Quality Testing of BT Cotton Seeds

An Industrial Training Report submitted for the partial fulfillment of Degree of Bachelor of Science

By

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[B.Sc. (Biotechnology), Semester VI]

Under the supervision of

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DOCTOR SEEDS

Date: 7th February 2023

CERTIFICATE OF ACCOMPLISHMENT

This is to Certify that

HENI P. DHOLARIA,

A Student of BSc. Biotechnology,

Atmiya University, Rajkot, Gujarat

Has Undertaken The Industrial Training At

Research & Development Centre of

Solar Agrotech Private Limited, Rajkot, on

'A Quality testing of Bt-Cotton Seeds'

From 5 January 2023 to 5 February 2023

Under the Guidance of Below Signatories.

SOLAR AGROTECH PVT. LTD.

Mr Denish T. Dholaria
Director,
Solar Agrotech Pvt. Ltd.

SOLAR AGRO TECH
PRIVATE LIMITED

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Acknowledgement

Words are indeed inadequate to convey my deep sense of gratitude to all those who have helped me in completing this training to the best of my ability. Being a part of this training has certainly been a unique and a very productive experience of my part.

The training opportunity I had with Solar Agrotech Pvt. Ltd. (SAPL) was a great chance of learning and professional development. Therefore, I consider myself as a very lucky individual as I was provided with an opportunity to be a part of it.

I express my deepest thanks to Dennis Dholaria, Director of company, for their invaluable guidance and support throughout my training. With their patience and openness, they created an enjoyable working environment.

It is indeed with a great sense of pleasure and immense sense of gratitude that I acknowledge the help of this individual.

I perceive this opportunity as a big milestone in my career development. I will strive to use gained skill and knowledge in the best possible way.

DECLARATION

I hereby declare that the work incorporated in the present training report, which is being submitted as a partial fulfillment of the Degree of Bachelor of Science in Biotechnology, is carried out by me during academic year 2022-2023.

I further declare that this training report written by me has not been previously submitted to any other College/Institute/University for any Certificate/Diploma/Degree

Date:

Name and signature of student

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Company Introduction

Solar Agrotech Pvt. Ltd. was established in 1994 by an agricultural technocrat with an aim to provide the solution to the agricultural problems to Indian farmer and better services to Indian agricultural by innovative research using the methods of breeding crop plant for higher yield with superior quality with increased profitability.

The founder Director of the company Dr. T. L. Dholaria has started Solar Agrotech Pvt. Ltd. and created a reputed brand Doctor Seeds and take company to its present incarnation as a high-tech seed company.

Using the BT-gene events carrying Cry1Ac and Cry2Ab company has developed wide range of cotton germplasm which will be used to develop the BT cotton hybrids suitable for varying agro-climatic condition in the country. To carry out the transgenic breeding successfully company has installed biotechnology research equipment in well-equipped laboratory.

Since 1994 company started extensive R&D work on new hybrid development. As a result company released non-transgenic researched cotton hybrid variety in year 1998 and started the marketing of hybrid cotton seed in Gujarat and Maharashtra.

In year 2007, the company has released BT Cotton hybrids for commercial cultivation after all necessary testing and required approval from competent authority.

Introduction

BT PROTEIN ANALYSIS LAB

BT-cotton refers to genetically modified cotton plants that have been engineered to produce a toxin called *Bacillus thuringiensis* (BT or Bt), which is harmful to certain insect pests that feed on cotton plants. BT-proteins are naturally occurring insecticidal proteins produced by *bacterium Bacillus Thuringiensis*. These proteins have been widely used in agriculture as a natural alternative to chemical pesticides.

BT-cotton was developed as a solution to the problem of pest infestation that has been a major challenge in cotton production, leading to significant crop losses and reduced yields. With BT-cotton, farmers can reduce the use of harmful pesticides, thereby reducing the negative impact on the environment and human health.

BT-cotton has been widely adopted in many cotton-producing countries, including India, China, and the United States, and has contributed to increased productivity and profitability in the cotton industry. However, there are concerns about the potential environmental and social impacts of genetically modified crops, and the debate around BT-cotton and its use continues to be a contentious issue.

BT Protein Analysis:-

BT-protein analysis refers to the process of analyzing the presence and concentration of Bt-protein in a sample, example Bt-protein produced from *CRY1AC* and *CRY2AB* genes.

