Detection of Cry1Ac & Cry2Ab gene in Gossypium hirsutum (cotton)

An Industrial Training Report submitted

for the partial fulfillment of the Degree of Master of Science

By

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[M.Sc. (Microbiology), Semester IV]

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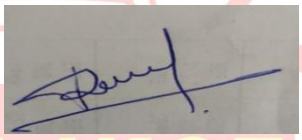
C E R T I F I C A T E

This is to certify that this training report entitled **"Solar Agtotech Pvt Ltd.(Doctor Seeds)"** was successfully carried out by **Ganjani Sagar** towards the partial fulfillment of requirements for the degree of Master of Science in Microbiology of Shree M & N Virani Science College (autonomous), Affiliated to Saurastra University, Rajkot. It is an authentic record of her own work, carried out by him under the guidance of **Dr. Denish Dholariya & Mr. Rahul Kothiya** for a period of 3 Month during the academic year of 1st February 2021 to 31st April 2021 .The content of this report, in full or in parts, has not been submitted for the award of any other degree or certificate in this or any other University.

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

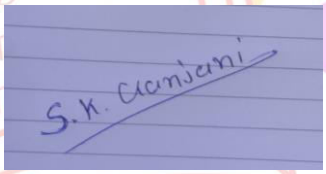


Name & Signature of the Head of the Department

Name & Signature of the supervisor

D E C L A R A T I O N

I hereby declare that the work incorporated in the present dissertation report entitled **"Solar Agtotech Pvt Ltd.(Doctor Seeds)"** is my own work and is original. This work (in part or in full) has not been submitted to any University for the award of any Degree or a Diploma.



04/05/2021

Date

(Name and signature of Student)

Acknowledgement

I have heartly thanks to first of all Shree M & N virani scince college for great opertunity for this internship in Solar Aggrotech Pvt Ltd in work on topic of Detection of Cry1ac & Cry2Ab gene in Gossypium hirsutum (cotton).

*For my guide **Dr. Denish Dholariya**(CEO of Solar Agrotech Pvt Ltd) & **Mr. Rahul Kothiya** (Quality Control Head) of DR SEED, a thank you seems such a small word for a person like him. We place on record, our gratefulness to him.*

*We would like to thank **Dr. K. D. Ladva** (Principal of Shree M. & N. Virani Science College), **Dr. Shivani Patel** (HOD of Life Science Dept.) for the continuous encouragement and for providing us with all the necessary facilities for the research.*

*With a special mention to **Savaliya Bansi, Patel Radhika, Vora Namrta, Pansara Juli** and to all other compony staff thanks.It was fantastic to have the opportunity to work with majority of them. What a cracking place to work!*

Finally, I express my very profound gratitude to our parents for providing us with unfailing support and continuous encouragement through the process of researching and writing this report. This

accomplishment would not have been possible without them. Thank You

Index

List of Table

Llist of Figure

Abstract

In germinaton the replication wise details of all categories of seedlings / seeds observed during evaluation are recorded in the seed analysis card. The result of germination test is calculated as the average of the replicates and is expressed as percentage by number of normal seedlings. The percentage is calculated to the nearest whole number. The percentage of abnormal seedlings, hard, fresh ungerminated and dead seeds is also calculated the same manner. If the result is nil for any of these categories it shall be reported as '0' instead of leaving the appropriate column blank.

Along with the increase market of the transgenic crops, the demand for testing GMOs and for certifying non-GMO foodstuffs has increased dramatically.Within the arena of expanding techniques for identification and quantification of transgenic crops, two major approaches for detecting GMOs are still applicable on large scale, namely ELISA based protein detection and PCR based gene identification.In present study, ELISA techniques was adopted to identify the specific Cry1Ac and Cry2Ab proteins in some transgenic cotton plants seed samples.

QIAGEN Multiplex PCR Kit is the first commercially available kit for multiplex PCR. Itminimizes the need for optimization, making the development of multiplex PCR assays bothsimple and fast. The kit contains a master mix whose composition and elements werespecifically developed for multiplex PCR applications. The master mix contains pre-optimized concentrations of

HotStarTaq DNA Polymerase and MgCl2, plus dNTPs, anda PCR buffer that contains the novel factor MP. Use of a master-mix format reduces time andhandling for reaction setup and increases reproducibility by eliminating many possiblesources of pipetting errors.

Introduction

1. **Purpose of germination:-** To ensure quality compliance and monitor effectiveness of quality management.
   The ultimate objective of seed germination testing is to obtain information with respect to the planting value of the seed and to provide results which could be used to compare the value of different seed lots.
2. **Scope:** Includes all core and supporting processes within the quality management of Bollgard® and Bollgard II® Cotton.
3. **Definitions**

Germination of a seed lot in a laboratory is the emergence and development of the seedling to a stage where the aspect of its essential structures indicates whether or not it is able to develop further into a satisfactory plant under favourable conditions in soil. These essential structures are a well-developed and intact root system, hypocotyl, plumule and one or two cotyledons according to the species. Seedlings cannot be evaluated in a germination test until these essential structures are clearly identifiable and the reported percentage germination expresses the proportion of seed, which have produced normal seedlings within the period specified (10d - 12d) for cotton species.

Authors have adapted ELISA technique to identify the specific Cry1Ac and Cry2Ab proteins in seed samples of some transgenic cotton plants from the Gujarat state of India. ELISA assumes more than one format; a micro well plate (or strip) format, and a coated tube format. The antibody-coated micro wells are used where protein is not denatured, but no extra information can be obtained

concerning the presence of transgene at the ingredient level in transgenic organism . A variation on ELISA, is the lateral flow strip technology where immobilized double antibodies, specific for the expressed protein, are coupled to a color reactant and incorporated into a nitrocellulose strip, which, when placed in a vial containing an extract from plant tissue harboring a transgenic protein, leads to an antibody sandwich with some of the antibody that is coupled to the color reagent .

Multiplex PCR is a powerful technique that enables amplification of two or more productsin parallel in a single reaction tube. It is widely used in genotyping applications anddifferent areas of DNA testing in research, forensic, and diagnostic laboratories. MultiplexPCR can also be used for qualitative and semi-quantitative gene expression analysis usingcDNA as a starting template .

As the name implies, it is a chain reaction, a small fragment of the DNA section of interest needs to be identified which serves as the template for producing the primers that initiate the reaction. One DNA molecule is used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e., the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates.

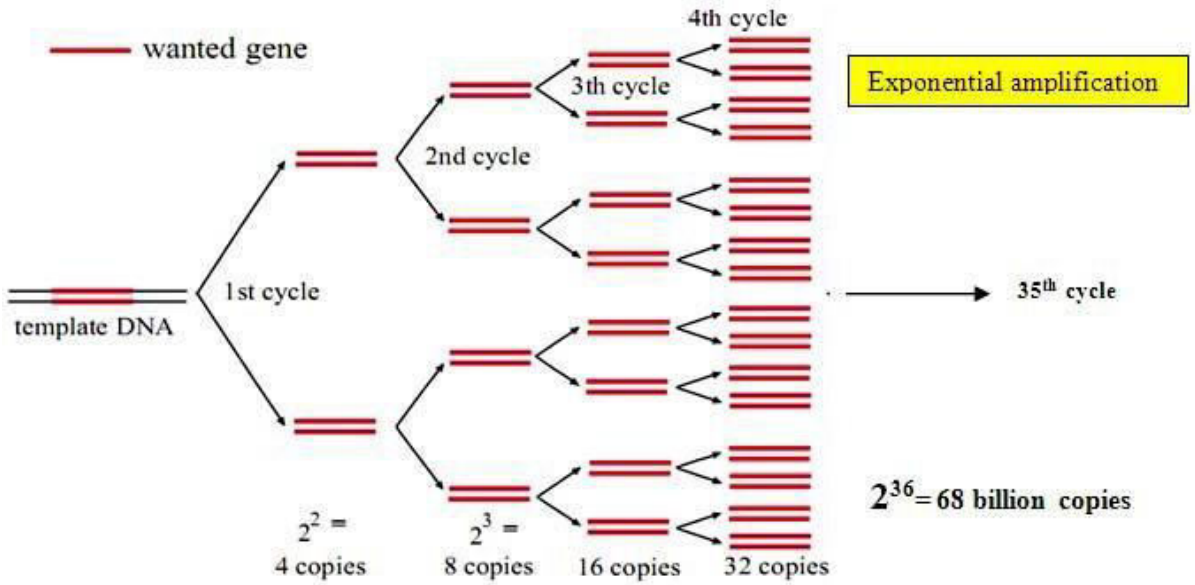


Fig no :-1 Number of copies of DNA obtained after 'n' cycles = 2(n+1)

