** Effect of *dev* gene silencing by RNAi on the corrected mortality rate of CBW insects; **(c)** Lethal phenotypes in CBW caused by *dev* RNAi-mediated silencing.

or gut (Swevers *et al.,* 2011). Multiple studies shown that the RNAi efficacy varies among insect species (Garbutt *et al.,* 2013; El-Halim *et al.,* 2016; Lin *et al.,* 2017). The Plant-Pest Molecular Interaction Laboratory (LIMPP - at Embrapa Genetic and Biotechnological Resources – Brasília – Brazil) is conducting studies that aim to understand both *in silico* and *in vivo* routes of RNAi in several insect species, among which CBW stands out. These studies are intended both to understand the differences between the RNAi machinery of insects and to develop biotechnological tools that increase the effectiveness of this technology for the control of insect pests. One of the objectives is to increase the stability of the dsRNA molecules administered to CBW to avoid degradation in the hemolymph and intestine of the insect. Wang and colleagues (2016) demonstrated that nucleases present in hemolymph and midgut of *Locusta migratoria* (Orthoptera), *Periplaneta americana* (Blattaria), *Spodoptera lituria* (Lepidoptera) and *Zophobas atratus* (Coleoptera) affect dsRNA. Previous studies have shown that this phenomenon also occurs in CBW (Grossi-de-Sa, unpublished data). Variations in the structure of exogenous dsRNA administered via nanoparticles or by expression in transgenic cotton plants can be extremely useful and cost effective. Also, the use of the RNAi approach can be pyramided with the use of the Cry10Aa toxin in the control of CBW, which would bring about a significant reduction in the emergence of resistant insect populations.

Final Remarks

Biotechnology has consistently provided new effective tools for safe and low-cost control of insect pests of crops, of which cotton is an important beneficiary. Successful development of transgenic cotton plants resistant to CBW demonstrate the growing need for constant research in the area, as well as a review of legislation that regulates transgenic research in several countries. These approaches could significantly improve cotton production so as to cater to the growing societal needs while reducing the negative impacts on environment.

Acknowledgements

This work was supported by grants from EMBRAPA, CNPq, CAPES and FAPDF.

References

Aguiar, RW; Martins, ES; Ribeiro, BM; Monnerat, RG. "Cry10Aa protein is highly toxic to *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), an important insect pest in Brazilian cotton crop fields". *Bt Research* 3.1 (2012).

Azambuja, R; Degrande, PE. "Thirty years of cotton boll weevil in Brazil". Arquivos do Instituto Biológico 81.4377-410 (2014).

Baum, JA; Bogaert, T; Clinton, W; Heck, GR; Feldmann, P; Ilagan, O; Johnson, S; Plaetinck, G; Munyikwa, T; Pleau, M; Vaughn, T; Roberts, J. "Control of coleopteran insect pests through RNA interference". Nature Biotechnology 25.11, 1322 (2007).

Bolognesi, R; Ramaseshadri, P; Anderson, J; Bachman, P; Clinton, W; Flannagan, R; Ilagan, O; Lawrence, C; Levine, S; Moar, W; Mueller, G; Tan, J; Uffman, J; Wiggins, E; Heck, G; Segers, G. "Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (*Diabrotica virgifera virgifera* LeConte)". PLoS One 7.10, e47534 (2012).

Burand, JP; Hunter, WB. "RNAi: future in insect management". Journal of Invertebrate Pathology 112, S68-S74 (2013).

Céleres. "2º levantamento de adoção da biotecnologia agrícola no Brasil, safra 2015/16". Informativo de Biotecnologia, 16.01. (http://www.celeres.com.br/2o-levantamento-de-adocao-da-biotecnologia-agricola-no-brasil-safra-201516/) (2016).

Coelho, RR; Souza-Jr, JDA; Firmino, AAP; Macedo, LLP; Fonseca, FCA; Terra, WR; Engler, G; Engler, JA; Silva, MCM; Grossi-de-Sa, MF. "Vitellogenin knockdown strongly affects cotton boll weevil egg viability but not the number of eggs laid by females". Meta Gene 9, 173-180 (2016).

Conab. "11º Acompanhamento da safra Brasileira – grãos – Safra 2016/17". Brasília, 4, 1−171 (http://www.conab.gov.br/OlalaCMS/uploads/arquivos/17_08_10_11_27_12_boletim_graos_agosto_2017.pdf) (2017).

Crickmore, N; Zeigler, DR; Feitelson, J; Schnepf, E; Van-Rie, J; Lereclus, D; Baum, J; Dean, DH. "*Bacillus thuringiensis* toxin nomenclature". http://www.btnomenclature.info/ (2016).

