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Analysis of DNA, Protein, and determination of Begomovirus infected chili plant.

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Abstract: Begomovirus is a serious threat to the dicotyledonous agricultural plant. The infection decreases the total yield of the vegetation. Chilli plant and its fruits are also useful in many therapeutic properties, it is a source of many minerals, vitamins, and different elements beneficial for health. This project is based upon the determination of begomovirus infected plant, and its molecular characterization. While determining the protein and DNA quantity of the infected chili plant the decreased percentage found between the healthy and the infected was quite a lot, this indirectly decreased the medical important components too. As this has become a serious threat to the agricultural plant, and the farmers. It is necessary and important to discover begomovirus-resistant crops, to save the normal flora and fauna of the agricultural plant. Also most importantly the cause of transmittance of this virus or the vector that carries this is the "White Fly" Bemisia Tabaci.

KEYWORDS: Chili leaf curl, Begomovirus, DNA, Jahangeerabad, Kursi road, Agarose gel electrophoresis, PCR: Polymerase chain reaction

1. INTRODUCTION

Plant viruses known as geminiviruses contain single-stranded circular DNA genomes that encode genes that diverge in opposite directions from the initiation of replication in the virion strand (i.e., geminivirus genomes are ambisense). They are defined as class II viruses by the Baltimore classification[1]. It is the single-stranded DNA virus family with the biggest known subgroup[1]. Around the world, these viruses have caused severe crop damage. Multiple variables, including the recombination of various geminiviruses infecting a plant, which allows for the development of novel, potentially lethal viruses, have contributed to the emergence of geminivirus disease epidemics[2].

The spread of infected plant material to new places, the extension of agriculture into new growing regions, and the migration and proliferation of the vectors that can transfer the virus from one plant to another are other contributory causes. The Gemini virus complex affects 300 plant species throughout 63 families of crops, with the Solanaceous crops such as tomato, pepper, chili, Cucurbita, tobacco, and cotton being among the most significant. Other contributing factors include the spread of infected plant material to new locations, the growth of agriculture into new growing zones, and the migration and expansion of the vectors that can transmit the virus from one plant to another[3]. 300 plant species from 63 different crop groups are impacted by the Geminivirus complex, with Solanaceous crops including tomato, pepper, chili, chili, tobacco, and cotton being among the most prominent. Within the nucleus of an infected plant cell, replication takes place. First, a double-stranded circular intermediate is created from single-stranded circular DNA. In this step, the viral genomic or plus-sense DNA strand is used as a template by cellular DNA repair enzymes to create a complementary negative-sense strand. The viral Rep protein cleaves the viral strand at a precise location located within the origin of replication in the following phase, the rolling circle phase, to begin replication.[4]

Although double-stranded unit circles can be isolated from infected plants and cells, this process in a eukaryotic nucleus can give rise to concatemeric double-stranded forms of replicative intermediate genomes.[4]

2. MATERIALS AND METHODS

2.1 Sample collection

For the collection of the sample we visited the field of "Jahangeerabad, Kursi Road Uttar Pradesh." It was the month of January and it was the time when the chili is grown largely. During this process, the first thing was to understand the symptoms that were shown by the infected chilly plant. During the collection, those plants that seem to be a dwarf and had curled leaves were collected as the

infected chili leaf, and those that have good growth and healthy leaves were collected for the healthy samples. The leaves were cut using scissors and were kept in different zip bags that were labeled before as healthy and unhealthy.

2.2 Processing of sample

After the collection, the sample was brought into the laboratory, and its storage was done for further use. The leaves were first removed and a 0.1% bleaching powder was made which is mixed with 500ml of double distilled water. leaves were first washed with tap water for the removal of dust, then soaked in 0.1% bleaching powder for exactly 1 minute. This is done to remove the fungus and other bacteria from the surface of the leaves. After 1 minute of soaking it in a bleaching powder, the leaves were strained and washed again with the distilled water twice to thrice times. Then the leaves were dried using the blotting paper packed in a zip lock bag and marked as healthy and infected. These zip lock bags containing the sample were now stored at -20 degrees Celsius for further use.