Compony Information

- ✓ Solar Agrotech Pvt Ltd was established in 1994 by agricultural technocrat with an aim To provide the solution to the agricultural problems to Indian farmer and better services to Indian agriculture by innovative research using the methods of breeding crop plants for higher yield with superior quality with increased profitability.
- ✓ The founder Director of the company Dr. T. L. Dholaria has started Solar AgrotechPvt. Ltd. and created a reputed brand Doctor Seeds and take Company to its present incarnation as a high-tech seed company.
- ✓ Solar Agrotech Pvt Ltd is a Private incorporated on 04 April 1994. It is classified as Nongovt. Company and is registered at Registrar of Companies, Ahmedabad. Its authorized share capital is Rs.2,000,000and

its paid up capital is Rs.419,000. It is involved in Growing of crops; market gardening; horticulture.

- ✓ Using the BT gene events carrying Cry 1Ac (Mon 531) and Carrying Cry1Ac & Cry2Ab (Mon 15985) 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 which is being upgraded every year.

❖ **First Cotton Hybrid Released**

- ✓ 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.

❖ **First Bt Cotton Hybrid Released**

- ✓ In the year 2007, Solar Agrotech Pvt. Ltd. has released Bt Cotton hybrids for commercial cultivation after all necessary testing and required approval from competent authorities.

❖ **Licensing for Bollgard II technology**

- ✓ Solar AgrotechPvt. Ltd. undergone for licensing process of Bollgard II technology from• Monsanto in the year 2008.

❖ **Success of Bollgard II Hybrids**

- ✓ Solar AgrotechPvt. Ltd. delivered Bollgard II hybrids optimised for wide spacing sowing• which became more popular among the Gujarat farmers. Dr.Dholaria's researched hybrids became a highly respected brand among the top seeds companies of India.
- ✓ Products of Solar Agro-biotech.

Fig 2- Seed from the Packet **Fig 3- Packet of cotton seeds** Fig 4 - **Information of packets**

- **<u>WORK PERFORMED DURING INTERNSHIP.</u>**

We have successfully worked on two methods at this company.

1. Germination of cotton seeds
2. ELISA for Protein expression detection in seed
3. Extraction & Isolation of genomic DNA from cotton plant.
4. PCR (Polymerase chain reaction) of the isolated DNA samples

Material & Method

1) <u>Germination</u>

Requirement

3.1.***Moisture determination*:** Seed moisture content is determined by using moisture meter. Seed moisture content is expressed as percentage by weight.

3.2.***Germination:*** Seeds sample, germination paper (substratum), racks and trays, fungicide and water.

3.3.***Suitable Substratum:*** The substrata serve as a moisture reservoir and provide a surface or medium for which the seeds can germinate and the seedlings grow. The commonly used substrates are paper, sand and soil.

3.3.1. ***Paper Substrate*:** Most widely used paper substrates are filter paper, blotter or towel (Kraft paper). These are easy to handle, versatile and comparatively cheap.

3.4.***Adequate Moisture or Water:*** High concentration of water at cellular level is necessary fix the seed to start germination. Mobilisation of food requires hydrolysis (breaking down process) to transport materials from storage to growing tissues. Moisture is supplied to the seeds through the substratum. (Generally, the moistened substrata is sufficient to rehydrate to 30-80%. However, the moment the radicle emerges, additional moisture contributes better seedling growth. Too much water would allow fungal growth and decay of seeds.

3.5.***Favourable Temperature:*** Germination occurs under different ranges of temperatures provided the seed is given adequate moisture. Temperature is not as critical as water requirement during the test. Seeds of most of agricultural and horticultural crops germinate in the temperature range of 10°C-15°C. Some seeds germinate better at constant temperature. Others require an alternating temperature. Temperature control does provide the comparable conditions exactly under which test can he repeated.

Temperature control is also necessary to overcome dormancy wherever it occurs. Exposure of seeds to the temperature at 40°C or higher, alternation of temperature, low temperature applications are the easiest and safest method to overcome seed dormancy although methods to overcome dormancy by chemical treatments do exist.

Therefore, the optimum temperature prescribed for cotton seeds germination on paper substrate is between 20-30°C (average 25°C).

*3.6.****Light***

There are crops for which light is not required during germination test. However, presence of light is desirable to enable the evaluation of

seedlings easier and with greater certainty. Other crops like lettuce and tobacco require light during germination on the test.

Procedure

1.1. **Working sample:** The submitted samples should be of at least 100 grams in weight. The accepted sample is registered for testing and is given a code number so as to maintain the secrecy about its identity during testing.

1.2. **Moisture meter method:** Here, the moisture content is determined using moisture meter which is calibrated and standardized against air oven

method. Moisture test is conducted in two replications and the average is recorded in the seed analysis card.

1.3. **Physical purity analysis:** The purity analysis is done on a working sample of prescribed weight drawn from the submitted sample. The working sample is separated into different components like pure seed, other crop seed, weed seed and inert matter by physical examination and the details are recorded in the seed analysis card (A4 paper sheet).

1.4. **Germination testing:** The pure seed fraction from the purity test is used for germination analysis. A minimum of one hundred (100) Cotton seeds for grower lots and four hundred (400) Cotton seeds for commercial lots are tested for germination.