The analysis is typically performed using techniques such as enzyme-linked immunosorbent assay (ELISA). This technique allows for the detection and quantification of BT-proteins in a seed or leaf sample.

In the SAPL Seed lab, a qualitative test for Bt-produced from *CRY1AC* and *CRY2AB* genes is conducted to find out the percentage of seeds containing these genes for a sample seed lot.

Only sample seed lot which has more than 90% of seeds containing *CRY1AC* and *CRY2AB* genes passes the test.

Lab includes: -

1. Germination and Moisture Test

2. ELISA Test (Enzyme Linked Immunosorbent Assay)

Moisture is a critical factor in seed germination and viability. Seeds require adequate moisture to initiate the germination process, and insufficient moisture can result in delayed or incomplete germination. However, excess moisture can also be detrimental to seed viability by promoting fungal growth and seed rot. Maintaining optimal moisture levels is key to ensuring successful germination and seedling establishment.

1. Germination and Moisture Test:-

The germination of a seed lot sample in laboratory is to emergence and development of the seedling to a stage where the aspect of its essential structures indicates whether it can develop further into satisfactory plant under favorable conditions.

These essential structures are a well-developed with intact root system, hypocotyl, plumule, and cotyledons, etc. according to species.

The germination process for BT-cotton seeds involves measuring of moisture content of seed by using moisture meter and then it is subjected to physical purity analysis. The seeds are pretreated with fungicide to prevent fungal growth. The cotton seeds are than placed in between the germination paper and kept in incubation at room temperature.

Seedlings cannot be evaluated in a germination test until the essential structures are clearly identifiable and the reported percentage of germination expresses the proportion of seed, which have produced normal seedlings within the period specified for cotton species which is usually of 10days-12days.

Seed Germination Test

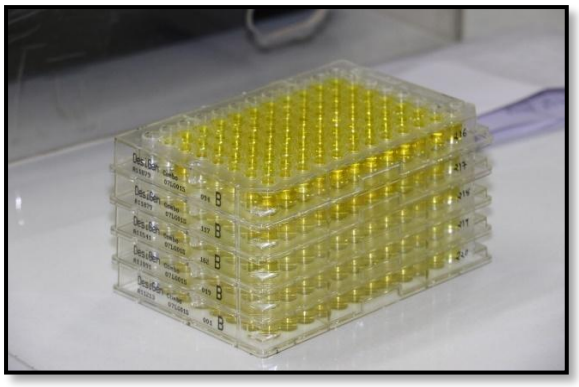
Moisture Meter

2. ELISA Test:-

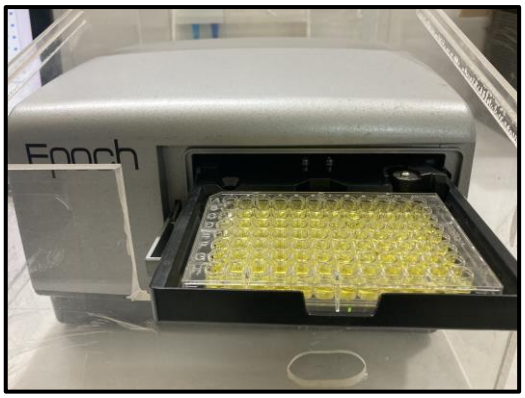
ELISA test for BT cotton seeds is used to detect two types of insecticidal proteins produced by bacillus thuringiensis i.e.Cry1Ac and Cry2Ab. These proteins are commonly used in genetically modified crops to confer resistance against certain insect pests.

In this assay, specific antibodies against these proteins are immobilized on a solid support such as a microtiter plate. The sample containing the protein of interest is added to the plate, and if the protein is present, it will bind to antibodies.

After washing away an unbound materials, a secondary antibody to which an enzyme is linked is added to the plate. This secondary antibody will bind to the immobilized antibodies that have captured the target protein. Any excess secondary antibody is then washed away. A substrate for the enzyme is added to the plate. The enzyme catalyzes a reaction that results in a colour change.

