

Curvularialunata - a foliar pathogen of Musa paradisiaca causing leaf spot disease and GC MS analysis of toxic metabolites

Papan Chowhan

Raiganj University

Arka Pratim Chakraborty

arka.botanyrgu@gmail.com

Raiganj University

Research Article

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Abstract

In the Uttar Dinajpur District of West Bengal, between December 2022 and January 2023, field-grown banana trees were found to have leaf spot disease. Initially, the symptoms showed up as yellow-brown dots on the periphery and center of the leaves. Lesions might be small and dispersed throughout leaves, or they can cover huge areas of leaves thoroughly. From the samples of diseased leaves that were gathered, one fungal infection was recovered. A TEM picture of the leaves was taken to compare the interior structures of healthy banana plant leaves with those of banana leaves affected with the fungal infection *C. lunata*. Chloroplast and vacuole structures changed, and certain balloon-shaped structures were discovered that may have been caused by fungal isolate infection in leaves. To find the hazardous compounds that the organism released, GC-MS analysis was performed on the *C. lunata* culture filtrate. The culture filtrate of *C. lunata* was found to contain a number of toxic compounds, including hexadecanoic acid, benzonitrile, 1- 15-hydroxypentadecanoic acid, 2, 3, 3, 3-pentafluoropropanoate, 5-tetradecene (E), 2-Dodecenc (Z), 2,4-D1-tert-butylphenol, 9-Octadecene (E), and 9-Eicosene (E), among others. The production of toxic chemicals by fungal pathogens may facilitate the diseases' ability to infect healthy plant sections and cause disease. The present study focuses on giving an idea of the most probable toxic metabolites responsible for the pathogenicity of the fungus *Curvularialunata*.

Introduction

The most widely eaten tropical fruit in world is banana (*Musa spp.*) (10). It has a lot of nutrients. In addition to its fruit, other plant parts such as the leaves, stem, and flowers can be used for cooking, eating, textiles, veggies, and a variety of other purposes. Banana plant also has cultural, religious significance. World largest blooming herbaceous plant is banana, serves both as a household's source of income and a staple aliment in many countries. They are growing in 135 different countries, primarily for its fruit. Nowadays, *Musa acuminata* and *Musa balbisiana*, two wild banana species, are the source of nearly all edible seedless bananas. Between December 2021 and January 2022, *Curvularialunata* was found to cause leaf spot of banana trees grown Uttar Dinajpur District, West Bengal. In January and December, the temperature varied from 9.0 to 24.0°C and 10.3 to 17.8°C, respectively. On the middle portion and around the leaf margins, the symptoms first showed up as yellowish brown patches. Lesions could have a yellow halo around them and frequently had a brown border. The symptoms varied from a few spots on leaves to lesions that were heavily distributed over large areas of leaves.

Materials and methods

Isolation of fungal pathogen

The diseased leaf sample, which was cut into slice between 1 to 1.5 centimeters in size, was surface sterilised with 0.1% HgCl₂ for two minutes, then ethanol for two minutes and then put on PDA medium. A fungus was isolated from affected leaf on PDA (Potato Dextrose Agar) treated with 5 ml of antibiotic Monocef-O 100 (5 ml of reconstituted solution contain cefpodoxime proxetil IP equal to 100 mg of cefpodoxime) after incubation period of 96 hrs. at 26°C. 18S rDNA partial sequencing was used to identify the fungus.

GC-MS analysis of culture filtrate

The GCMS study was done by Environcheck in West Bengal. The two solvents used in the GCMS analysis were hexane and chloroform. A 10 ml amber vial was filled with a 2 ml sample of sterile culture filtrate was obtained after centrifuging the fungal broth culture in PDB (potato dextrose broth) media was taken. The material then was combined with two milliliters of hexane/chloroform. After then, the mixture was extracted until it was clear that the two layers were distinct. The hexane/ethylacetate layer (1:1) was pipetted after the anhydrous sodium sulphate layer used as inert drying

agent, helping to remove water from organic solutions. The hexane and ethylacetate layer (1:1) were filtered using a syringe filter before being subjected to GCMS. To find the toxic metabolites, the AGILENT 7890B System was utilized for gas chromatography mass spectrometry (GCMS) study. The initial temperature (hold time zero) was 75°C, and the input temperature was 225°C. The temperature ranges for Ramps 1 and 2 were 25 °C/min to 150 °C and 10 °C/min to 280 °C (Hold time 10 min), respectively.