1.5. **Methods of using paper substrata:**

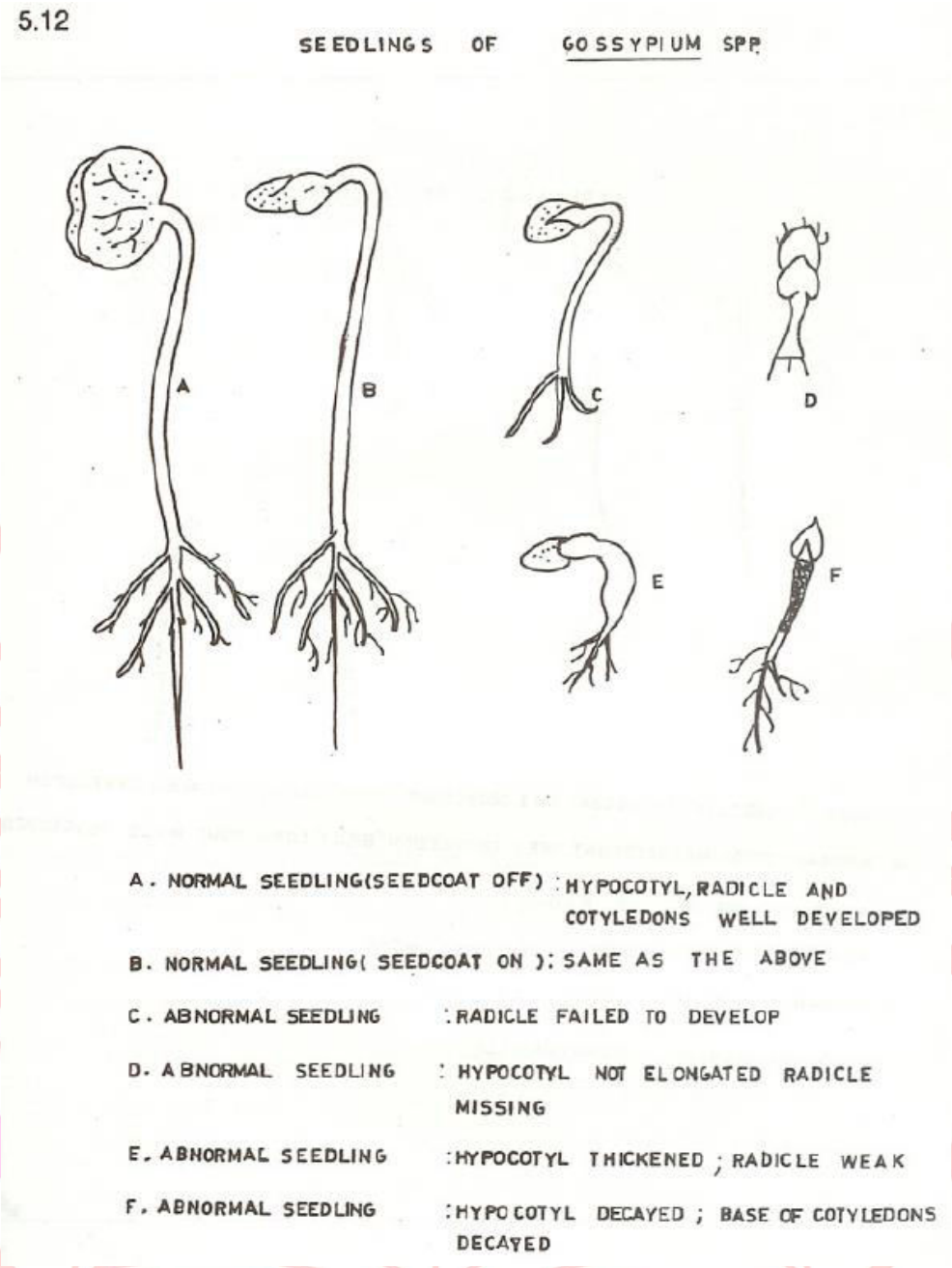
1.5.1. **Between paper (B.P.):** Cotton Seeds are placed in between two moist germination papers and rolled together to look like a rolled towel. The rolled towels are placed inside the germination cabinet/racks and trays and kept in incubation at room temperature (25°C).

1.6. Pre-treatments for Germination: Germination papers are treated with fungicide (Carboxin & Thiram, Brand name: Vitavax by Dhanuka Agritech Ltd.) to prevent fungal growth on seeds.

1.7. **Duration of the test:** The duration of sample incubation varies from crop to crop. For Cotton 'first count' is taken on the 4th or the 5th day and the final count between 10^{th} days. The time for count is fixed in such a way that it is sufficient to allow seedlings to reach a stage of development which allows for accurate evaluation.

1.8. **Seedling Evaluation:**

Seedlings which have reached a stage when all essential structures can be accurately assessed, shall be removed from the test at the first or any other intermediate counts. Badly decay seedlings should be removed in order to reduce the risk of secondary infection, but abnormal seedling with other defects should be left on the substrate until the final count.



TRIALS OF CYTOZYME (811 SEEDS)	TRETMENT DAY	TRETMENT DAY		2 DAY AFTER	REMARK
	05/02/2021	05/02/2021	08/02/2021	08/02/2021	

Fig 5:- Different type of seedlings

sr. no.	code id	seeds weight	Polymer &vitavax & Thio	cytozyme weight	dm weight	GERMINATION	GERMI ID		GERMI ID	
1	811 T0	100 GM		0	0	200 SEEDS	5 811 T0	200 SEEDS	8 811 T0	
2	811 T1	100 GM		300 MG	700 MG	200 SEEDS	5 811 T1	200 SEEDS	8 811 T1	
3	811 T2	100 GM		500 MG	500 MG	200 SEEDS	5 811 T2	200 SEEDS	8 811 T2	
4	811 T3	100 GM		1000 MG	0	200 SEEDS	5 811 T3	200 SEEDS	8 811 T3	
5	811 T4	100 GM	treated	0	0	200 SEEDS	5 811 T4	200 SEEDS	8 811 T4	
							14/02/ 2021		17/02 /2021	
							OBS		OBS	

Table no 1:- Procedure of germination

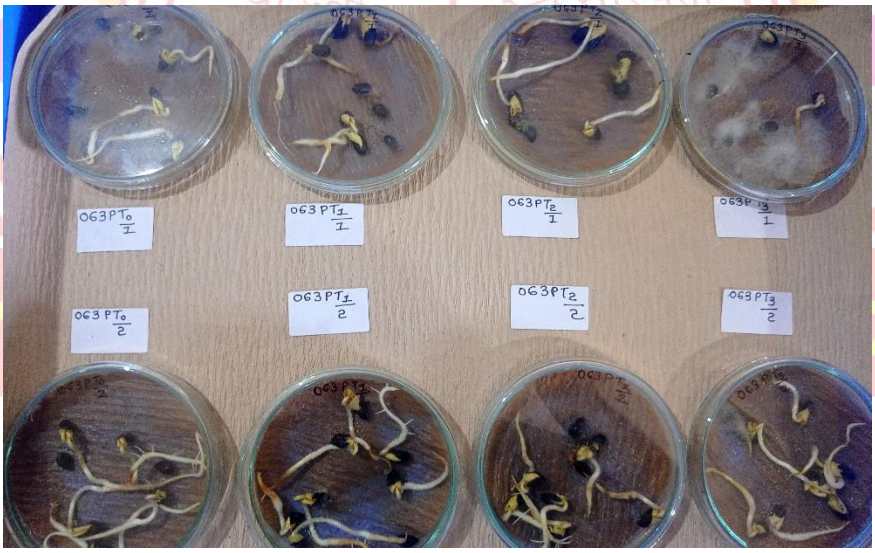


Fig 6:- Treatmet seed germinate in Petriplate

Fig 9:- 200 seeds on paper seperate

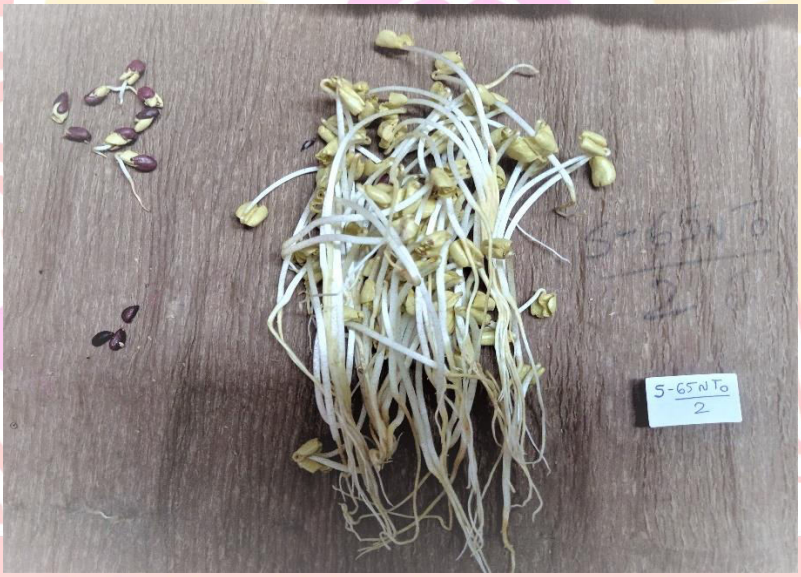


Fig 10:- Normal Germination is observed

Result & Conclusion

1) Varity of "811" seeds

sr. no.	code id	NO. OF SEEDS		OBS DATE	Normal seeds	Abnormal seeds	Dead seeds	%
1	5 811 T0	200		14/02/2021	158	22	20	79
2	5 811 T1	200		14/02/2021	160	24	16	80
3	5 811 T2	200		14/02/2021	159	21	20	79.5
4	5 811 T3	200		14/02/2021	153	20	27	76.5
5	5 811 T4	200		14/02/2021	159	23	18	79.5
6	8 811 T0	200		17/02/2021	157	21	22	78.5
7	8 811 T1	200		17/02/2021	161	25	14	80.5
8	8 811	200		17/02/20	158	26	16	79