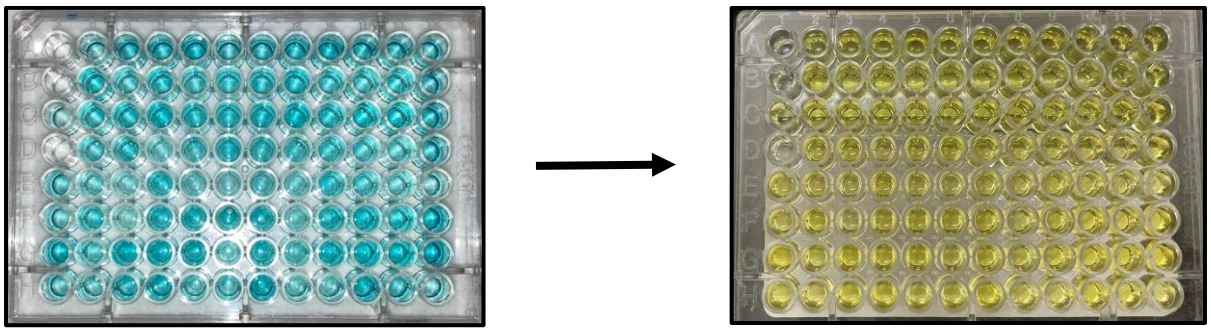


Microtiter Plate

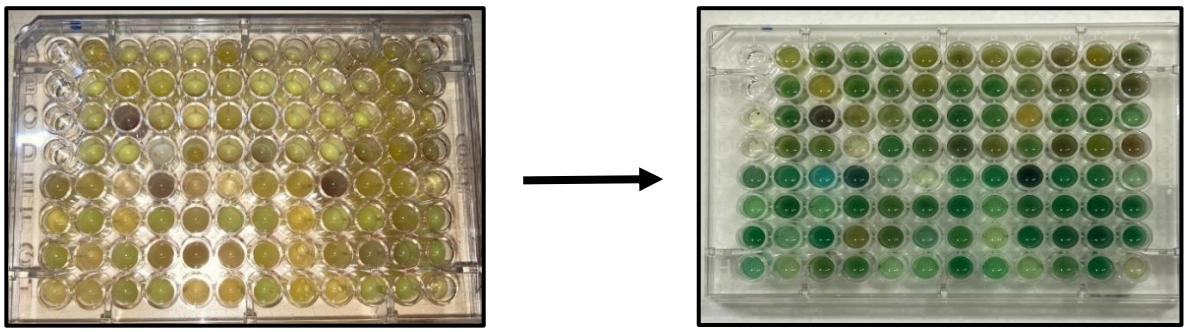


BIOTEK EPOCH Microplate Absorbance Reader

Cry1Ac Result:-



Cry2Ab Result:-



ELISA result observed at 450nm for Cry1Ac:-

DOCTOR SEEDS LAB

Assay: Combo Cry1Ac 450 Reading Date/Time: 09-Jan-23 2:49:48 PM

Wavelength: 450 Plate ID:

Result

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols
	-0.017	0.570	0.969	0.940	1.012	0.741	0.858	0.963	1.101	0.943	0.821	0.812	Blank 450
	BLK	SPL5	SPL13	SPL21	SPL29	SPL37	SPL45	SPL53	SPL61	SPL69	SPL77	SPL85	Well ID
B	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols
	0.017	1.214	0.803	1.035	0.819	0.900	0.952	1.150	0.966	0.905	1.021	0.824	Blank 450
	BLK	SPL6	SPL14	SPL22	SPL30	SPL38	SPL46	SPL54	SPL62	SPL70	SPL78	SPL86	Well ID
C	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols
	0.413	1.033	1.071	1.243	1.054	1.015	1.073	1.313	0.936	1.040	1.195	1.012	Blank 450
	PC	SPL7	SPL15	SPL23	SPL31	SPL39	SPL47	SPL55	SPL63	SPL71	SPL79	SPL87	Well ID
D	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols
	-0.024	0.832	0.982	0.560	0.802	1.016	1.025	0.843	0.875	0.837	0.745	0.913	Blank 450
	NC	SPL8	SPL16	SPL24	SPL32	SPL40	SPL48	SPL56	SPL64	SPL72	SPL80	SPL88	Well ID
E	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols
	0.756	0.719	0.506	1.030	0.606	0.472	0.749	0.659	1.017	0.839	0.716	0.494	Blank 450
	SPL1	SPL9	SPL17	SPL25	SPL33	SPL41	SPL49	SPL57	SPL65	SPL73	SPL81	SPL89	Well ID
F	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols
	0.656	1.070	0.472	1.106	1.136	1.046	1.082	0.589	1.144	1.236	1.008	1.188	Blank 450
	SPL2	SPL10	SPL18	SPL26	SPL34	SPL42	SPL50	SPL58	SPL66	SPL74	SPL82	SPL90	Well ID
G	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols
	1.467	0.795	1.409	1.384	1.484	0.733	1.197	0.846	1.271	1.394	1.418	1.504	Blank 450
	SPL3	SPL11	SPL19	SPL27	SPL35	SPL43	SPL51	SPL59	SPL67	SPL75	SPL83	SPL91	Well ID
H	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols
	0.837	0.673	1.042	1.189	0.734	0.918	1.414	0.692	0.648	1.293	0.959	0.546	Blank 450
	SPL4	SPL12	SPL20	SPL28	SPL36	SPL44	SPL52	SPL60	SPL68	SPL76	SPL84	SPL92	Well ID

