"QUALITY TESTING & ANALYSIS OF Bt-COTTON HYBRID SEEDS"

AN INTERNSHIP/TRAINING REPORT SUBMITTED

FOR THE PARTIAL FULFILMENT OF THE DEGREE OF MASTER OF SCIENCE

BY

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[M.SC. BIOTECHNOLOGY]

UNDER THE SUPERVISION OF

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2022-23

(On letterhead of the Department)

C E R T I F I C A T E

This is to certify that this training report entitled "**QUALITY TESTING & ANALYSIS OF Bt-COTTON HYBRID SEEDS**" was successfully carried out by Mr. VINIT VIMALBHAI PANDYA towards the partial fulfilment of requirements for the degree of **Master of Science in Biotechnology** of Atmiya University, Rajkot. It is an authentic record of his own work, carried out by him under the guidance of Mr. DENNIS DHOLARIA for a period of 3 months during the academic year of **2022-23**. 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.

Dr. Nutan Prakash Vishwakarma
Name & Signature
Head of the Department

Mr. Dennis Dholaria
Name & Signature
Supervisor

DECLARATION

I hereby declare that the work incorporated in the present internship/training report entitled **"QUALITY TESTING & ANALYSIS OF Bt-COTTON HYBRID 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.

Pandya Vinit Vimalbhai

Date: April 13, 2023 (Name and Signature of Student)

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INTRODUCTION OF THE COMPANY:

Solar Agrotech Private Limited

- **Solar Agrotech Private Limited, Rajkot**, one of India's **leading seed producing company** & No. 1 seed producing company in Gujarat, was founded by an agricultural technocrat **Dr. Thobhan L. Dholaria (CEO)**, who has a degree of M.Sc. (Agriculture) & Ph.D. (Plant Breeding and Genetics).
- Dr. Dholaria had a bright academic career and awarded the research fellowships of CSIR as well as ICAR during his postgraduate and doctorate study.
- The company was incorporated in **1994** aiming to fulfil the needs of farming community to provide the **High-tech** and **High yielding quality seeds** of various crops under the registered trademark of "**Dr. Seeds®**".
- Dr. Dholaria having bright academic career, so he established the state-of-the-art **R&D facilities** at Solar Agrotech Private Limited.
- The company had earned very good reputation in large farming community and won the confidence of trade partners in last **25 years** and Dr. Dholaria has earned the trust of farmers as "**The Trusted Seedsman**".
- Dr. Dholaria provides needful information for **increasing crop production** and **protect (resist)** the crop under **varying climatic and insects' diseases conditions** through **Dr. Dholaria Agricultural Foundation** which was established for providing services to the farming community.

- "Dr. Seeds" sees good future business using the **Biotechnology** and strengthening R&D company's young Director, Dennis Dholaria (M.Sc. Plant Biotechnology from Australia), for **advancements in biotech crops breedings** to support the breeding work of team headed by company's Director of Research, Dr. J. M. Kamani (Ph.D. in Plant Breeding and Genetics) along with Mr. Rahul Kothiya (M.Sc. Plant Biotechnology) and Dr. M. K. Bhalala (ex-University Registrar) as an advisor to the company in the government matters.
- **2001:** Bt-cotton technology (BGII GMO Pest Resistance) is allowed to use in **India**.
- **2005:** Dr. T. L. Dholaria purchased Bt-cotton **license/agreement** from **Monsanto-Mahyco** – Monsanto Technology for insect tolerance in Bt-Cotton (BGII – Cry1Ac & Cry2Ab).
- Solar Agrotech Private Limited works on **Cotton**, Castor, Wheat, Bajra, Vegetables, etc.
- Solar Agrotech Private Limited is providing services to Cotton growers in **9 states of India**.
- Further, in order to provide the farmers with good quality pesticides, Dr. Dholaria went into the business of pesticides to fulfil the farmers needs of good quality low-cost pesticides. The pesticides manufacturing company name is **Cistronics Technovations Private Limited** and marketed pesticides under **Cistronics Pesticides LLP**.

OBJECTIVES:

- To understand the **basic principles** of Plant Biotechnology, Molecular Biology and Plant Pathology.
- To understand about the **integration** of two disciplines – Agriculture & Biotechnology.
- To know about the **research opportunities** in the Agricultural Biotechnology field.
- To understand the **concepts & techniques used** to make healthy plants in a safe environment for food, non-food, feed and health applications.

WORK PLAN:

Areas/Units: R&D, Testing, QA-QC, Production & Packaging, etc.

