Gone Bananas: An Overview of the Role of Phospholipase C in Banana Anthracnose Infection

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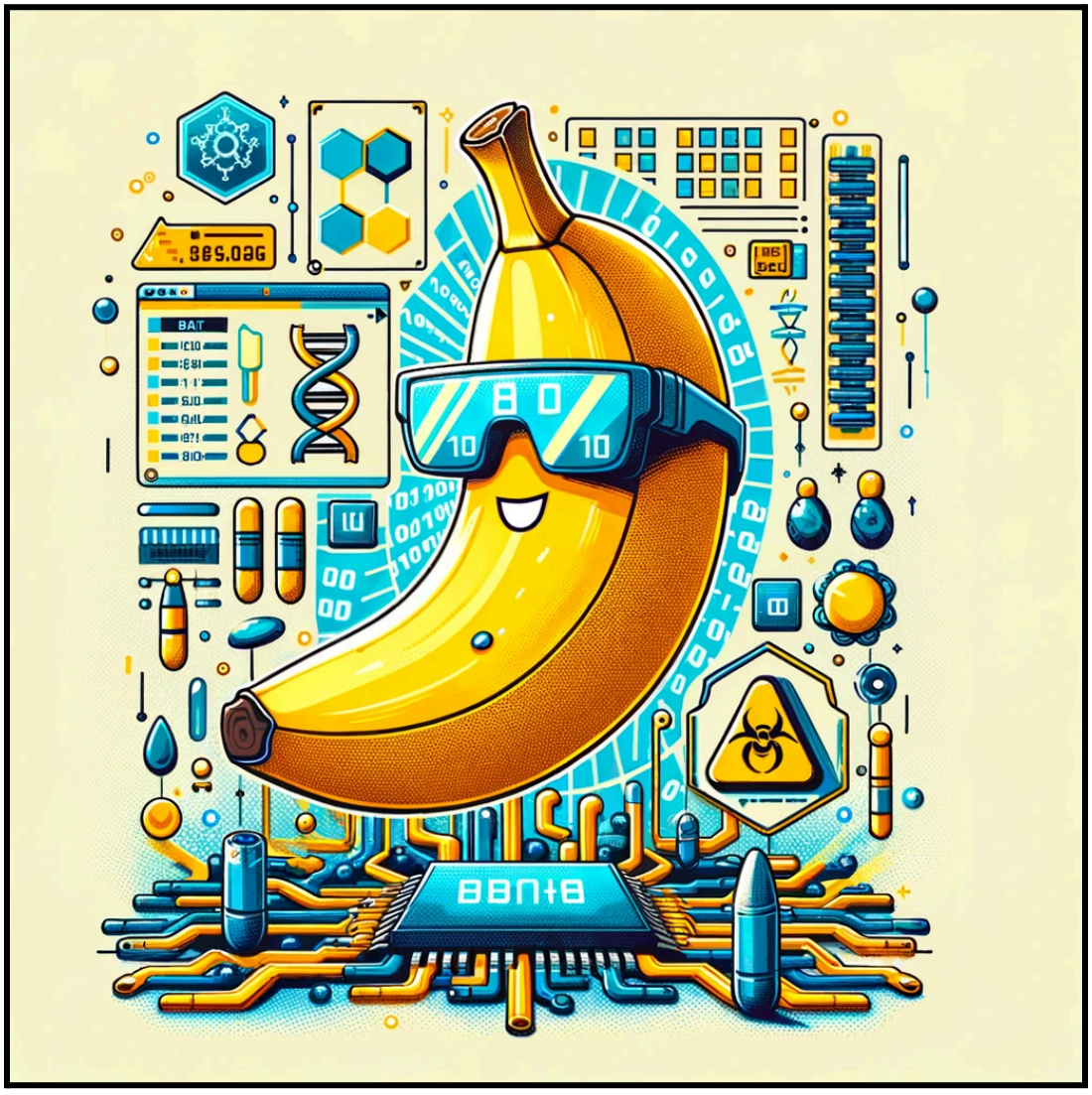

[1] Biochemistry and Molecular Biology - Wrote conclusion + methods
[2] Biochemistry and Molecular Biology - Wrote introduction + abstract
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The image was created using DALL-E given the prompt “Create an image for a team working with AI and Banana.”