	T2			21				
9	8 811 T3	200		17/02/2021	152	24	24	76
10	8 811 T4	200		17/02/2021	157	23	20	78.5

Table No 2:- Normal result of varity "811"

AVERAGE %	
T0	79
T1	80
T2	79
T3	76
T4	79

2) Varity of " S-65N" Seeds

sr. no.	code id	NO. OF SEEDS	OBS DATE	1	2	3	%	REMARK
1	9 S-65N T0	200	18/02/2021	174	21	5	87	
2	9 S-65N T1	200	18/02/2021	176	23	1	88	
3	9 S-65N T2	200	18/02/2021	176	21	3	88	
4	9 S-65N T3	200	18/02/2021	171	26	3	85.5	
5	12 S-65N T0	200	21/02/2021	168	23	9	84	
6	12 S-65N T1	200	21/02/2021	173	24	3	86.5	
7	12 S-65N T2	200	21/02/2021	172	25	3	86	
8	12 S-65N T3	200	21/02/2021	169	21	10	84.5	

Tavle no 3:- Normal result of varity "9 S—65N"

AVERAGE %	
T0	85.5
T1	87.25
T2	87
T3	85

3) Varity of " S-65N P" Seeds

sr. no.	code id	NO. OF SEEDS	OBS DATE	1	2	3	%	REMARK
1	5 S-65N PT0	200	11/03/2021	178	21	1	89	
2	5 S-65N PT1	200	11/03/2021	177	17	6	88.5	
3	5 S-65N PT2	200	11/03/2021	178	21	1	89	
4	5 S-65N PT3	200	11/03/2021	173	25	2	86.5	
5	8 S-65N PT0	200	16/03/2021	174	25	1	87	
6	8 S-65N PT1	200	16/03/2021	175	20	5	87.5	
7	8 S-65N PT2	200	16/03/2021	181	17	2	90.5	
8	8 S-65N PT3	200	16/03/2021	174	26	0	87	

Table no 4:- Normal reasult of varity " 8 S-65N & 5 S-65N"

AVERAGE %	
T0-	88
T1	88
T2	89.75
T3	87

4) Varity of "22" Seeds

sr. no.	code id	NO. OF SEEDS	OBS DATE	1	2	3	%
1	22T0	200	03/02/2021	135	34	31	67.5
2	22T1	200	03/02/2021	134	34	32	67
3	22T2	200	03/02/2021	127	38	35	63.5
4	22T3	200	03/02/2021	136	37	27	68
5	24T0	200	06/02/2021	131	43	26	65.5
6	24T1	200	06/02/2021	144	35	21	72
7	24T2	200	06/02/2021	141	30	29	70.5
8	24T3	200	06/02/2021	138	35	27	69
9	26T0	200	09/02/2021	136	29	35	68
10	26T1	200	09/02/2021	137	36	27	68.5
11	26T2	200	09/02/2021	140	45	15	70
12	26T3	200	09/02/2021	136	42	22	68

Table 5:- Normal result of varity "24" in seeds

AVERAGE %	
T0	67

T1	**69.16**
T2	**68**
T3	**68.33**

ELISA(Enzyme Linked Immuno Sorbent Assay)

Material & Method

Procedure

1) Add 50 µl Cry1Ac - Cry2Ab enzyme conjugate in each well.

2) Add 50 µl of working extration buffer in blank well.

3) Add 50 µl of Cry1Ac - Cry2A positive control in two seperate wells .
   Add 50 µl of Cry1Ac - Cry2A negative control in two seperate wells.
   Add 50 µl of each sample to reset of wells.

4) Incubate for 45 minutes at room temperature.

5) Remove content of the wells by decating into a sink or a waste water container. Add 300 µl/well wash solution to all wells and then empty wells by inveting the plate. Repeting washing procedure two more times.

6) Alternately , performe four washes by using microtiter plate washer. after last wash, tap the inverted microtiter plate on paper towel to remove as much liquid as possible.

7) Add 100 µl substarate #1.

8) Incubate for 30 minutes at room temperature.

9) Take absorbance at 405 nm with 630 nm as secondary filter . Presence of yellow colocr indicates that samples are positive for Cry2A .

10) Wash the plate in the same maner as indicate in step 4 above.

11) Add 100 µl Substrate #2.

12) Incubate for 30 minutes at room temperature.

13) Add 100 µl stop solution.

14) Take absorbance at 450 nm with 630 nm as secondry filter. Presence of yellow color indicates that samples are positive for Cry1Ac.