MOLECULAR BIOLOGY LAB FOR ZYGOSITY TESTING

ZYGOSITY TESTING:- Zygosity testing in cotton for the *Cry1Ac* gene is an important tool for cotton breeders and growers to ensure the effectiveness of genetically modified (GM) cotton varieties. The *Cry1Ac* gene is responsible for producing the Bacillus Thuringiensis (Bt) toxin, which provides resistance against certain insect pests in cotton.

By testing the zygosity of the *cry1Ac* gene in cotton, breeders and growers can determine the exact copy number and expression levels of the gene in the plant, which can affect the level of pest resistance.

Zygosity testing can also help identify off-types and segregants in the population, which can impact the uniformity and performance of the cotton crop.

Overall, zygosity testing for the Cry1Ac gene is a valuable tool for ensuring the efficacy and uniformity of GM cotton varieties.

At SAPL Molecular Biology Lab, zygosity of Bt-genes such as Cry1Ac and Cry2Ab are performed to find the zygosity state of the plant such Cry1Ac homozygous, Non-Bt homozygous or heterozygous in order to eliminate identify off-types and segregants and select homozygous plant during the plant breeding process to develop Pure parental lines, which are required to be in homozygous state.

Lab includes:-

1. DNA Isolation and Gel Electrophoresis
2. PCR (Polymerase Chain Reaction)

The zygosity testing in cotton for Cry1Ac gene generally includes:-

1. DNA Isolation

2. PCR Amplification

3. Gel Electrophoresis

4. Analysis

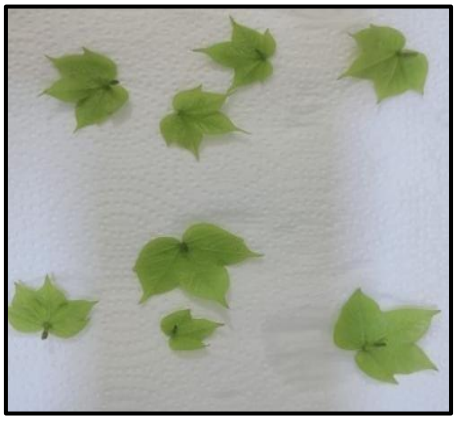
1. DNA Isolation:- DNA isolation is the process of extracting DNA from a sample such as plant tissue. In the case of cotton, the DNA is extracted from the leaves of Bt cotton.

To confirm the presence of the Cry1Ac gene in genetically modified (GM) cotton, DNA isolation is necessary. It is isolated by using a Qiagen DNA extraction kit.