- **Testing:** Seeds germination test, Cry1Ac-Cry2Ab (Bt-protein) Sandwich ELISA Qualitative test, Zygosity testing (RT-PCR), Moisture testing, etc.
- **Lot Passing Criteria/Range:** Germination 75-80%, ELISA Cry 95%, 6-8% moisture (by weight)
- **Treatment after Passing:** Coating of Polymer, Insecticide, Fungicide on seeds
- **Packaging:** Solar 76 BG II Hybrid Cotton – 450 gm BT + 25 gm Non-BT Weeds
- **Parameters:** Field, Rain/Water, Environment
- *Gossypium hirsutum* (F1 Hybrid Bt-Cotton)
- Bt **Transgene** = *Bacillus thuringiensis* **Delta-endotoxin (Cry Protein) Natural Insecticide** = Against **Pink bollworms**

STEP-1: SAMPLES

- **Performance Experimental Trials:** Farmers of North Gujarat are using F1 Hybrids cotton species, made from pure lines - The company is having very rich (more than 2000) germplasm lines in cotton (homozygous trait, from wild type cotton Male flowers & Female flowers – 1:2999 different crosses for 3000).
- **Ginning:** Cotton & Seeds separation.
- **Seeds treatment:** Acid (Pure, Commercial grade H2SO4) & Base (lime) treatment, washing, drying.
- **Grading of seeds:** By Gravity air **OR** Gradient.
- **Seeds Transport:** Testing Laboratory, Rajkot.

Figure-1: Seed Lots

STEP-2: SEEDS PHYSICAL PURITY & GERMINATION TESTING (SOP POLICY)

Purpose:

- 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**.

Scope:

- Includes all core and supporting processes within the quality management of **Bollgard®** and **Bollgard II®** Cotton.

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 **(10-12 days)** or cotton species.
- Also, to review the **findings for trends**.

General requirements:

- **Moisture determination:** Seed moisture content is determined by using **moisture meter**. Seed moisture content is expressed as percentage by weight.
- **Germination:** Seeds sample, germination **paper** (substratum), racks and trays, fungicide and water.
- **Suitable substratum:** The substratum 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.

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

Adequate moisture or water:

- High concentration of water at cellular level is necessary fix the seed to **start** germination.
- Mobilization 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.
- The general specifications for water are:

i. It should be free from organic or inorganic impurities.

ii. The pH value should be within the range of 6.0 to 7.5.

iii. If the usual water supply in the laboratory is not satisfactory, distilled, deionized water may be used.

Favourable temperature:

- Germination occurs under different ranges of temperatures provides 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 10-15 °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 be 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).

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:

- **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.
- **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.
- **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. The composition of different components is expressed either in percentage by weight or as numbers per kilogram.
- **Germination testing:** The pure seed fraction from the purity test is used for germination analysis. A minimum of **100** Cotton seeds for grower lots and **400** Cotton seeds for commercial lots are tested for germination.
- **Methods of using paper substrata:**

Between paper: 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 ℃).

- **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.

Dip germination paper rolled up with seeds in a solution of Vitavax 1% solution (1g/100mL water) and keep it for 5 minutes so that germination paper gets thoroughly wet.

- **Duration of the test:** The duration of sample incubation varies from crop to crop. For cotton, **first count** is taken on the 4^{th} or the 5^{th} 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. The test can be

terminated before the end of prescribed test period, if the sample under test meets the minimum limits of germination during the first count.

- **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.

Categories of seedlings (Germination steps):

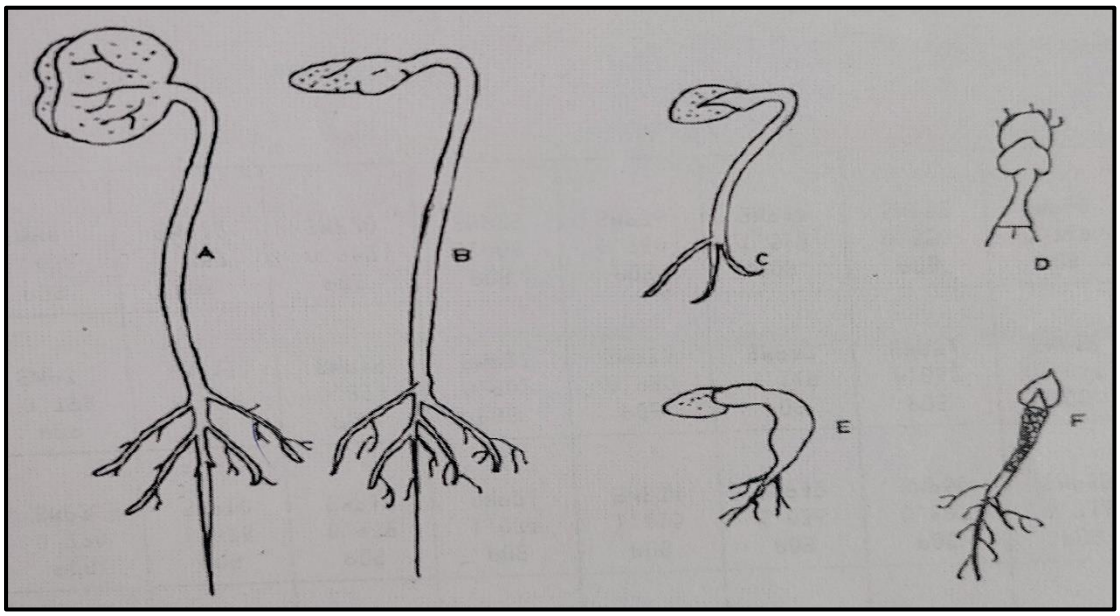

Figure-2: Seedlings of *Gossypium spp.* (seednet.gov.in)

(A) **Normal seedling (seedcoat off):** Hypocotyl (shoot), Radicle (root) and cotyledons (leaf) well developed.