Transmission Electron Microscope (TEM)

To find out the changes between the internal structures of leaves of healthy banana plant and banana leaves infected with fungal pathogen- *Curvularia* sp. The healthy leaf samples as well as infected banana leaves with fungal pathogens were sent to AIIMS New Delhi after fixation in 2.5% of glutaraldehyde solution. CRYO-TEM (TALOS S) Microscope from the company THERMO SCIENTIFIC was used for the transmission electron microscopic images. Samples of both healthy and diseased leaves (1–2 mm) washed using 0.1M pH 7.4 sodium phosphate buffer and shifted right away to eppendorf tubes with 2.5% glutaraldehyde for two to twelve hours at room temperature. In addition to two changes in absolute alcohol at one-hour intervals at 4° C, dehydration was carried out in progressive grades of alcohol (30%, 50%, 70%, 80%, and 90%) at 30-minute intervals. Two infiltrations in LR White resin (London Redin Co. Ltd.) were carried out in absolute alcohol (1:1) at 4° C for one hour each. Following their dip in LR White, the samples were kept overnight at 4° C. They were kept at 27°C for three hours. A fresh batch of LR white was prepared and kept at 56° C for 36 hours. The sample moulds were roughly trimmed using a block trimmer (Reichert TM 60) fitted with a revolving milling cutter. Several thick slices were cut using Belgium glass strips in a microtome (Leica EM UC7) so that the selected blocks could be seen under an optical microscope.

Tissue fixation for electron microscopy

Perfused the tissue with 0.9% saline for 10 minutes and later fix with 4% paraformaldehyde for 20 minutes. Dissected the leaf tissue samples into 1–2 mm size for TEM (a must for proper fixation to occur). Fixed samples in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, first at room temperature for 20 minutes and later at 40°C for 4–6 hours.

Preparation of fixative for Transmission Electron Microscopy

Preparation of 0.2 M phosphate buffer (PB; pH 7.4)

Two solution was used for the making of phosphate buffer, Sol A: Sodium dihydrogen phosphate dihydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; MW 156)- 3.12 gm /100 ml distilled water and Sol B: Disodium hydrogen phosphate anhydrous (Na_2HPO_4 ; MW 142)— 2.84 gm/100 ml in distilled water. Mixed 19 ml of soln A with 81 ml of soln B, final pH is adjusted with HCl or NaOH.

Preparation of Paraformaldehyde solution

It is prepared in 100 ml distilled water by dissolving 4 grams paraformaldehyde powder (EM grade) at 60°C (do not boil). Added few drops 0.1N sodium hydroxide while dissolving. Warmed the solution for 5–6 min, when paraformaldehyde will start dissolving out. The solution is cooled at room temp and filtered. It must be a clear solution.

A. Added equal volume (100 ml) of 0.2 M PB to the paraformaldehyde solution to make it a 2% solution in 0.1 M PB (the molarity of PB is now reduced to 0.1 M).

B. Added the required quantity of glutaraldehyde (from the concentrated 25% stock solution, EM grade) in the final paraformaldehyde solution. If a milky precipitate occurs, it indicates impurity of the glutaraldehyde stock solution. For any convenience, added 10 ml glutaraldehyde (from 25% stock solution) to 90 ml of 2% paraformaldehyde solution. The final concentration of the fixative is 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB. It is a clear solution with having pH 7.4.

C. Washed in the buffer for 3 times, each for one-hour duration at 40°C.

D. For transportation, samples kept inside a thermos flask using ice cool pack separated by a thin barrier.

Experimental protocol for SDS page

Pour polyacrylamide gel

First the gel sandwich was put together. Plates, spacers, and combs cleaned thoroughly and rinsed with 70% ethanol. Then the glass plate sandwich was put together. Stood upright after sliding the plate assembly into the plate clamp assembly. The assembly was sealed when the plate clamp was carefully pushed up until the glass plate sandwich was 1 to 2 mm below the clamp's edge. As a result, the sandwich won't leak the unpolymerized resolving gel solution. The whole assembly then put in the gel casting stand.

Pour separating gel (5–12%)

For protein analysis, a 12% resolving gel was used. Smaller proteins and peptides require higher acrylamide percentages (> 15%), while larger proteins require lower gel percentages (< 10%). 100 µl of 10% APS (up to 10 µl TEMED if the gel percent was less than 8%) and 5 µl of TEMED were added. Carefully the tube inverted to thoroughly mix recalling that the polymerisation reaction was inhibited by oxygen. Oxygen was present in bubbles. The gel sandwich was gently covered with 70% ethanol and 0.01% SDS after the solution was put into it until the proper level (5.5–6.5 cm) was attained using a Pasteur pipette (running it down a side spacer). At room temperature, the gel polymerise after 30 to 60 minutes. The gels can be taken out of the clamps, covered with resolving gel buffer (it can be kept at 4°C for two to three days if they won't be run that day).

Pour stacking gel (4%)

Rinsed with ddH₂O after removing the separating gel's aqueous layer. The ingredients were mixed for the stacking gel. Covered the separating gel with the stacking solution. To prevent bubbles from forming on the ends of the teeth, carefully inserted the comb into the stacking gel. Gave the gel 30 to 60 minutes to polymerise.

Clamp gel onto electrophoresis tank

The comb and binding clips were taken out of the gel. The glass plate and gel were encased within the electrophoresis core. The short glass plate was either within core or facing center. Placed the buffer dam on the other side of the core; or placed glass plate sandwich on the other side. The operational tank was filled with the core component. 1X electrophoresis buffer was applied to core. Buffer must be included to top of the assembly. Lastly, 1 to 2 inches of 1X electrophoresis buffer were added to the operating tank. The buffer-filled wells were washed before the sample was loaded.

