UNIVERSITY OF CAPE COAST

ANTI-PHYTOPHTHORA ACTIVITY OF *CARICA PAPAYA* LINN EXTRACTS: A CASE OF THE TWO MAJOR COCOA BLACK POD PATHOGENS IN GHANA

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BY

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DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part it has been presented for another degree in this University or elsewhere.

Candidate's Signature…… Date……

Name: Woasiedem Kudzo Tamekloe

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature…… Date……

Name: Dr. Isaac Asiamah

Co-Supervisor's Signature……Date…….

Name: Dr. Francis Ackah Armah

ABSTRACT

Background: *Theobroma cacao* (Cocoa) is a major foreign exchange earner for Ghana. Over the recent decades, there has been a consistent decline in cocoa production in the country. A major contributing factor is the cocoa black pod disease caused by *Phytophthora sp*. Synthetic fungicides have been used to control the pathogens, but recent calls for organic cocoa devoid of residues from synthetic fungicides, which are said to pose several health challenges as well as a negative impact on the environment, have gained attention, thus inviting investigations into more environmentally friendly and sustainable alternatives. Objective: This study explored the inhibitory potential of crude extract from *Carica papaya* Linn. against cocoa black pod disease caused by *Phytophthora sp*. Material and Methods: An initial *in silico* assessment was done by using compounds previously isolated and characterized from *Carica papaya* in the literature. These compounds were used as ligands against a phytophthora effector protein. Crude extracts were obtained from the plant materials using 70% ethanol as the extraction solvent. The crude Green Leaf Extract (GLE), Aging Induced Chlorophyll Deficient Leaf Extract (AICDLE), and Matured Black Seed Extract (SDE) were tested *in vitro* against precultured *P. palmivora* and *P. megakarya* using the poison food technique. GLE was modulated with Delco, a synthetic fungicide, at different ratios, which were tested *in vitro* and *in vivo*. Results: 16 of the ligands showed binding affinity higher or equal to the standard ligand alliin, suggesting *Carica papaya* is likely a repository of anti-Phytophthora agents. Assessment of crude extracts at concentrations 5–20 mg/ml showed fungistatic activity against *P. palmivora* and *P. megakarya in vitro*. Modulation of GLE with Delco produced some combinations that made GLE fungicidal *in vitro* against *P. palmivora* and *P. megakarya* with fractional inhibition concentration indices between 0.51 and 0.65, interpreted as partially synergistic. *In vivo* assessment of combination D (comprising Delco and GLE in a ratio of 0.3mg:3mg per ml), demonstrated effectiveness comparable to Delco alone at the recommended dosage of 5mg/ml. Conclusion: This study shows *Carica papaya* in a new light as a potential material that could be formulated into a natural product-based fungicide for combating Phytophthora-induced black pod disease.

KEY WORDS

Black pod disease

Carica papaya

Cocoa

Ligand

Molecular docking

Phytophthora

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DEDICATION

To my friends: Joseph, Daniel, Richard, Bismark and Justice.

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LIST OF ABBREVIATIONS

AICDLE	Aging Induced Chlorophyll Deficient Leave
ANOVA	Analysis of Variance
cm	Centimeters
Conc	Concentration
CRIG	Cocoa Research Institute of Ghana
GLE	Green Leave Extract
Lab	Laboratory
mg	Milligram
ml	Milliliters
PDB	Protein Data Bank
PGE	Triethylene glycol
PI	Percentage Inhibition
Pm	*Phytophthora megakarya*
Pp	*Phytophthora palmivora*
RMK	Remark
RMSD	Root Mean Square Deviation
SDE	Matured Seed Extract
SDW	Sterile Distilled Water
SEM	Standard Error of Mean

CHAPTER ONE

INTRODUCTION

Cocoa, a primary export commodity for Ghana, has played a crucial role in the country's economic growth for over a century (Bailey & Meinhardt, 2016). Over the past few decades, Ghana has experienced a significant decrease in cocoa production, which has had adverse effects on its economy (Oduro *et al*., 2020). This decline has been primarily attributed to cocoa diseases, with the black pod disease being one of the most prevalent (Akrofi, 2015). The current management of diseases relies heavily on synthetic fungicides, which have been associated with inducing environmental hazards and inadvertently attacking beneficial organisms (Bailey & Meinhardt, 2016). This reliance on synthetic fungicides poses a significant challenge in achieving sustainable and environmentally friendly disease management practices (Bailey & Meinhardt, 2016).

Natural products derived from plants, microbes, and other biological sources offer the potential to limit disease infections. This study aims to explore the anti-black pod activity of a widely used medicinal plant in Ghana. Success in this work will lead to the production of biopesticides from locally available materials, reducing reliance on synthetic fungicides.

1.0 Background to the Study

The multifaceted influence of cocoa on Ghana's economy is evident in various aspects, encompassing historical, political, technological, and industrial contexts. Historical records indicate that cocoa has been a part of human interaction for at least 3000 years (Bailey & Meinhardt, 2016). Cocoa has demonstrated sufficient potential to be an agent of poverty alleviation when the

necessary investments are made (Vivek *et al*., 2019). Ghana's cocoa industry has a long-standing history that dates back over a century (Kuusaana *et al*., 2021).

Literature suggests two narratives on how cocoa was introduced in Ghana and how it became a major force in the country's agricultural sector. Cocoa, a crop that would later become vital to Ghana's economy, is believed to have been introduced by Dutch missionaries in the 19th century (Bailey & Meinhardt, 2016; Kuusaana *et al*., 2021). However, it was not until Tetteh Quashie's significant contribution that cocoa production gained commercial attention and prominence in the country (Kuusaana *et al*., 2021).

The agriculturalist Tetteh Quashie was a key figure in the growth of Ghana's cocoa industry. He brought cocoa seeds back to Ghana from Fernando Po, which is now Equatorial Guinea, in the late 19th century. Recognizing the potential of cocoa cultivation, he established a nursery to propagate cocoa plants (Darkwah & Verter, 2014). Tetteh Quashie's nursery quickly became the primary supplier of raw planting material for cocoa farms not only in Ghana but also in neighboring countries. His efforts were instrumental in promoting the widespread adoption of cocoa farming throughout the Western African region.

Tetteh Quashie's initiative and the subsequent growth of cocoa farming propelled Ghana to become one of the world's leading cocoa producers (Darkwah & Verter, 2014). For many people in Ghana, the cocoa industry is a major source of employment. Approximately 800,000 farmers nationwide are actively engaged in the production of cocoa, either through their own farms or cooperative groupings (Akoto *et al*., 2017; Maguire-Rajpaul *et al*., 2020).

Producing cocoa serves as the primary income source for these farmers, enabling them to support their families and uphold their livelihoods. In addition to its significant contribution to employment, the cocoa industry in Ghana makes a substantial economic impact. The annual revenue generated from cocoa exports and related activities exceeds $2 billion (Akoto *et al*., 2017; Vivek *et al*., 2019). The income generated from cocoa production is vital to Ghana's economy, as it contributes to foreign exchange earnings and enhances the nation's overall GDP. Ghana's success in cocoa production can be attributed to its robust agricultural heritage, built upon generations of farming knowledge and practices, which has provided a strong foundation for cocoa production. The country's farmers have long benefited from the favorable conditions, including fertile soils that are conducive to cocoa cultivation, and the presence of regular rainfall, which supports crop growth (Etaware, 2022).

Climate change has brought about shifts in natural weather patterns, posing challenges to cocoa farmers. Changes in temperature, shifts in rainfall patterns, and increased occurrences of extreme weather events have disrupted the typical growing conditions for cocoa (Anning *et al*., 2022; Tham-Agyekum *et al*., 2023). To mitigate the effects of climate change, cocoa farmers in Ghana have adopted complementary practices to support their crops. Soil water conservation techniques, such as mulching and the construction of water-holding structures, help retain moisture in the soil during periods of reduced rainfall or drought, ensuring that cocoa trees have access to sufficient water (Anning *et al*., 2022; Tham-Agyekum *et al*., 2023).

The application of organic fertilizers has become increasingly important as farmers seek to maintain soil fertility and nutrient levels in the face of

changing climatic conditions. Organic fertilizers, such as compost and animal manure, provide essential nutrients to cocoa trees and improve soil health, promoting sustainable cocoa production (Amponsah-Doku *et al*., 2022). Farmers have recognized the benefits of intercropping leguminous plants, such as beans or groundnuts, alongside cocoa trees. Legumes help to enhance soil fertility through nitrogen fixation, provide shade to cocoa trees, and contribute to diversifying farmers' income sources (Acheampong *et al*., 2023).

Politically, the Ghanaian government acknowledges the value of the cocoa sector and its contribution to the country's economy both before and after independence. Policymakers have thus paid close attention to the production of cocoa (Asuming-Brempong & Kuwornu, 2013; Kuusaana *et al*., 2021). During the pre-independence period, Ghana implemented policies aimed at cultivating industrial crops to meet the demand of manufacturing industries in Europe (Asuming-Brempong & Kuwornu, 2013). These policies led to significant advancements in the infrastructure, including the construction of extensive road and rail networks connecting various towns and villages that specialized in producing cocoa and other crops. By 1957, Ghana emerged as a dominant force in export-driven economic growth among its neighboring West African counterparts (Asuming-Brempong & Kuwornu, 2013). The implementation of tax laws at the beginning of the 20th century resulted in a surge of small-scale farmers transitioning to cash crop cultivation, specifically focusing on cocoa, due to the financial advantages it offered.

Ghana's initial documented cocoa export took place in 1885. This rapid transformation enabled Ghana to surpass other nations and become the leading global producer of cocoa in 1911 (Kuusaana *et al*., 2021). Due to its

substantial contribution as a primary source of foreign exchange for the country, a specialized governing authority was established to effectively oversee the responsibilities associated with the cocoa sector. The entity initially known as the Cocoa Marketing Board, later renamed Cocoa Board or COCOBOD, has been entrusted with the responsibility of managing and supervising Ghana's cocoa sector (Asuming-Brempong & Kuwornu, 2013; Maguire-Rajpaul *et al*., 2020). Its operations are carried out through regional offices located throughout the country. COCOBOD is authorized to secure funding both domestically and internationally in what is known as the cocoa syndicated loan to purchase cocoa beans from farmers as well as subsidize agricultural inputs for farmers (Boakye, 2021; Tröster *et al*., 2019). Additionally, it is responsible for developing and implementing various activities including but not limited to extension services, research initiatives, and fumigation efforts as deemed necessary. It is noteworthy that a considerable portion of cocoa revenues has been allocated toward supporting education through the provision of scholarships (Kuusaana *et al*., 2021).

Research-focused technological advancements and innovative approaches played a crucial role in driving success. In 1938, the British Gold Coast Department of Agriculture established the Central Cocoa Research Station with the goal of enhancing cocoa quality standards (Kuusaana *et al*., 2021). Following its renaming as the West Africa Cocoa Research Institute (WACRI) in 1944, the Central Cocoa Research Station extended its activities by establishing branches in Sierra Leone and Nigeria (Bailey & Meinhardt, 2016; Kuusaana *et al*., 2021). Following Ghana's independence, WACRI was disbanded in 1962 and renamed the Cocoa Research Institute of Ghana (CRIG)

(Bailey & Meinhardt, 2016; Kuusaana *et al*., 2021). This transformation marked the formation of CRIG as the new institution responsible for cocoa research in Ghana. CRIG conducted thorough research on cocoa diseases, focusing on identifying the range of hosts, patterns of transmission, and effective strategies for managing them. CRIG's research has played a critical role in developing cocoa varieties with enhanced resistance to diseases such as swollen shoots and cocoa black pod disease. Additionally, researchers at the institute provide valuable recommendations for disease prevention strategies based on their extensive studies (Akrofi *et al*., 2015; Bailey & Meinhardt, 2016). CRIG has been instrumental in the advancement and enhancement of cocoa varieties. Concerning agronomic practices, CRIG has researched diverse agronomic practices aimed at optimizing cocoa cultivation (Asamoah *et al*., 2015).

Research efforts covered various areas such as fertilization methods, pruning techniques, shade management, and intercropping systems, and the knowledge gained from these investigations was effectively translated into manuals utilized by extension services (Obeng Adomaa *et al*., 2022). To improve the quality of cocoa beans, CRIG has made improving post-harvest processing procedures a top priority. Extensive research on fermentation, drying methods, and storage conditions has yielded improved practices in post-harvest processing (Ackah & Dompey, 2021). CRIG has actively engaged in training cocoa farmers, extension officers, and other stakeholders on cocoa production and management practices (Asamoah *et al*., 2015).