2.3 DNA isolation from healthy and infected Chilly plant leaves.

An important attribute of the cationic detergent CTAB is its ability to precipitate nucleic acids. The C.T.A.B buffer is used to keep the DNA's surroundings from degrading while also assisting in the separation of the DNA from other cell constituents. Tris keeps its pH steady. The metal ion chelator EDTA. A reducing chemical called beta mercapto ethanol guards the DNA from quinotiesdisulfide peroxide and polyphenol oxidase. The sodium ions in sodium chloride prevent the DNA phosphates that carry negative charge from binding to them. DNA molecules repel one another because of their negative charges. The phosphates carry a negative charge on the DNA and will create an ionic connection with the sodium ions, balancing the charges and enabling them to approach. Materials that were used during the process were a mortar pestle, beaker, double distilled water, micropipette, tip, tissue paper roll and discarded, gloves, aluminum foil, and Eppendorf tubes.

2.4 Initial preparation of the experiment.

1. The mortar and pestle were chilled at -20 degrees Celsius and left overnight.
2. The water bath was set to 65˚C and 2XCTAB was preheated to use.
3. Beta - mercaptoethanol was added to CTAB initially before use, for 1ml of CTAB 2 microlitre of beta–mercaptoethanol was added.
4. 70 % ethyl alcohol was made by adding 70 ml of ethyl alcohol to 30 ml of distilled water.
5. 1:24 ratio was taken for isoamyl alcohol and chloroform. To prepare this 1 ml of isoamyl was added to 24 ml of chloroform.

3. RESULTS

3.1 Results For Nanodrop Spectrophotometer Readings For Healthy And Unhealthy Chilli Leaves Samples.

3.1.1 Healthy chili leaves

Table 1: the result of nanodrop spectrophotometer for healthy chili leaf.

Absorbance

Sample no.	230nm	260nm	280nm	260/280nm	260/230nm	Conc (mg/ml)
1.	31.804	60.427	28.076	2.15	1.90	3021.4
2.	31.442	59.886	27.449	2.18	1.90	2994.3
3.	29.102	53.891	24.691	2.18	1.85	2694.5
4.	30.326	57.325	26.411	2.17	1.89	2866.2
5.	26.335	49.353	22.365	2.21	1.87	2467.6
6.	30.584	56.905	26.185	2.17	1.86	2845.3

3.1.2 Unhealthy Chilli Sample:

The concentration of the unhealthy chili DNA was measured through a nanodrop Spectrophotometer.

Table 2: Result of nanodrop spectrophotometer for unhealthy chilly leaf.

Absorbance

Sample no.	230nm	260nm	280nm	260/280nm	260/230nm	Conc (mg/ml)
1.	31.842	67.291	33.629	2.00	2.11	3364.5
2.	33.037	69.339	34.577	2.01	2.10	3467.0

3.	33.153	64.310	31.843	2.02	1.94	3215.5
4.	30.322	63.382	31.327	2.02	2.09	3169.1
5.	33.268	69.029	34.278	2.01	2.07	3451.5
6.	31.037	64.460	31.881	2.02	2.08	3223.0

3.2 Agarose Gel Electrophoresis of DNA.

Agarose gel electrophoresis is a process that separates the DNA or RNA according to its mass charge per ratio, in this the DNA that is lighter in molecular mass and size migrates towards the positive side in very less time but the larger size DNA molecule has a higher molecular weight and does not travel very fast and thus this molecule is found in or near to the well. The gel that is formed by the agarose is a mesh-like structure and which allows the smaller molecule to pass faster than the larger molecule due to the friction.

This process also allows us to visualize the bands according to their molecular mass just by loading a molecular ladder which is the standard DNA according to its known molecular masses. The ladder is also applied to check the gel function.

3.2.1 Agarose gel electrophoresis procedure :

- Agarose, was weighed 160 mg (0.16 gram) and dissolved 20 ml of 1X TAE buffer.
- Agarose was dissolved using a hot plate.
- Then added 1 microlitre of ETBR in it.
- Now the solution was poured into the gel casting tray from one corner very carefully and slowly so that there is no formation of bubbles in the gel, and then the comb was placed gently.
- Now the solution is left for solidification at least for 30 minutes.
- The comb was then removed with care so that the wells formed by the comb were not ripped.
- Then a paraffin wax strip was placed and 2 microlitres of bromophenol dye was taken and mixed with a 10 microlitre DNA sample.
- Now the gel chamber is kept properly and the electrode is connected to the power supply and then the supply was turned on.
- The voltage was set to 65 volts for 15 minutes and 90 volts for 45 minutes.
- Check the power supply readings and allow the gel to run until the bromophenol dye reaches 1/3rd of the total length of the gel.
- After this switch the power supply off.
- To visualize the DNA, the gel is placed on a UV trans-illuminator for the visualization of the bands.