Prepare samples

At room temperature, protein samples were rapidly thawed in a water bath. Protein samples were cooked in a 95°C dry bath for 5–10 minutes after 1/5 volume 6X Sample buffer was added. For five to six minutes, the samples were spun down in a microfuge.

Separate protein samples by PAGE

The wells were filled with samples using a 200 μ L pipet tip carefully not to push the tip of the glass plate too deep into the well, as this could cause it to separate. Typically, each well can hold 25 μ L of sample. 1X Sample dye was poured into the empty wells. In order for proteins to move towards the anode (+) electrodes were affixed. Run the gel at 100 and 200 V until the dye front reaches gel's bottom, running time was influenced by the gel and buffer composition's % cross linking.

Protein bands on a stain gel

After removing the gel sandwich from the tank, the electrodes were detached, and the electrophoresis buffer was drained. After removing the side spacers, pulled apart the plates to make sure gel remains on one. The staining solution was poured into pipet tip box. Invert the gel-containing plate into staining solution, then gently let the gel to "float" off the plate into it. Covered the gel with plastic wrapped, carefully agitate it on the gel rocker for 15 to 30 minutes. The greater the time, the more sensitive it will be, but the longer the staining period as well. To get rid of extra stain, the gel rinsed with H_2O after removing the staining solution, which could be used repeatedly. Putting a stream of water on the gel itself was avoided. Filled the pipet tip box with water, then gently rinsed the gel surface. For ten to fifteen minutes, added the destaining solution and shake the gel rocker. the destaining solution was altered and stir until the desired degree of destaining was reached.

Result and Discussion

The 18S rDNA fragment sequence of the fungal pathogen strain MLP-01 was uploaded to GenBank and assigned the accession number ON246070. Both the Agharkar Research Institute in Pune, India (accession number: NFCCI 5361) and the institute's NAIMCC culture collection division (accession number: NAIMCC-F-04338) recognized *Curvularia lunata* as the fungus based on its morphology (Fig. 1). Using cotton blue staining and a light microscope, the mycelial invasion of the pathogen in the infected leaf was verified. Using scanning electron microscopy, the surface morphology of both healthy and *C. lunata*-inoculated leaves was examined. SDS-PAGE testing revealed differences in the protein band pattern between healthy and *C. lunata*-infected leaves, treated or infected leaf showed higher intensity of protein bands compared to healthy leaves with the size of the proteins being between 40 and 50 KDa (Fig. 5). A number of toxic compounds produced by *Curvularia* sp. were found in the culture filtrate, including benzonitrile, hexadecanoic acid, 1-hydroxypentadecanoic acid, 2,3,3,3-pentafluoropropanoate, 5-tetradecene (E), 2-Dodecenc (Z), 2,4-D1-tert-butylphenol, 9-octadecene (E), and 9-Eicosene (E), among others (Fig. 2, Fig. 3).

These toxic compounds, released by fungal pathogens may help the pathogen to establish disease in healthy plant parts (Tables 1 and 2). TEM images of the leaves were recorded (Fig. 4). There were changes in the structures of chloroplast, vacuoles and some balloon shaped structures were found that might be due to infection by the fungal isolates in leaves.

Conclusion

The structural changes in banana leaves that have been found and the presence of poisonous chemicals generated by *C. lunata* indicate that these variables contribute to the pathogen's capacity to infect and damage the plant. The disease may have a more severe effect on healthy banana leaves due to the changed structure of cells and harmful toxic compounds. By comprehending these relationships, focused management plans to prevent fungal infections in banana plants and lessen the effects of leaf spot diseases in the area can be developed.

Declarations

Acknowledgments

We acknowledge The Agharkar Research Institute is located in Pune, Maharashtra, India. For morphological identification of the fungal pathogen and light microscopic images of the fungus *Curvularialunata*. We also like to acknowledge Sophisticated Analytical Instrument Facility (SAIF) AIIMS, Delhi, India, for taking the transmission electron microscopic images, Edison Life Science, Kolkata, India, for molecularly identifying the fungal isolate and ENVIROCHECK, Kolkata, India for GC-MS analysis.

Data Availability Statement

This research work did not involve in the generation or analysis of any kind of separate datasets.

Ethical approval

The leaf samples used in the present study were procured from Ramganj, Uttar Dinajpur, between November 2022 and January 2023. samples are collected during survey programs of Banana cultivated fields in the local area, permitted and approved by the university and samples are documented properly. As an Assistant Professor of the Department of Botany of Raiganj University, the Corresponding author was fully authorized to collect, conserve, and study the local crop plant diseases and research the causal organisms. No human or animal was harmed by the participants in the research that was presented. The collection of the plants used in the study complies with local or national guidelines with no need for further affirmation.

Funding

No funding support was taken.

Informed consent

No informed consent was required for this publication.