Christiaens, O; Swevers, L; Smagghe, G. "dsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay". Peptides 53, 307-314 (2014).

Cuadrado, GA. "*Anthonomus grandis* Boheman (Coleoptera: Curculionidae) en la Zona Central y Sur Oeste de Misiones, Argentina: polen como fuente alimenticia y su relación con o estado fisiológico en insectos adultos". Neotropical Entomology, Londrina, v. 31, n.1, p. 121−132, jan/mar (2002).

El-Halim, HMA; Alshukri; BMH; Ahmad, MS; Nakasu, EYT; Awwad, MH; Salama, EM; Gatehouse, AMR; Edwards, MG. "RNAi-mediated knockdown of the voltage gated sodium ion channel TcNav causes mortality in *Tribolium castaneum*". Scientific Reports 6, 29301 (2016).

Fire, A; Xu, S; Montgomery, MK; Kostas, SA; Driver, SE; Mello, CC. "Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*". Nature 391.6669, 806 (1998).

Firmino, AAP; Fonseca, FCA; Macedo, LLP; Coelho, RR; Souza-Jr, JDA; Togawa, RC; Silva-Junior, OB; Pappas-Jr, GJ; Silva, MCM; Engler, G; Grossi-de-Sa, MF. "Transcriptome analysis in cotton boll weevil (*Anthonomus grandis*) and RNA interference in insect pests". PLoS One 8.12 (2013): e85079.

Freire, IS; Miranda-Vilela, AL; Barbosa, LCP; Martins, ES; Monnerat, RG; Grisolia, CK. "Evaluation of cytotoxicity, genotoxicity and hematotoxicity of the recombinant spore-crystal complexes Cry1Ia, Cry10Aa and Cry1Ba6 from *Bacillus thuringiensis* in Swiss mice". Toxins 6, 2872-2885. (2014).

Gallo, DN; Nakano, O; Neto, SS; Carvalho, RPL; Baptista, GC. "Manual de entomologia agrícola". Instituto Brasileiro do Café, Rio de Janeiro (Brasil) (2002).

Garbutt, JS; Reynolds, SE. "Induction of RNA interference genes by double-stranded RNA; implications for susceptibility to RNA interference". Insect Biochemistry and Molecular Biology 42.9, 621-628 (2012).

Garbutt, JS; Bellés, X; Richards, EH; Reynolds, SE. "Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: evidence from *Manduca sexta* and *Blattella germanica*". Journal of Insect Physiology 59.2, 171-178 (2013).

Grossi-de-Sa, MF; Guimaraes, LM; Batista, JAN; Viana, AAB; Fragoso, RR; Rocha, TL. "Compositions and methods for modifying gene expression using the promoter of ubiquitin conjugating protein coding gene of cotton plants". U.S. Patent No. 8,227,588. ID US 20090320153 A1. 05 fev. (2007).

Gullan, PJ; Cranston, PS. "The insects: an outline of entomology". John Wiley & Sons (2014).

James, C. "Global status of commercialized biotech/GM crops: 2016". Ithaca: The International Service for the Acquisition of Agri-biotech Applications (ISAAA). (2017).

Lin, Y; Huang, JH; Liu, Y; Bellés, X; Lee, HJ. "Oral delivery of dsRNA lipoplexes to German cockroach protects dsRNA from degradation and induces RNAi response". Pest Management Science 73.5, 960-966 (2017).

Luo, Y; Wang, X; Yu, D; Chen, B; Kang, L. "Differential responses of migratory locusts to systemic RNA interference via double-stranded RNA injection and feeding". Insect Molecular Biology 22.5, 574-583 (2013).

Macêdo, JA; Castellani, MA; Santos, FAR; Oliveira, PP; Maluf, RP. "Fontes alternativas de pólen utilizadas pelo bicudo-do-algodoeiro em duas regiões produtoras de algodão na Bahia". Revista Caatinga, Mossoró, v. 28, n. 3, p. 255−262, jul/set (2015).

Macedo, LLP; Souza-Jr, JDA; Coelho, RR; Fonseca, FCA; Firmino, AAP; Silva, MCM; Fragoso, RR; Albuquerque, EVS; Silva, MS; Engler, JA; Terra, WR; Grossi-de-Sa, MF. "Knocking down chitin synthase 2 by RNAi is lethal to the cotton boll weevil". Biotechnology Research and Innovation (2017).