(B) **Normal seedling (seedcoat on):** Hypocotyl (shoot), Radicle (root) and cotyledons (leaf) well developed.

(C) **Abnormal seedling:** Radicle failed to develop.

(D) **Abnormal seedling:** Hypocotyl not elongated, Radicle missing.

(E) **Abnormal seedling:** Hypocotyl thickened, Radicle weak.

(F) **Abnormal seedling:** Hypocotyl decayed, base of cotyledons decayed.

During each count, the germinated seedlings and the remaining ungerminated seeds are classified into any of the following categories:

A. **Normal seedlings:** Seedlings which shows the capacity for continued development into mature plant when grown under favourable conditions and having well developed root and the shoot system.

B. **Abnormal seedlings:** Seedlings with any abnormality in their root or shoot system thus which do not have the capacity to develop into a normal plant when grow.

C. **Ungerminated seeds:** Seeds which have not germinated by the end of test period under the prescribed conditions.

- **Hard seeds:** Seeds which remain hard at the end of the prescribed test period because of the non-absorption of moisture due to the presence of impermeable seed coat. Usually, hard seeds are seen in leguminous crops.
- **Fresh ungerminated seeds:** Seeds other than hard seeds, which remain firm, fresh and apparently viable, even at the end of the test period.
- **Dead seeds:** Seeds which at the end of the test period are neither hard nor fresh and have not produced seedlings are classified as dead seeds. Dead seeds often show the symptoms of decaying and fungal growth which can be felt by pressing the seed under test.

Resting:

- When the test results are unsatisfactory due to out of tolerance performance between replicates or wrong evaluation of seedlings or errors in testing conditions, a **second test** is conducted by the same method or by alternative method to confirm the results before they are declared.

Reporting results:

- **Purity & germination percentage:** 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.

- **Moisture percentage:** Moisture percentage calculated using **moisture meter** should be recorded on the seed analysis card. Moisture percentage should be **6 or less**.

Seedling descriptions: As per the International Seed Testing Association **(ISTA) Rules** (1985 Para 5.2.4.A. and 5.2.5.A.), the following is the detailed description for normal and abnormal seedlings. These have to be taken into account while evaluating the seedlings.

Reference:

- SL agreement **Exhibit B**
- http://seednet.gov.in/Material/Handbook_of_seed_testing/Chapter%205.pdf

Table-1: Marking on the Cellulose paper (for Germination testing)

On samples	On cellulose papers
Variety / Lot No.	Variety
No. of Bags	Lot No.
Weight	

- On **cellulose paper** (substratum & moisture reservoir), count 100 seeds and band this cellulose paper in prescribed pattern. This method is called **"Paper method"** of seeds germination test.
- Spray water (daily, twice in a day) & fungicide (one time, on day-1) on these cellulose papers during the germination incubation period (5-10 days). Incubation temperature should be 20-30 °C.
- **Germination steps - seedling:** Root (Radicle), Shoot (Hypocotyledon) & Cotyledon (Leaf)

Figure-3: Research Station

(Performance Experimental Trials)

Figure-4: Seeds Germination Test

STEP-3: SAMPLE PREPARATION FOR ELISA TEST

Prepared & Tested **313 seed lots** samples.

1) Take 96 wells (12-horizontal x 8-vertical) box.
2) Add **seeds** in all wells.
3) Add metal balls in all wells.
4) Add 750 µL of 1x Protein Extraction Buffer (Contains Deionized/Distilled Water + Tween-20) in all wells.
5) Crush in **QIAGEN® TissueLyser II** (Fast & High-throughput process of protein extraction from cotton leaves/seeds) for 5 minutes.
6) Mix well & incubate at room temperature for 30-60 minutes or overnight.
7) Allow particles to settle and use only the supernatant (contains crude protein) to do the ELISA test.

Note: Wash & rinse grinding equipment carefully between samples to avoid cross contamination.

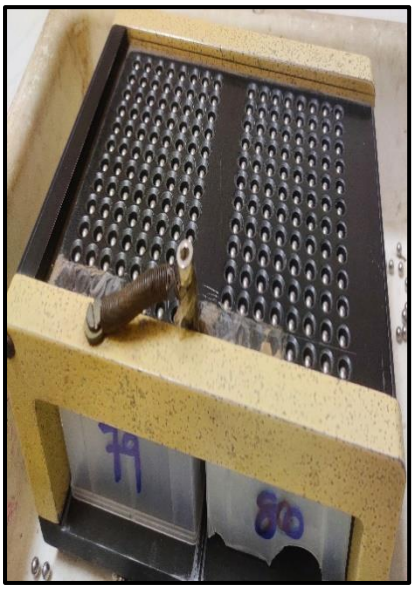

Figure-5: Addition of Metal Balls

Figure-6: Crusher

Figure-7: Crusher 17.5 Frequency 1/s

STEP-4: QUALITATIVE ELISA TEST

Cry1Ac-Cry2A in-vitro ELISA Kit (eurofins Technologies)

Intended use:

- The **Bollgard**II** combination kit is used for simultaneous **qualitative** detection of Cry1Ac and Cry2A protein in cotton leaf or seed samples with both analytes, measured in the same well of the assay plate.
- The kit is ideally suited for testing large numbers of seed lots for Cry1Ac and Cry2A simultaneously.