Abstract

The bulk of the economies of major developing countries are supported by the export of tropical fruit, such as bananas. However, as bananas are most susceptible to disease post-harvest, farmers are becoming more reliant on innovative breakthroughs to protect their yields from diminishing. Allocating resources and time to identifying disease more easily could offer a way for farmers to save feasible bananas and eradicate heavily infected crops. Recent studies have been able to connect the spread of the fungal infection by *Colletotrichum musae*, known as anthracnose, in bananas to the functions of the phospholipase C (PLC) communication pathway. As a group, we plan on **validating the effect of induced and suppressed PLC expression variation on the spread of anthracnose** in Cavendish bananas. **We also plan on utilizing AI software to analyze the diseased cell count** and predict the salvageability of the banana after various stages of infection and modifier application. In order to accomplish this, our group first determined a financially feasible method to capture and culture anthracnose through potato dextrose agar (PDA) plating of phenotype samples. We isolated six different microbial phenotypes and performed a DNA extraction to characterize the samples to their respective organisms. While being unable to perform inoculation experiments with the identified culture, our group asserts that further experimentation with the isolated culture could show that samples of bananas at various time points could be observed to determine the longevity of a banana with and without chemical treatment. We anticipate that such data could be used to teach an AI model to classify bananas that are worth saving with respect to their natural aging process and disease progression after treatment.

Introduction

The Cavendish banana is a key crop export for developing countries, such as Benin, which implies that growers are constantly in search of ways to improve resistance of the fruit to fast-spreading fungal infection, such as anthracnose.[1] Current methods for treating anthracnose often involve the use of chemical fungicides, such as benzimidazoles, which have been reported as having potential carcinogenic risks.[2,3] Considering that these treatments must span large masses of crop, the extent of these risks would be multiplied, increasing the need for a safer treatment alternative.

As such, there presents the need for the investigation into more sustainable and safe methods for treating or reducing the spread of this disease that do not pose a health risk to

humans or present the possibility of fungicide resistance. A potential option could be found in pre-existing mechanisms that contribute to this growth in Cavendish bananas. For instance, phospholipid cleaving molecules such as phospholipase C (PLC) have been connected to plant defense and therefore present a potential avenue for exploring novel treatment.[4] However, discerning the relationship between the spread of anthracnose and phospholipase activity first requires understanding the mechanisms by which PLC functions.

The specific role of PLC in the activity of anthracnose piqued the interest of scientists. In 2020, Shuai, et al. explored the mechanism of anthracnose infection by evaluating key phospholipid cleaving molecules involved in plant signaling, particularly regarding expression of PLC genes, as well as DAG and IP3 content of infected bananas using spectrophotometric analysis. This evaluation revealed a significant uptick in PLC expression after anthracnose infection with *C. musae,* a correlation that favored the notion that PLC was likely involved in either the spread or defense against anthracnose infection.[5]

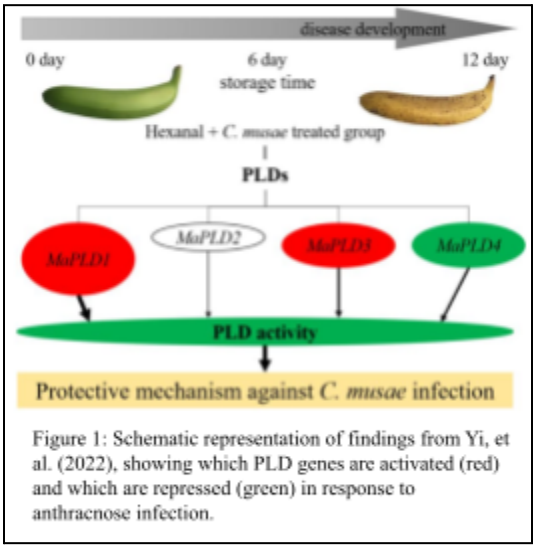


Figure 1: Schematic representation of findings from Yi, et al. (2022), showing which PLD genes are activated (red) and which are repressed (green) in response to anthracnose infection.

Since the discovery of increased PLC expression during anthracnose progression in Shuai, et al., several other studies have expanded on the connection between phospholipases and anthracnose index. For example, hyperexpression of varying phospholipase D (PLD) genes, as seen in Figure 1 above, have been derived by evaluating the RNA content of genes MaPLD1-4 from infected bananas exposed in response to hexanal, a known inhibitor of PLD.[6] However, this observation conclusion indicated a selectivity of regulation for certain PLD genes during anthracnose infection, which could indicate the existence of an experimental lead towards means of resistance, in contrast to the role of PLC, according to the authors.