Fig 09:- Grinding of seed sample

Fig 10:- Add sample into the well

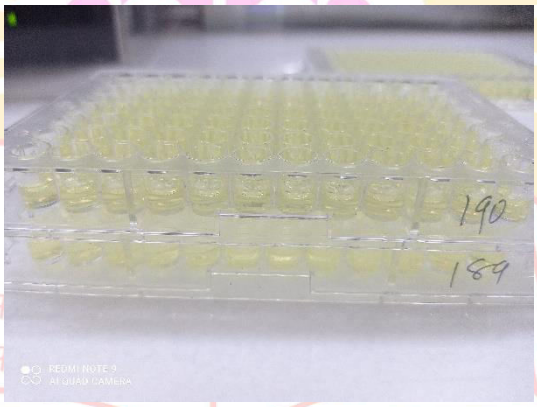


Fig 11:-Add Pnpp Solution

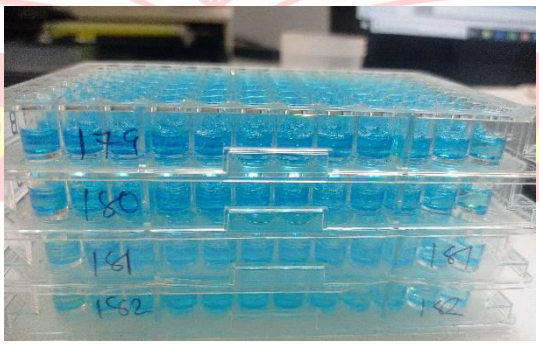


Fig 12:-Add TMB substrate

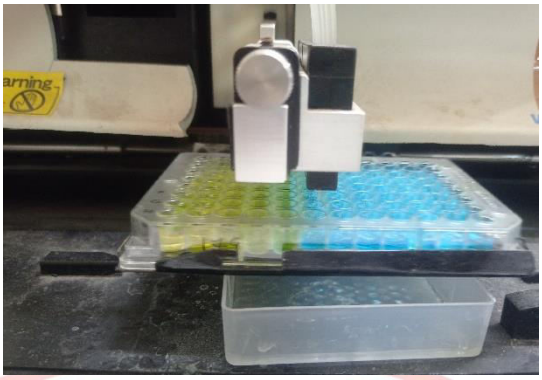


Fig 13:-Add Stop solution

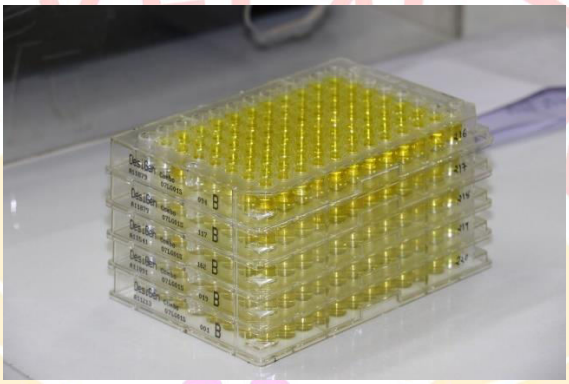


Fig 14:-TMB Substrate

Result & Disscusion

- The interpretations of results were done based on the mean absorbance data of the individual samples (Table 2). The prescribed cut off value for the presence or absence of Cry1Ac and Cry2Ab proteins in the samplewas '+1.0'. The samples having absorbance <'+1.0' were considered to be as negative for the presence of both Cry1Ac and Cry2Ab proteins, while the samples having absorbance ≥ '+1.0' were considered to be as positive for the presence of both Cry1Ac and Cry2Ab proteins.
- From the studies so far, it can therefore be postulated that the technique of ELISA for identification of Cry1Ac and Cry2Ab proteins in the sample is quite handy and easily adoptable. But there are certain inherent disadvantages of ELISA-based techniques as they require significant lead-time for method development, have high up-front costs for assay development, and cannot discriminate between different transgenic events that express similar protein characteristics.

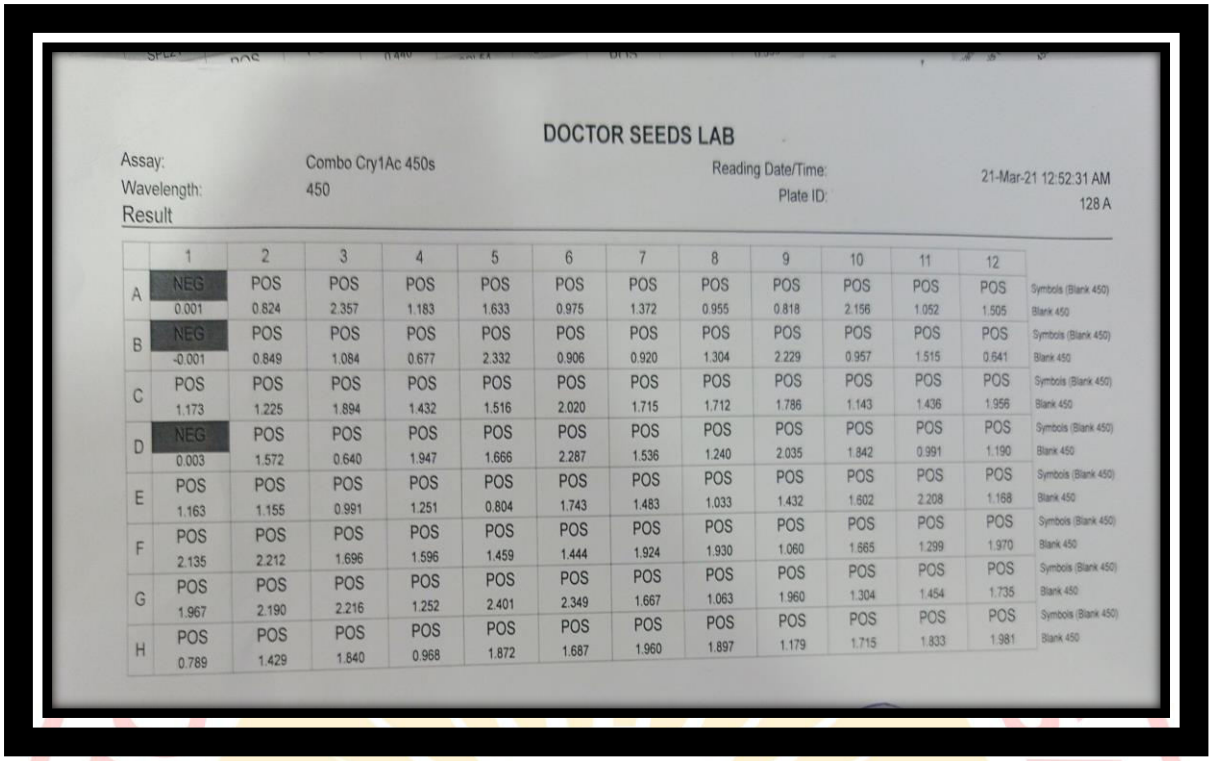
- In ELISA technique Sandwitch ELISA based method are performed in this technique observed two result on a one is 405 nm and another one is 450 nm. This technique is based on qualitative results either its positive or negative.

DOCTOR SEEDS LAB

Assay: Combo Cry2Ab 405s
Wavelength: 405
Reading Date/Time: 21-Mar
Plate ID:
Result

	1	2	3	4	5	6	7	8	9	10	11	12
A	NEG	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	-0.024	-0.012	0.509	0.518	0.520	0.684	0.480	0.242	0.202	0.362	0.600	0.426
	BLK	SPL5	SPL13	SPL21	SPL29	SPL37	SPL45	SPL53	SPL61	SPL69	SPL77	SPL85
B	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	0.024	0.501	0.482	0.569	0.298	0.384	0.443	0.320	0.497	0.427	0.232	0.503
	BLK	SPL6	SPL14	SPL22	SPL30	SPL38	SPL46	SPL54	SPL62	SPL70	SPL78	SPL86
C	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	0.780	0.576	0.511	0.346	0.455	0.343	0.462	0.287	0.478	0.538	0.390	0.423
	PC	SPL7	SPL15	SPL23	SPL31	SPL39	SPL47	SPL55	SPL63	SPL71	SPL79	SPL87
D	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	-0.008	0.411	0.474	0.471	0.384	0.496	0.498	0.535	0.459	0.280	0.604	0.612
	NC	SPL8	SPL16	SPL24	SPL32	SPL40	SPL48	SPL56	SPL64	SPL72	SPL80	SPL88
E	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	0.457	0.592	0.376	0.506	0.598	0.478	0.512	0.244	0.345	0.321	0.429	0.345
	SPL1	SPL9	SPL17	SPL25	SPL33	SPL41	SPL49	SPL57	SPL65	SPL73	SPL81	SPL89
F	POS	POS	POS	POS	POS	POS	POS	NEG	POS	POS	POS	POS
	0.367	0.448	0.401	0.304	0.471	0.472	0.717	0.178	0.502	0.392	0.554	1.245
	SPL2	SPL10	SPL18	SPL26	SPL34	SPL42	SPL50	SPL58	SPL66	SPL74	SPL82	SPL90
G	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	0.430	0.366	0.562	0.247	0.706	0.275	0.601	0.298	0.478	0.446	0.626	0.350
	SPL3	SPL11	SPL19	SPL27	SPL35	SPL43	SPL51	SPL59	SPL67	SPL75	SPL83	SPL91
H	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	0.333	0.488	0.354	0.573	0.531	0.594	0.325	0.297	0.484	0.359	0.429	0.346
	SPL4	SPL12	SPL20	SPL28	SPL36	SPL44	SPL52	SPL60	SPL68	SPL76	SPL84	SPL92

Fig 15:- ELISA result observed at 405 nm for combo Cry2Ab



DOCTOR SEEDS LAB

Assay: Combo Cry1Ac 450s
Wavelength: 450
Result
Reading Date/Time: 21-Mar-21 12:52:31 AM
Plate ID: 128 A