Principle of the test (Sandwich ELISA):

- In the test, cotton sample extracts are added to test wells coated with antibodies raised against Cry1Ac and Cry2A proteins.

- Any Cry1Ac or Cry2A protein present in the sample extract binds to the antibodies. This is detected by addition of alkaline phosphatase-labeled Cry2A antibody **OR** horseradish peroxidase-labeled Cry1Ac antibody.
- After simple wash step, the results of the Cry2A assay are visualized via the addition of a pNPP substrate.
- Once the yellow color develops and is read, the wash step is repeated, and TMB substrate is added.
- The Cry1Ac results are visualized via the development of the resulting blue color which, subsequently, becomes yellow on addition of stop solution.
- Cry2A = AP + pNPP = Yellow
- Cry1Ac = HRP + TMB = Blue
- **Coating:** Anti-Cry1Ac & Cry2A antibody

Step-1: Take 96 wells Anti-Cry1Ac & Anti-Cry2A Combo ELISA Plate (eurofins Technologies)

Step-2: Add 45 µL Enzyme/Antibody conjugate (Cry1Ac & Cry2Ab combined) in all 96 wells

Step-3: Add 50 µL Cry1Ac/Cry2A Positive control (in-house) in C1 well, Cry1Ac/Cry2A Negative control in D1 well & keep A1-B1 as Blank (only extraction buffer)

Step-4: Add 50 µL supernatant of crushed samples in all wells (according to the markings), except wells A1-B1-C1-D1

Step-5: Incubate for 60 minutes at room temperature

Step-6: Wash-1 (2 times) (Wash solution contains deionized/distilled water + Tween-20)

Step-7: Add 70 µL Substrate-1 (pNPP) & incubate in dark for 30 minutes (Yellow color will appear)

Step-8: Absorbance-1 for Cry2A at 405 nm (With 630 nm as secondary filter)

Step-9: Wash-2 (1 time)

Step-10: Add 90 µL Substrate-2 (TMB) & incubate in dark for 30 minutes (Blue color will appear)

Step-11: Add 100 µL Stop solution (27 mL of 98% H2SO4 in 973 mL of deionized/distilled water)

Step-12: Absorbnce-2 (Yellow) for Cry1Ac at 450 nm (With 630 nm as secondary filter)

Figure-8: Sample addition (Multichannel Pipette)