Additionally, chemical activators and inhibitors have also been investigated. Methyl jasmonate (MeJa) has been reported to induce expression of defensive enzymes such as chitinase and β-1,3-glucanase.[7] On the other hand, salicylic acid (SA) has been established as a key player in regulating infection response, and crosstalk between the two pathways has been identified to show that each influences the other in a variety of ways, such as through activation or blocking

of further signaling depending on the order of gene expression.[8] Previous studies have characterized the difference between the two molecules on disease progression in bananas, ultimately showing that both salicylic acid and jasmonic acid/methyl jasmonate application results in reduced incidence of fungal disease.[9,10,11] While methyl jasmonate is shown to increase PLC and PLD activity, salicylic acid appears to inhibit it.[12]

By building off of the prior work done in this field we aim to affix a more clear time point to the progression of the disease in the context of these chemical applications with the use of artificial intelligence tools that make understanding this progression and possible treatment plans more clear. We hypothesize that by recognizing the changes in the PLC levels upon treatment of anthracnose using chemical agents, we could better track the disease progression in bananas.

Methods

Culturing Anthracnose

In order to accomplish our experimental goals, we plan to use the following procedures. Our first experiment will be to evaluate a positive and negative control of either anthracnose-inoculated or uninoculated bananas. Doing so will provide us with a basis for regular disease progression and also allow us to start training our AI model to better evaluate images and identify diseased cell counts. We will start by obtaining a pure culture of *Colletotrichum musae*, which will be isolated from infected banana tissues. We plan to grow the fungus on Potato Dextrose Agar (PDA) plates at 37 ℃ and 95% humidity to obtain actively growing fungal colonies. Once the fungal culture is actively growing, we will prepare the inoculum by gently scraping fungal spores from the edge of the formed colonies on the surface of the agar plate. We will then transfer the spores to a sterile container and suspend them in distilled water, where we will use a spectrophotometer to achieve a spore density of 10^6 spores/mL.

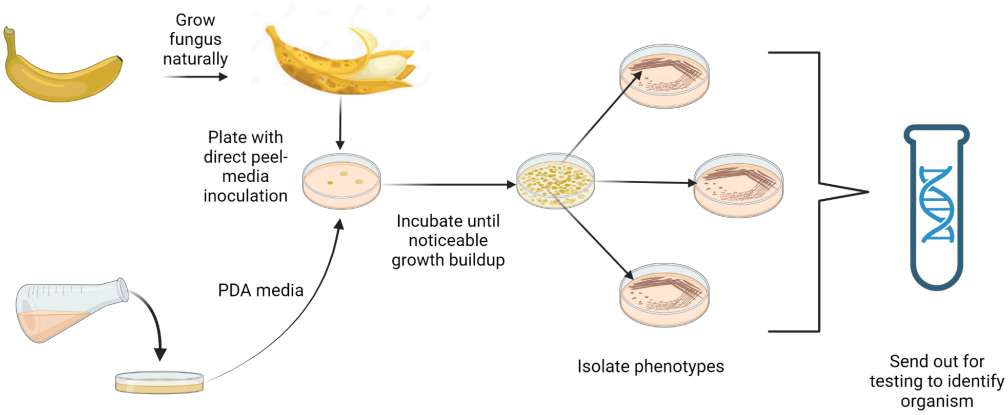


Figure 2: Schematic representation of methods used to isolate and identify anthracnose culture from plain bananas.

Preparation of banana samples will start with the selection of healthy, unripe bananas for inoculation. Using a sterile needle, we will carefully apply the fungal spore suspension onto the exterior of the banana fruits, making sure to distribute the spore suspension evenly over the peel surface. Control bananas can be mock-inoculated with sterile water as a comparison. After inoculation, bananas will dry briefly under sterile conditions to promote spore adherence. We will divide the bananas into groups as follows based on days after inoculation (dai). Inoculated bananas will be transferred to a humid chamber or growth chamber set to the optimal conditions for *Colletotrichum musae* growth (typically warm temperatures around 25-28°C with high humidity). Bananas will be regularly monitored visually for disease development, and symptoms such as lesion formation, fungal growth, and disease progression over time will be recorded. In addition, images of the banana will be taken with a digital camera and uploaded to AI software for machine learning, where the model will learn to identify patterns within images to accurately identify diseased regions.