Competing Interest declaration

There are no Competing Interests

Consent of publication

I, Papan Chowhan performed the experiment and surveyed the cultivated fields. The photos belong to me. I have no objection if any image is used for publication in purposes of this article

Clinical Trial

No clinical trial was performed during the study.

References

1. Adebisi O, Dolma SK, Verma PK, Singh B, Reddy SE (2019a) Volatile, non-volatile composition and insecticidal activity of *Eupatorium adenophorum* Spreng against diamondback moth, *Plutellaxylostella* (L.), and aphid, *Aphis craccivora* Koch. *Toxin reviews* 38(2): 143-150
2. Adebisi O, Dolma SK, Verma PK, Singh B, Reddy SE (2019b) Volatile, nonvolatile composition and biological activities of *Ageratum houstonianum* Mill. against diamondback moth, *Plutellaxylostella* (L.) and aphid, *Aphis craccivora* Koch. *Indian J. Exp. Biol.* 57: 908-915
3. Adesanwo JK, Egbomeade CO, Moronkola DO, Akinpelu DA (2019) Chemical, toxicity and antibacterial studies on methanol extracts of *Melanthera scandens*, *Ageratum conyzoides*, *Aspilia Africana* and *Synedrellanodiflora*. *Journal*

of Exploratory Research in Pharmacology 4(1): 1-7

4. Ali MS, Ravikumar S, Beula JM, Anuradha V, Yogananth N (2014) Insecticidal compounds from Rhizophoraceae mangrove plants for the management of dengue vector *Aedes aegypti*. *Journal of Vector Borne Diseases* 51(2): 106-114
5. Babarinde GO, Babarinde SA, Ojediran TK, Odewole AF, Odetunde DA, Bamido TS (2019) Chemical composition and toxicity of *Jatropha curcas* seed oil against *Sitophilus zeamais* Motschulsky as affected by pre-extraction treatment of seeds. *Biocatalysis and Agricultural Biotechnology* 21: 101333
6. Dehpour AA, Yousefian M, Jafary Kelarijani SA, Koshmoo M, Mirzanegad S, Mahdavi V, Javad Bayani MJ (2012) Antibacterial activity and composition of essential oils of flower *Allium rotundum*. *Adv Environ Biol*, 6(3):1020-1025
7. Ezeobiora CE, Igbokwe NH, Amin DH, Okpalanwa CF, Mota'a SC, Mendie UE (2023) Antibacterial Potential of Endophytic Fungi from *Xylopia aethiopica* and Metabolites Profiling of *Penicillium* sp. XAFac2 and *Aspergillus* sp. XAFac4 by GC-MS. *Tropical Journal of Natural Product Research*, 7(7)
8. Karanja LN, K'Owino IO, Wangila PT, Ramkat RC (2021) Phytochemical Composition and Antibacterial Activity of Fruit Extract of *Solanum incanum* L. against *Ralstonia solanacearum*. *Asian Journal of Applied Chemistry Research*, 9(4): 1-16
9. Kluchinsky Jr TA (2001) Identification of CS-derived compounds formed during heat dispersion of CS riot control agent and the temperature ranges associated with their formation. *Uniformed Services University of the Health Sciences*.
10. Mendoza AR, Sikora RA (2009) Biological control of *Radopholus similis* in banana by combined application of the mutualistic endophyte *Fusarium oxysporum* strain 162, the egg pathogen *Paecilomyces lilacinus* strain 251 and the antagonistic bacteria *Bacillus firmus*. *BioControl* 54(2):263-272
11. Nair RR, Gangaprasad A (2017) GC-MS analysis of methanolic stem extract of *Gynochthodes ridsdalei* Razafim. and B. Bremer, an endemic, endangered medicinal plant of southern Western Ghats. *Int J Curr Pharm Res* 9(3): 98-101
12. Odion EE, Ambe DA, Dunkwu J, Odiete EC (2023) PHYTOCHEMICAL ANALYSIS OF THE METHANOL LEAF EXTRACT OF *FICUS SUR FORSSK* (MORACEAE). *Journal of Pharmaceutical & Allied Sciences* 20(4)
13. Oyekunle DT (2017) Analysis of the chemical composition of the essential oil extracted from *Thevetia peruviana* seeds using gas chromatography analysis. *Am J Eng Res (AJER)*, 6(10): 51-55
14. Premalatha K, Vellaikumar S, Shanmugam PS, Harish S, Jayarajan Nelson S (2023) Laboratory Studies on Chromatographic Profile, Toxicity and Repellent Activity of Custard Apple (*Annona squamosa* L.) Seed Extract. *International Journal of Environment and Climate Change* 13(10): 2949-2955
15. Sivakumaran G, Prabhu K, Rao MR, Jones S, Sundaram RL, Ulhas VR., Shruthi Dinakar, Vijayalakshmi, N (2019) Gas chromatography-mass spectrometry analysis of one ayurvedic oil, anuthailam. *Drug Invent Today* 11: 2675-8
16. Tyagi T, Agarwal M (2017) Ethnomedicine analysis of bioactive constituents in ethanolic leaf extract of *Pistia stratiotes* L. and *Eichhornia crassipes* (Mart.) Solms by GC-MS. *Adv. Biores* 8: 204-211
17. Ugboogu EA, Akubugwo IE, Ude VC, Gilbert J, Ekeanyanwu B (2019) Toxicological evaluation of phytochemical characterized aqueous extract of wild dried *Lentinus squarrosulus* (Mont.) mushroom in rats. *Toxicological research* 35: 181-19

Table 1 and 2

Table 1 and 2 are available in the Supplementary Files section.