CRIG's dedication to providing technical expertise and sharing knowledge has empowered cocoa farmers with valuable skills. This

commitment ensures that Ghana's cocoa sector continues to thrive as a vital source of income.

Cocoa holds great importance in the industrial sector as the primary raw material for producing a wide range of goods and byproducts, such as chocolate, cocoa powder, cocoa liquor, animal feed, jelly, cosmetics, alcoholic beverages, soap and various others (Beg *et al*., 2017; Boakye-Yiadom, 2020; Obuobisa-Darko, 2015). Despite the enormity of benefits derived from cocoa coupled with the implementation of policy and technical interventions aimed at maximizing production, there has been a persistent decline in cocoa production in the last few decades (Bailey & Meinhardt, 2016).

The significant influence of black pod disease largely accounts for the decline in cocoa production in Ghana (Akrofi, 2015). The decreasing trends in production have significant consequences for various stakeholders, including the national economy, cocoa farmers, and the global chocolate industry, among others. Ghana is a major global producer of cocoa beans, and any disruptions in its production have a significant impact on the whole chocolate supply chain (Adeniyi, 2019; Akrofi, 2015; Wessel & Quist-Wessel, 2015).

Cocoa beans from Ghana are a primary supply for chocolate manufacturers (Beg *et al*., 2017). Decreased availability and quality of Ghanaian cocoa impact their ability to meet consumer demands and maintain product standards (Teye & Nikoi, 2021). These implications can result in shifts in sourcing preferences among chocolate manufacturers, leading to diversification of supply sources (Lazzarini *et al*., 2022).

1.1 Problem Statement

Black pod disease of cocoa, which is linked to several Phytophthora species, is said to be responsible for 30% of crop losses, costing cocoa farmers and investors $4 billion worldwide (Bailey & Meinhardt, 2016). In Africa, farmers face staggering annual crop losses ranging from 30% to 90% due to black pod infection (Adeniyi, 2019).

In the first few days of black pod infection, the cocoa beans do not suffer significant damage. However, as the infections progress, the pathogen penetrates the internal pod tissues, leading to discoloration and shrinking of the cocoa beans. The result is a reduction in the quality of the beans. Despite significant efforts to control black pod disease, there remains a substantial gap in identifying sustainable management options to ease the challenges faced by over 5 million smallholder farmers and more than 40 million individuals reliant on cocoa for their livelihoods (Beg *et al*., 2017).

Phytophthora is typically controlled by spraying synthetic fungicides regularly (Wessel & Quist-Wessel, 2015). Some farmers find it challenging to use this control strategy because of the high cost of synthetic fungicides and the financial inputs into spraying (Wessel & Quist-Wessel, 2015). Experts on black pod disease predict that without fungicides, the incidence of the disease on cocoa farms will reach unacceptably high levels (Akrofi, 2015; Sonwa *et al*., 2008).

Farmers with the financial means to purchase commercially available synthetic fungicides also encounter specific challenges. In a survey of 306 cocoa farmers from the Western North and Central regions, 81% reported using synthetic pesticides to control cocoa diseases and pests. However, exposure to

the chemical residues has led to health concerns such as skin and eye irritation, along with unintended consequences such as harm to essential pollinators like bees (Osei-Owusu *et al*., 2022).

Researchers are voicing concerns about the profound environmental impact caused by the overuse of synthetic fungicides. They highlight problems such as fungicide residues contaminating cocoa beans and contributing to air, water, and soil pollution. These residues pose significant risks to human health, soil microorganisms, and aquatic life (Denkyirah *et al*., 2016; Miyittah *et al*., 2022).

A viable attempt to mitigate these risks is the encouragement of farmers to use biopesticides derived from natural sources such as insects, nematodes, microorganisms, and plants. Examples include *Paecilomyces spp., Rhizopus spp., Trichoderma spp., Rhinoceros beetle,* and *Ganoderma spp*. (Takyi *et al*., 2019). This approach leverages local resources for effective pest and disease management. Ghana's rich biodiversity includes many flora and fauna with medicinal properties (Adeniyi *et al*., 2019).

Despite Ghana's wealth of natural products, commercial-level biopesticides for managing cocoa black pod disease are still scarce. This gap persists despite the pressing demand for environmentally friendly alternatives to synthetic fungicides. *Carica papaya,* well-known in traditional medicine, contains bioactive compounds like alkaloids, saponins, flavonoids, and phenols, which have antifungal properties. Notwithstanding these recognised advantages, there is still a limitation in our understanding of *Carica papaya's* efficacy against black pod pathogens. This study aims to conduct

comprehensive computational, *in vitro*, and *in vivo* evaluations to assess *Carica papaya's* potential as a natural fungicide for managing black pod disease.

1.2 General Objective

The general objective of this study is to assess the anti-Phytophthora activity of *Carica papaya* Linn extracts against black pod disease.

1.3 Specific objectives

i. Perform *in silico* studies to assess the potential interactions between compounds previously isolated and characterized from *Carica papaya* in the literature and a well-characterized target protein of Phytophthora species.

ii. Conduct *in vitro* evaluations of *Carica papaya* crude extracts to determine their antifungal activity against Phytophthora.

iii. Modulate the *Carica papaya* extract with a synthetic fungicide to optimize the performance and assess the antifungal efficacy.

iv. Perform *in vivo* evaluations to assess the effectiveness of the best-performing *Carica papaya* extract and synthetic fungicide combination against Phytophthora infections.

1.4 Significance of the Study

Cocoa black pod disease is a significant challenge in the cocoa industry, causing losses in time and money for farmers, governments, and investors. This issue affects the entire cocoa value chain and requires attention for the industry's sustainability. Ghana, the second-leading producer of cocoa globally, has its fair share of challenges with *Phytophthora palmivora* and *Phytophthora megakarya*-induced black pod disease.

A favorable anti-Phytophthora activity from this study could be optimized for commercial use on farms. This will increase the drive towards biological and environmentally cost-effective Phytophthora control as opposed to the relatively high cost of synthetic fungicides and challenges associated with their residue levels, which are concerns of health matters for cocoa importers.

The result of this research will also add to the literature as a baseline study of the use of *Carica papaya* extracts in controlling Phytophthora species associated with cocoa black pod disease.

1.5 Delimitations of the Study

The study was limited to the following: the use of ethanolic extracts of green leaves, aging induced chlorophyll deficient leave, and matured seeds of *Carica papaya* from Cape Coast, Ghana. *Phytophthora palmivora* and *Phytophthora megakarya* were used as representatives of cocoa black pod pathogens.

1.6 Limitations of the study.

Using only ethanolic extracts does not provide extensive information about the potential activity of extracts of other solvents of *Carica papaya*.

Limiting the *in vitro* and in vivo investigation to *Phytophthora palmivora* and *Phytophthora megakarya* means that the findings of the study may not apply to cocoa black pod disease caused by other Phytophthora species.

1.7 Definition of Terms

Black pod disease: is a destructive disease that primarily targets cocoa pods, though it can also affect cocoa flowers, stems, and roots. It is caused by oomycete species such as *Phytophthora palmivora* and *Phytophthora*

megakarya. The disease manifests as brown, water-soaked lesions on cocoa pods, which eventually turn black, rendering the beans unsuitable for use. Infection begins when Phytophthora spores land on the pod surface, germinate, and penetrate the tissue. Spread occurs through wind, rain, and contaminated pods, leading to substantial yield reductions and financial hardships for cocoa farmers (Bailey & Meinhardt, 2016).

Phytophthora: a plant damaging oomycete genus Phytophthora contains species that have the potential to destroy natural ecosystems and inflict significant economic damage on crops throughout the world (Oduro *et al*., 2020).

Effector protein: a molecule secreted by a pathogen, such as a bacterium, virus, or parasite, that interacts with the host cell to manipulate its physiology and promote the survival and replication of the pathogen (García & Hirt, 2014).

Molecular docking: is a computational method employed in drug development and design to forecast the interaction between a small molecule, like a drug candidate, and a target protein or receptor. The objective of molecular docking is to determine the optimal binding position and alignment between the small molecule and the target, offering insights into the potential effectiveness and specificity of the drug candidate (Chen, 2014).

Carica papaya: also called papaw or pawpaw, succulent fruit of a large plant in the family Caricaceae. Has a diverse history of use as medicine for several human disease conditions (Igwe, 2015).

Plant extracts: Plant extracts are concentrated preparations of bioactive compounds, such as phenols, terpenes, and alkaloids, derived from plant materials (Manousi *et al*., 2019).

Phytochemicals: Chemical compounds produced by plants, generally to aid in their resistance against pathogens, including viruses, bacteria, and fungi, as well as other pests (Gruessner *et al*., 2019).

Biological control agents: are naturally occurring or genetically modified microorganisms that effectively decrease the occurrence or severity of diseases caused by plant pathogens. They demonstrate antagonistic activity against specific phytopathogens (Gupta *et al*., 2021).

1.8 Organization of the Study

This research has a five-chapter format. An introduction, the background of the research, the problem statement, its objectives, its significance, its limitations and limitations, and the definitions of terms are all included in the first chapter. A review of the relevant works on this field of research is included in Chapter 2. The materials and methods used for the work are described in depth in Chapter 3. The findings and their relationship to the body of literature were described in Chapter 4. The last chapter, Chapter five, offers a summary of the results, conclusions, and suggestions for further research.

CHAPTER TWO

LITERATURE REVIEW

2.0 Introduction

This chapter presents a comprehensive overview of the significance of cash crops, particularly cocoa, to the economy of Ghana. It explores various studies and literature that highlight the importance of cocoa and delves into the diseases that commonly affect cocoa plants, with a specific focus on the damaging Phytophthora-induced black pod disease. The review examines the existing management strategies employed to combat this disease, considering both their advantages and disadvantages. Additionally, it introduces *Carica papaya*, commonly known as papaya, as a medicinal plant that holds great potential for effectively countering Phytophthora and its detrimental effects on cocoa crops.

2.1 Cash Crops

A cash crop is a type of agricultural crop that is grown specifically for sale in the market rather than for personal or subsistence use (Hashmiu *et al*., 2022; Sharp & Busse, 2019). The term is typically used to refer to crops that are in high demand and bring a good price at market, such as coffee, cocoa, cotton, tobacco, and certain fruits and vegetables (Chauvin & Porto, 2011). These crops are a significant source of income for numerous countries and farmers as they are frequently cultivated for export to different countries (Minch, 2017; Monteiro *et al*., 2017).

2.2 Major cash crops of economic importance

There are many cash crops that are economically important across the world; some of the most significant include: Grains: rice, wheat, and maize

(Chaudhary *et al*., 2020). Pulses such as chickpeas, lentils, and beans (Meena *et al*., 2019). Oilseeds: soybeans, rapeseed, and palm oil (Sabajo *et al*., 2017). Sugar crops: including, sugar cane and sugar beet (Manavalan, 2021). Coffee: Arabica, Robusta (Campuzano-duque *et al*., 2021). Cotton: Upland cotton and Pima cotton (Reddy *et al*., 2020). Tobacco: flue-cured, burley, and dark air-cured (Sifola *et al*., 2021). Rubber: *Hevea brasiliensis* (Benya *et al*., 1980). Tea: *Camellia sinensis* (Liu *et al*., 2021). Fruits: bananas, oranges, apples, mangoes, grapes (Wani, 2018).

These crops have a notable impact on the worldwide economy and are essential for ensuring food security as well as sustaining the livelihoods of millions of individuals. Nevertheless, the particular cash crops cultivated in a given area frequently hinge on local conditions such as climate, soil fertility, water resources, and market needs.

2.3 Cash crops cultivated In Ghana

Ghana is known for growing a variety of cash crops, including*:* *Theobroma cacao* (Cocoa): Ghana stands as a significant contributor to global cocoa production, with the crop serving as a primary source of revenue for its farmers (Zakaria, 2017). Oil palm: After Nigeria, Ghana ranks as the second-largest producer of palm oil in Africa (Murphy *et al*., 2021). Coffee holds significant economic importance in Ghana, with the nation being a prominent producer of both Arabica and Robusta coffee beans (Hirons *et al*., 2018).