3.2.2 Results For Agarose Gel Electrophoresis :

The following observations were made during the qualitative analysis through agarose gel electrophoresis and visualization of the band through a UV transilluminator.

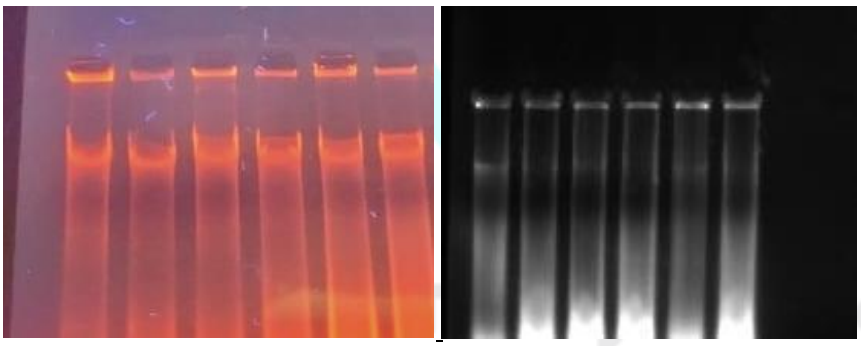

FIG 1: A clear set of bands was visualized in every lane of the well, under the UV-transilluminator for healthy chili leaves sample and the gel documentation of the result.

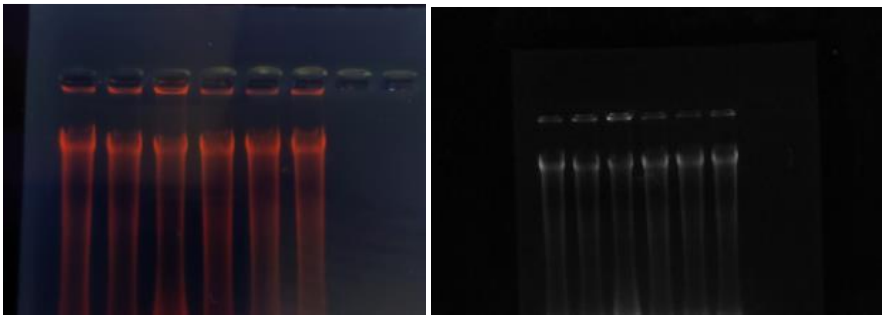

FIG 2: A very light band was visualized in every lane of the well, under the UV-transilluminator for unhealthy chili leaves sample and the gel documentation result.

3.3 Chili leaf Protein estimation and isolation using the Lowry method.

One of the most popular colorimetric tests that are used by the biochemists is Lowry test for protein estimation and isolation. In this process, a two-color is formed, which makes this procedure very delicate. It makes use of the Biuret reaction, in which the peptide bond combines with copper ion in the presence of a base to produce a deep blue color. A complex mixture of inorganic salts which reacts with tyrosine and tryptophan residues in Folin-Ciocalteu chemistry, results in vivid blue-green color.

3.3.1 Analytical reagents.

REAGENT - A: 2 grams of sodium carbonate was dissolved in 100 ml of distilled water to make a 2 % sodium carbonate solution and 0.4 grams of sodium hydroxide was dissolved in 100 ml of distilled water to make a 0.1 normality sodium hydroxide solution.
REAGENT B: To make 1 % of the Sodium-Potassium-tartrate solution 1 gram is dissolved in 100 ml of distilled water.
REAGENT C: to make 0.5 % of copper sulfate solution, 0.05 grams is dissolved in 10 ml of distilled water.
REAGENT D: this reagent is prepared freshly while experimenting, for this 2 ml of each of reagents B and C is added to 96 ml of reagent A.
REAGENT E: reagent E is a folic ciocalteau solution, the commercial solution is of 2 normality. To prepare this reagent an equal volume of it is added to an equal volume of distilled water.