Figure-9: ELISA Microplate Washer

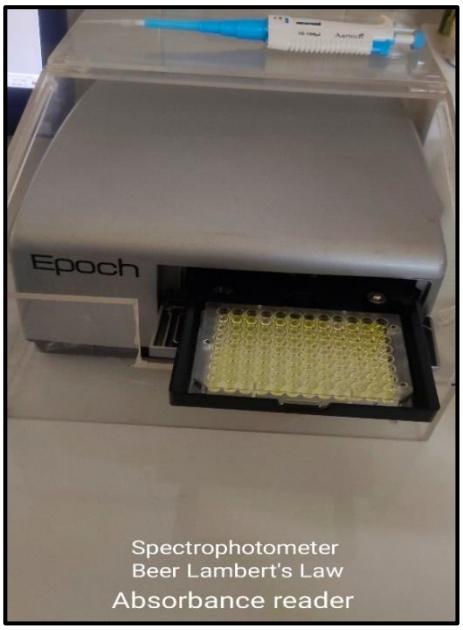

Figure-10: ELISA Microplate Reader with 450/620 nm Filter

Figure-11: Biotechnology Laboratory-1

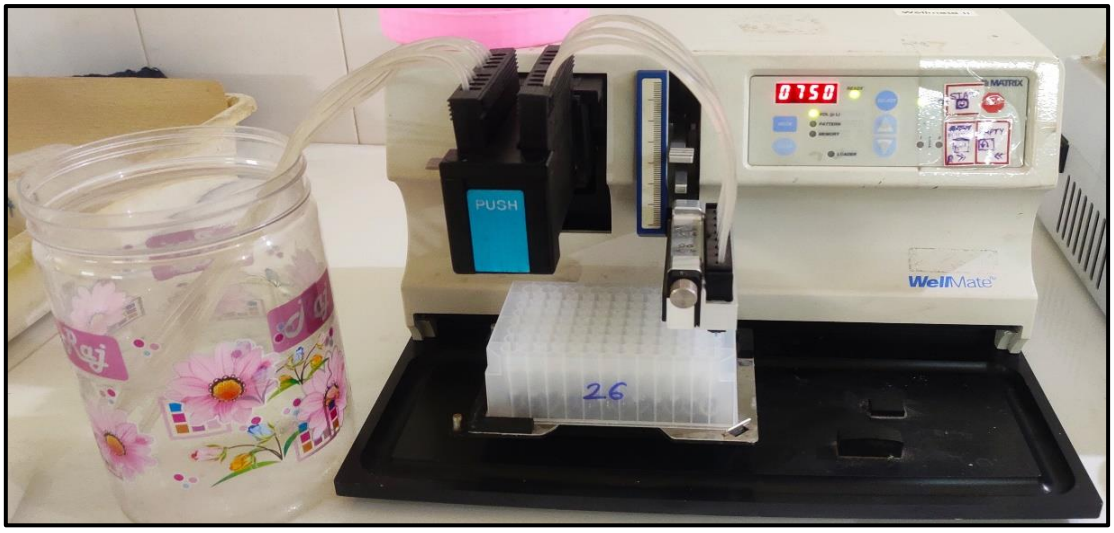

Figure-12: 1x Protein Extraction Buffer

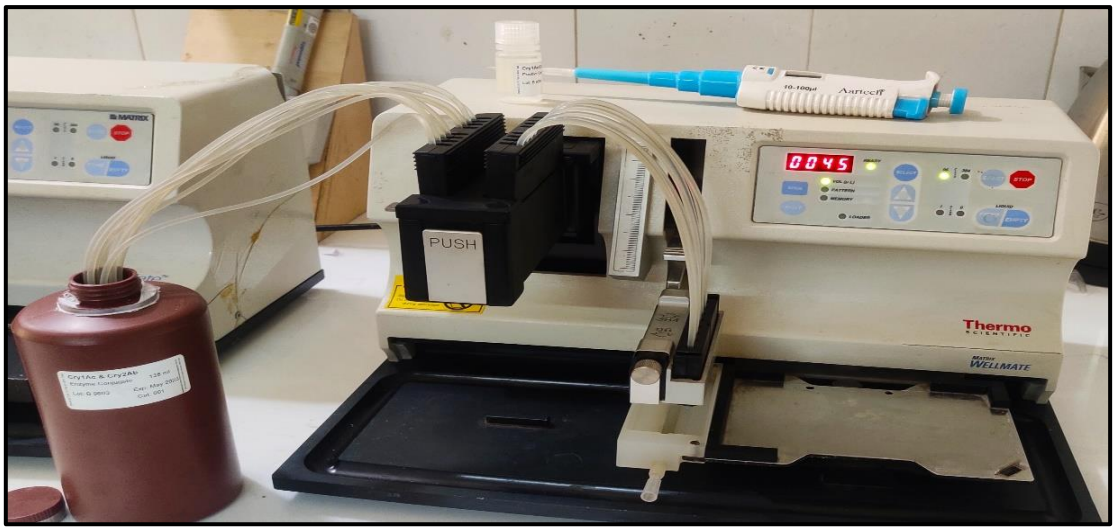

Figure-13: Cry1Ac-Cry2Ab Enzyme Conjugate

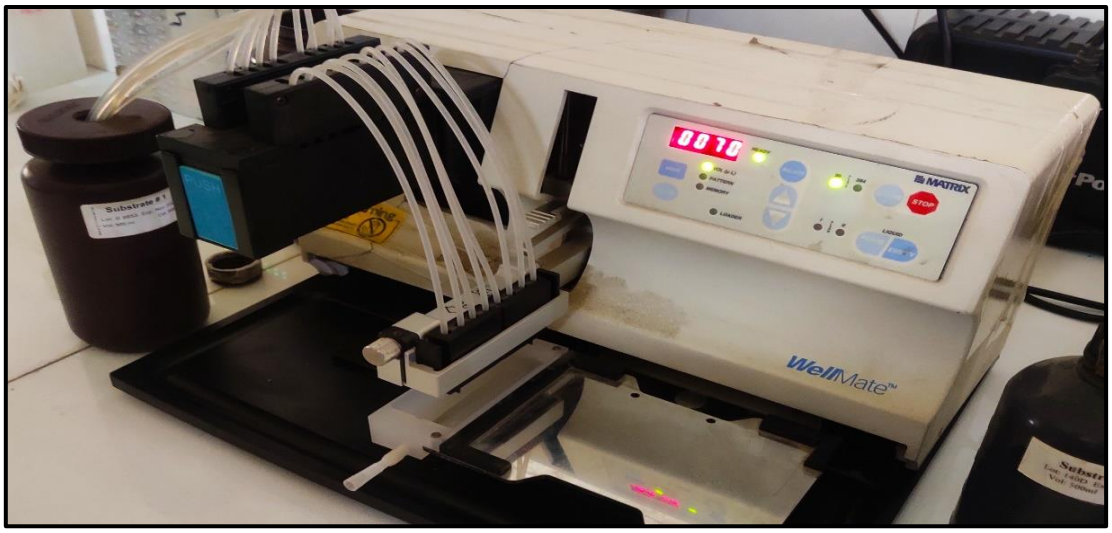

Figure-14: Substrate-1 (pNPP)

