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Quantitative Assessment of Phenolic Compounds from Cotton Seed Extract with PCR-Based Identification of GMO plant varieties

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Abstract

The purpose of the present study was to isolate and perform preliminary qualitative and quantitative assessment of gossypol, a phenolic phytochemical constituent present in cotton seed. Gossypol was extracted from cotton seeds from non-genetically modified source as well as Bt cotton seeds. The PCR approach is used in this study to specifically distinguish genetically modified organisms (GMOs) that from Bt cotton seeds which are genetically modified. The four pairs of primers that were used to detect the DNA of genetically modified cotton seeds include P-35S, T-Nos, *nptII*, and *cry1Ac* respectively. DNA was extracted from cotton seed samples and then amplified using PCR with four set of primers. Gel electrophoresis was used to examine amplified DNA fragments for the presence of GMO-specific sequence. PCR technique was successful to detect the genetic modified cotton seeds. Diethyl ether extracts obtained from Bt and non- Bt seeds were subjected for qualitative and quantitative assessment. Preliminary assessment of gossypol was accomplished with chemical tests, TLC, and UV spectrophotometry. In qualitative analysis lead acetate tests produced a yellowish precipitate, and TLC showed a bright violet line, confirmed presence gossypol in both extracts. The preliminary quantitative analysis using UV spectrophotometry at 290 nm showed higher absorbance in non-*Bt* extracts (1.3) compared to Bt extracts (0.6), indicating less amount of gossypol present in *Bt* cottonseeds. The results of present research study positively leverage PCR based GMO detection techniques, enabling both qualitative and quantitative evaluation of the phytochemical constituents found in plant-based sources.

Key words: Genetic modified organism (GMO), cetyltrimethylammonium bromide (CTAB), Polymerase chain reaction (PCR), Gossypol Thin Layer Chromatography (TLC) and Spectrophotometry.

1. Introduction

Cotton seeds are the small but essential parts of the cotton plant that, although incredibly relevant and adaptable across wide range of industries. Scientific names for the commodity are *Gossypium hirsutum*, *Gossypium barbadense*, *Gossypium arboretum*, and *Gossypium herbaceum*. (Malhotra SK *et.al.*, 2017). Cotton seeds have a strong nutritional profile and can produce essential oils, making them valuable for industrial uses, animal feed, and human nutrition. Additionally, the extraction of its oil produces valuable byproducts including cottonseed hulls, which are used in a variety of industrial and agricultural processes, and cottonseed meal, which is fed to animals. (Liu Q *et.al.*, 2012). Cotton seeds, the foundation of the cotton business, continue to have a big influence on innovation, global agriculture, and the economy. Cottonseed oil's neutral flavor and high smoke point make it widely used in the food sector for cooking, frying, and baking. (Richard DOB *et.al.*, 2002) In addition, it is an essential component of many industrial operations like the production/ manufacturing of lubricants and soap as well as cosmetics and pharmaceuticals. Cotton-producing regions benefit economically from the production and processing of cotton seeds, which create jobs and revenue for farmers, processors, and other businesses. (Murugkar M *et.al.*, 2007)

Cotton seed belongs to the *Malvaceae* family. Cotton seeds are oval in shape and range in length from 3.5 to 10 mm. They are extensively covered in shorter hairs (linters) as well as long, fuzzy, white or rusty hairs, also known as lint, which is the major material utilized in cotton textile production. (Liu Q *et.al.,* 2012). Cotton seeds can be stored for up to a year in optimum conditions. To prevent mycotoxin production, the seeds must be dried and stored with a moisture level of no more than 10%. Because of their high hygroscopicity, the relative air humidity in the storage facility must be kept under control. (Ibragimov M *et.al.,* 2021). An air-cooling system is essential to keep seeds from heating up while in storage. To limit further degradation, seeds must be chilled to 15.6°C.