Figures

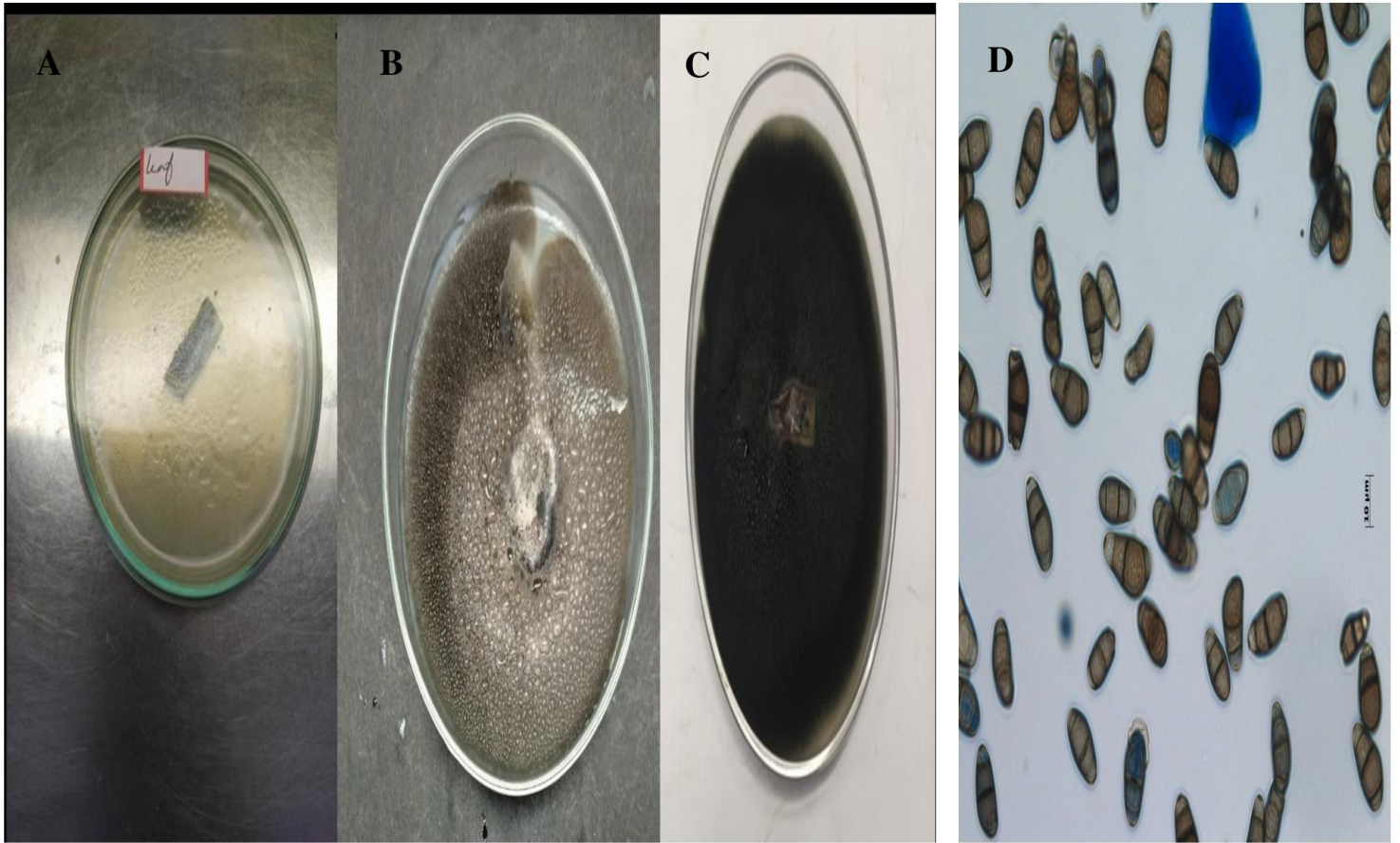


Figure 1

A: Isolation of fungal pathogen MLP-01 on PDA medium amended with an antibiotic Monosef, B: 7 days old culture of Pathogen, C: Mature sporulated culture of Pathogen , D: Observation of Curvularialunata spores under light microscope