Rubber is another important cash crop in Ghana, with the country being a significant producer of both natural and synthetic rubber. Pineapple is a popular cash crop in Ghana, with the country being a major producer of both fresh and processed pineapple products (Schneider *et al*., 2010). Cashew is a relatively

new but rapidly growing cash crop in Ghana, with the country being a major producer of both raw and processed cashews (Dadzie *et al*., 2020). These are just a few examples of the many cash crops grown in Ghana, which is known for its diverse and productive agricultural sector. It proves challenging to pinpoint an exact total income figure generated by cash crops in Ghana, given the considerable variability influenced by factors such as weather conditions, pest occurrences, demand shifts, and fluctuations in global market prices.

Cash crops like cocoa, oil palm, and coffee are considered to be some of the most important contributors to the country's economy, providing significant income for farmers and supporting the growth of other industries, such as processing and export (Hashmiu *et al*., 2022). As an illustration, the Ghana Cocoa Board stated that cocoa represents the primary agricultural export of the country, comprising around 60% of the total agricultural exports and contributing roughly $2 billion in foreign exchange earnings annually (Tröster *et al*., 2019). Similarly, oil palm is considered to be a key driver of economic growth in Ghana, with the industry supporting the creation of thousands of jobs and contributing over $700 million to the country's economy each year (Darkwah Osei *et al.*, 2021). Cash crops are pivotal in fostering the economic advancement of Ghana and offering sustenance for numerous farmers and their households.

2.4 Economic and nutritional importance of cocoa production

The mean annual production of cocoa beans worldwide is approximately 4 million tons, corresponding to a monetary value of about $12 billion since 2010 (Beg *et al*., 2017; Vivek *et al*., 2019). Peprah argues that cocoa production is important because it serves as food, a source of employment and income, a

source of raw materials for industries, and a means of poverty reduction (Peprah, 2019). Exported cocoa beans in the form of whole raw, broken, or roasted were valued at $ 8.8 billion in 2017 (Vivek *et al*., 2019). Between 2019 to 2025, the worldwide cocoa bean market is expected to reach 16.3 billion (Vivek *et al*., 2019). In 2017 the chocolate retail market was valued at $ 106.19 billion and is projected to reach over $ 190 billion by 2026 (Boakye, 2021; Vivek *et al*., 2019).

Cocoa is a plant that is widely consumed for its flavorful and nutritious seeds, which are processed to make chocolate (Tan *et al*., 2021). Some nutritional advantages of cocoa: Antioxidant Effect: Cocoa possesses abundant flavonoids, potent antioxidants that help protect the body against damage caused by free radicals. (Afoakwa *et al*., 2015). Minerals in cocoa: Cocoa serves as a valuable reservoir of various essential minerals such as iron, magnesium, and zinc. Among these, magnesium plays a particularly vital role in supporting bone health and cardiovascular well-being (Herreros-Chavez *et al*., 2019). Cocoa includes dietary fiber, essential for digestive well-being and assisting in the regulation of blood sugar levels (Soares & Oliveira, 2022). Cocoa contains compounds such as theobromine and phenylethylamine, which are thought to have mood-enhancing effects and may improve cognitive function (Dutta & Mitra, 2021). Cocoa contains compounds like epicatechin and procyanidins, which have demonstrated beneficial effects on cardiovascular health (Chaudhari *et al*., 2018).

The nutritional value of cocoa is subjected to variation based on process of production which subsequently affects the nutritional benefits of cocoa products such as chocolate deduced from cocoa (Achaw & Danso-Boateng,

2021). Dark chocolate is generally considered to be the most nutritious form of chocolate, as it contains a higher percentage of cocoa and fewer added sugars and fats. Apart from chocolate, cocoa is used in the production of other products such as cocoa butter, cocoa powder, and cocoa liquor, which are further processed into various food and cosmetic items, including confectioneries, baked goods, ice creams, cosmetics, and personal care products (Boakye-Yiadom, 2020).

2.5 Biological description of the cocoa plant

The cocoa plant, scientifically named *Theobroma cacao*, is a tropical evergreen tree indigenous to Central and South America (Beg *et al*., 2017). It is grown in West Africa, Latin America, Southeast Asia, Caribbean, South Asia, and Oceania (Benya *et al*., 1980). It is a small tree that typically grows to a height of 5-12 meters, with a trunk that can reach up to 40 centimeters in diameter (Chumacero de Schawe *et al*., 2018).

Leaves: The leaves of the cocoa plant are large and glossy, and they grow to a length of 15-30 centimeters (Essola *et al*., 2017). They are dark green in color and are arranged alternately along the branches of the tree. The flowers of the cocoa plant are small and yellowish-white in color. They develop in clusters on the trunk and larger branches of the tree, typically pollinated by midges or other small insects (Malhotra & Elain Apshara, 2017). The fruits of the cocoa plant are large, football-shaped pods that grow to a length of 15-30 centimeters. They are green when unripe, and they turn yellow, orange, or red as they mature.

The seeds, or beans, of the cocoa plant are found inside the fruit, surrounded by a sweet, white flesh. The roots of the cocoa plant are shallow and

fibrous, and they spread widely to absorb nutrients and water from the soil. The plant is also capable of developing prop roots, which help support the tree and improve its stability (Saleh *et al*., 2022).

The cocoa plant is a tropical plant that grows best in areas with high annual rainfall, high humidity, and temperatures between 20-30°C (Beg *et al*., 2017). It is highly sensitive to frost and drought and requires protection from strong winds (Gateau-Rey *et al*., 2018). The cocoa plant is an important crop for many countries, it is widely cultivated for its seeds, which are processed to make chocolate, cocoa butter, and other products (Achaw & Danso-Boateng, 2021).

Cacao propagation commonly begins with seeds, which are obtained from open-pollinated or biparental crosses, also known as "hybrid" crosses. These seeds are sourced from seed gardens, farmers' own farms, or local communities. Many cacao farmers grow seedling trees on their farms as part of their propagation process (Bailey & Meinhardt, 2016). Other methods of propagation, such as using cuttings or grafting, enable farmers to cultivate plants that are genetically identical and possess desirable traits (Kuusaana et al., 2021).

2.6 Cocoa production in Ghana

One can barely discuss cocoa production in Ghana without reference to the legendary statesman and agriculturalist, Tetteh Quarshie (Darkwah & Verter, 2014; Ebewore, 2020; Kuusaana *et al*., 2021). Historical records indicate that in 1876, Tetteh Quarshie introduced cocoa from Fernando-Po Island in Equatorial Guinea. He established a cocoa plantation in Akwapim Mampong, located in the Eastern region of Ghana, utilizing the seeds he

brought. For 65 years (1911-1976) Ghana was the largest producer of cocoa in the world, a position the country lost for the past 46 years when it maintained the title of the second largest producer after Cote d'Ivoire (Kuusaana *et al*., 2021).

2.7 Challenges with cocoa production

Numerous challenges facing cocoa production, both within and beyond Ghana's borders, are linked to diseases and pests, resulting in significant economic losses for farmers and other stakeholders in the cocoa industry. These diseases have played a significant role in Ghana's loss of its status as the world's largest cocoa producer.

2.8 Plant pathogens

There are many plant pathogens that can affect cash crops worldwide. Plant pathogens, such as fungi, bacteria, viruses, and nematodes, can have a significant impact on crop yield (Wilson, 2016; Zhang *et al*., 2022). They can cause a range of diseases in crops, which can result in reduced growth, stunted development, and ultimately, lower yields. For example, fungal pathogens such as Fusarium and Late blight (*Phytophthora infestans*), powdery mildew (*Podosphaera xanthii*), downy mildew (*Peronospora sp*.) can reduce the overall health and productivity of the plant (Shuping & Eloff, 2017).

Bacterial diseases, such as Bacterial diseases: Bacterial wilt (*Ralstonia solanacearum*), bacterial blight (*Xanthomonas campestris*), fire blight (*Erwinia amylovora*) can cause shoots to wilt and die, leading to reduced yield (Kevin *et al*., 2019). Viral diseases such as Tobacco mosaic virus, tomato spotted wilt virus , cucumber mosaic virus can also have a significant impact on crop yield, as they can cause stunted growth, yellowing of leaves, and decreased resistance

to other pathogens (Dombrovsky *et al*., 2010). Nematode infestations including Root-knot nematodes (*Meloidogyne spp.*), cyst nematodes (*Globodera spp.*), stem and bulb nematodes (*Ditylenchus spp*.). Root damage can result in diminished capacity for water and nutrient absorption by the plant, ultimately causing a decline in yield (Abd-elgawad *et al*., 2019). Collectively, these pathogens can cause significant economic losses to farmers and have implications for food security.

It is important to implement measures to prevent and control their spread and to manage plant pathogens effectively to minimize their impact on crop yield. Recommendations to attenuate the effects of pathogens have been encouraged through the use of cultural practices such as rotating crops and improvising sanitation. Fungicides and insecticides, for example, may be used in some cases to manage plant pathogens, protect crop yielding soil health, and select disease-resistant varieties (Adu-Acheampong *et al*., 2015; Blessina *et al*., 2022).

2.9 Major Pest and diseases of cocoa in Ghana

The dominant cocoa pests within West Africa are the *Distantiella theobroma and Sahlbergella singularis*. These pests have earned their reputation because research reveals that they are responsible for about 35% of crop losses in Côte d'Ivoire and a little below 30% in Ghana (Wessel & Quist-Wessel, 2015). The pest feed on cocoa flush leaves and young twigs resulting in canopy deterioration and possible death of plant (N'Guessan *et al*., 2013). The insects are noted to be ubiquitous at unshaded or less shaded regions of cocoa presumably owing to the production of fresh flushers. Adequate shade management has been found to competently resolve the issues however

additional use of pesticides have been touted as an extra measure with more desirable effect (Padi & Owusu, 1998).

Farmers however are discouraged from the use of chemical control means thus insecticides because several field studies from Ghana suggest that cost of labor, spraying equipment and the chemical insecticides are usually above the means the farmers can pay for (Akesse-Ransford *et al*., 2021). In the Ghanaian context, it has been reported that the government organizes regular mass spraying exercises in targeted areas to help farmers fight against the challenges brought by the insects (Sonwa *et al*., 2008).

Several complains from the targeted communities on the fact that the pesticides could pose significant health problems to them in the near future puts the government and cocoa farms into a tight corner (Affum *et al*., 2018; Akesse-Ransford *et al*., 2021; Imoro *et al*., 2019). Some experts have proposed the adoption of Integrated Pest Management Systems; this was anticipated to blend cultural practices and the insect resistant cocoa species with a mild use of pesticide a level just sufficient to reduce pest density (Adu-Acheampong *et al*., 2015; Antwi-Agyakwa *et al*., 2015).

2.10 Cocoa Swollen Shoot Virus Disease

Swollen shoot virus disease has been documented in every West African nation that produces cocoa (Benya *et al*., 1980). In the immediate past, very virulent strains of swollen shoot were responsible for the destruction of significant acreages of the crop across Ghana (Thresh *et al*., 1988). Such incidents have led to the lessons culminating into the intensified action of rigorous identification and removal of infected plants from field and subsequently replacing them with disease resistant cocoa varieties (Ameyaw *et*

al., 2014). While that strategy gains some success, it was not sufficient to prevent the rise of new viral strains and their outbreaks in cocoa farming areas.

Researchers argue that cocoa farms are usually huge and that visual inspection may not reveal symptoms on trees that are actually carrying the virus before considering eliminating such infected plants (Domfeh *et al*., 2016). Many experts now align with the school of thought that development of virus resistant breeds seem to be the most viable means of tackling the menace. Some researchers suggest the taking of extra precaution of growing non-CSSVD host crops including coffee, citrus and oil palm to serve as barriers between cocoa farms to prevent spread of disease should an outbreak emerge from a nearby cocoa farm (Andres *et al*., 2017; Domfeh *et al*., 2016).

2.11 Black pod disease and Phytophthora

Despite the numerous species, only 4 are considered to be economically important with respect to cocoa, these are *P. palmivora, P. capscici, P tropicalis, P. megakarya* and *P. citropthtora* (Bailey & Meinhardt, 2016). *P. megakarya* known to produce profuse aerial mycelium. *P. megakarya* is more virulent because it has larger nuclei in the gametangia less number of chromosome and longer sporangia. It is the most lethal fungal pathogen of *Theobroma cacao* and has gained adequate acknowledgement to destabilize cocoa production and the central and western Africa (Akrofi *et al*., 2015).