One of India's major cash crops is cotton. Because cotton seed contains gossypol, which is toxic compound, (Quezia BC et.al., 1991) and because It's an excellent source of protein, fats and vitamins, its leftovers are used as animal feed. (Ganapathyswamy H et.al., 2021). Gossypol is a polyphenolic chemical that is naturally present in cotton plants, especially in the seeds, Because of its hazardous qualities and possible therapeutic uses, it has significant implications in pharmacology, animal nutrition, and agriculture. Chemical Formula: $C_{30}H_{30}O_8$, Molecular Weight: 518.56 g/mol, Gossypol is a yellow pigment having a structure of a binaphthyl dialdehyde including two hydroxyl, two methyl, and two aldehyde groups. It can be found in both bound and free forms in the cotton plant. Gossypol is largely present in cotton plants' pigment glands, which include the seeds, leaves, and stems. Gossypol appears in cotton seeds in both free and bound forms, with the free form being more toxic. Generally speaking, gossypol content in cotton seeds ranges from 0.4% to 1.6% of the seed weight, depending on the cotton type and growing environment.

The GMO (Genetically Modified Organism) cotton seeds are the subject of the current study. Examining GMO cotton seeds usually tries to evaluate how they affect agriculture, human health, and the environment. These seeds are modified to improve desired qualities including pest resistance, herbicide tolerance, and higher yield by adding genetic material from other species that has been altered by the insertion or deletion of genes. The creation of genetically modified cotton seeds seeks to decrease the need for chemical pesticides, cut manufacturing costs, and enhance cotton farming's environmental sustainability. These seeds are now an essential tool in contemporary agriculture, helping to manage pests and maximize resource efficiency while satisfying the world's expanding cotton demand. Cotton seeds labeled as genetically modified organisms (GMOs) can be identified using a variety of techniques that look for certain genetic alterations inserted into the cotton plant. These techniques are essential for resolving customer concerns about GMOs, maintaining accuracy in product labeling, and complying with regulations. The following methods are frequently used to identify genetically modified organisms (GMOs) in cotton seeds: digital PCR, enzyme-linked immunosorbent assay (ELISA), southern blotting, real-time PCR, conventional PCR, DNA sequencing, and immunoassays.

In present research study the genetic modification in cotton seeds was confirmed with PCR. The solvent extraction from cotton seeds was further subjected to preliminary quantitative analysis of gossypol using chemical test, thin layer chromatography (TLC) and the spectrophotometer method.

PCR techniques are frequently applied attributing to their dependability, sensitivity, and specificity. In PCR Specific DNA sequence unique to GMO of interest that are *Agrobacterium tumefaciens nopaline* synthase terminator (T-Nos), the amplified cloning vector genes coding for resistance to ampicillin and neomycin/kanamycin (nptII), and the cauliflower mosaic virus (CaMV) 35S promoter or terminator (P-35S) were the specific DNA sequences that were amplified in PCR. Among the first crops to be genetically engineered was *Bt*-Cotton which is commercially distributed globally. It is derived from a gene found in the soil bacteria *Bacillus thuringensis (Bt)*, which produces proteins that are toxic to several insect pests, especially bollworms, which pose a serious danger to cotton harvests in India. Since its introduction in the early 2000s, *Bt* cotton has become widely used in the nation because of its efficiency in reducing pests and raising output. Additionally, we used *cryAc1*, a *Bt* gene-specific primer, in this investigation. (Bennett RM *et.al.,* 2005). Information about the primers listed in Table No. 1. The isolation of gossypol process involved extraction from cotton seeds. Qualitative analysis to find gossypol in seed extract samples,

particular chemical tests were conducted it provide valuable insights and spectrophotometric analysis facilitated the quantification of gossypol.

2. Materials and method

2.1. Plant material:

Cotton seeds sample were collected from local authentic organic supplier from Mumbai, Maharashtra, India

2.2. Chemicals & Other Reagents (DNA Extraction)

The DNA extraction of cotton seeds was performed using cetyltrimethylammonium bromide (CTAB).