Oliveira, GR; Silva, MCM; Lucena, WA; Nakasu, EYT; Firmino, AAP; Beneventi, MA; Souza, DSL; Gomes-Jr, JE; Souza-Jr, JDA; Rigden D; Ramos, HB; Soccol, CR; Grossi-de-Sa, MF. "Improving Cry8Ka toxin activity towards the cotton boll weevil (*Anthonomus grandis*)". BMC Biotechnology 11.1:85 (2011).

Oliveira, CM; Auad, AM; Mendes, SM; Frizzas, MR. "Crop losses and the economic impact of insect pests on Brazilian agriculture". Crop Protection 56: 50-54 (2014).

Oliveira, RS; Oliveira-Neto, OB; Moura, HFN; Macedo, LLP; Arraes, FBM; Lucena, WA; Lourenço-Tessutti, IT; Barbosa, AAD; Silva, MCM; Grossi-de-Sa, MF. "Transgenic cotton plants expressing Cry1Ia12 toxin confer resistance to fall armyworm (*Spodoptera frugiperda*) and cotton boll weevil (*Anthonomus grandis*)". Frontiers in Plant Science 7 (2016).

Ribeiro, TP; Arraes, FBM; Lourenço-Tessutti, IT; Silva, MS; Lisei-de-Sa, ME; Lucena, WA; Macedo, LLP; Lima, JN; Amorim, RMS; Artico, S; Alves-Ferreira, M; Silva, MCM; Grossi-de-Sa, MF. "Transgenic cotton expressing Cry10Aa toxin confers high resistance to the cotton boll weevil". Plant Biotechnology Journal (2017).

Ribeiro, PA; Sujii ER; Diniz, IR; Medeiros, MA; Salgado-Labouriau, ML; Branco, MC; Pires, CSS; Fontes, EMG. "Alternative food sources and overwintering feeding behavior of the boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) under the tropical conditions of Central Brazil". Neotropical Entomology, Londrina, v. 39, n. 1, p. 28−34, jan/fev (2010).

Silva, CRC; Monnerat, R; Lima, LM; Martins, ES; Melo-Filho, PA; Pinheiro, MP; Santos, RC. "Stable integration and expression of a *cry1Ia* gene conferring resistance to fall armyworm and boll weevil in cotton plants". Pest Management Science (2015).

Silvie, P; Bélot, JL; Michel, B. "Manual de identificação das pragas e seus danos no cultivo do algodão". (2007).

Swevers, L; Liu, J; Huvenne, H; Smagghe, G. "Search for limiting factors in the RNAi pathway in silkmoth tissues and the Bm5 cell line: the RNA-binding proteins R2D2 and Translin." PloS One 6.5, e20250 (2011).

Tay, WT; Mahon, RJ; Heckel, DG; Walsh, TK; Downes, S; James, WJ; Lee, SF; Reineke, A; Williams, AK; Gordon, KHJ. "Insect resistance to *Bacillus thuringiensis* toxin Cry2Ab is conferred by mutations in an ABC transporter subfamily A protein". PLoS Genetics 11.11, e1005534 (2015).

Wang, K; Peng, Y; Pu, J; Fu, W; Wang, J; Han, Z. "Variation in RNAi efficacy among insect species is attributable to dsRNA degradation *in vivo*". Insect Biochemistry and Molecular Biology 77, 1-9 (2016).

Wu, Y. "Detection and mechanisms of resistance evolved in insects to Cry toxins from *Bacillus thuringiensis*". Advances in Insect Physiology 47, 297-342 (2014).

Brief History of Boll Weevil Eradication in the United States Challenges and Lessons Learned

James A. Schoenholz, Associate Executive Director, Field Operations (retired). U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Plant Protection and Quarantine

The dream of boll weevil eradication probably began in 1892 when cotton producers in the USA discovered what was destroying their crops. Many things were tried while the weevil marched across the south where, in 1922, it found its limit in Virginia. All efforts to control had some, but not lasting, success in eliminating the pest.

The first real steps to look at a solution were taken in the late 1950's when the House and Senate Agriculture Committees asked the Office of the Secretary of Agriculture to make recommendations to find a solution for the boll weevil problem. This request included research and facility needs, a compilation of status on current research from Federal, State and industry and information on what the Federal emphasis concerning the boll weevil problem should be.

In 1958 a committee consisting of various USDA agencies and the National Cotton Council presented their findings to Congress,

which lead to appropriations to fund cotton research with the goal for developing technologies to initiate boll weevil eradication programs within the United States. To further this effort, the National Cotton Council of America, the national organization for the cotton industry and producers, appointed a committee to look at areas in the Southeast, South and Southwest that could be used to test the program assumptions and technologies.