Nigeria, Togo and Cote d'Ivoire reported the invasion of their territories by *P. megakarya* in 1979, 1982, 1985 and 2003 respectively (Akrofi, 2015). Every development stage of cocoa plant is susceptible to *P. megakarya* resulting in either black pod disease, leaf and root rots and cankers of branches, stems and chapons among others (Bailey & Meinhardt, 2016). The infection is

facilitated by humid conditions which is one of the requirements for optimal growth (Akrofi, 2015).

A classical phenotypic evidence of Phytophthora infection in the stem water soaked lesion with either dark or brown patches on cocoa trunk (Akrofi, 2015). In the case of the pod multiple lesions are inflected on the pod which occurs as a results on infections spores upon landing on the pods transforming to brown and dark and undergoes accelerated growth to cover the entire pod in less than 15 days post infection (Bailey & Meinhardt, 2016).

P. megakarya has gained an infamous reputation as the main yield declining factor in Ghana as it is in West and Central African production regions. Conservative estimates place the distractive power of *P. megakarya* between 60-100% (Akrofi, 2015). The Ghanaian government, cocoa farmers and major actors in the cocoa value chain received a shock when 212,500 metric tonne a 25% of annual cocoa bean production equivalent to GHC 7.5 million was losses with *P. magakarya* suspected to be playing an advanced role in the occurrence (Akrofi, 2015; Bailey & Meinhardt, 2016).

Cultural practices alone have not demonstrated the ability to singly handle *P. megakarya* once an infection is established the use of fungicide is required to recede the pathogenic effect (Akrofi *et al*., 2015; Etaware, 2022). Recent studies show that when cocoa is infected by *P. megakarya* the plant invokes metabolic apparatus to reduce biotic stress, physical evidence of this biochemical process is thought to be formation of pod necrosis in the case of pod infection (Bailey & Meinhardt, 2016). This theory has been verified as experiment reveal correlation between artificially inoculated cocoa pods resistant to *P. magakarya*. It is thought that this stress release process by the

plant involves peroxidases which work to cross link cell walls optimize lignification and tuberization which fortifies the cell walls to resist pathogenesis from viruses, bacteria and fungi (Simo, 2018).

P. palmivora showed 9-12 small chromosomes at metaphase when grown in the dark on carrot media. S type when they grow, they produce stallate patterns very little aerial mycelial growth. *P. palmivora* is thought to have a global presence and causes loss of 20-30% of cocoa beans and 10% of cocoa trees deaths, until 1985 it was the leading cause of black pod disease in Ghana (Akrofi *et al*., 2015).

P. palmivora has a wide host range numbering into over 200 species (Perrine-walker, 2020). It was first identified and reported in 1909. It has been identified in every country where cocoa is grown (Bailey & Meinhardt, 2016)

P. capscici and *P. citrophthora* have broad host range but their geographical distribution is narrower than *P. palmivora*. They are restricted to the Western Hemisphere. *P. capscici* is present in the Americas and Carribean and has also been reported in Cameroon. India, Brazil and Indonesia have recorded economic loss in their cocoa production due to *P. citrophthora* (Bailey & Meinhardt, 2016).

2.12 Similarities and Differences between Phytophthora and Oomycetes

Phytophthora among oomycetes share similar growth patterns as such oomycetes were previously classified as fungi. Apart from growth similarity they also share a common infection strategy (He *et al*., 2020; Thines, 2018).

Basic differences have now been established among the fungi and oomycetes. Due to misclassification, control strategies were misleading and not

yielding the needed results. Classical examples were when chitin was mentioned to be a minor component in cell walls of oomycetes but it is now known that chitin is a major component of hyphal tips, hence making them susceptible to chitin synthase inhibitors (Brown *et al*., 2022).

When oomycetes produce motile forms, they typically possess two distinct types of flagella: a whiplash flagellum and a tinsel flagellum adorned with small hairs (Thines, 2018). The vegetative stages of oomycetes, which include the resting spores called oospores, are commonly diploid or polyploid in nature. In contrast, the majority of fungal species are either haploid or have two haploid nuclei (dicaryotic) within their cells throughout most stages of their life cycle (Thines, 2018).

The manipulation of hosts to facilitate a persistent infection is primarily accomplished by intracellular effectors, which are predominantly recognized by specific resistance proteins (Chepsergon *et al*., 2021). In Phytophthora and obligate biotrophic downy mildews, these intracellular effectors often possess distinctive motifs (RxLR or LFLAK) that exhibit a high degree of conservation. Consequently, computational methods make it relatively straightforward to identify these conserved motifs and detect intracellular effectors (Chepsergon *et al*., 2021).

Most identified oomycete species rely on the living cells of flowering plants, engaging in obligate biotrophy, wherein they obtain nutrients through specialized haustoria structures (Thines, 2018). While certain oomycetes may induce only mild symptoms or none at all, they can still be transmitted to subsequent generations of their host plants by infiltrating the seeds (Thines, 2018). Conversely, some oomycetes cause the death of their host plants to

facilitate the degradation and nourishment of the host tissue. This may occur either following a biotrophic phase (hemibiotrophy) or immediately (necrotrophy).

Oomycete pathogens possess a wide array of effectors that they release into their host organisms. These effectors have diverse roles, either staying external to host cells to hinder nonspecific defense mechanisms or being transported into host cells to perform their functions within various organelles such as the cytoplasm, nucleus, chloroplasts, or mitochondria (Thines, 2018). A specific function of numerous intracellular effectors is to inhibit cell death induced by the detection of foreign components, often achieved by interfering with plant catalases (Thines, 2018). Additionally, other effectors produced by oomycetes are targeted at suppressing general defense mechanisms, including the deposition of callose.

2.13 Life cycle of Phytophthora

Phytophthora sp. is a group of plant pathogens that cause significant economic damage to a wide range of crops, including fruits, vegetables, ornamental plants, and forest trees. The life cycle of *Phytophthora sp*. typically includes the following stages:

Sporangia: Sporangia are reproductive structures that contain sporangiospores. They are produced on infected plant tissue or in water, and they are dispersed by water, wind, or animals.

Germination: When the sporangiospores come into contact with a suitable host plant, they germinate and produce a germ tube, which grows into the plant tissue.

Infection: Once the germ tube enters the plant tissue, the pathogen grows into the plant and begins to cause damage. This stage is characterized by the development of root rot, stem rot, and foliar blight symptoms.

Reproduction: As the pathogen continues to grow and spread, it produces more sporangia, which are dispersed and can infect new plants. This cycle can continue for several generations, causing widespread damage to crops and forests.

Overwintering: Some species of *Phytophthora* can overwinter in soil or plant debris, surviving through the dry season and infecting new plants in the wet season.

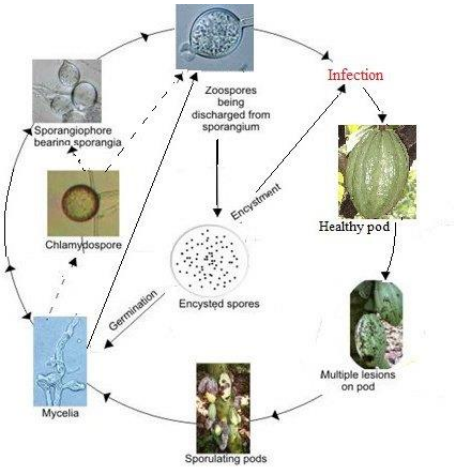


Figure 2.1: Life cycle of *Phytophthora megakarya*
Source: Nembot et al. (2017)

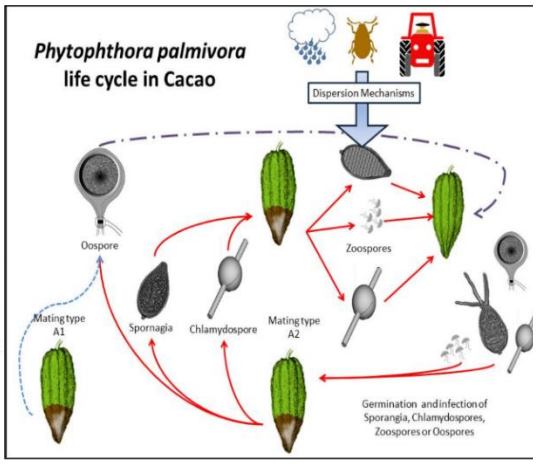


Figure 2.2: Life cycle of *Phytophthora palmivora*
Source: Torres-Londono (2016)

The life cycle of *Phytophthora megakarya* (Figure 2.1) as reported by (Nembot *et al*., 2017) and *Phytophthora palmivora* (Figure 2.2) as reported by (Torres-Londono, 2016). The life cycle of *Phytophthora sp*. is complex and can vary depending on the species and environmental conditions. The fungal pathogen *Phytophthora megakarya* is highly aggressive and represents a significant threat to cocoa production in West and Central Africa, as it is known

to be the most destructive pathogen affecting *Theobroma cacao* L. plants (Akrofi *et al*., 2015).

Effective control of *Phytophthora sp*. requires a combination of cultural practices, such as crop rotation and soil drainage, as well as chemical controls, such as fungicides. In some cases, biological control methods, such as the use of beneficial bacteria or fungi, can also be effective in reducing the impact of *Phytophthora sp*. on crops.

2.14 Management of black pod disease in cocoa

Phytosanitary activities including pod removal, pruning of trees at emergence of the new season to prevent infected pods serving as secondary inoculum (Ndoungue *et al*., 2018). This process lacks any bit of automation and has to be manually executed which makes it laborious, time consuming and expensive venture. Some experts are of the view that the main strategy to control black pod disease is the breeding lead genetic control (Andres *et al*., 2017; Malhotra & Elain Apshara, 2017).

Genetic hybrid that possess dual ability to be tolerant to and also resistant to black pod pathogens (Navarro *et al*., 2017). Those for genetic enhancement either through conventional breeding or modern methods. There is an assumption that genetic control will increase profitability of cocoa production by minimizing incidence of black pod disease (Bailey & Meinhardt, 2016). Experts from this school of though think that chemical control is expensive, limited in ability to discriminate between pathogens and beneficial organisms, and lacks the character of environmental friendliness (Simo, 2018).

Those opposed to genetic control argue that farmers who have already heavily invested in large plantations of genetically susceptible cocoa varieties

will use synthetic fungicides extensively to protect their investment. For these farmers, financial concerns often outweigh environmental risks (Simo, 2018).

2.15 Use of synthetic fungicides

The rising global population is driving an increased demand for crop production, projected to surpass current levels by nearly 40% by the year 2050 (Dijk *et al*., 2021). As a result, there has been a twentyfold rise in pesticide usage to accommodate the demand-driven increase in crop production (Popp *et al*., 2013).

Globally, around 2 million metric tons of pesticides are applied each year, with distribution as follows: Herbicides accounting for 30%, insecticides for 44%, fungicides for 21%, and other integrated pest management strategies for 5% (Aktar *et al*., 2009).

Since 1882, copper has been employed as a fungicide in France, where it was discovered that a mixture of copper sulfate and calcium effectively reduced *Plasmopara viticola* fungi on grape plants (Battiston *et al*., 2019; Koledenkova *et al*., 2022). Subsequently several copper-based agrochemicals like copper oxychloride, copper hydroxide, and cuprous oxide were developed and used in agriculture. However, due to their limited solubility in water, these fungicides are applied in substantial quantities to adequately control phytopathogenic fungi (Chrisfield *et al*., 2022).

The mechanism of action of copper fungicides involves the availability of free Cu^{2+} ions, which are utilized by growing fungal spores through the secretion of malic acid and amino acids (Sajid *et al*., 2022). The toxicity of copper-based agrochemicals stems from their ability to convert between Cu(I)/Cu(II) states, enabling reactions that produce reactive oxygen species

(ROS) like lipid peroxidation, protein oxidation, and DNA damage (Y. Y. Cao *et al*., 2019).

There several contact synthetic fungicides used in Ghana including; Cleanomil with a composition of 600 g per kg of copper oxide and 120 g per kg of metalaxyl (Essola *et al*., 2017). Ridomil (metalaxyl 12% and cuprous oxide 60%). Delco is a 75% copper hydroxide-based fungicide approved for commercial use in Ghana for the control of black pod disease in cocoa (Larmie, 2023). Its high effectiveness and relatively lower toxicity, classified as Hazard Class III, make it a competitive choice among other brands (Larmie, 2023).