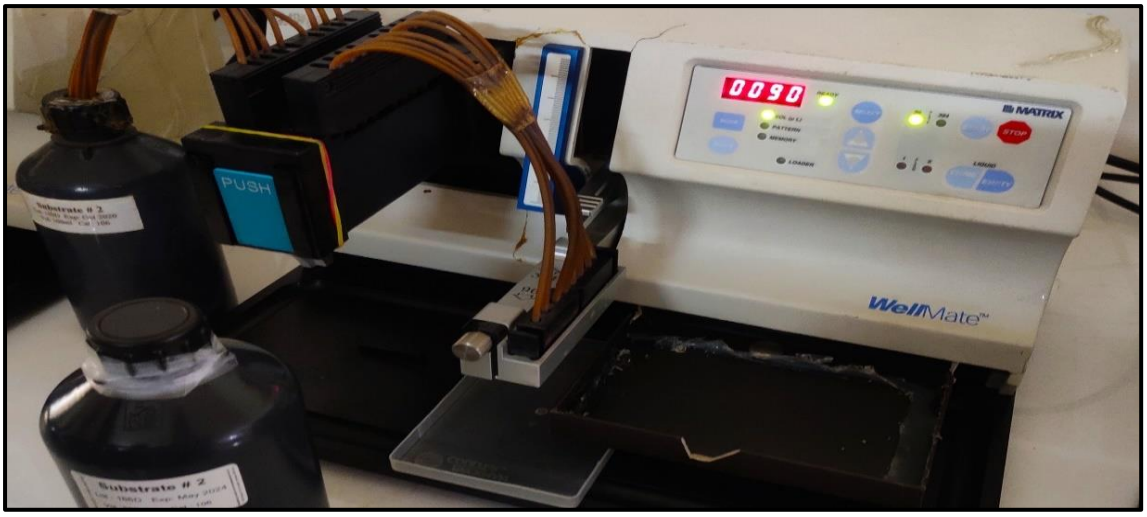

Figure-15: Substrate-2 (TMB)

Figure-16: Stop Solution

STEP-5: QUALITATIVE ELISA TEST RESULTS

Interpretation of Results

General test criteria (Absorbance value):

- **Blank:** should not exceed 0.1/0.15
- Absorbance value of blank should be subtracted from the absorbance values of positive control, negative control and samples.
- The coefficient of variance between the positive control, in duplicate, should be less than 15%
- **Positive control:** at least 0.5 (1 or more than 1)
- **Mean negative control:** below 0.1-0.2

Note: If above criteria are not met, the test is invalid and should be repeated.

Negative: less than 0.150

Cut-off value: (Mean absorbance of negative control + 0.1)

- All negative seeds should give absorbance below 0.2
- Negative control should give absorbance below 0.1