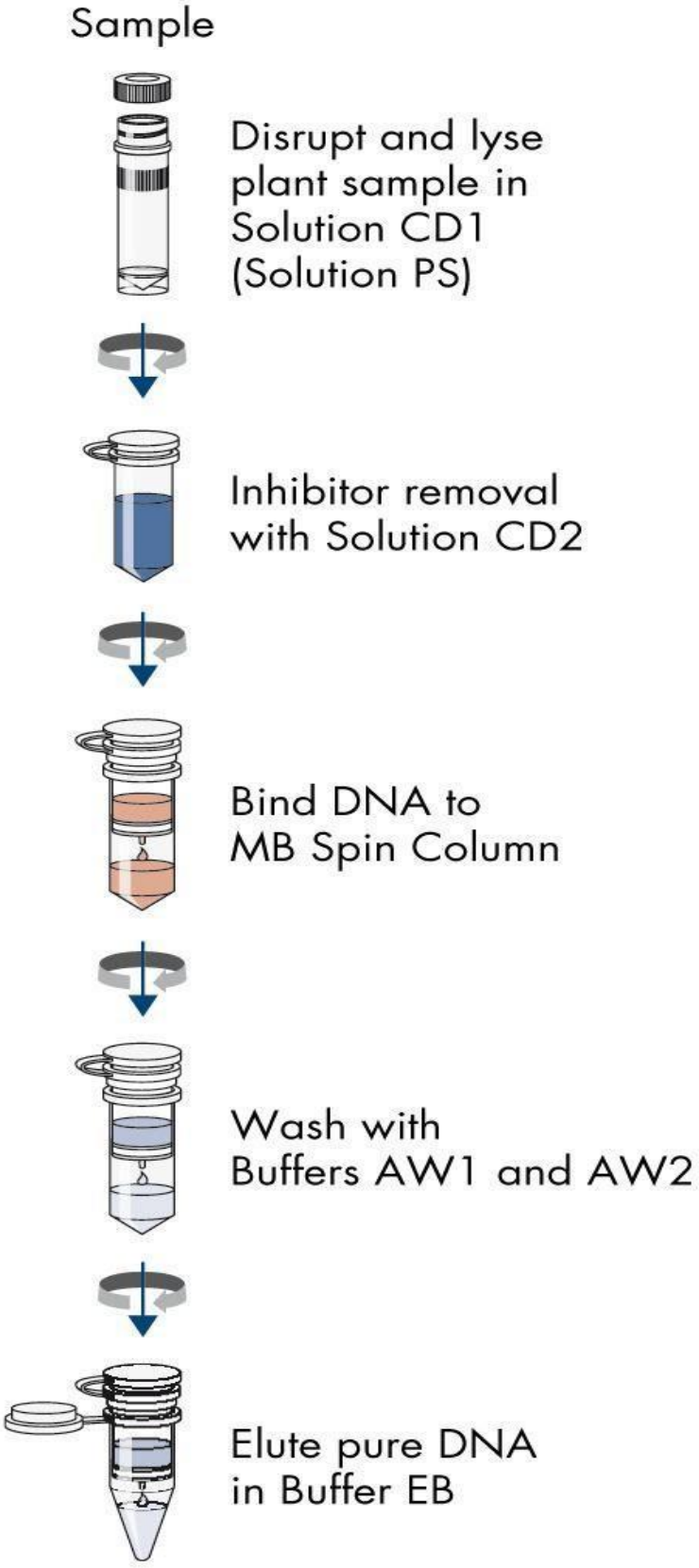
In DNA isolation, the plant tissue was cut into small pieces by adding buffer solution and was homogenize using a TissueLyzer II . TissueLyzer II lysis the cells and denature the proteins that may interfere with DNA isolation. The sample is added to a MB spin column that has silica-based membrane which binds the DNA while other impurities are removed through washing step. The column is washed with buffer to remove impurities and then DNA is purified by washing with alcohol.

The purified DNA is eluted with buffer or water which disrupts the interaction between silica-based column and DNA. This DNA sample is stored at -20°C.

TissueLyzer II Apparatus



Leaf Sample


Sample
Disrupt and lyse plant sample in Solution CD1 (Solution PS)
Inhibitor removal with Solution CD2
Bind DNA to MB Spin Column
Wash with Buffers AW1 and AW2
Elute pure DNA in Buffer EB

2. PCR Amplification:-

Polymerase Chain Reaction is commonly used molecular biology technique that can amplify specific DNA sequence. In case of cotton, it is used to amplify Cry1Ac gene, which encodes the protein that confers resistance to insect pests.

It involves three steps :- **1. Denaturation**

2. Annealing

3. Extension

1. Denaturation:-

The double-stranded DNA sample is heated to a high temperature(90-94°C) to break hydrogen bond between two strands, separating the DNA into single strand.

2. Annealing:-

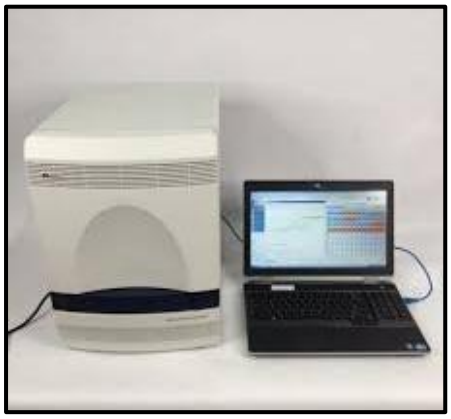
The temperature is lowered to around 50-65°C and the primers (small DNA fragment that are specific to target DNA) are added to reaction mixture. The primers will attach to the single-stranded DNA , binding to complementary target sequence and acts as starting point for Taq polymerase to synthesis new strand of DNA in both the direction i.e. towards the 3'-end of the primer on one strand and towards the 5'-end of primer on the opposite strand.