West Africa mainly use synthetic fungicides containing either copper compounds or metalaxyl or both (Adeniyi *et al*., 2019; Bailey & Meinhardt, 2016). Copper-based agrochemicals have demonstrated efficacy in combating late blight of tomato, a significant disease caused by the oomycete *Phytophthora infestans*. The copper fungicides are believed to form a chemical blockage to prevent infection, the combination of metalaxyl and copper is used with the aim of exploring multiple sites of pathogen target so as to reduce the incidents of resistance. Frequent application of copper or copper-metalaxyl are necessary for the effective control of infection (Ouattara *et al*., 2020).

Metalaxyl based fungicide are utilized to combat plant diseases caused by Oomycete fungi (Lamichhane *et al*., 2020). It is available in different formulations such as granules, wettable powders, dusts, and emulsifiable concentrates (Heshmati *et al*., 2020). Metalaxyl is a versatile anti-fungal that can be administered using different techniques such as foliar or soil incorporation, surface spraying, drenching, and seed treatment (Feng *et al*., 2019). It is present in authorized products either as the primary active ingredient

or in combination with other active substances such as captan, mancozeb, copper compounds, or carboxin (Seloma, 2021). Due to its broad-spectrum efficacy, metalaxyl is utilized worldwide on diverse fruit and vegetable crops (Bailey & Meinhardt, 2016). The main ways in which metalaxyl is metabolized involve the hydrolysis of the methyl ester and the oxidation of the methyl groups on the ring structure.

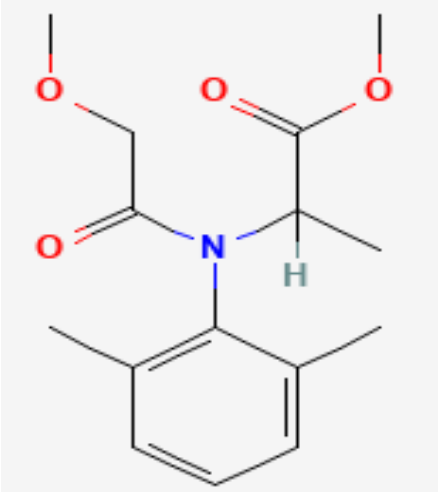


Figure 2. 3: Structure of metalaxyl

Source: Bailey and Meinhardt (2016)

These methyl groups serve as precursors for conjugates in both plants and animals. In soil, the most important metabolite is metalaxyl acid, which is primarily produced by microorganisms present in the soil. Metalaxyl dissipation occurs through various processes such as plant uptake, microbial degradation, photodecomposition, and leaching. Metalaxyl has a tendency to migrate towards deeper soil layers, potentially contaminating groundwater, especially in soils with low levels of organic matter and clay content (Bailey & Meinhardt, 2016). In the event of a significant increase in metalaxyl usage, it becomes necessary to reassess the risk of its occurrence in groundwater (Sukul & Spiteller, 2000).

The use of synthetic fungicides in cocoa production in Ghana is widespread, as they help control diseases that can reduce yields and lower the quality of the crop (Lin Marcellin *et al*., 2018). Excessive use of these chemicals can have negative effects on the environment, including soil and water pollution, as well as human health risks. Some farmers in Ghana have resolved to using more environmentally friendly alternatives, such as integrated pest management practices that rely on natural predators and cultural techniques to control pests and diseases (Larbi-Koranteng *et al*., 2020).

2.16 Need for responsible use of fungicides

By reducing crop losses due to disease and maintaining high yields and quality, the use of fungicides can help farmers increase their profits. This is particularly important for small-scale farmers who rely on their crops for their livelihoods. For countries that rely on crop exports, the use of fungicides can be critical in maintaining the quality and reputation of their products. This can help to maintain and increase demand for their crops, contributing to the country's overall economic development. The use of fungicides on crops is important for disease control, food safety, profitability, and protection of exports.

2.17 Environmental and Human Health Hazards of synthetic fungicides

Synthetic chemical fungicides can have negative effects on non-target species, such as birds, insects, and aquatic life (Shafeeque *et al*., 2020). They can also contaminate soil and water, leading to long-term ecological damage (Weldeslassie *et al*., 2018). Overuse of chemical fungicides can lead to the evolution of fungicide-resistant strains of fungi, making them less effective over time (Baibakova *et al*., 2019). Some fungicides can be toxic to humans and animals and can cause a range of health problems, including skin and eye

irritation, headaches, nausea, and in severe cases, respiratory problems or cancer (Kim *et al*., 2017). Chemical fungicides can reduce the population of beneficial microorganisms in the soil, leading to a decline in soil health and fertility (Baweja *et al*., 2020). Chemical fungicides can be expensive and may not be cost-effective in the long run due to resistance and environmental damage (Abbey *et al*., 2019).

Specific copper toxicity in humans includes impairment in systems responsible for antioxidation as well as membrane and DNA of red blood cells (Husain & Mahmood, 2019). Ejaz reported the link of copper toxicity to Alzheimer's disease (Ejaz *et al*., 2020). Balancing plant protection and phytotoxicity remains a topic of debate. Over use of synthetic pesticides have been found to induce cardiovascular, cancers and endocrine diseases (Rani *et al*., 2021; Sabarwal *et al*., 2018). Researchers are making conscious efforts to develop natural and sustainable products with fewer hazardous chemicals (Aktar *et al*., 2009). More than one million deaths and chronic illnesses worldwide are linked to pesticide poisoning (Dassanayake *et al*., 2021).

2.18 Rejection of crops due to chemical residues

Rejection of cash crops due to high levels of synthetic pesticides can have significant economic impacts for farmers. Some potential consequences include: Farmers may face significant financial losses if their crops are rejected, as they may not be able to sell the contaminated produce.

Rejection of crops due to pesticide contamination can harm a farmer's reputation and make it harder for them to sell their produce in the future. If a large number of crops are rejected due to pesticide contamination, it can lead to a market disruption and potentially cause prices to increase. In some cases,

regulatory agencies may take action against farmers who use excessive amounts of pesticides, which could result in fines or other penalties. To avoid rejection of their crops, farmers may need to shift to alternative methods of pest control, such as integrated pest management or organic farming, which may require additional investments and training.

High levels of pesticide residues in crops can also raise concerns among consumers about the safety and quality of the food they are eating, leading to decreased demand for the affected crops. India rejected shipments of Australian wheat due to the detection of unapproved chemical residues, including the herbicide glyphosate (Guerrera, 2006). In 2020, the European Union rejected shipments of South African citrus fruit due to the detection of high levels of a fungicide called carbendazim (EU, 2020). These examples highlight the importance of following international standards and regulations for pesticide use in agriculture, as well as the need for rigorous testing and monitoring of chemical residues in exported crops to avoid trade disruptions and maintain consumer confidence in the safety of imported food.

2.19 Use of Natural Products in the control of plant diseases

The necessary but sometimes excessive use of fungicides in protection of plants and plant products on and off the farm goes in tandem with increasing resistance by pathogens, environmental pollution and health hazards. Many scientists the world over have shown much interest in finding low toxic, effective, safe and sustainable fungicides. Thus, intensifying race identification of naturally occurring inorganic and biological entities with desired characteristics needed for outwitting pathogens (Shuping & Eloff, 2017).

Pierre-Marie-Alexis Millardet was a French botanist and mycologist who was convinced that copper sulfate was a naturally occurring compound that was able to reduce the growth and distruction of mildew on crops including grapes (Dassanayake *et al*., 2021). Biological control of plant diseases involves utilizing living organisms, such as bacteria, fungi, or parasites, to manage the proliferation and dissemination of plant pathogens (Tamò *et al*., 2012). This approach provides an alternative to employing chemical fungicides and herbicides, adhering to the principles of integrated pest management.

Some plant pathogens are naturally controlled by predators, parasites, that feed on the pathogen or its spores. Certain bacteria and fungi have the capability to generate antibiotics that are harmful to plant pathogens, or they can engage in competition with these pathogens for nutrients and resources in the soil. Biopesticides offer a means of managing plant diseases by introducing beneficial microbes into the soil or applying them directly to the plants (Smith *et al*., 2015). Some plants have been genetically modified to produce antibiotics that are toxic to plant pathogens, effectively providing a natural form of pest control (Shuping & Eloff, 2017). *Streptomycetes avermitilis* was used to produce avermectin (Cao *et al*., 2018).

Saccharopolyspora spinosa used to produce spinosyn which were effective in paralysis of pest insects. Glufosinate broad spectrum herbal agent produced from *Streptomyces sp.* (Tothova *et al*., 2010). Antimycin produced *Streptomycetes spp* was used to produce Fenpicoxamid (Tothova *et al*., 2010). This method functions by suppressing the cellular respiration of fungi. The combined yearly revenue generated from fenpicoxamid and glufosinate surpasses $1 billion (Dassanayake *et al*., 2021).Breeding or selecting crops that

are naturally resistant to specific diseases can be an effective form of biological control (Granke *et al*., 2012). Biological control methods can be more sustainable and environmentally friendly than chemical controls, and they can also reduce the risk of the evolution of pesticide-resistant strains of pathogens (Nboyine *et al*., 2022).

2.20 Examples of microbial active ingredients for biopesticides

Biopesticides are a type of biological control that use living organisms or natural substances to control pests and diseases (Atri *et al*., 2022). Some examples of biopesticides include: *Bacillus thuringiensis* (Bt), a bacterium that produces a Bt-toxin with detrimental effects on various insects, including caterpillars, mosquitoes, and beetles, has been documented (Zhang *et al*., 2017).

Beauveria bassiana, a fungus known as a natural enemy pathogen of many insects, is a biopesticide effective in controlling pests like whiteflies, thrips, and beetles (Maina & Dauda, 2018). *Trichoderma spp*.: This fungus is commonly found in soil and is known for its ability to compete with plant pathogens for nutrients and resources (Thakur, 2021). It can also produce antibiotics that are toxic to plant pathogens. Neem oil: This oil is extracted from neem tree seeds and has been utilized in traditional agriculture for centuries (Gowda, 2019). It is a natural insecticide and fungicide that can control a wide range of pests and diseases. Serenade: Derived from the bacterium Bacillus subtilis, this biopesticide is utilized for managing fungal infections like powdery mildew and downy mildew (Marrone, 2002).

Biopesticides like Double Nickel, Prestop, Mycostop, and RootShield Plus, which utilise active ingredients derived from microbes, are recommended for combating Phytophthora blight (Cornell, 2022).

2.21 Phytochemicals

Phytochemicals are naturally occurring chemical compounds found in plants that serve to safeguard the plants against diseases and damage when they encounter environmental threats (Shuruq *et al*., 2017). Phytochemicals are also responsible for the color, taste, flavor and aroma of a plant among others. The classification of phytochemicals may either be based on the role they play in plant metabolism as a primary or secondary metabolite or based on characteristics of physical, chemical or protective function. Simple sugars, amino acids, proteins, chlorophyll among others are primary phytochemicals (Altemimi *et al*., 2017).

All other phytochemicals are secondary they include alkaloids, phenolics, steroids, terpenes, glucosides, saponins, flavonoids (Agidew, 2022). Phytochemicals accumulate at different part of the plant such as stem, root, leaves, seeds, bark, fruit, flowers among others. The pigment molecules such as cyanin and flavonoid are concentrated in the outer layers of plant especially the leaves and fruits of vegetables (Sabah, 2021). The phytochemical concentration and distribution vary from plant to plant and from stage of growth as well as the environmental conditions (Dou *et al*., 2017).

2.22 Extraction solvents

Plant materials are subjected to extraction to obtain their phytochemical constituents. Several extraction solvents exist, they perform their roles based on polarity (Altemimi *et al*., 2017). Thus polar, mid polar and non-polar solvents such as Water, Ethanol, methanol, DMSO, chloroform and hexane among others (Adedokun *et al*., 2018). Each solvent has their advantages and limitations on potential quantity and quality of extraction.

2.23 Identification of phytochemicals

Preliminary qualitative phytochemical analysis is conducted to identify compounds such as alkaloids, flavonoids, phenolics, tannins, saponins, steroids, glycosides, terpenes, and others present in an extract (Shaikh & Patil, 2020). Experts then use techniques such as column chromatography, GC-MS, HPLC-MS, TLC-bioautography, and NMR, among others, to identify active ingredients in natural products (Chen *et al*., 2019).