In the fall of 1969, the committee recommended that a pilot boll weevil eradication experiment be initiated in 1970. Adequate funding was made available from USDA, the 'Cooperative State Research Service' and the 'Cotton Incorporated' representing industry. The experiment began in July 1971. It was centered in Mississippi and was conducted in cooperation with the Departments of Agriculture of Alabama, Louisiana, Mississippi, respective experimental stations, Cooperative Extension Services along with the National Cotton Council, Cotton Incorporated and USDA agencies including the Agricultural Research Service, Animal and Plant Health Inspection Service, the Agriculture Stabilization and Conservation Service, which assisted with identifying cotton field locations, and other co-operative agencies.

The operation began with diapause treatments in the fall. The following spring it was found that the emerging populations were greater than expected due to heavy 1971 populations and a mild winter. The Program tested trap crops treated with aldicarb around the field border rows and then treated all acreage with insecticide twice. Sterile insects were also tested, but were ineffective. This was attributed to dry weather and late-diapause weevil emergence. There were 5 in-season applications and 13 diapause applications. Trap catches in the spring of 1973 revealed low weevil survival. The Experiment ended in August 1973 and was deemed a success to indicate that Boll Weevil Eradication was feasible and ecologically acceptable.

A Technical Advisory Committee had been appointed through the National Cotton Council in 1972. In December of 1973 the Committee recommended to the Secretary of Agriculture that a Boll Weevil Eradication Trial be conducted. The Agriculture Act of 1973 instructed the Secretary to initiate a Program to eliminate boll weevil, pink bollworm and other cotton pests considered feasible for eradication.

Funding was made available for the boll weevil eradication trial to begin in northeast North Carolina with a buffer area in central North Carolina. The trial ended in 1981 and was considered successful both biologically and technically. With the passage of a referendum to expand the Program further as a full eradication effort in 1981, USDA sponsored a 2- year program on boll weevil containment in the original buffer zone in central North Carolina. A referendum is a legally binding vote of registered farmers in a defined area to determine if there is majority support to continue a program. The farmers agree to a fee schedule, one in the spring and the final yearly payment in the fall to cover the cost of a program facilitated by the government.

A positive referendum in 1983 led to the beginning of the expansion of boll weevil eradication from the southeast to California and all states in between. Expansion has had its challenges since grower referendums are necessary since growers bear the bulk of the costs. As in the Carolinas, grower economic concerns delayed expansion into Georgia and Alabama. The positive referendum in 1987 began the beginning of continued positive grower responses.

This success led to further expansion in Alabama in 1990, 1992 and into the Tennessee River Valley in 1994. There were doubters in these areas when the efforts began in 1987, but they saw the economic benefits quickly and wanted to get on board.

In the West, California control was initiated at the pin-head square stage rather than the diapause-treatment program in 1985. Arizona also started their program in western Arizona adjacent to the California border. These Programs also included the Mexicali and San Luis Rio Colorado areas in the States of Baja and Sonora, Mexico respectively. The California program was managed by the California Department of Food and Agriculture, and the Arizona program by the Arizona Cotton Research an Protection Council of the state of Arizona. The responsibility of management of respective Programs shifted from the USDA, as in the southeast, to state or State Grower Foundations which has been the norm for all states west of Alabama, except for Kansas, which is managed by USDA and the Kansas Department of Agriculture. The Mexican Program areas were managed by USDA and USDA International Services funds. In 1986, the remainder of Arizona was included, along with Sonoita, Sonora and Mexico. Mexican producers in these areas did not contribute funding to Program operations. Later expansions in Mexico began in 1988 in the State of Chihuahua and in the Caborca area in Sonoro. Growers there, and in the remaining expansions in Mexico, contributed to their 70% cost share.

In 1994, Texas initiated their eradication program in the Southern Rolling Plains zone under the management of the Texas Boll Weevil Eradication Foundation. Currently, boll weevils in 15 of the 16 zones are eradicated. The remaining zones, the Lower Rio Grande Valley, along with the State of Tamaulipas, Mexico continue their efforts to eliminate the weevil.

In the mid 1990's through the early 2000's, boll weevil eradication programs were established in all states and have completed their active Program activities. All are in the post-eradication phase, monitoring reduced trap lines to detect any migration or other introduction of boll weevils into their respective States or zones.