3.3.2 PROCEDURE ADOPTED FOR THE ISOLATION OF PROTEIN

- Fresh leaves were collected from the field and preserved at -20 degree Celsius.
- These leaves were soaked in the blotting paper properly before use.
- Remove the midrib ad veins from the leaf.
- Then weigh 1 gm of the leaf.
- The leaf was put on the mortar and pestle and crushed the leaves in 10ml sodium phosphate buffer.
- Then the homogenate was filtered with a muslin cloth.
- Now I have taken 1ml of homogenate in each Eppendorf tube.
- Now the sample was centrifuged at 5000rpm for 10 minutes.
- Supernatant was taken for protein precipitation.
- And 10 % T.C.A with exactly the equal volume of sample (1ratio1) was added for the protein precipitation, and leave the solution for 30 minutes.
- Then the samples were centrifuged at 5000rpm for 5 min to obtain protein pallet.
- Now, the pellet is washed three times with acetone to remove chlorophyll along with it was centrifuged for 5 minutes at 5000rpm.
- The pellet was dissolved in 0.1 normality sodium hydroxide solution.
- Now the next step is to quantify or estimate the protein using the Lowrys method.

3.3.3 PROCEDURE FOR ESTIMATING THE PROTEIN:

- I have a set of 14 tubes, where 7 tubes were for the standard solution and 7 for the sample solution.
- B.S.A Solution was made by adding 25mg B.S.A in distilled water 25 ml.
- Now from BSA and protein solutions, I have pipette out .1 ml, .2 ml, .4 ml, .6 ml, .8 ml, and 1 ml protein solution to all the seven tubes, and one blank was prepared.
- Now I have added 5ml of Reagent D and mixed it well.
- Now the solution is left for 10 minutes at room temperature for the incubation.
- I have now added 0.5 ml of Reagent E to all the tubes and vortexed each tube immediately for proper mixing.
- now tubes are incubated at room temperature for at least 30 minutes in the dark.
- Now I have determined the optical density of each sample at 660 nm using a double beam spectrophotometer.

3.3.4 OD AND CONCENTRATION OBSERVED THROUGH DOUBLE BEAM SPECTROPHOTOMETER.

BSA standard solution (table 4):

S.no	B.S.A conc (mg/ml)	water (ml)	Alkaline copper sulfate (ml)	lowry reagent (ml)	Optical density (660 nm)
blank	0.0	1.0	5.0	0.5	0.0000
1.	0.1	0.9	5.0	0.5	0.3118
2.	0.2	0.8	5.0	0.5	0.5101
3.	0.4	0.6	5.0	0.5	0.8527
4.	0.6	0.4	5.0	0.5	1.1625
5.	0.8	0.2	5.0	0.5	1.3640
6.	1.0	0.0	5.0	0.5	1.6154

Healthy chili leaf protein (table 5):

S.no	Infected leaf protein (ml)	water (ml)	Alkaline copper sulphate(ml)	Folin calteau reagent (ml)	Optical density (660 nm)	Protein concentration. (mg/ml)
blank	0.0	1.0	5.0	0.5	0.0000	0.0000
1.	0.1	0.9	5.0	0.5	0.2193	0.2193
2.	0.2	0.8	5.0	0.5	0.3351	0.3351
3.	0.4	0.6	5.0	0.5	0.5904	0.5904
4.	0.6	0.4	5.0	0.5	0.7955	0.7955
5.	0.8	0.2	5.0	0.5	0.9216	0.9216
6.	1.0	0.0	5.0	0.5	1.0830	1.083

3.3.5 OD AND CONCENTRATION OBSERVED THROUGH DOUBLE BEAM SPECTROPHOTOMETER.

BSA standard solution (table 6):

S.no	B.S.A concentration (Mg/ml)	water (ML)	Alkaline copper sulfate (Ml)	Folin plateau reagent (ML)	Optical density (660 nm)
blank	0.0	1.0	5.0	0.5	0.0000
1.	0.1	0.9	5.0	0.5	0.3118
2.	0.2	0.8	5.0	0.5	0.5101
3.	0.4	0.6	5.0	0.5	0.8527
4.	0.6	0.4	5.0	0.5	1.1625
5.	0.8	0.2	5.0	0.5	1.3640
6.	1.0	0.0	5.0	0.5	1.6154

Infected chili leaf protein (table 7):