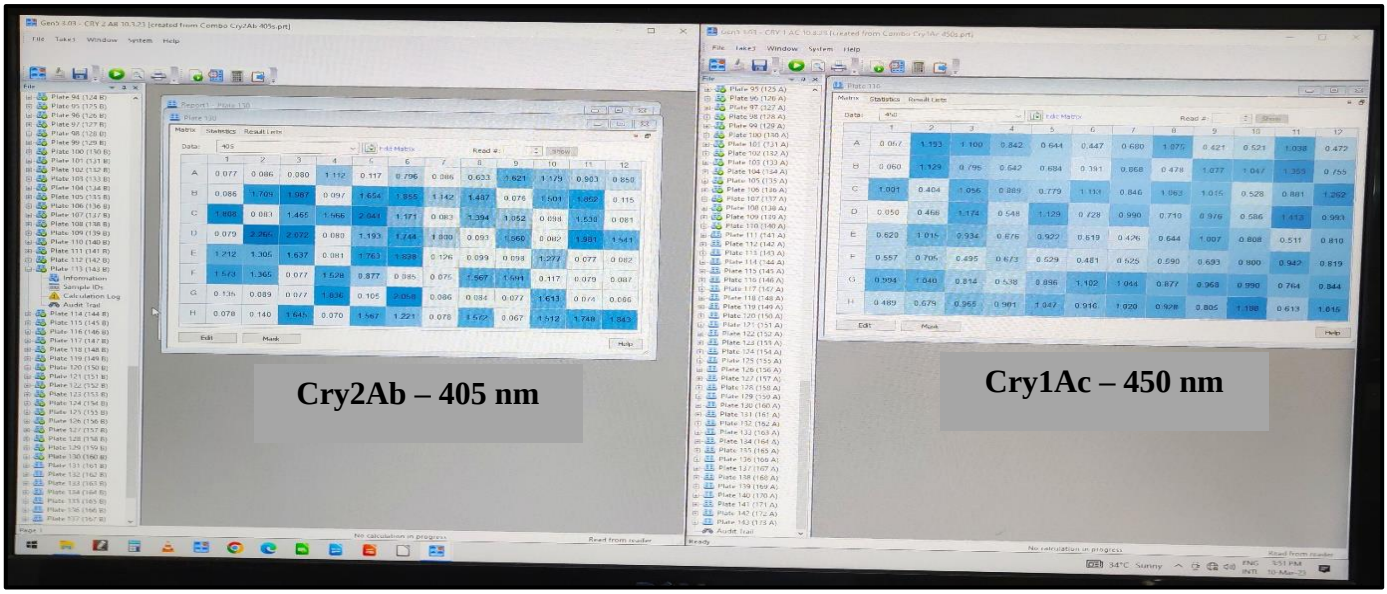

Figure-17: Gen5 3.03 Software

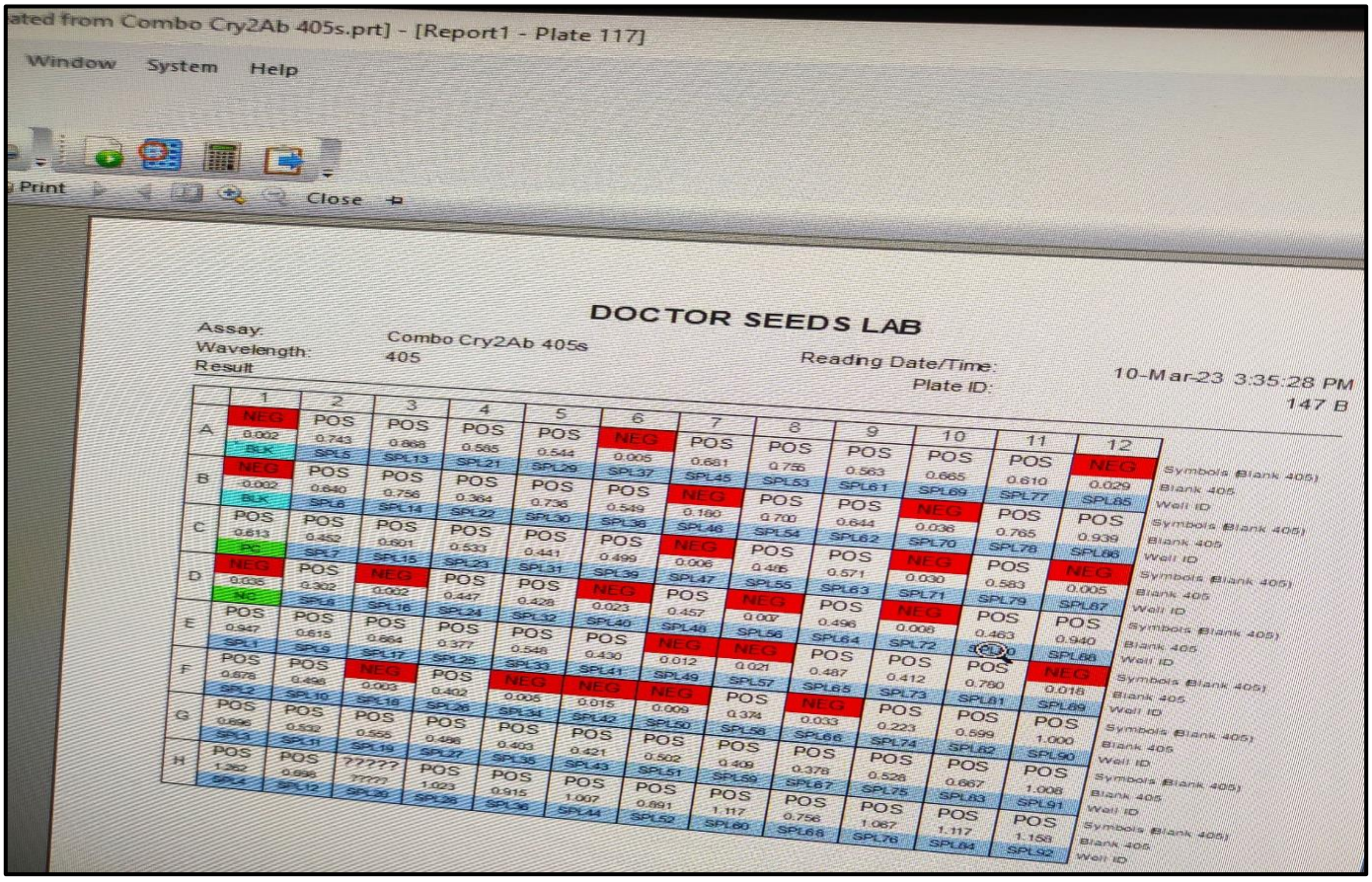

Figure-18: Cry2Ab – 405 nm

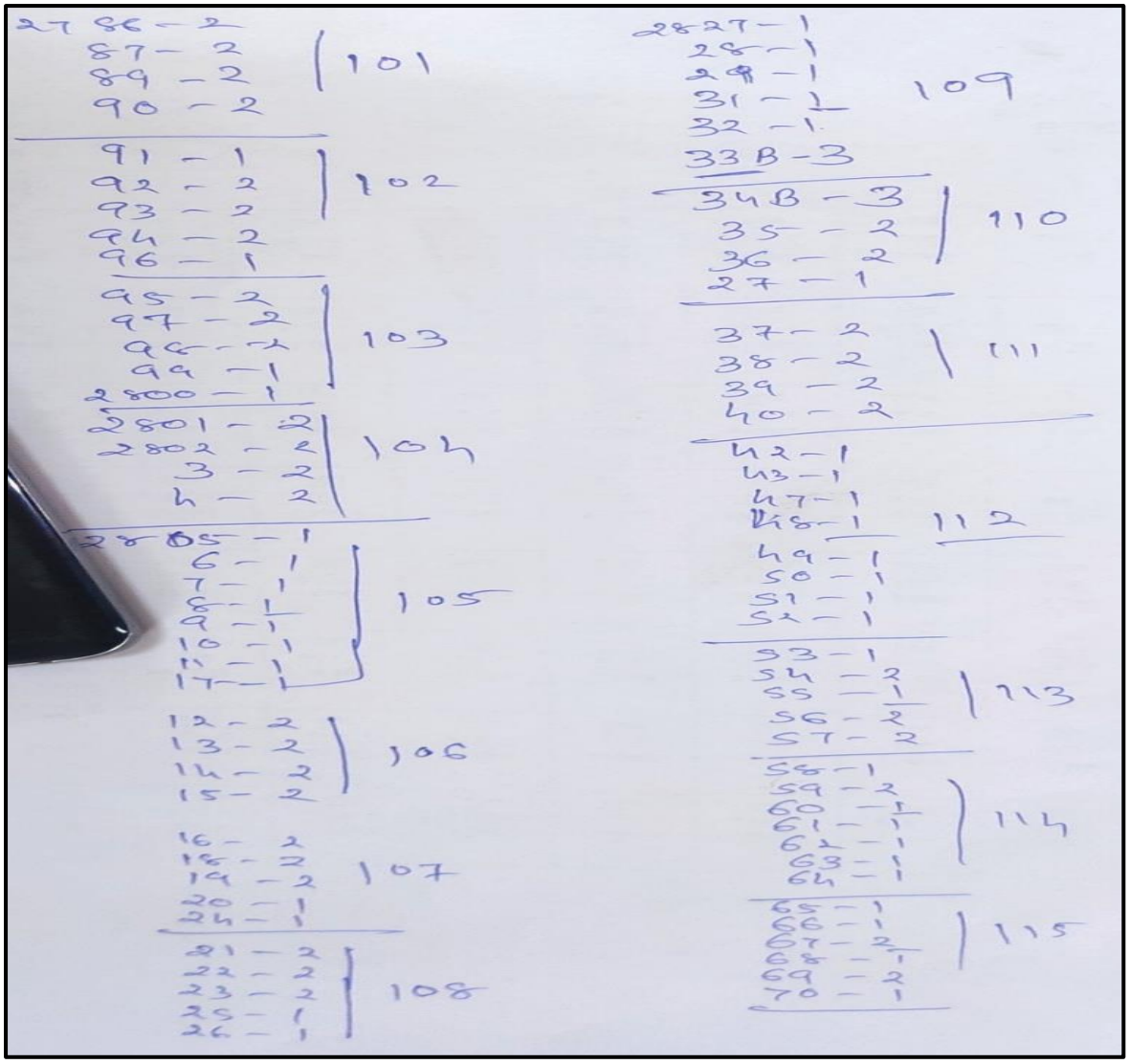

Figure-19: Seed lots samples distribution in wells

STEP-6: FIELD TESTING & ZYGOSITY TESTING

Figure-20: Biotechnology Laboratory-2

- May (Haryana), June, July (Gujarat).
- Zygosity test (field) – Visual/Physical.

STEP-7: PROCESSING OF SEEDS

Figure-21: Seeds Processing

(Polymer Coating + Fungicide + Pesticide)

Figure-22: Seed Loader (for seeds processing-coating)

STEP-8: PACKAGING

Figure-23

Figure-24

Figure-25

Figure-26

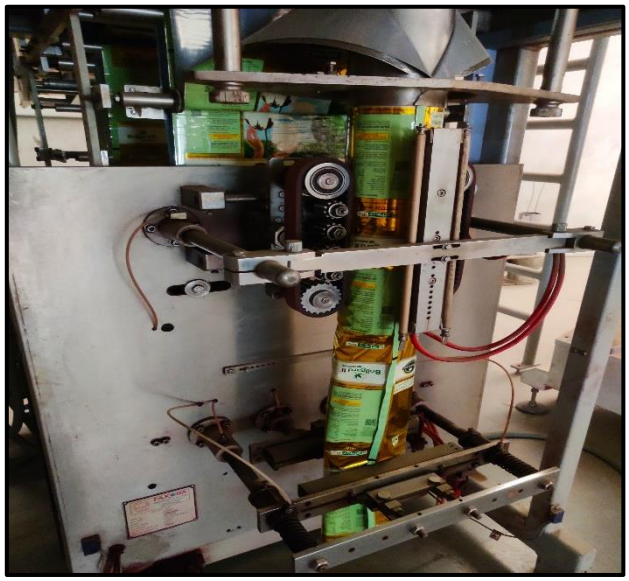

Figure-27

Figure 23 to 27: Packaging Units

PRODUCTS:

SALIENT FEATURES OF HIROSHI BGII & SOLAR 76 BGII

Regional Adaptability:

Medium maturity segment for cultivation in Central and South India.

Advantages:

- *High yield potential, bottom to top boll load.*
- *Good boll opening and Ease in picking.*
- *Compact plant type.*
- *Big boll combined with good boll weight (5 to 5.5 g per boll).*
- *Good Ginning out turn percentage.*
- *Good fiber quality hence preferential buying by the traders.*
- *Long Lasting Greenness.*

REFERENCES:

- Indian Seeds Act, 1966
- International Seed Testing Association (ISTA) Rules
- www.doctorseeds.com

“Investments in agriculture are the best weapons against hunger and poverty, and they have made life better for billions of people.”

Bill & Melinda Gates Foundation (UN-SDG)

----------END OF REPORT----------