Steps for a Successful Eradication Program

The most important steps for a successful boll weevil eradication program are listed below:

- Close cooperation and collaboration between industry, government, growers, researches and appropriate universities
- Enabling legislation
- Dedicated leadership at the local, state and federal levels
- A detailed plan of action to establish procedures and goals
- Inclusive training for all field employees conducting the operations
- Commitment to short and long term goals

- Post eradication strategy

Challenges

There are some challenges that are uncontrollable, but most challenges have solutions that can be provided by operational controls:

- Weather—wind/rain cannot be controlled but usually have short term affects. A big exception here is the Lower Rio Grande Valley and Tamaulipas Programs where spring winds have serious impacts on spraying conditions and schedules. Timely treatments, especially at pin head square are the foundation of boll weevil eradication success.
- Undetected cotton fields- sometimes fields are planted after maps have been prepared or were missed during the mapping process. Undetected fields create an insectary that can eventually overwhelm program operations.
- Volunteer cotton (Cotton that grows like weeds in unplanted areas) - this is the great devil to the program. This can occur on field edges or road shoulders as cottonseeds are dropped on the ground and germinate. Program personnel must be vigilant to detect this situation quickly. Harder to find and the most problematic are volunteer plants that result from improper plow down and sprouting plants in over-cropped fields (fields that have been planted to a successive crop, such as corn, without first ensuring that all cotton has been destroyed). It is extremely difficult to see cotton plants in rows of an alternate crop and "roguing" such plants (removing them by hand one at a time) can be difficult. Another problem is that your options may be limited in what should be sprayed and how many times you can spray the crop. Undetected insectary is yet another more serious problem.
- Public reaction to Program Operations.

Technology

The basic tools and concepts utilized at the beginning of boll weevil eradication, for the most part, remain the same but have been improved. The concepts of 'map, trap and treat' are still the foundation of an eradication program. Traps and chemicals remain vital tools to detect and fight the boll weevil.

- Traps: The trap is the detection tool that is critical to locating boll weevil populations. It has evolved from a handmade flimsy drink cup base and stamped-screen tool to a snap-together durable tool that may be used for more than one season.
- Lure: The pheromone dispenser has evolved from an impregnated cigarette filter, functional for maybe 7 days to the current dispensers that are effective for 14 days to 30 days.
- Chemical: Malathion remains the pesticide of choice. It is highly effective with low mammalian toxicity, 5-7 day effective residual and, while some mid-season resistance has been observed, research over the years indicate that this resistance is not passed on to the next generations.
- Data Collection: This is one area that has improved greatly. All data is collected and recorded in the field using computers rather that hand recording and transcription. The Texas eradication program records all information in the field using tablets, and this data can be remotely transmitted to a headquarters location. This process has improved reporting and reaction times so necessary to a successful program operation.

Lessons Learned

- Strong local, state, national, research and industry leadership is critical to sustain Program goals and success.
- Training needs to be inclusive and timely, aimed to when it can be applied as immediately as possible.
- Ultra-low-Volume (ULV) malathion is the chemical of choice
- Timely treatments are critical
- Undetected fields and volunteer cotton prolongs the program and increases costs
- Crop destruction immediately following harvest reduces food source for diapausing weevils and reduces survival.
- Grower cost incentives for early plow-down saves program costs in the short term
- Debrief at the end of each season to find best practices and solutions to problem areas.

Conclusion

Boll weevil eradication is no longer a dream but a reality. The concepts are simple but it requires patience and commitment, not only by program operation people but especially by producers, industry and government.

In the U.S, the producers have been required to bear a majority of the program costs, and this has been the major contribution to the success of the eradication effort. Grower support has kept their respective programs on course to completion, taking only a few years to reach their goal. After eradication is achieved, grower costs are significantly reduced.

Boll weevil eradication is not for the faint of heart. It's hard work and will never be completely over once achieved because a surveillance operation will be necessary to ensure re-infestation doesn't occur. It's much cheaper than fighting the pest year after year.

References

El-Lissy, O. and Bill Greffenstette, 2001. Boll Weevil Eradication in the U.S., 2001. 2002 Beltwide Cotton Conference, Atlanta, GA.

Cross, W. H. 1979. Boll Weevil Behavior and development of Traps. Boll Weevil Research Laboratory, Starkville, MS

Parencia, C.R. Jr. 1978. One Hundred Twenty Years of Research on Cotton Insects in the United States. Agriculture Handbook No. 515. Agriculture research Service, United States Department of Agriculture, pp. 62-65

Ganyard, M. Justin Dillier and J.R. Brazzel. The Boll Weevil Eradication Trial. 1981