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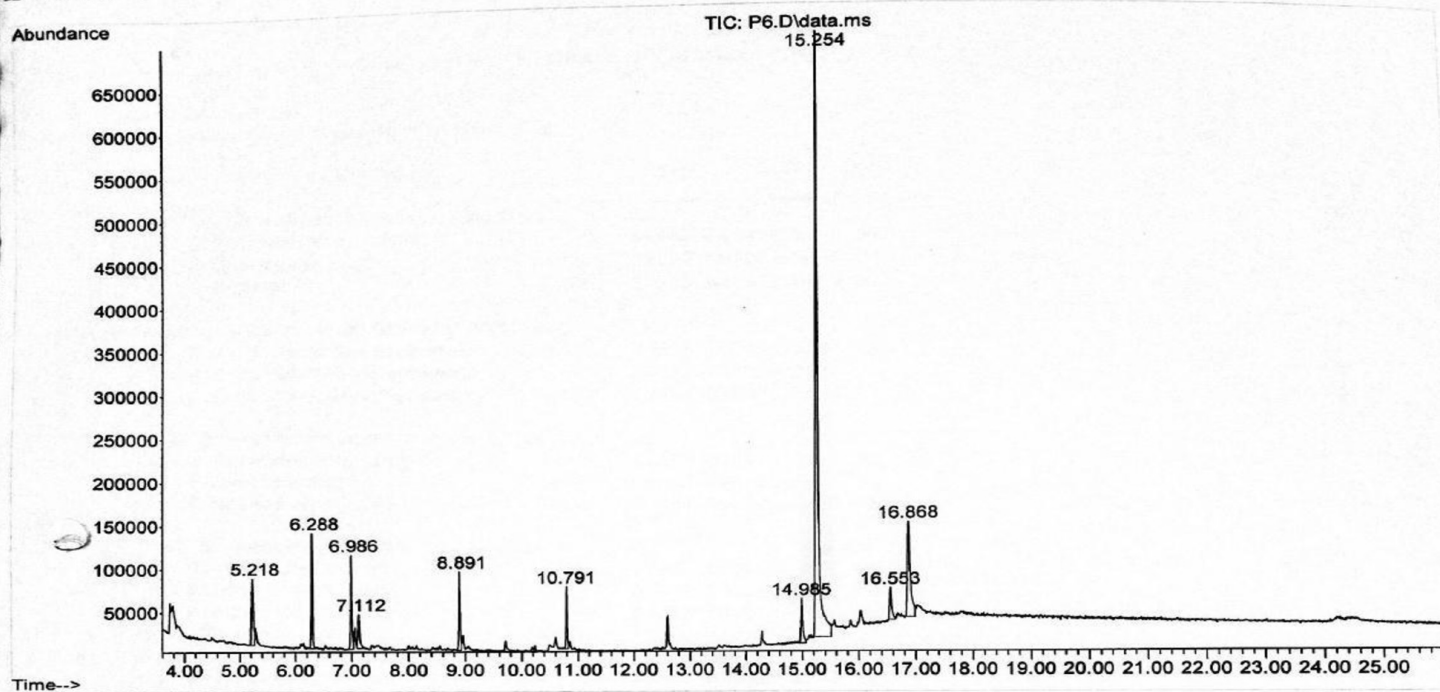


Figure 2

Chromatogram of GCMS analysis carried out the fungal broth culture by using Ethyl acetate solvent

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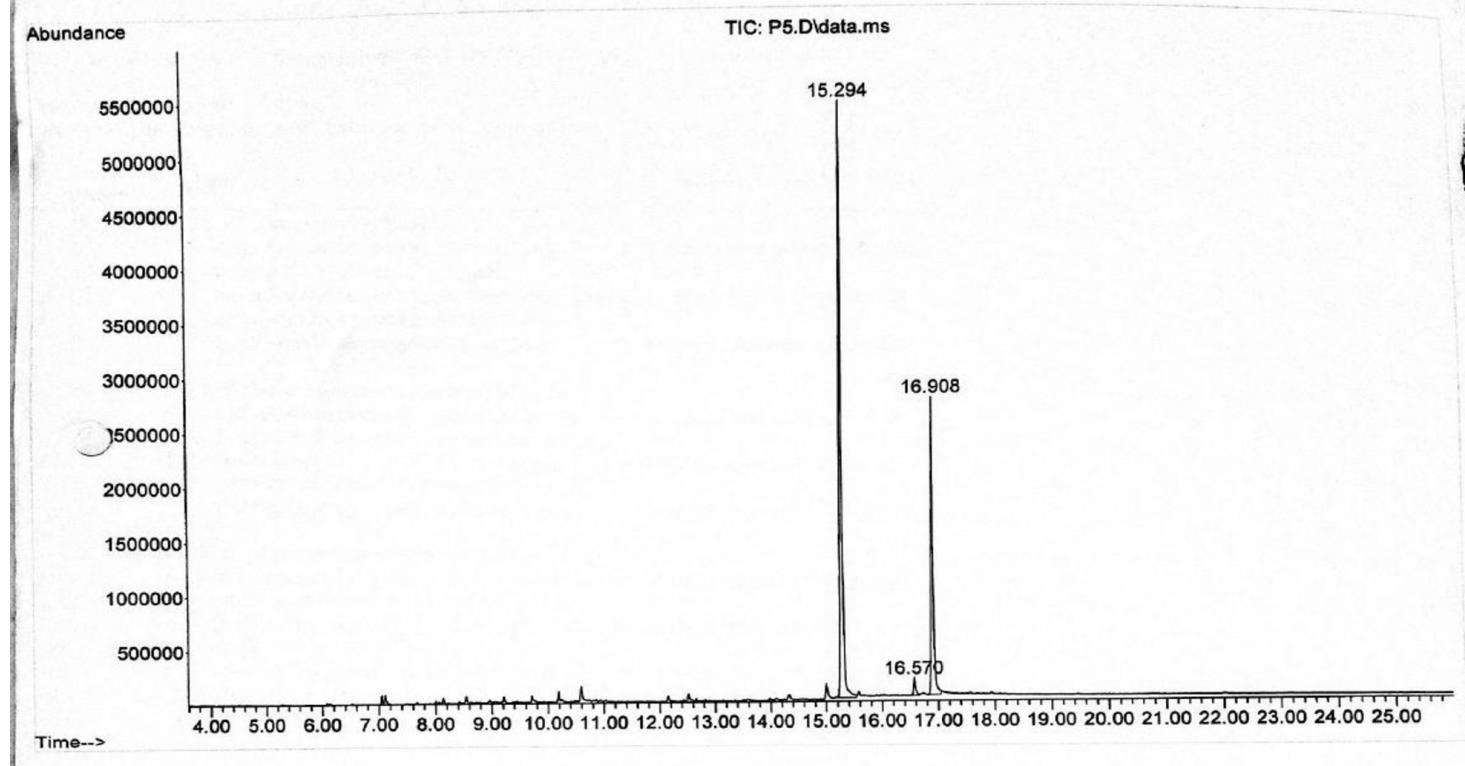