Manipulating PLC expression with either Methyl Jasmonate or Salicylic Acid

Once a basis for typical inoculation parameters are obtained, we planned to go on to inoculate bananas once more in conjunction with spraying of either 2mM SA or 0.1mM MeJa, concentrations derived from a similar experiment done by Shan et.al, to isolate the effects of either treatment on the expression activity and levels of PLC.[13] We planned on using a range of concentrations up to 50% above and below these parameters (e.g. 0.05-1.5mM MeJa). In addition, we planned on applying the spray a varying days after inoculation in a similar manner

as the infection initially using To verify the presence of PLC in these samples, we planned on conducting a colorimetric assay of PLC activity by measuring the absorbance through spectrophotometry.

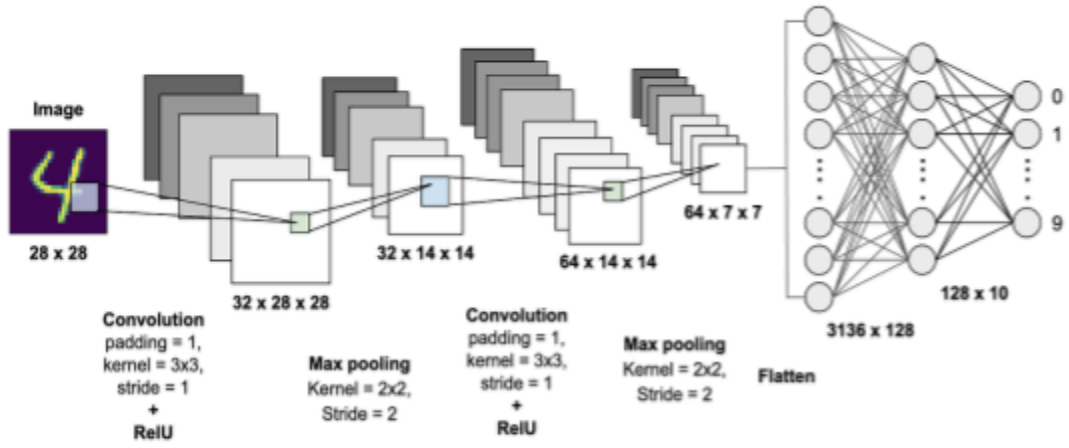
Determining age of the banana at peak potency of Anthracnose infection

This test would allow for the correlation of the aging rate and the peak potency/level of anthracnose infection in the sampled bananas. By comparing the typical aging rate of a banana sample placed in an oxidizing environment (controlled for external factors such as temperature) to the aging rate of a sample inoculated with the anthracnose fungi, a fold change that influences the aging rate in the inoculated sample is anticipated to be observed. The test will proceed in three “observed” stages: Green (preliminary stage) to yellow (mature), and final yellow stage (right before first spots of brown appear). We plan on identifying when the anthracnose is at its most potent level or at its highest spread at the aging stage of the banana to see whether treatment or eradication would be the best approach to combating the anthracnose. For instance, if it is found that the banana shows high anthracnose spread and activity in a banana that is nearing its aging stage, then the model would justify killing the crop and identifying surrounding crops that might be affected (determining whether to treat or kill them as well based on their age). By looking at infection spread and level of infection with relation to a healthy banana’s decay, we could validate using this model to determine when a banana crop is past the stage of saving. The healthy banana prior to ripening, would be sterilized in the process to ensure a less than significant chance of anthracnose traces present on the surface.

Constructing an AI model

For the data collection phase, a diverse dataset of banana images infected with anthracnose is gathered by acquiring multiple samples (approximately 5 to 7) on each day of experimentation. Following data collection, data preprocessing steps are employed to enhance data quality and minimize noise. Techniques such as resizing, normalization, and augmentation will be used to clean and prepare the image data for analysis. Additionally, Meta’s Segment Anything Model (SAM) will be utilized for the visual segmentation of banana cell images. This eliminates the need for manual description of unhealthy cells, as SAM effectively distinguishes between normal and infected banana cells. Given SAM's recent release to open source and its

advanced application in identifying brain lesions, its incorporation into this study has a potential of providing insight into studies with a similar scope.[14]



A simplified flowchart of how a CNN processes an image

For model selection, we plan on using a Convolutional Neural Network (CNN) architecture as it is the most suitable deep learning model for the task at hand. In this phase, we plan on developing and training the model by feeding prepared images into the CNN model. Techniques such as batch training, learning rate adjustment, and early stopping will be employed to optimize the model's performance. These strategies ensure efficient utilization of computational resources and facilitate the convergence of the model towards our desired performance metrics.