	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 (Blank 450)
	0.001	0.824	2.357	1.183	1.633	0.975	1.372	0.955	0.818	2.156	1.052	1.505	Blank 450
B	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols (Blank 450)
	-0.001	0.849	1.084	0.677	2.332	0.906	0.920	1.304	2.229	0.957	1.515	0.641	Blank 450
C	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols (Blank 450)
	1.173	1.225	1.894	1.432	1.516	2.020	1.715	1.712	1.786	1.143	1.436	1.956	Blank 450
D	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols (Blank 450)
	0.003	1.572	0.640	1.947	1.666	2.287	1.536	1.240	2.035	1.842	0.991	1.190	Blank 450
E	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols (Blank 450)
	1.163	1.155	0.991	1.251	0.804	1.743	1.483	1.033	1.432	1.602	2.208	1.168	Blank 450
F	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols (Blank 450)
	2.135	2.212	1.696	1.596	1.459	1.444	1.924	1.930	1.060	1.665	1.299	1.970	Blank 450
G	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols (Blank 450)
	1.967	2.190	2.216	1.252	2.401	2.349	1.667	1.063	1.960	1.304	1.454	1.735	Blank 450
H	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	Symbols (Blank 450)
	0.789	1.429	1.840	0.968	1.872	1.687	1.960	1.897	1.179	1.715	1.833	1.981	Blank 450

Fig 16:- ELISA result observed at 450 nm for combo Cry1Ac

3) **Isolation of genomic DNA FROM PLANT (COTTON)**

- **REQUIREMENTS**
  - **Reagents**

1. Solution CD1
2. Solution CD2
3. Solution of PS buffer
4. Buffer APP
5. Lysate solution
6. Buffer AW1 (concentrate)
7. Buffer AW2 (concentrate)
8. Ethanol
9. Distil water (D/W)
10. Agarose,
11. TAE buffer

- **INSTRUMENTS & GLASS WARE:**

1. Tissue disruption tubes orEppendorf tubes
2. Flask
3. Beaker
4. Mastering cylinder
5. Collection tubes 1.5 ml & 2 ml
6. Centrifuge
7. TissueLyser II (for crushing of leaf)
8. Vortex
9. Micro pipettes & tip-box
10. Weighing balance
11. Electrophoresis
12. UV transilluminator
13. Microwave

❖ PROCEDURE

Twenty-four samples were chosen for DNA extraction namely T1 to T24 using following method.

❖ Important points before starting

- Ensure that the tissue disruption tubes rotate freely in the centrifuge, without rubbing against the sides.
- Perform all centrifugation steps at room temperature (15–25°C).

1. Add 5–100 mg of plant tissue and 400µl Solution CD1&100µl Solution PS to a 2 ml tissue disruption tube. Vortex briefly to mix.

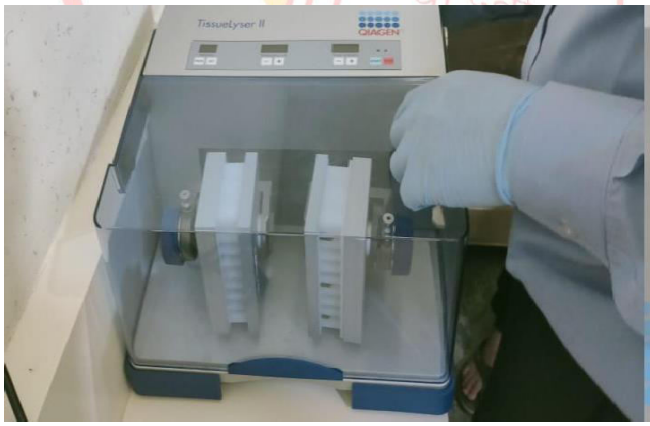
Fig 17:- Plant leaf cutting

The tissue was cut into small pieces before loading into the bead tube.

1. Homogenize using one of the following methods:

A. <u>TissueLyser II:</u>

Most plant samples can be lysed with the TissueLyser II, using the TissueLyser Adapter Set 2 x 24: Place samples in TissueLyser II and run at 24 Hz for 2 min Reorient the adapter so that the side that was closest to the machine body becomes furthest from it, and then run the TissueLyser again at 24 Hz for another 2 min.



(A)

(B)

Fig 18 :- (A)TissueLyser II, (B)Block/adapter for 1.5/2mL tubes

B. Vortex:

Secure tissue disruption tubes to a Vortex Adapter (cat. no. 13000-V1-24) and vortex atmaximum speed for 10 min.

2. Centrifuge the Tissue Disruption Tubes at 12,000 x g for 3 min.
3. Transfer the supernatant to a 1.5 ml collection tube.

Note: Expect 350–450µl. The supernatant may still contain some plant particles.

4. Add 250µl Solution CD2 and vortex for 5 s.
5. Centrifuge at 12,000 x g for 1 min at the room temperature. Avoiding the pellet, transferthesupernatant to a 1.5 ml collection tube (provided)

Note: Expect 400–500µl.

6. Add 500µl Buffer APP and vortex for 5 s.
7. Load 600µl of the lysate onto an MB Spin Column and centrifuge at 12,000 x g for1min.
8. Discard the flow-through and repeat step 8 to ensure that all lysate has passed throughtheMB Spin Column.
9. Carefully place the MB Spin Column into a clean 2 ml collection tube (provided). Avoid splashing any flow-through onto the MB Spin Column.
10. Add 650µl Buffer AW1 to the MB Spin Column. Centrifuge at 12,000 x g for 1 min.
11. Discard the flow-through and place the MB Spin Column back into the same 2ml collection tube.
12. Add 650µl Buffer AW2 to the MB Spin Column. Centrifuge at 12,000 x g for 1 min.
13. Discard the flow-through and place the MB Spin Column into

the same 2 ml collectiontube.

14. Centrifuge at up to 16,000 x g for 2 min. Carefully place the MB Spin Column into anew

    1.5 ml collection tube (provided).

15. Add 75µl of Buffer EB to the center of the white filter membrane.

16. Centrifuge at 12,000 x g for 1 min. Discard the MB Spin Column.

    The DNA is now readyfor downstream applications.

    The extracted DNA from the samples were was stored at –20°C.