2.24 Secondary metabolites

Secondary bioactive compounds confer antioxidant properties, modulation of detoxification processes, stimulation of the immune system, and regulation of hormone metabolism (Dey *et al*., 2022).

Secondary metabolites contain antimicrobial compounds that respond to threats from pathogens (Zaynab *et al*., 2018). These secondary metabolites have been successfully extracted from plants and tested in various dose-dependent studies to understand how they exert their antimicrobial effects (Lelario *et al*., 2018).

At bactericidal or fungicidal concentrations, plant extracts cause membrane damage, disrupt energy production, impair enzyme function, and lead to leakage of cellular contents, resulting in impaired cellular physiology and cell death (Baazeem *et al*., 2021).

Recent research has shown that at sub-lethal or sub-inhibitory concentrations, plant extracts influence the virulence of Gram-positive and Gram-negative bacteria, as well as fungal pathogens (Álvarez-Martínez *et al*., 2020; Samreen *et al*., 2022).This effect is suspected to occur through altering gene transcription, protein expression, or quorum sensing (Bouyahya *et al*., 2022). Thus, plant extracts present a promising strategy for developing anti-virulence agents that target pathogen attacks.

The use of plant extracts in tackling cocoa fungal diseases is a promising alternative to synthetic fungicides in cocoa production (Rachmawaty *et al*., 2018). Many plant extracts have been shown to have antifungal properties and can be used to control diseases such as black pod, frosty pod, and Phytophthora rot, which are common in cocoa production (Dooh *et al*., 2014).

Some of the most commonly used plant extracts in cocoa disease control include neem, lemongrass, and eucalyptus (Coulibaly *et al*., 2021; Mboussi *et al*., 2016; Yousaf & Subhani, 2019).

The use of plant extracts can have several benefits, including reduced cost, reduced environmental impact, and improved human health compared to synthetic fungicides (Bolanle, 2017). It is crucial to acknowledge that the efficacy of plant extracts in managing cocoa fungal diseases varies based on the particular disease and extract employed, and may not always match the effectiveness of synthetic fungicides (Soković *et al*., 2013). Additionally, the use of plant extracts in agriculture requires proper application techniques and adequate research to ensure their safe and effective use (Abbey *et al*., 2019).

Although numerous plant extracts have been evaluated for their effects on cocoa black pod pathogens, several important medicinal plants, such as *Carica papaya*, have not yet been studied.

2.25 Modulating Phytochemicals with synthetic fungicides

In stances where plant extracts alone could not control pathogens to desirable level, there are reports of such extracts being optimized by combination with other natural products or with synthetic fungicides (Chtioui *et al*., 2022; Dassanayake *et al*., 2021). The combination of plant extract tea saponins and prochloraz (chemical synthetic fungicide) was found to improve the activity of the plant extracts and reduce the use of prochloraz (Chen *et al*., 2019).

2.26 Plant description: *Carica papaya* Linn.

Caricaceae is a family of small flowering plants found in Africa and the Neotropics. It is renowned for its fruit crop, and various parts of plants from this

family are utilised both as food and in the pharmaceutical industry. *Carica papaya* is perhaps the most prominent member of this family (Antunes Carvalho et al., 2015).

Carica papaya, commonly referred to as papaya or pawpaw, is a tropical fruit originating from Central America and Mexico (Hernández-Salinas *et al*., 2022; Ming & Moore, 2014). It is known for its sweet and slightly musky flavor and has a soft and juicy texture (Ruiz-Coutiño *et al*., 2019). Papaya contains abundant vitamins and minerals, such as Vitamin C, Vitamin A, and potassium, and has been utilized for medicinal reasons in certain societies (Ruiz-Coutiño *et al*., 2019). The fruit is typically eaten raw, but can also be used in smoothies, sauces, and marinades (Das *et al*., 2010; Hasballa, 2015).

Figure 2.4: A *Carica papaya* plant

Source: Fieldwork (2021)

Papaya is a fruit that contains a diverse range of beneficial compounds derived from plants, known as phytochemicals. These phytochemicals include various enzymes, carotenoids, alkaloids, phenolics, and glucosinolates. Among the numerous phytochemicals present in papaya, some important ones include lycopene, beta-carotene, benzylisothiocyanate, beta-cryptoxanthin, benzylglucosinolate, chlorogenic acid, caffeic acid, protocatechuic acid, and quercetin (Ali *et al*., 2011).

2.27 Cultivation of *Carica papaya*

Papaya propagation primarily involves using seeds. Farmers typically gather high-quality fruits from their orchards and extract the seeds for future planting. Within the fruits, numerous black seeds are surrounded by a gelatinous sarcotesta. Typically, seed germination occurs within 3-5 weeks, but this

process can be accelerated to 2-3 weeks by removing the sarcotesta. To remove the gelatinous substance, the seeds undergo washing and air drying. Once seedlings reach a height of 15-20 cm, they are prepared for transplantation into the field.

Essential agricultural practices such as applying fertilizer and irrigation are carried out as needed (Silva *et al*., 2007). Micropropagation has become increasingly important for the efficient multiplication of specific sex types of papaya and for the application of genetic transformation techniques. Notably, significant advancements have been made in the use of organogenesis and somatic embryogenesis for this purpose. The most commonly employed method for micropropagation involves the culture of shoot tips or axillary buds, with explants typically measuring around 20 mm in length. This method is considered the most dependable and reliable approach for papaya micropropagation (Silva *et al*., 2007).

2.28 Therapeutic uses of *Carica papaya*

Carica papaya has been used in traditional medicine for various purposes, including as a digestive aid, to treat wounds, and to support the immune system (Yogiraj *et al*., 2011). The seeds, leaves, and latex of the papaya plant contain papain, an enzyme with anti-inflammatory and digestive properties (Gunde & Amnerkar, 2016; Singh *et al*., 2020).

Papaya extract has also been shown to have wound-healing properties, and some studies suggest that it may help to prevent certain types of cancer and heart disease (Kong *et al*., 2021). Extracts of *Carica papaya* have been used to treat various pathogens, including bacteria and viruses (Girish & Prabhavathi, 2019; Hossain, 2018). The papain enzyme, found in the fruit, leaves, and seeds

of the papaya plant, has been shown to have antimicrobial properties (Airaodion *et al*., 2020; Akujobi *et al*., 2010).

In vitro studies have shown that *Carica papaya* can effectively inhibit the growth of bacteria such as *E. coli*, *S. aureus*, and *P. aeruginosa* (Peter *et al*., 2014). Papaya extracts have also been found to have antiviral properties, and have been studied for their potential use in treating viral infections such as dengue fever and human papillomavirus (Haber *et al*., 2022; Jethinlalkhosh, 2017; Sarker *et al*., 2021).

In vitro studies have shown that *Carica papaya* can effectively inhibit the growth of fungi such as *Candida albicans*, *Aspergillus niger*, and *Cryptococcus neoformans* (Baskaran *et al*., 2012; Srivastava & Singh, 2016). Papaya leaf extract has also been found to have antifungal activity against dermatophytes, a type of fungus that causes skin infections (Bhadane *et al*., 2014). It is obvious that most of the studies investigating the medicinal properties of *Carica papaya* are biased towards human pathogens.

A limited number of studies have explored the potential of *Carica papaya* in combating plant pathogens. Both *in vitro* and *in vivo* investigations have indicated that extracts from papaya leaves can successfully suppress the proliferation of various plant fungal pathogens, including *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, and *Phytophthora infestans* (Amienyo & Onuze, 2015; Gonza *et al*., 2011).

While *Carica papaya* has shown therapeutic effectiveness against human and some plant pathogens, its potential against pathogens causing cocoa black pod disease remains inadequately understood. Such a situation presents an opportunity for medicinal chemists, drug discovery scientists, academic

institutions, and independent researchers to undertake comprehensive research and fully unlock the untapped potential of *Carica papaya*. Utilising extracts from *Carica papaya* to develop antifungals against *Phytophthora sp*, which are responsible for inducing black pod disease in cocoa, would mark a significant achievement.

2.29 Chapter Summary

The second chapter of this research presents an extensive review of the significance of cash crops, notably cocoa, to the economy of Ghana. It then shifts its focus to cocoa diseases, specifically black pod disease, and advocates for the medicinal potential of *Carica papaya*. It proposes the utilization of *Carica papaya* against black pod disease in cocoa.

CHAPTER THREE

MATERIALS AND METHODS

3.0 Introduction

This third chapter of the research outlines the organization of the methods through which the objectives were achieved. Five sections were thus outlined. The first section is an *in silico* study that examines the interactions between ligands that have been previously isolated and characterized from *Carica papaya* and a well-characterized effector protein of *Phytophthora sp*. The second section dealt with the extraction and phytochemical screening of the *Carica papaya* materials. The third section entails conducting an *in vitro* inhibitory study using ethanol extracts from three materials from the *Carica papaya* plant. The fourth section deals with a modulation study of the extract. The fifth section deals with in vivo assessment of selected, best-performing modulated formulations.

3.1 Molecular Docking

An *in silco* study was conducted using some reported compounds from *Carica papaya* (Table 3.1) against a well-characterized Phytophthora effector protein.

3.2 Target protein

The Protein Data Bank serves as a vital repository for protein deposits, including crystal structures that are essential for various *in silico* studies, such as molecular docking (Joosten *et al*., 2011; Zardecki *et al*., 2022). In the present study, there is a focus on *Phytophthora palmivora* and *Phytophthora megarkaya* and their roles in cocoa black pod disease pathogenesis. However, it is unfortunate that the validated crystal structures of effector proteins from these

two pathogens have not been deposited in the PDB. Nevertheless, a relative of these pathogens, *Phytophthora capsici*, has been extensively studied. One of its effector proteins can serve as a suitable starting point to establish *in silico* evidence of the effectiveness of compounds from *Carica papaya* against Phytophthora pathogens that are responsible for cocoa black pod disease pathogenesis.

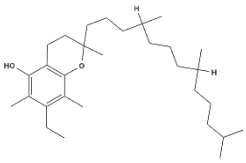
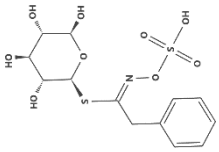
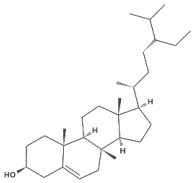
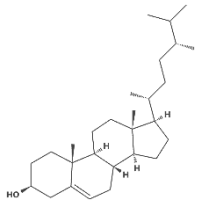
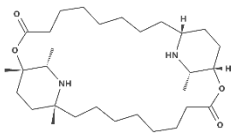
Phytophthora capsici is a recognized pathogenic species that causes disease in *Theobroma cacao* (Pakora *et al*., 2018). One of its RXLR effector proteins, Avr3a11, has been extensively characterized and its corresponding code, 3ZR8, has been deposited in the Protein Data Bank (Boutemy *et al*., 2011; Rani *et al*., 2017). This effector protein has been utilized in various research studies with the aim of developing therapeutic interventions against the aforementioned pathogen (Boutemy *et al*., 2011). The protein's crystal structure was determined using X-ray diffraction, revealing a high-resolution structure at 0.90 Å. The agreement between the observed data and the refined model was excellent, as indicated by low R-values: R-value free of 0.149, R-value work of 0.126, and R-value observed of 0.127 (Boutemy *et al*., 2011). The protein was minimized using the default settings in UCSF Chimera (Dwivedi *et al*., 2021), and subsequently loaded into PyRx, where it was designated as a macromolecule.