Results

The figure below illustrates the photographs of the six phenotypes isolated from the bananas that were sampled and incubated for 24 hrs at 37 ℃.

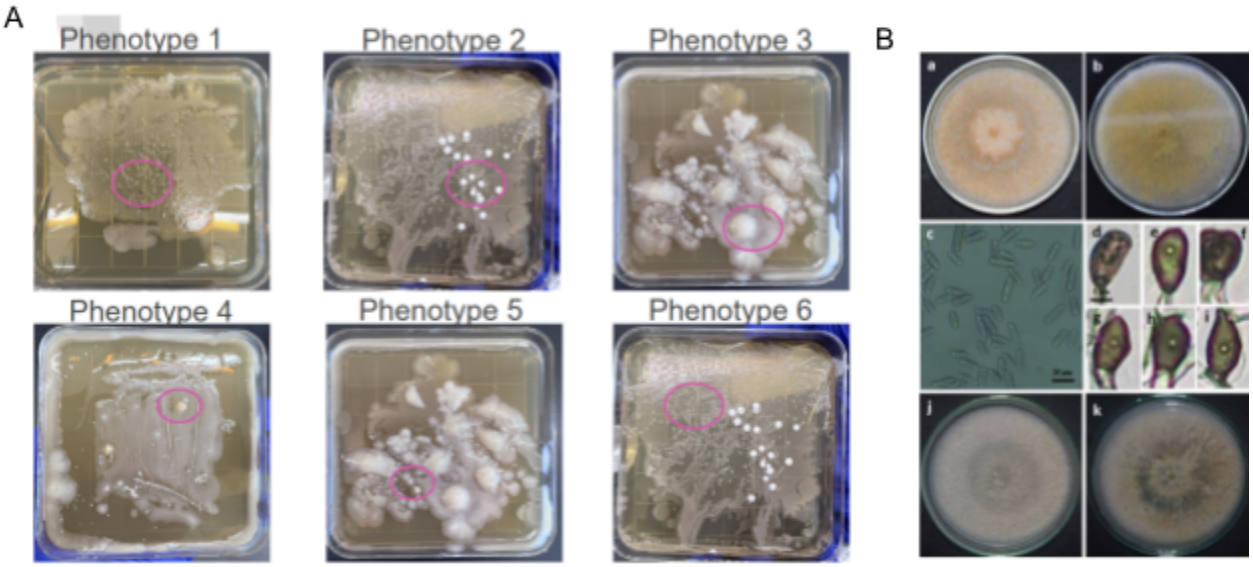


Figure 3: YPDA Plates obtained after being swabbed with various contacts of banana lesions and cultured for 48 hours. (B) Data obtained from potato dextrose agar cultured *C. musae* from Kurera, et al., 2023

Samples from each of the six phenotype cultures then underwent a DNA extraction procedure using the Invitrogen™ Purelink Microbiome DNA Extraction Kit. The 260/280 and 260/230 ranges listed in the table below are the ideal absorbance ranges for the DNA that was purified and extracted. Starred values in the table indicate phenotypes that did not fall within the indicated absorbance ranges.

	Phen. 1	Phen. 2	Phen. 3	Phen. 4	Phen. 5	Phen. 6
Amount (ng)	630	80	970	2700	700	2,650
260/280	1.87	1.95	1.87	1.85	1.91	1.82
260/230	3.04	3.94	2.73	1.09*	3.54	1.60*

Table 1: Absorbance ratio values for the extracted and purified DNA of the six phenotypes cultured from banana extremities

Discussion

As a group we chose to isolate six different phenotypes of microbial culture (Figure 3A) based on similarity in color, shape, and texture to known anthracnose cultures in PDA (Figure 3B). We then isolated the cultures by streaking, and performed a DNA extraction using an Invitrogen™ Purelink Microbiome DNA Extraction Kit. As seen in Table 1, our data revealed that all phenotypes were purified within ideal absorbance ranges except for phenotype 4 and 6. In addition, although Phenotype 2 produced pure DNA according to 260/280 and 260/230 ratios, the quantity of DNA was only 80 ng, causing us to consider this sample null and void for the purposes of our investigation. An additional control sample containing just media was also analyzed and revealed less than 100 ng of sample present and impure absorbance ratios with <1 for each (not pictured).