CTAB buffer (PH-8):- 100 mM Tris-cl (PH-8.0); 24 mM EDTA; 0.35 M Sodium bisulfite; 1.5 M NaCl; 2.5% CTAB; (0.02ml)0.2% B- mercaptoethanol (v/v) (added immediately before use) and 1% Polyvinylpyrrolidone (w/v) (added immediately before use).

Phenol:Chloroform:Isoamylalchohol (25:24:1); Chilled distilled ethanol; ethanol, o.1X TE buffer containing 10mM Tris (pH-8) and 1mM EDTA. $2H_2O$ (pH-8)

2.3. DNA Isolation:

Approximately 0.24 grams of cotton seed powder was obtained and transfer it to polypropylene tube. 1000 µl of CTAB extraction buffer was added. Mixed the content by inversion**.** Incubated the same at 55^0C-60^0C in a shaking water bath for 60 minutes. Added 100 µl of Phenol: Chloroform:Isoamylalchohol (25:24:1) and mix gently by inversion for 15 minutes. Same mixture centrifuge at 10,000 rpm at room temperature for 10 minutes. The aqueous layer was collected and transferred in new tube (repeated the step 3 and 4 twice). Added chilled isopropanol to the collected aqueous layer, gently mixing by inversion. and let mixture stand for RT for 5-10 minutes. Allowed the DNA threads to appear. Dry the pallet (in vacuum for 15 minutes or overnight air dry) and dissolved the pallet in TE buffer 80µl and store the isolated DNA samples at -20^0C. Added 20µl RNase A and incubate at 37^0C for 30 minutes. Purified and transferred the again in TE buffer and stored the samples at – 20^0C till use. (Khadye SV *et.al.,* 2012 and Moyo M *et.al.,* 2008)

DNA quantification: - DNA Purity and Agarose Gel Electrophoresis:

The genomic DNA was resolved on a 0.8% agarose gel in 1X TAE buffer using internal ethidium bromide staining, and the electrophoresis was run for two hours at 65 V under a UV transilluminator. By measuring the absorbance at 260nm and 280nm, the purity of the DNA was verified. The ideal OD ratio range is between 1.6 and 2.0. (Moyo M et.al., 2008)

Table no. 1- Details of primer sequence used for PCR analysis:
P-35S(19bp) F:GCTCCTACAAATGCCATCA R:GATAGTGGGATTGTGCGTCA
nptII (21bp) F:GAGGCTATTCGGCTATGACTG R:ATCGGGAGCGGCGATACCGT
T-Nos (20bp) F:GAATCCTGTTGCCGGTCTTG R:TTATCCTAGTTTGCGCGCTA

cryIAc (26bp) F:GCCAATGCCTCGTGATTGTTCTCTGC R:GATTTGCGAGGCTGGCCAGCTCCACG

2.4. Polymerase Chain Reaction:

Utilizing the previously reported primers p-35S, *nptll*, T-Nos, and *cry1Ac*, PCR analysis of cotton seeds was performed.

In a 25 µl final volume reaction mixture, PCR was performed using 10X Taq DNA Polymerase Buffer and 15 mM $MgCl_2$; 2.5mM dNTPs; 0.25mM of each primer- Forward Primer, Reverse Primer; 100ng of Template DNA; 3U of Taq DNA Polymerase.

PCR for p-35S, *nptll* and T-Nos primers was carried out. The thermocycler was set up for 3 minutes of initial denaturation at 94^0C, the cycling conditions for 12 cycles were: denaturing at 94^0C for 30 sec, Annealing at 54^0C for 40 sec (for p-35); at 57^0C for 40s (for *nptll*); at 55^0C for 40 sec (for T-Nos) and extension at 72^0C for 30 sec. The final extension was at 72^0C for 10 min.