Figure 3

Chromatogram of GCMS analysis carried out the fungal broth culture by using Hexane solvent

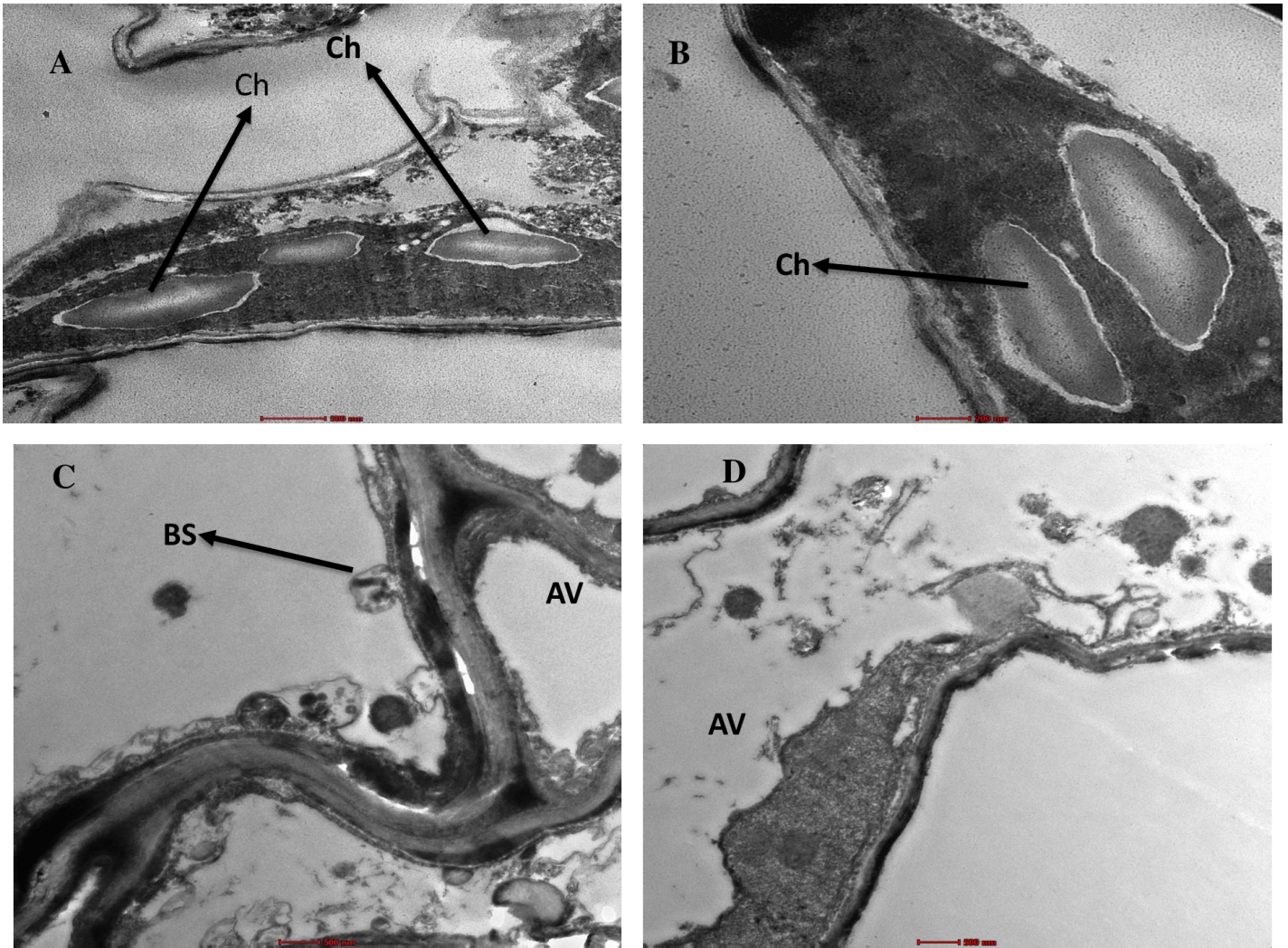


Figure 4

A, B healthy leaf of banana and B, C, infected leaf of banana, Ch- chloroplast , BS- ballon shaped structure, AV- air vacuole

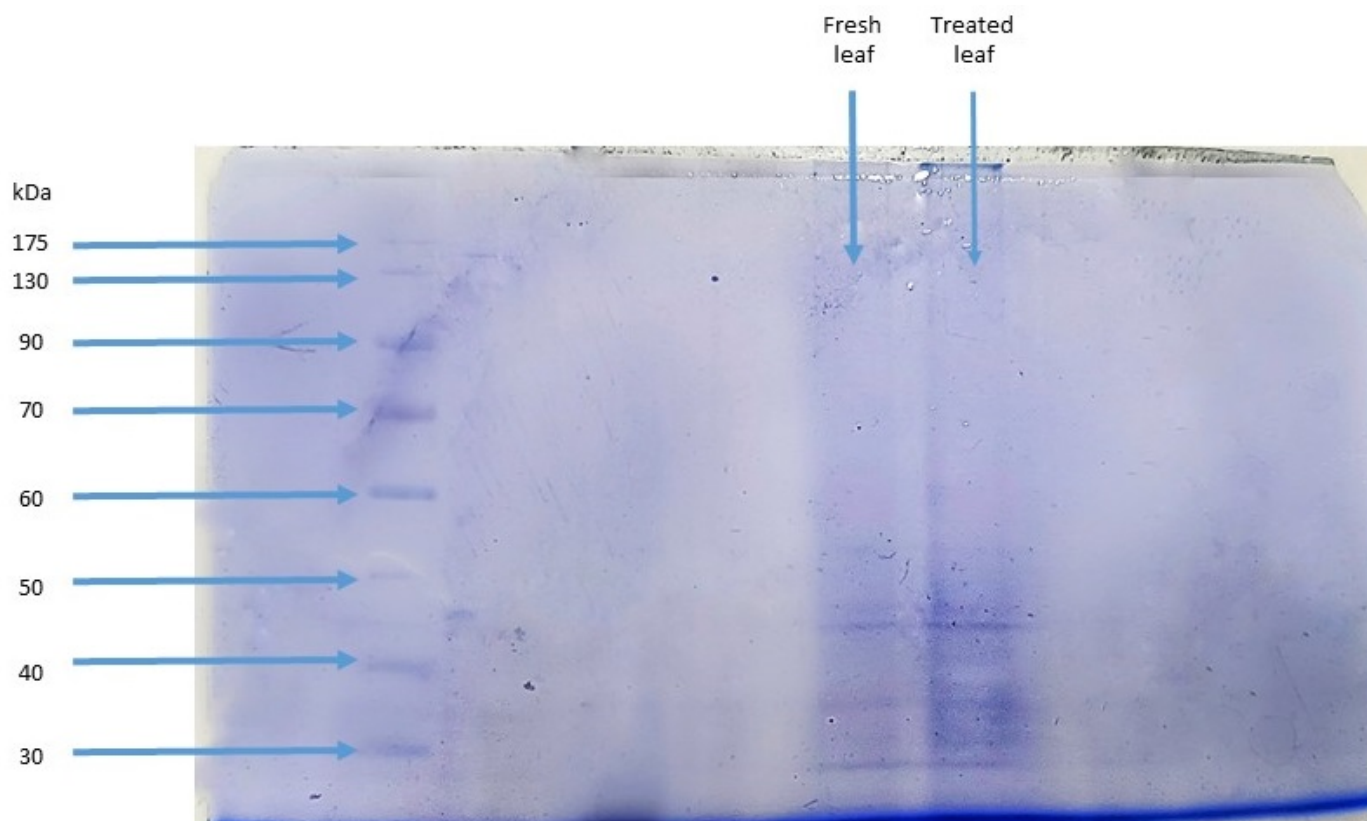


Figure 5

SDS-PAGE testing revealed differences in the protein band pattern between healthy and *C. lunata*-infected leaves, treated or infected leaf showed higher intensity of protein bands compared to healthy leaves with the size of the proteins being between 40 and 50 KDa

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1and2.docx](#)