Our team ultimately did not have enough time to continue our research progress after this experiment, resulting in these sole pieces of data. Though we did not accomplish our initial objectives of analyzing PLC inhibitor and activator effects on anthracnose infected bananas, as well as using AI to make decisions about banana longevity and lifetime, our team asserts that we were on track to produce valuable and usable results had we had more time to finish our experimental objectives. Our next steps would have involved using PCR to sequence and mass produce DNA in the internalized transcribed spacer (ITS) region of DNA using universal ITS primers 1 and 4. This region, located between the genes encoding for the RNA components of the ribosomal subunits in microorganisms, is unique to each organism, and can be used to identify microbes with great accuracy. We then would send these PCR-generated DNA molecules in to be sequenced. Upon reception of this data and subsequent confirmation of *C. musae* collection, we would then be able to proceed with the second stage of our experiment - treating our banana samples with the cultured anthracnose and administering the MeJa and SA treatments accordingly to track their growth.

Conclusion

Considering the crucial role of bananas in maintaining the economic stability of producing and purchasing nations, a deeper analysis of the upstream mechanisms and molecules involved in the progression of anthracnose infection, such as those associated with the phospholipase C (PLC) protein could aid in clarifying the route of infection and offer a broader range of detection and mitigation strategies. By understanding the extent to which the role of phospholipase C plays in the progression of anthracnose, it would make it more feasible to track

the mechanism and speed of infection growth from an earlier stage. This could allow for the formation of strategized and conservative methods for fungicide and pesticide use, ultimately offering a more specific and refined approach to ensuring the health and useability of banana crop as opposed to traditional methods of mass fungicidal treatment.

Previous approaches to address this knowledge gap have not yet evaluated an *induced* expression to verify the effect of PLC, nor fully evaluated the context of other complexes involved in the PLC communication pathway, such as diacylglycerol (DAG) and inositol triphosphate (IP3), which are products involved downstream of phospholipase cleavage. In order to further the development of these methods, our research poses the purpose of developing a stronger understanding of phospholipase activity, specifically phospholipase C, with respect to the spread of anthracnose in a mature banana candidate. To address these questions, we first plan to measure the effect of a proposed PLC inhibitor (salicylic acid) and stimulant (methyl jasmonate) on mature banana cells inoculated with and without anthracnose infection. Based on these models, we hypothesize, for example, that the cells infused with increasing concentrations of the stimulant would display fewer regions of anthracnose infection. Over time, data collected at varying concentrations of either the stimulant or the repressor solutions would be explained to the AI model in order to teach it how disease index changes under these varying conditions. Factoring in these changes and corresponding conditions, as well as accounting for the natural aging process, might allow our AI model to effectively predict the rate of which the infection would spread in our samples, as well as evaluate the chance of survival of a banana under varying aging/disease conditions.

The potential impact of our study extends beyond immediate stakeholders to encompass broader societal and environmental benefits. By enhancing our understanding of anthracnose resistance mechanisms and reducing reliance on chemical interventions, we promote sustainable agricultural practices and contribute to global food security efforts. Additionally, our study may yield visual products such as image datasets and AI models, which can be shared with the scientific community for further research and application. The learning outcomes for our team members include gaining practical experience in interdisciplinary research, data analysis, and experimental design, preparing them for future academic and professional endeavors.

Looking ahead, the outcomes of our study lay the groundwork for future research endeavors, including capstone projects, senior design projects, or interdisciplinary initiatives.

These projects could build upon our findings to explore additional aspects of anthracnose resistance, investigate alternative disease management strategies, or further refine AI-based diagnostic tools for plant pathology. Overall, the successful completion of our study holds the potential to advance scientific knowledge, promote sustainable agricultural practices, and empower stakeholders with innovative solutions for combating anthracnose infection in bananas.

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