S.no	Infected leaf protein (ML)	water (ML)	Alkaline copper sulfate (ML)	Folin calteau reagent (ML)	Optical density (660 nm)	Protein concentration. (mg/ml)
blank	0.0	1.0	5.0	0.5	0.0000	0.0000
1.	0.1	0.9	5.0	0.5	0.1603	0.0977
2.	0.2	0.8	5.0	0.5	0.2601	0.1586
3.	0.4	0.6	5.0	0.5	0.4319	0.2634
4.	0.6	0.4	5.0	0.5	0.6217	0.3791
5.	0.8	0.2	5.0	0.5	0.7448	0.4542
6.	1.0	0.0	5.0	0.5	0.8094	0.4935

3.3.6 GRAPHICAL REPRESENTATION OF PROTEIN CONCENTRATION VS ABSORBANCE.

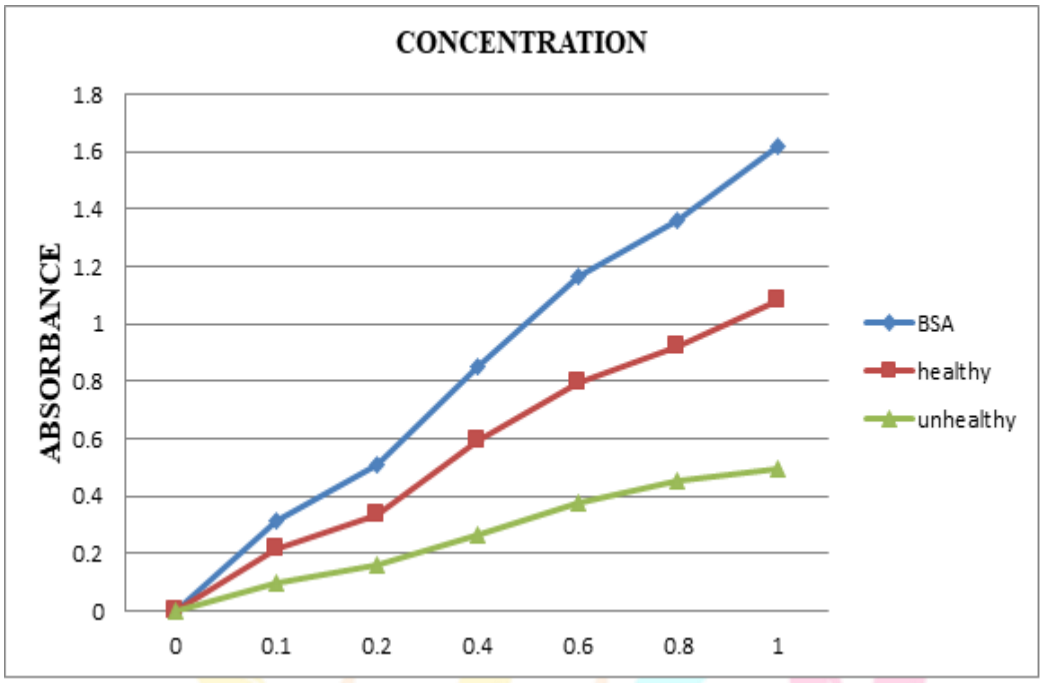

FIG 8: Graphical representation of absorbance and concentration of BSA, healthy and unhealthy chili leaf protein sample through double beam spectrophotometer.

4 VIRAL GENOME AMPLIFICATION

4.1 PCR (POLYMERASE CHAIN REACTION) USING DEGENERATIVE PRIMER

PCR is one of the potent methods for amplifying DNA sequences using the idea of enzymatic replication of the nucleotides. This technique is quick and easy to understand and confirms the genetic test for the viruses. The three steps of PCR are typically repeated 39 times. The quantity of synthesized DNA is doubled after each cycle of three stages, continuing until all of the reaction's components have been consumed. The PCRs are heated and cooled repeatedly using thermal cycler equipment. In several scientific fields, including virology, the polymerase chain reaction is the new benchmark in identifying a wide range of templates. The technique uses two artificial oligonucleotides or primers, each of which hybridizes to create a single strand of complementary target DNA and this part is multiplied many times. A Taq polymerase uses the hybridized primers to make a complementary strand by adding deoxynucleotide sequence-wise. A programmed thermal cycle regulates the pace of temperature change, the time taken for incubating at, each temperature, and the number of times each cycle is performed. Following agarose gel electrophoresis can be performed to separate the amplified DNA fragment, the bands are visible by Ethidium Bromide when exposed to UV lights the DNA appears orange colored. Certain sets of primers, specific primers, and random primers are being used to amplify different target DNA, they vary in annealing temperature.