3. Extension:-

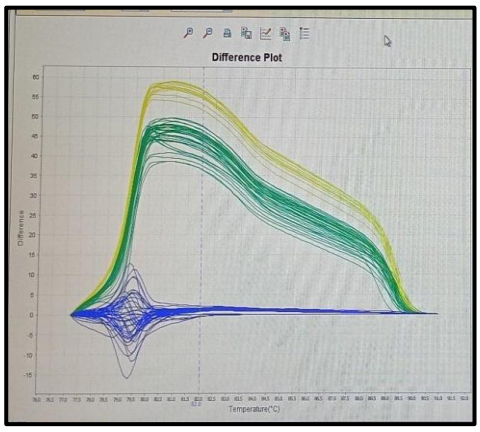
The temperature is raised to around 72°C and the Taq polymerase enzyme is added to the mixture. The enzyme synthesizes new stand of DNA by using single-stranded DNA as a template, adding new nucleotides in a sequence that complements the opposite strand. The mixture is then run on thermal cycler.

To amplify the target DNA (Cry1Ac), the cycling process is repeated multiple times usually 30-45 cycles, with each cycle doubling the amount of DNA in sample. PCR amplification conditions and cycling parameters may vary depending on specific primer set and equipment used.

Initial Enzyme Activation Step	Time	Temperature
	5 minutes	95˚C
3-step cycling (30-45 cycles)		
Denaturation	30s	95˚C
Annealing	90s	60˚C
Extension	90s	72˚C
Final Extension Stage	10 minutes	68˚C



Thermal Cycler



Observed positive and negative result

In graph,

Yellow Colour Line:- it indicates the homozygous Cry1Ac is present

Blue Colour Line:- it indicates the homozygous Non-BT is present

Green Colour Line:- heterozygous Cry1Ac and Non-BT both present

3. Gel Electrophoresis:-

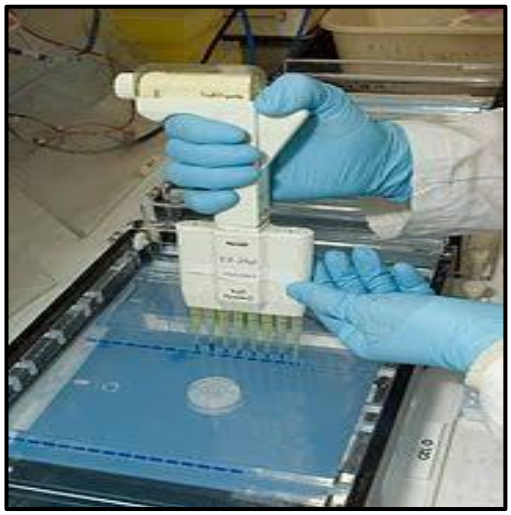
Gel electrophoresis is a technique which separates The DNA fragments based on their size. It involves the uses of electric field to move charged DNA molecules through a gel matrix.

After PCR amplification, gel electrophoresis is often used to separate and visualize the amplified DNA fragments. In particular, agarose gel electrophoresis is commonly used for this purpose. This involves pouring a gel made of agarose powder mixed with buffer solution into a gel tray and adjust the comb in gel matrix, set at cathode end. Allow the gel to solidify.

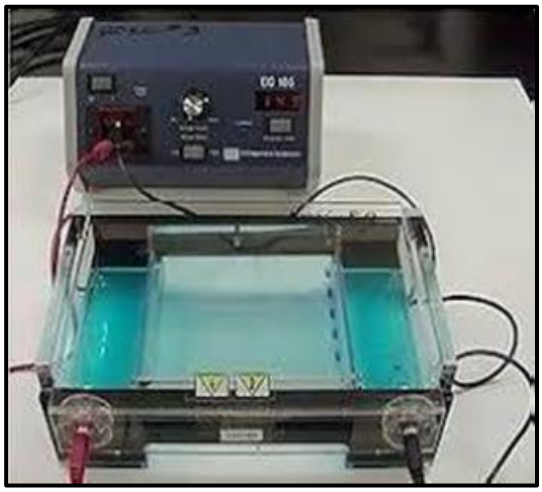
The amplified PCR products can be visualized by adding a fluorescent dye i.e. ethidium bromide to the gel that binds to the DNA and will fluoresce under ultraviolet light.