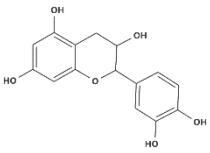
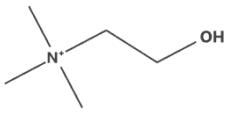
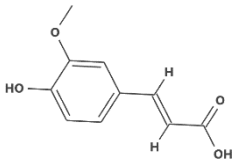
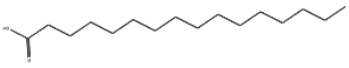
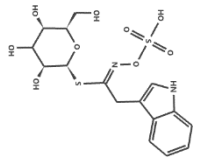
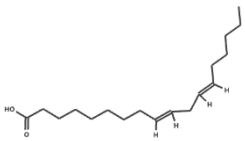
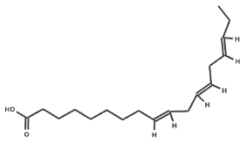
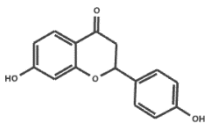
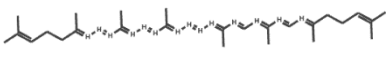
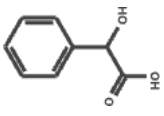
3.3 Ligands

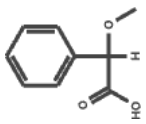
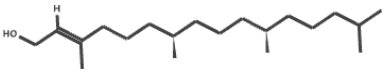
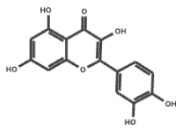
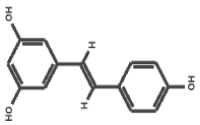
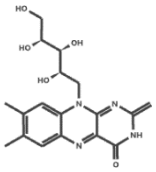
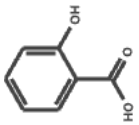
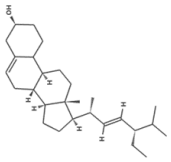
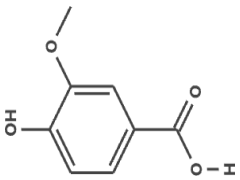
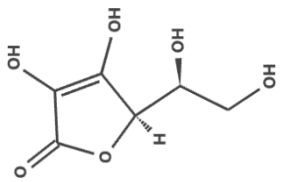
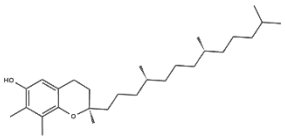
Twenty-six (26) compounds, which have been isolated and characterized from *Carica papaya* and deposited in the PubChem library, were used to build the ligand library following recommendations by (Shimu *et al*., 2022). The use of *in silico* docking studies to investigate the potential

interactions between bioactive compounds and effector proteins may provide valuable insights into the mechanism of pathogen-host interactions and aid in the development of new strategies for plant disease control (Asiamah *et al*., 2023). The SDF files were imported into PyRx by utilizing the Open Babel plugin (Hassan *et al*., 2019). The ligands were then subjected to default minimization settings and subsequently converted into PDBQT format to facilitate docking (Zothantluanga *et al*., 2022).

Table 3. 1: *Carica papaya* compounds used in this study

Compound Name	Pubchem CID	Structure	Reference
α-tocopherol	2116		(Maisarah *et al*., 2014)
Benzylglucosinolate	9548605		(Castro-Vargas *et al*., 2016)
β-sitosterol	222284		(Khaw *et al*., 2020)
Campesterol	173183		(Sani *et al*., 2020)
Carpaine	442630		(Pertiwi *et al*., 2019)

Catechin	9064		(Haber *et al.*, 2022)
Choline	305		(Gunde & Amnerkar, 2016)
Ferulic acid	445858		(Kong *et al.*, 2021)
hentriacontane	12410		(Haber *et al.*, 2022)
Hexadecenoic acid	985		(Sani *et al.*, 2020)
Indolylglucosinolate	6537198		(Gonza *et al.*, 2011)
Linoleic acid	5280450		(Haber *et al.*, 2022)
Linolenic acid	5280934		(Haber *et al.*, 2022)
Liquiritigenin	114829		(Muntholib *et al.*, 2020)
Lycopene	446925		(Ali *et al.*, 2011)
Mandelic acid	1292		(Sani *et al.*, 2020)

Methoxyphenylacetic acid	107202		(Gonçalves Rodrigues *et al*., 2019)
Phytol	5280435		(Igwe, 2015)
Quercetin	5280343		(Kong *et al*., 2021)
Resveratrol	445154		(Feng *et al*., 2022)
Riboflavin	493570		(Yogiraj *et al*., 2011)
Salicyclic acid	338		(Jindal & Singh, 1975)
Stigmasterol	5280794		(Haber *et al*., 2022)
Vanillic acid	8468		(Gogna *et al*., 2015)
Ascorbic acid	54670067		(Airaodion *et al*., 2020)
ɑ-Tochopherol	92729		(Kong *et al*., 2021)

Source: Literature

3.4 Validation of Docking Protocol

The docking protocol was validated using the approaches of redocking of the co-crystallized ligand, superimposition of the co-crystallized ligand (PGE), and the redocked ligand and computation of the root squared mean deviation (RSMD). The co-crystallized ligand had a conventional hydrogen bond interaction with ASP 119 and three van der Waals interactions with GLY 116, ARG 120, and ASN 123 at the active site of 3ZR8 (Figure 4. 1). When Triethylene glycol (PGE) was separated and redocked using PyRx 0.8 at default settings, PGE maintained interaction with ASP 119 through a carbon-hydrogen bond and GLY 116 through a conventional hydrogen bond. Other interactions via conventional hydrogen bonds at the active site were TYR 118, LEU 110, and SER 112 (Figure 4. 1). When the native and redocked PGE was superimposed an RMSD generated by Pymol was 1.067, an RMSD of ≤ 2 is generally preferred for higher accuracy of docking (Xu *et al*., 2021). The redocked and RMSD outputs give validity to the docking protocol.

3.5 Blind docking

Blind docking is a computational method used to predict the binding mode and affinity of ligands to target proteins (Liu *et al*., 2022). It involves the simulation of the interaction between a ligand and a protein by computationally exploring the conformational space of the ligand and protein to identify the most energetically favorable binding pose (Liu *et al*., 2022). This docking is preferred over other docking methods because it allows for the simultaneous exploration of a large number of potential binding poses, which can capture the flexibility of both the ligand and the protein. This makes it more accurate in predicting the binding affinity and mode of ligands compared to other docking methods that

use rigid protein structures or limit the conformational space of the ligand (Kharisma *et al*., 2022). Recognizing its advantageous features, the blind docking method was employed in this research study.

The freely available docking software, PyRx version 0.8, was utilized to perform the docking. The docking grid was centered at the coordinates X: 15.04279 Å, Y: 15.4508 Å, and Z: 55.7605 Å of the receptor protein 3ZR8, and it had dimensions of X: 28.8321 Å, Y: 37.0923 Å, and Z: 26.6333 Å.

3.6 Analysis of Docking interaction

Discovery Studio is a software suite that offers a wide range of tools for the computational analysis of biomolecules (Accelrys, 2008). One of the applications of Discovery Studio is in the analysis of molecular docking results. Molecular docking is a computational technique used to predict the binding affinity and orientation of a ligand (small molecule) to a target protein. Once a set of docking poses has been generated using a docking software, Discovery Studio can be used to analyze and visualize the results.

One of the key features of Discovery Studio is its ability to generate 3D visualizations of the docked complexes (Accelrys, 2008). This can help identify potential binding modes and interactions between the ligand and protein. Additionally, Discovery Studio offers various tools for analyzing the binding energy, hydrogen bonding, and other factors that contribute to the stability of the complex (Accelrys, 2008). Another useful feature of Discovery Studio is its ability to cluster the docking results based on similarity. This can help identify the most representative docking poses and can help to prioritize compounds for further experimental validation (Accelrys, 2008).

3.7 Collection and extraction of plant materials

Plant materials from *Carica papaya* Linn were obtained from the botanical gardens of the University of Cape Coast at a GPS coordinate of (Latitude: 5.1167, Longitude: -1.2945) and subsequently validated for authenticity by Mr. Felix Fynn at the Herbarium of the School of Biological Sciences at the same University. This work was the first to deposit *a Carica papaya* Linn sample under collection number TKW 001 at the University of Cape Coast Herbarium.

3.8 Preparation of plant extract

Fresh green leaves, aging induced chlorophyll deficient leave extract (yellow leaf), and mature pawpaw seeds were subjected to air-drying for 14 days and subsequently pulverized into fine powder using a household Binatone blender. To extract the plant materials, 900 g of green leaves, 700 g of aging induced chlorophyll deficient leaves, and 300 g of pawpaw seeds were separately extracted using 2 liters of 70% v/v ethanol in water for six days.

The beakers containing the plant materials and solvent were shaken several times each day during the extraction process. The resulting filtrates were concentrated using the Ecochyll rotary evaporator system. The percentage yields of the green leaf extract, aging induced chlorophyll deficient leave extract (yellow leaf), and pawpaw seed extract were found to be 11.4%, 10.3%, and 6.3%, respectively.

3.9 Phytochemical Analysis

Phytochemical analysis was performed on *Carica papaya* leaves and seed extracts to detect the presence of different phytoconstituents. The analysis was conducted using previously reported methods (Nandini *et al*., 2020; Nayak

et al., 2012; Tabiri Henneh, 2019). This information is important because phytoconstituents are bioactive compounds that contribute to the medicinal properties of plants. The identification and quantification of these compounds in *Carica papaya* can provide valuable insight into the potential therapeutic benefits of the plant. The results of this analysis can help in the standardization of the plant extract and its use in the development of new drugs or as a natural alternative to conventional fungicides.

3.10 Test for Alkaloids (Drangendroff's method)

To test for alkaloids in a sample, leaves and seeds were first dissolved in 5 mL of dilute hydrochloric acid and filtered. 2 mL of the resulting filtrate was then taken and mixed with 1 mL of potassium bismuth iodide solution using Drangendroff's method. The addition of the potassium bismuth iodide solution drop by drop led to the formation of an orange-red precipitate, which is indicative of the presence of alkaloids in the sample.

3.11 Test for Saponins (Foam test)

Samples weighing 0.5 g were subjected to agitation with 2 mL of water in a test tube. The persistence of foam produced over a period of ten minutes was taken as an indication of the existence of saponins.

3.12 Test for Tannins

In the experiment, two drops of $FeCl_3$various reagents were added to 2 mL of the extract. The existence of tannins was confirmed by the development of a deep blue-black coloration upon the addition of a 0.1% solution of FeCl3.

3.13 Test for Flavonoids (Lead Acetate test)

The procedure involved dissolving an extract weighing 0.5 g in water, followed by filtration. Next, 2 mL of the filtrate were taken and mixed with 1

mL of lead acetate solution, and the resulting mixture was observed for the formation of a yellow-colored precipitate.

3.14 Test for Glycosides (Kellar-Killiani test)

0.5 ml of sample solution was mixed 0.5 ml with of glacial acetic acid, few drops of ferric chloride and concentrated sulfuric acid were added to the mixture. Appearance of reddish-brown ring at junction indicates presence of glycosides.

3.15 Test for Terpenoids

The procedure involved the dissolution of 0.5 g of the extract in 5 mL of chloroform, followed by the cautious addition of 3 mL of concentrated sulphuric acid to the chloroform solution. The resultant mixture was thoroughly mixed, and the development of a reddish-brown coloration at the interface confirmed the existence of triterpenoids.

3.16 Test for Phytosterols (Lieberman Burchardt's test)

To investigate the presence of steroids, samples weighing 0.5 g were subjected to extraction using chloroform. A few drops of acetic anhydride were then added to the extract, followed by heating on a water bath for a duration of 30 minutes. The resulting mixture was allowed to cool and concentrated H_2SO_4 (1 mL) was added with care. The mixture was shaken and allowed to stand, and the presence of steroids was confirmed by the appearance of a bluish-green color.

3.17 *Carica papaya in vitro* Anti-Phytophthora Activity

In order to investigate the anti-phytophthora activity of *Carica papaya* extracts, it is crucial to create an appropriate growth medium for the pathogen. The extract is introduced into the medium, followed by the inoculation of the

pathogen to observe its effects on the pathogen's growth and survival (Vazquez-Muñoz *et al*., 2020). Carrot agar is a suitable medium for cultivating Phytophthora species because it contains nutrients and compounds conducive to their growth and reproduction (Schmitthenner & Bhat, 1994). The poison food technique involves introducing the extract into the medium after autoclaving it for sterilization. This approach prevents any interference with the sterilization process (Bolanle, 2017).

Using this technique allows the extract to directly interact with the pathogen, providing adequate feedback on its impact on the pathogen's growth and survival.

The incubation of these inoculated samples enables the observation of changes in the pathogen's growth and morphology over time.

Daily measurements help detect and quantify any inhibitory or stimulatory effects the extract has on the pathogen. This process aids in evaluating the extract's effectiveness against Phytophthora.

3.18 Antifungal Activity using the Poison Food Technique

The poisoned food technique is a common method used in antifungal activity studies to evaluate the efficacy of potential antifungal compounds or extracts and their modes of action. In this technique, the antifungal substance is incorporated into the growth medium. This medium is usually a nutrient-rich agar medium, and its purpose is to create a concentration gradient (Balamurugan, 2014; Blessina *et al*., 2022; Tian *et al*., 2011). The technique has some limitations. For example, it may not accurately reflect the activity of the compound in vivo, and the results may vary depending on the specific fungal strain and growth conditions used. The poisoned food technique enjoys high

popularity and usage because it is a relatively simple and effective method for evaluating the antifungal activity of potential compounds (Martins, 2022).