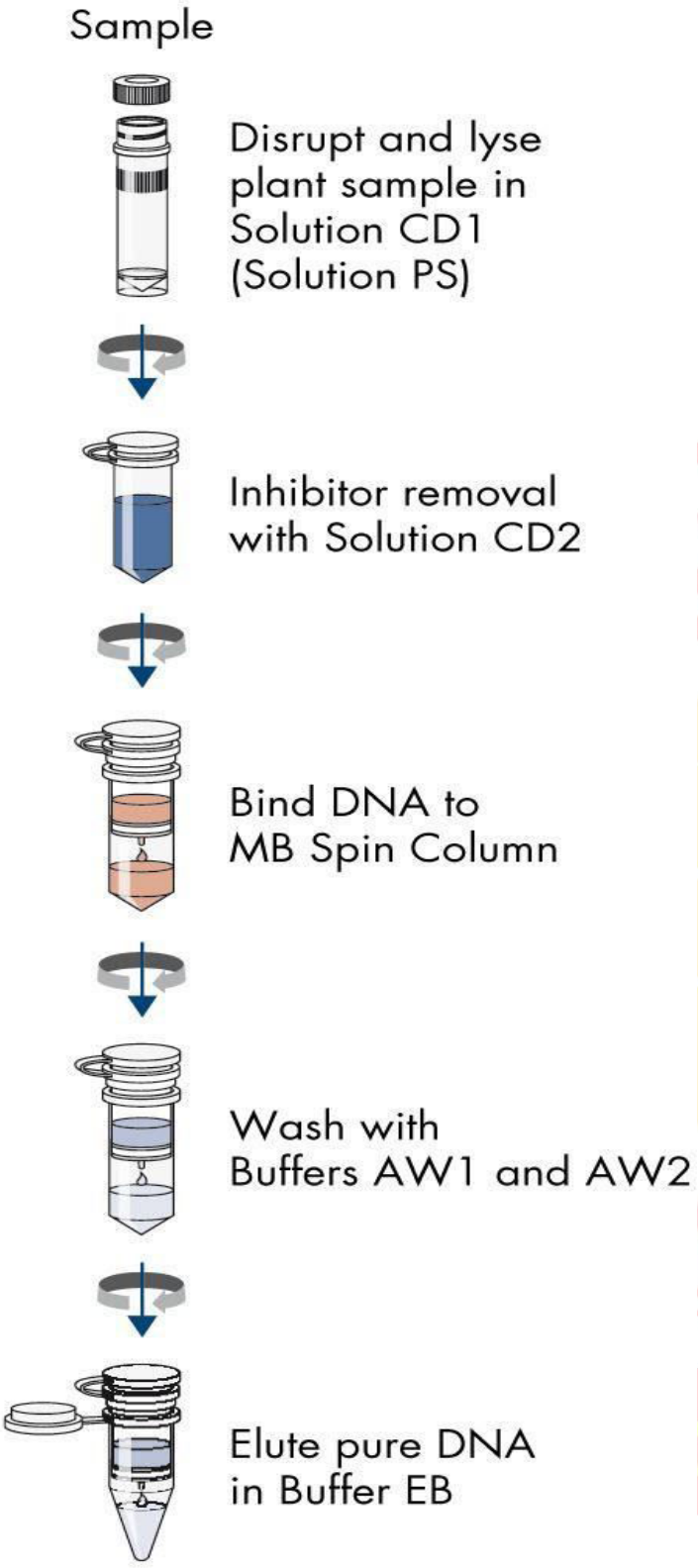


Fig 20 :- **Procedure of leaf extractio**

Procedure of Gel Electrophoresis

1. 1%Agarose gel was prepared by dissolving 0.5 gm of agarose in 50 ml $1x$ TAE buffer and boiling to microwave, the suspension unit a clear solution results, Allow it to cool.

Fig 20:- Microwave Ovan

2. Meanwhile adjust the comb in the electrophoresis, set at cathode end.
3. After the gel is cooled sufficiently and appropriate amount of ethidium bromide solution, mix and pour it slowly in to the gel tank without creating air bubbles, gently removes, it to the side with the help of micro tip.
4. Keep the set distributed till the agarose solidifies.
5. Gently lift the combs in an up word direction carefully without breaking the gel.
6. 18µl of DNA sample was loaded in separate wells with 2µl of loading dye.

Fig 21:-Loading dye & DNA sample

7. Anode and cathode were connected to power unit so volts of electric current was appliedand allowed to run for 2-3 hours.

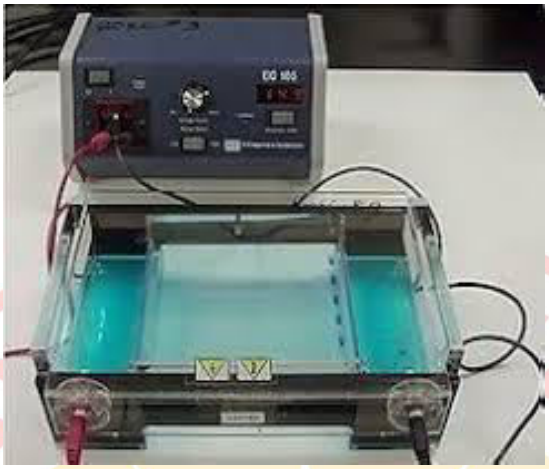


Fig 22:- Electrophoresis

8. The gel was removed from electrophoresis, set and observed under UV transilluminator.

Fig 24:- **UV transilluminator and observe in it.**

[W.R.9]

Result and Discussion

Samples were loaded in wells in an agarose gel of 1%. Sample names corresponding to well it was loaded into is shows in table below.

Gel was placed onto the UV transilluminator for visualization. By observing gel under UV transilluminator, it was observed that DNA samples had all the band intact of cotton specie***Gossypiumhirsutum***. Thus, the DNA was successfully extracted and isolated from the 25 samples namely T1 to T94 as seen in figure 25.

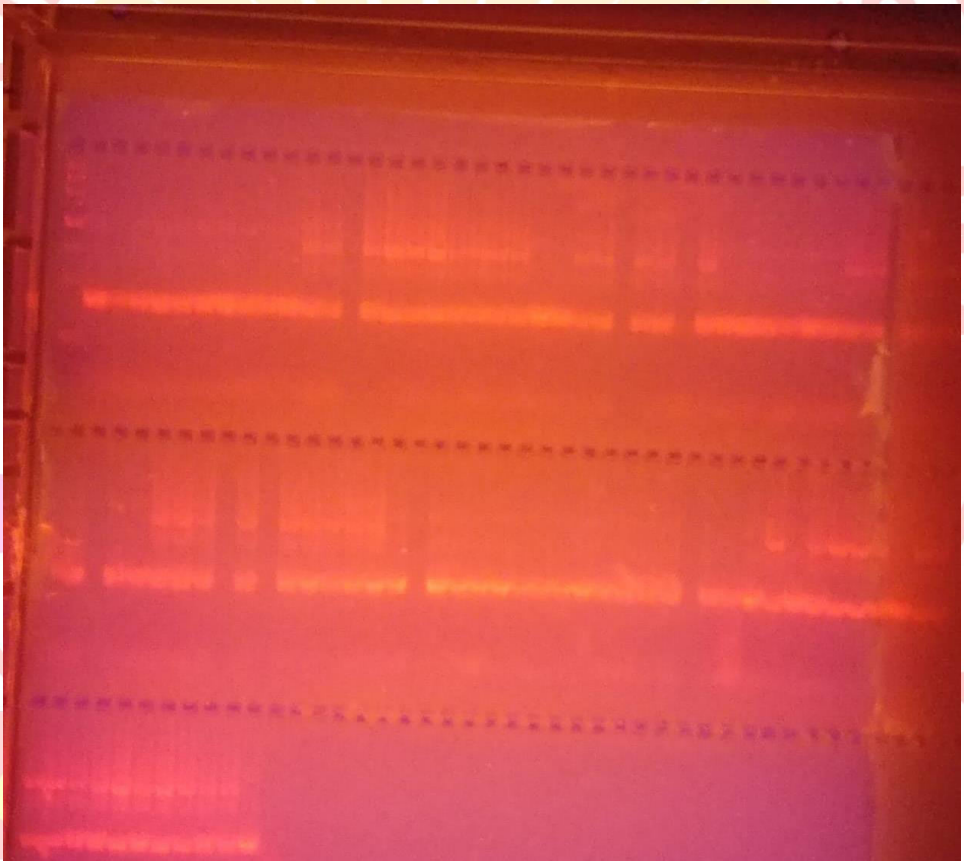


Fig 25:-Shows gel run time was about 2 hour at 100 volts in TAE buffer.

4) Polymer Chain Reaction (PCR)

REQUIREMENTS

- **REAGENTS**
  1. DNA Template
  2. NF-H2O
  3. Coral Load Dye
  4. Q-Solution (5x)
  5. Primer F
  6. Primer R
  7. Qiagen Multiplex+

- **INSTRUMENT & GLASS WARE**
  1. Beaker
  2. Flask
  3. Electrophoresis
  4. UV transilluminator
  5. ABI 7500 FAST RTPCR

PROCEDURE

Thaw the reagents and other frozen reaction components to RT. Mix thoroughly,centrifuge briefly to collect solutions at the bottom of tubes.