4.1.1 FOR PCR AMPLIFICATION

working concentration **(1x)**

FOR 50 ML OF VOLUME IN MASTERMIX. (Table 8)

nuclease-free water.	39.5 µl
Taq Buffer.	5 µl
dNTP mix.	1 µl
Forward Primer.	2 µl
Reverse Primer.	2 µl
Taq DNA polymerase.	0.5 µl
Template.	2.5 µl

- A master mix was prepared to add nuclease-free water, taq buffer,dNTP mix, forward and reverse primer and Taq DNA polymerase, and a total volume of 50 µl was made.
- Now as the master mix has a total volume of 50 µl, for setting up 4 tubes of PCR, I have transferred 12.5 µl of master mix to each PCR tube using the micropipette.
- And then added 2.5 µl of template DNA that I isolated to the master mix of each tube.
- Now each PCR tube contains a total volume of 15 µl each.

4.1.2 PROCEDURE :

This mixture was now spun down for 30 sec at 6000 rpm in the cold centrifuge of 4'C. Now add 50 microlitres of mineral oil on top of the mixture, this can be avoided if we have a manual PCR machine where we can adjust the temperature of the lid. Before proceeding with the experiment the protocol was set in the PCR machine. To set up the protocol in the PCR machine we need to go to the nucleic acid section and that edit protocol to set the new protocol, the denaturation, annealing, and elongation temperature and time in the given format suitable for the sample and the primer, enzymes, and reagents used.

4.1.3 PROTOCOL SET ON THE PCR (for degenerative primer)

- PRIMER USED **:** PALIVI979 (forward primer)

  PARIC715 (reverse primer)

- PRODUCT AMPLICON SIZE : 1.6KB

4.1.4 RESULT FOR PCR PRODUCT IN AGAROSE GEL.

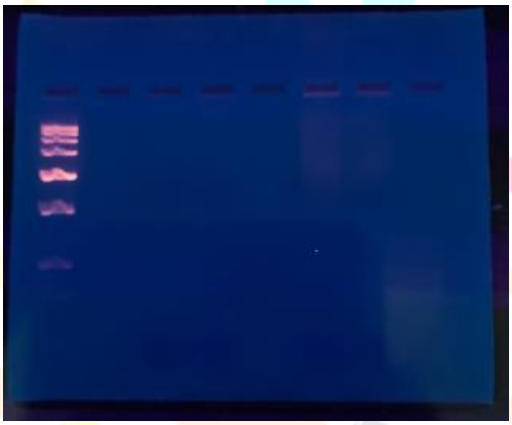

FIG 9: Final result of PCR product

Negative result: no amplification; Positive result: Positive amplification

4.1.5 OBSERVATION

A very light band was visualized in the lane 6 and 7 of the well with a very little amount of smearing of DNA, therefore, the result can be interpreted as the DNA is amplified and a band is found above the 1500 bp ladder that is the first band shown in the ladder, hence it can be said that the product size might be 1.6 KB.

5. DISCUSSION

The Begomovirus is the main cause of disease in agricultural plants and its species cause a wide variety of agricultural significant plants infection moreover in every part of the world. According to a few sources, the virus infection causes a 60% to 100% of the total decrease in yield. This is a serious threat to the farmers and this should be a global motive to discover some virus-free plant or seed. In this process, the white fly known as Bemisia Tabaci is the vector that carries these viruses and spreads over every plant. Therefore, understanding the disease symptoms and its infection-causing agent increased my interest in my chosen topic. As per my topic, the chili plant that I have chosen is of great use and has very good nutritional value in it.

The viral genome was amplified using degenerative primer, as a degenerative primer is a set of primers that are used majorly in the amplification of Begomovirus genetics. material and also its amplification is shown via Agarose gel electrophoresis, this has shown a positive result by showing two visible light bands, as the expected size of the product was 1.6 kb and the bands shown were in the well, therefore disease was confirmed as per the ladder that was applied.

Apart from the viral DNA estimation, also the concentration of DNA was decreased in the infected, as a comparison to the healthy ones, and the protein that was estimated both from the healthy and infected leaves of chili plant was shown to have a decrease in the

percentage of protein in infected ones, which simply signifies that the infection might have led to the decrease in the protein concentration in chili plant.

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