PCR for the (*Bt* gene) was performed with *cry1Ac* primers. The thermocycler was set up for 3 minutes of initial denaturation at 940C, the cycling conditions for 12 cycles were: denaturing at 94^0C for 30 sec, annealing at 77^0C for 40 sec (extension time after annealing 20 sec), Extension at 72^0C for 30 sec. The final extension was at 72^0C for 10 min. (Vidhya SS *et.al.*, 2012)

DNA quantification: - The PCR amplicons were examined using silver-stained 2% agarose gel electrophoresis, which was run for 10 to 15 minutes at 100V. (Khadye SV *et.al.*, 2012).

3. Extraction of Gossypol

About 100 gm of each dried

cotton seed powder from each source was soaked separately in 100 ml of separate diethyl ether solvent flasks and at low temperature extracted using Soxhlet apparatus till the complete solvent evaporated. Resulting concentrated extracts were preserved in sterilized air tight bottles at 4 ^{0}C until required for further phytochemical assessment.

Qualitative Analysis of Gossypol

Qualitative analysis of gossypol was done using chemical tests and Thin layer Chromatography (TLC).

4.1 Gossypol detection using chemical test

Five milliliters of both seed extract were dissolved in a small quantity of ethanol in a 25milliliter conical flask, and additional ethanol was added to reach the desired final volume. Each test tube was filled with two milliliters of the sample solution, and then an equal volume of lead acetate was added and thoroughly mixed. (Similarly, antimony chloride and stannic chloride were used in different types of assays.) In the instance of antimony chloride, a turbid reddish complex emerged after 15 minutes. When stannic chloride was used in the test, a reddish precipitate formed after 10 minutes, and lead acetate produced a yellowish precipitate after 20 minutes. (Chandrashekar R. *et.al.*, 2013)

3.2 Thin Layer Chromatography

Silica gel was used to prepare TLC plates. The plates had a thickness of 0.02 mm. 5 milliliters of diethyl ether were used to dissolve one gram of each sample.

Then, using a fine capillary jet, an equal volume of the Cotton seed and *Bt* Cotton seed extract were spotted on the plates.

Chromatography plates were developed using a solvent system consisting of 91:10:4 acetic acid, pet ether, and ethyl acetate. After being removed from the chamber, the plates were allowed to air dry completely. After Plates dried completely Observed under UV Light. (Chandrashekar R. *et.al.,* 2013)

4. Quantitative Analysis of Gossypol Using Spectrophotometric Measurements.

A UV-visible spectrophotometer was used to record the absorption spectra both of cottonseed extract. This method allows for the direct quantification of gossypol in sample extracts without the need for a chromogenic reagent reaction because the usual analytical band at 300 nm can be measured. To do this, 100 ml of Chloroform were used to dissolve one gram of each sample. Every sample's absorbance was measured at 250, 270 and 290 nm.

In order to exclude interference from other aromatic compounds, which typically exhibit absorbance at about 270 nm, the spectrophotometric investigations were conducted directly at 290 nm. (Chandrashekar R. *et.al.,* 2013)

5. Results

In the PCR-based identification of genetically modified traits in cotton seeds, four primers targeting specific genetic elements were employed: *Cry1Ac*, P-35S, T-NoS, and nptII. The amplification results revealed successful PCR products for the *Cry1Ac*, P-35S, and T-NoS primers, indicating the presence of the corresponding genetic sequences in the tested cotton seed samples. However, amplification was not accomplished using the *nptII* primer. This result indicate that the tested cotton cultivars do not contain this particular transgene, or that there may be issues with primer specificity. Based on the PCR results, it indicates that the cotton seeds tested positive for genetic modification according to presence of P-35S, T-Nos, and *cry1Ac* sequences. However, the absence of a positive result for *nptII* indicates that the seeds may not contain that specific transgene.