An voltage is applied to the gel which causes the DNA fragment to migrate across the gel towards the positive electrode based on their size. The smaller fragments migrate further towards the gel than larger fragments.

The number of PCR product and their size can indicate the copy number and expression levels of Cry1Ac gene in the cotton plant.

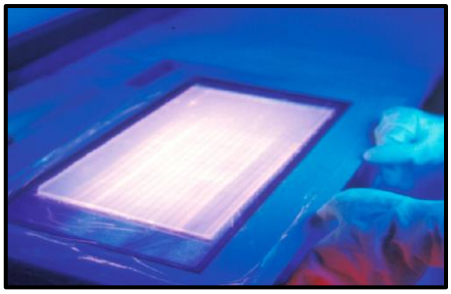


Loading the sample

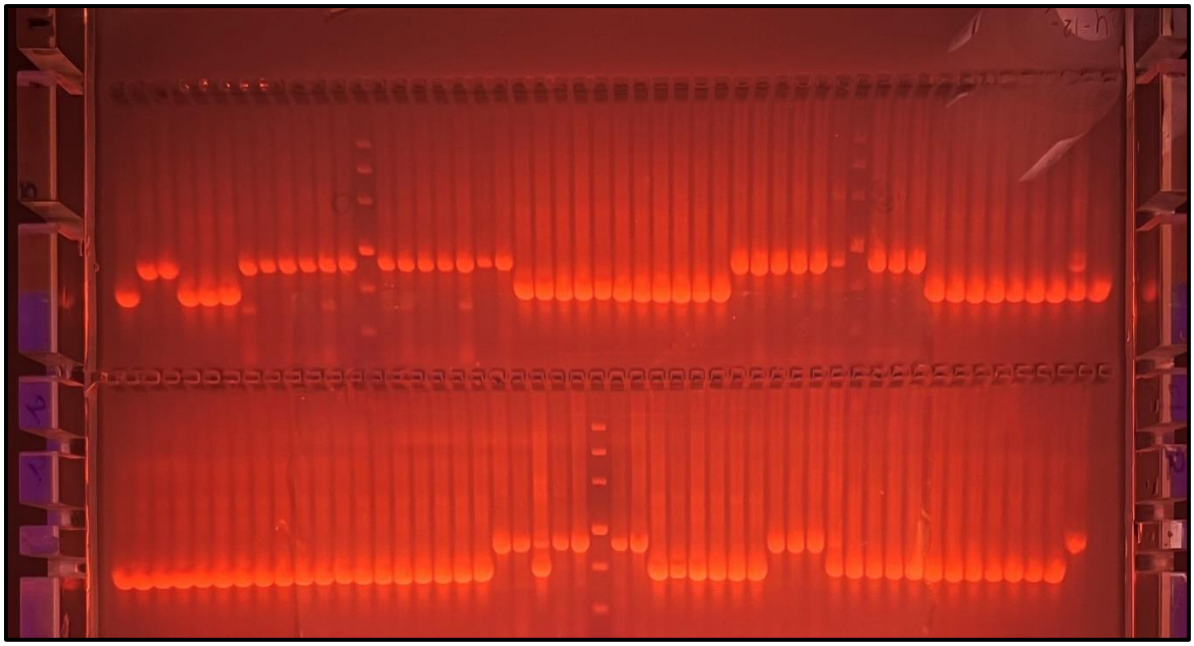


Gel Electrophoresis

UV Transilluminator



Observing under UV light



Separation of DNA fragment based on their size

4. Analysis:-

The PCR products are visualized and analyzed using specialized software or by manual inspection. The results are interpreted to determine the zygosity of the cry1Ac gene in the cotton plant.

Reporting:-

The results of the zygosity testing are reported to the breeder or grower, who can use the information to make informed decisions regarding seed selection, crop management, and pest control strategies.

Overall, zygosity testing for the cry1Ac gene in cotton is a valuable tool for ensuring the effectiveness and uniformity of GM cotton varieties.

References

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