⇩

Prepare master mix for all qPCR reactions by adding all required components, except theDNA template.

⇩

Mix the master mix thoroughly to ensure homogeneity and dispense equal aliquots intoeach PCR tubes.

⇩

Add DNA samples(and nuclease free water, if needed) to the PCR tubes containingmaster mix, seal the tubes and vortex for 30sec. spin the tubes to remove any air bubbles.

⇩

Program the thermal cycling protocol on the real-time PCR instrument.

⇩

Load the PCR tubes into RT-PCR instrument and start the PCR run.

⇩

Perform data analysis according to the instrument specific instructions.

Each Primer Final Conc. (675nM)	**Date:-5th April 2021**		
EVENT ID:WY21	**WY21**	**MM-1**	**MM-2**
Factor	**Rxn vol.30µl**	**For 50 Rxn**	**50x tube mgmt**
Qiagen Multiplex+(MM)	**12.069**	**603.45**	**603.45**
Type-IT HRM (MM)EVAGREEN	**2.414**	**120.69**	**120.69**
Q-Solution(5x)	**1.062**	**53.10**	**53.10**
SEQ18	**4.055**	**202.76**	**202.76**
SEQ19	**4.055**	**202.76**	**202.76**
SEQ20	**4.055**	**202.76**	**202.76**
DNA Template	**2.000**	**100.00**	**100.00**
NF-H_2O	**0.29**	**14.48**	**14.48**
Total rxn vol	**30.00**	**1500.00**	**1500.00**

Table No 6:- Master Mix of PCR

Primer Information:

Forward Primer:
CTCGCACACGGCTTCG
AReverse Primer:
GATTGTTCTCTGCAAA

Initial Enzyme Activation Step	**Time**	**Temp.°C**
	5 min	**95°C**
3-step cycling (38 cycles)		
Denaturation	**30s**	**95°C**
Annealing	**90s**	**60°C**
Extension	**90s**	**72°C**
Final Extension stage	**10 min**	**68°C**

Table No 7:- Steps of PCR

- **Instrument of PCR**

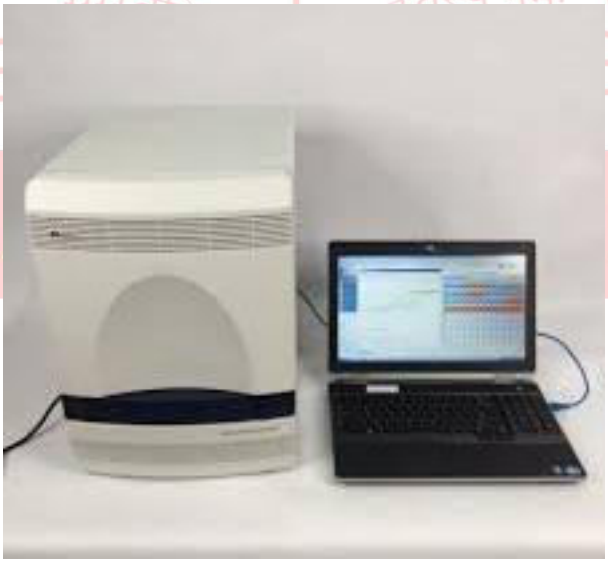


Fig 25 :- PCR Instrument Thermal Cycler

Result & Disscusion

In conclusion, DNA from the 94 samples namely T1 to T94 using DNeasy Plant Pro Kitbased on the silica spin columnwere successfully extracted and isolated. The silica-based spin column method that was used for this experiment was fast,reliable, accurate and consumes less time, which was about 3-4 hours. The silica-based spin column method is highly is recommended. Further, after performing PCR of the T1 to T94 samples,bands on the gels of size around 600bp were observed indicating the presence of CRY1Ac. There was no band visible for the sample T94 indicating the absence of Cry1Acgene. Thus, all the 94 samples were analysed successfully using the methods mention in this experiment

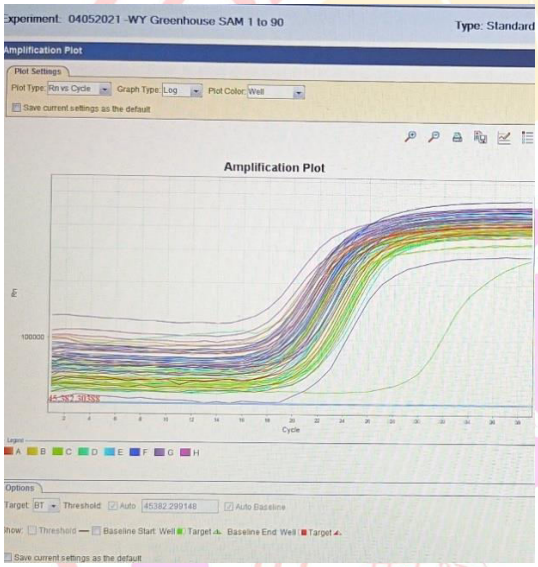


Fig 26:- PCR result in different sample Gene

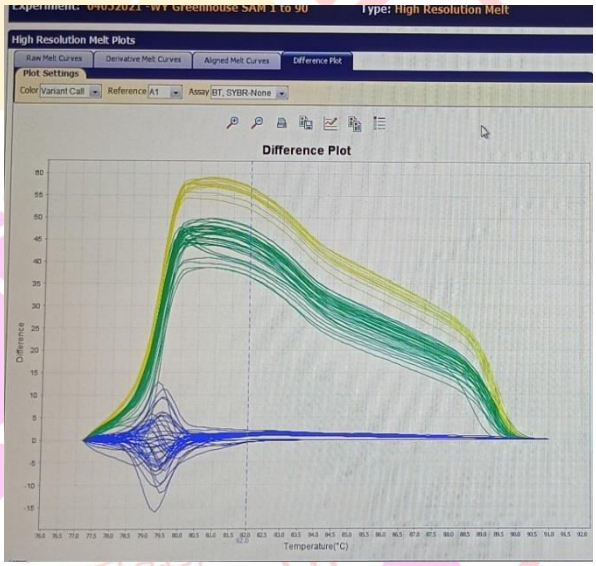


Fig 27:- Observed Positve & Negative

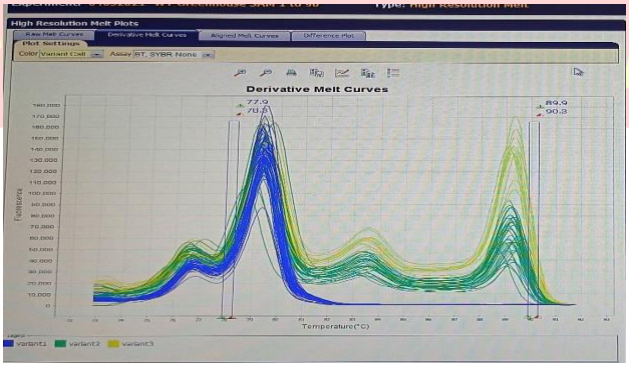


Fig 28 :- Observed homologus gene

Conclusion

- The germination technique is the required for the pass the commercial lot of farmer by treatment of cryoenzyme. The growth of seed is based on treatment of 5 to 10 days in dark area between 1 to 2 days observation.
- In ELISA technique Sandwitch ELISA based method are performed in this technique observed two result on a one is 405 nm and another one is 450 nm. This technique is based on qualitative results either its positive or negative.
- The silica-based spin column method is highly is recommended. Further, after performing PCR of the T1 to T94 samples, bands on the gels of size around 600bp were observed indicating the presence of CRY1Ac. There was no band visible for the sample T94 indicating the absence of Cry1Ac gene. Thus, all the 94 samples were analysed successfully using the methods mention in this experiment.

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