Specific chemical tests using lead acetate were conducted to detect gossypol in the samples of *Bt* and non-*Bt* cottonseed extracts. After 20 minutes, a yellowish precipitate was produced by lead acetate in both the extract. The color intensity increased over time. because gossypol is most frequently found in cottonseed. The sample's TLC analysis reveals a bright violet color line on the TLC plate, which suggests that gossypol is present in the sample.

The presence of spectral maxima at 290nm in this UV light spectrum indicates the presence of gossypol in the samples. Since other aromatic compounds typically exhibit absorbance at around 270nm, the spectrophotometric investigations were conducted directly at 290nm to prevent interference.

Table No. 2 UV Spectrophotometric Absorbance of *Bt* Cotton and Non *Bt* cotton Seeds extract

Extracts	**Absorbance (nm)**		
	250	270	290
Non *Bt* Cotton Seeds	1.2	1.2	1.3
Bt Cotton Seeds	0.2	0.3	0.6

Because the OD values of non-Bt seed extracts are higher than those of Bt seed extracts, it was shown during these investigations that there was less gossypol in Bt cotton seed extracts. Table 2 indicates that the low level of OD values may indicate a low amount of component present in the extracts.

6. Discussion

Certain genetically modified features may be present in the analyzed samples, as indicated by the successful amplification of the *Cry1Ac*, P-35S, and T-NoS genetic elements in the cotton seed PCR study. The existence of the *Cry1Ac* gene indicates the potential incorporation of insect resistance traits, commonly found in genetically modified cotton varieties to confer protection against pests such as bollworms. Similarly, the identification of P-35S and T-Nos sequences indicates the presence of regulatory elements commonly necessary for transgene expression and utilized in transgenic plant development. However, the absence of amplification for the neomycin phosphotransferase II (*nptII*) gene, a commonly used selectable marker in genetic engineering, raises intriguing questions regarding the genetic composition of the tested cotton varieties. While the absence of *nptII* indicates that this specific transgene may not be present in the tested samples, potential variations in the primer binding sites, or suboptimal PCR conditions affecting primer annealing or amplification efficiency.

These findings highlight the importance of rigorous molecular screening techniques in the evaluation of genetically modified organisms (GMOs). Understanding the precise genetic makeup of transgenic cotton varieties is essential for assessing their safety, environmental impact, health, and regulatory concerns associated with their cultivation and commercialization. (Shah DK. *Et.al.*, 2012)

The gossypol content of *Bt* and non-*Bt* cottonseed extracts differs noticeably, according to the Results. With a distinctive bright violet line on the TLC plate, gossypol was confirmed to be present by lead acetate tests and TLC analysis. Higher absorbance for non-*Bt* extracts (1.3) at 290 nm than for *Bt* extracts (0.6) was demonstrated by UV spectrophotometry, indicating less gossypol content in *Bt* cottonseeds. *Bt* cotton's genetic alterations, which may change secondary metabolite pathways, are probably responsible for this reduction. Practical advantages could result from lower gossypol levels in *Bt* seeds, particularly in terms of reducing toxicity in animal feed.

7. Conclusion

In the current investigation, PCR was used to identify the transgene present in GM cotton. The protocol developed can be used to detect the transgenes in most of the GM crops. These findings demonstrate the application and stability of genetic modification techniques in conferring desirable traits such as herbicide tolerance and insect resistance in cotton cultivation. This study underscores the importance of molecular characterization in assessing transgenic crops. Future studies could explore alternative methodologies or verify these results through complementary analytical approaches to enhance the reliability and comprehensiveness of GMO identification efforts.

Gossypol was successfully detected and measured in *Bt* and non-*Bt* cottonseed extracts by the study utilizing a combination of spectroscopic, chromatographic, and chemical techniques. The results confirmed that gossypol levels in *Bt* cottonseeds are substantially lower than those in non-*Bt* cottonseeds. *Bt* cotton has less gossypol, which could help lessen the toxicity of the chemical in animal feed applications.

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