The poisoned food technique as used in this study is grouped into three major steps:

i. Preparation of Carrot Agar

200 g of carrots were washed, peeled, and finely chopped into small, uniform pieces. A 2L beaker was filled with 1L of distilled water. The chopped carrots were introduced into the water, and the beaker was placed on a hotplate, where the contents were brought to a boil.

After reaching a boil, the mixture was allowed to simmer for 45 minutes until the carrots were softened. The resulting carrot mixture was gently blended using a household Binatone blender and then sieved through a fine mesh strainer to remove any solids. 1.8L of the carrot-infused water was measured and poured into a clean, 2L capacity beaker. To reach the 2L mark, 0.2L of sterile distilled water was added. Then, 40 g of agar was added to the carrot-infused water. The mixture was subjected to gentle heating in a water bath, with constant stirring, until all traces of agar were completely dissolved. Finally, the resulting mixture was dispensed into 20 shoulder bottles, each holding 100 ml. These bottles were then autoclaved for 15 minutes.

ii. Preparation of poison medium and allocation of petri using completely randomized approach

2 g of GLE were weighed and dissolved in 100 ml of carrot agar stored in a shoulder bottle held in a water bath. This action resulted in the preparation of a 20 mg/ml GLE concentration. The same approach was followed to produce 15 mg/ml, 10 mg/ml, and 5 mg/ml GLE concentrations. Additionally, the four

concentrations of 20 mg/ml, 15 mg/ml, 10 mg/ml, and 5 mg/ml for AICDLE and SDE were prepared using the described method.

Each of the 100 ml concentrations was dispensed into six 9 cm Petri dishes. Three Petri dishes were allocated as replicates for *P. palmivora*, while the other three were designated as replicate plates for *P. megarkaya*. The process was repeated for AICDLE and SDE.

For the positive controls, Delco (at a recommended dose of 5 mg/ml) was amended into the carrot agar as earlier described for the plant extracts. Unamended carrot agar was used as the negative control.

iii. Inoculation, incubation and reading

Aseptic protocols were observed to ensure sterile conditions. Three-day-old pre-cultures of *Phytophthora palmivora* (GH-20-ER-417) and *Phytophthora megakarya* (GH-20-WNR-524) were obtained from CRIG. The cultures were then inoculated onto the prepared media in a Petri plate using a sterile 0.2 cm cork borer. The Petri plates were immediately covered after each procedure to prevent contamination. Incubation of Petri plates was conducted at 28 °C for *P. palmivora* and 25 °C for *P. megakarya*. Daily readings were taken until complete growth was observed in the negative control.

3.19 Determination of Percentage Inhibition

The Vincent formula is a method employed for determining the percentage inhibition of a specific substance or compound on the growth of a microorganism (Mishra & Gupta, 2012). To calculate the percentage inhibition of a substance, one compares the growth of a microorganism in the presence of that substance to its growth in the absence of that substance. The formula is as follows:

Percentage inhibition = $\frac{(C-T)}{C} * 100$

Where:

C = the growth of the microorganism in the control (untreated) sample

T = the growth of the microorganism in the treated sample (in the presence of the substance)

The formula's output signifies the extent to which the substance inhibits the microorganism's growth, expressed as a percentage. A higher % indicates a more significant impact of the substance on the microorganism's growth.

3.20 Protocol for *in vitro* testing of modulated plant extract on black pod pathogen

i. The poison food technique, as described earlier, was used to determine the minimum fungicidal concentration (MFC) of Delco on *Phytophthora sp.*

ii. Ten different combinations of Delco-GLE as presented in Table 3.2. The various Delco-GLE combinations are referred to as Combinations A, B, C, D, E, F, G,H, I, and J.

iii. Three-day-old *P. megakarya* and *P. palmivora* cultures were aseptically punched with a 0.2 cm cock borer and inoculated on gelled carrot agar media containing the combinations (A-J).

iv. Pathogen growth is recorded.

v. Combinations that exhibited 100% inhibition *in vitro* were selected for *in vivo* testing.

Table 3. 2: Formulation of Delco to GLE ratios for modulation studies

Number	Combination (Delco: GLE) mg/ml
1	A(0.3:0.3)
2	B(0.3:0.9)
3	C(0.3:1.5)
4	D(0.3:3)
5	E(0.01:5)
6	F(0.03:5)
7	G(0.07:5)
8	H(0.15:5)
9	I(0.2:4)
10	J(0.2:5)

Ten combinations (A-J) were created containing the mass of Delco and GLE per ml of carrot agar media.

Source: Lab work (2022)

3.21 Protocol for *In vivo* Testing (Amoako-Attah *et al.*, 2021)

The following steps were carried out to conduct the *in vivo* pod test of the fungicides using relatively young, disease-free pods:

i. Relatively young, disease-free pods, approximately three months old, were obtained from the research farm at CRIG, Tafo, Ghana.

ii. Transferred into the laboratory and washed thoroughly with sterile distilled water to remove external contaminants.

iii. Pods were arranged in clean plastic trays in replicate consisting of five pods.

iv. Four of the best modulated Delco-GLE combinations, which had exhibited 100% inhibition *in vitro*, were chosen for the experiment.

v. The respective masses of synthetic fungicides and extracts for the selected combinations were dissolved in 100 ml of sterile distilled water to achieve the desired concentration in mg/ml as provided in Table 3.2.

vi. The fungicide concentrations A, B, C and D were transferred into a spray gun and evenly sprayed onto the pods arranged in the plastic trays.

vii. Mycelial plugs from pre-cultured *P. palmivora* and *P. megakaya* were inoculated onto the pods using a 0.2 cm cork borer and sterile rod.

viii. Humidity was created by providing water in beakers positioned within the trays. The trays, and sellotapes were applied to firmly hold the system in place, ensuring proper containment of the pods and pathogens.

ix. The trays were left in the laboratory under room-temperature conditions to incubate for a period of 7 days.

x. On the seventh day, the pods were observed for symptoms arising from the sites of pathogen inoculation. The presence or absence of infection was noted as infected or not infected.

xi. In cases where infection occurred, lesions formed on the pods. Since the pods had an oval shape, a flexible tape measure was used to obtain quantitative measurements of the lesion sizes.

xii. The spreadsheet data was used to calculate the average lesion size of the five pods for each fungicide and each pathogen, providing quantitative results for analysis.

3.22 Data Analysis

All collected data from the study was collated with Microsoft Excel 2019 and later copied to the Statistical Package for Social Sciences for further analysis.

Quantitative pod lesion data were normalized by taking their square roots. Data were expressed as mean ± SEM, and the difference in means was determined by the one-way analysis of variance (ANOVA). Values were considered significant when they were less than 0.05. Where there were significant differences, Tukey's post hoc test was conducted to find the segment of the data responsible for such differences.

3.23 Chapter Summary

The molecular docking method was utilized to assess the binding affinity and interaction of compounds from *Carica papaya* with the pathogen target effector protein, as described previously. Crude *Carica papaya* extracts were obtained and tested against black pod inducing Phytophthora isolates using the poison food technique. Optimization of the most effective crude extract was conducted by varying ratios in combination with a synthetic fungicide (Delco), also using the poison food technique. Promising combinations were further evaluated *in vivo* on detached pods.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.0 Introduction

The primary objective of this study was to assess the efficacy of *Carica papaya* extracts against black pod disease caused by Phytophthora species. The aim was to identify effective and environmentally friendly alternatives to synthetic fungicides, which are known to pose health and environmental risks. To achieve this, an *in silico* assessment using molecular docking was conducted to evaluate known compounds present in *Carica papaya against* a validated black pod-inducing target protein.

Based on the promising results from the *in silico* assessment, crude extractions of *Carica papaya* leaves and seeds were performed. These extracts were then tested against *P. megakarya* and *P. palmivora* using the poison food technique in carrot agar. The objective was to determine the best performing extract among the tested samples. Following the identification of the most effective extract, it was further combined or modulated with a synthetic fungicide to optimize its performance. Initially, this optimization was evaluated *in vitro* using appropriate methods. Subsequently, four combinations of the *Carica papaya* extract and the synthetic fungicide were selected for further assessment *in vivo*, specifically using detached cocoa pods.

4.1 Results

The 2D and 3D docking interactions between the native ligand, its redocked form, and 3ZR8 have been illustrated in the diagram below.

4.2 Molecular docking

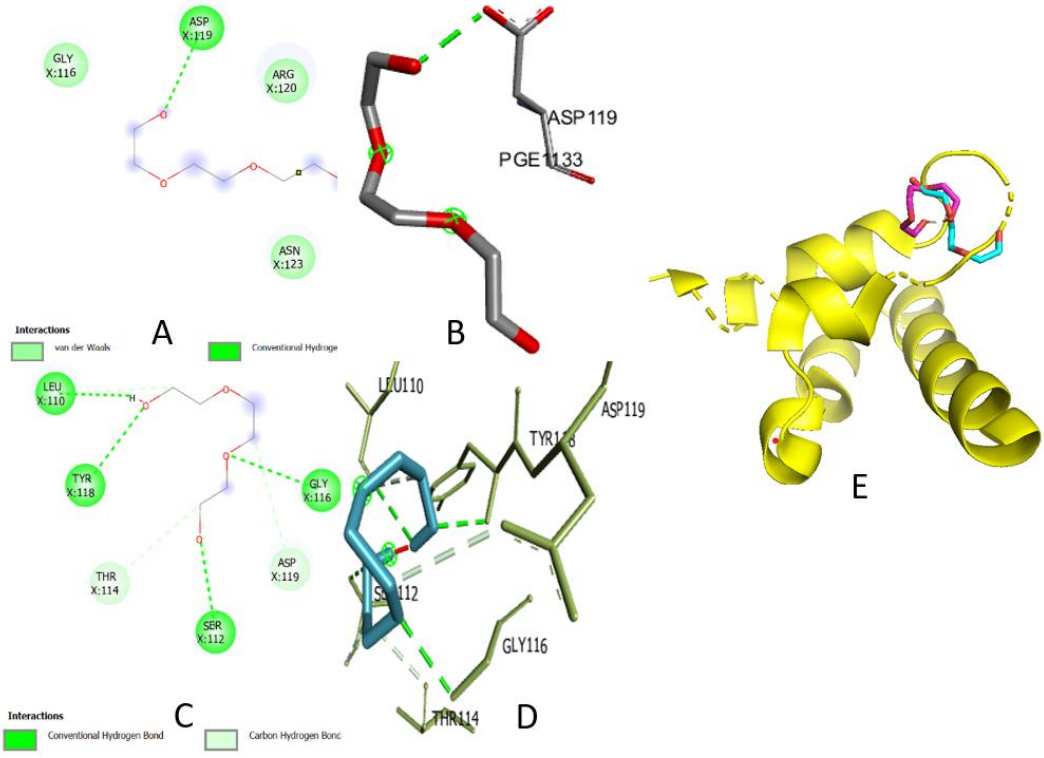


Figure 4. 1: Docking validation

A and B are 2D and 3D interaction of co-crystalized 3ZR8 protein with Triethylene glycol (PGE). C and D are 2D and 3D interaction redocked PGE against 3ZR8 protein. E represents superimposition of original co-crystalized PGE and redocked PGE.

Source: *in silico* work (2022)

Alliin is an L-alpha amino acid that has been demonstrated to effectively inhibit 3ZR8. Therefore, it was utilized as the reference inhibitor in the docking studies. The PyRx docking results revealed that the maximum binding affinity obtained from alliin was -5.3 kcal/mol. After conducting PyRx docking experiments, it was found that carpaine exhibited the highest binding activity of -7.2 kcal/mol, whereas a-tocopherol, ferulic acid, and Mendelic acid exhibited a binding affinity of -5.4 kcal/mol.

Ligands with binding affinities greater than -5.3 kcal/mol, including benzylglucosinolate, quercitin, Riboflavin, catechin, liquiritigenin, vanillic acid, b-sitosterol, campesterol, indolylglucosinolate, Ascorbic acid, lycopene, resveratrol, were selected as potential inhibitors of 3ZR8 (Table 4. 1). The observation that 16 out of the 26 isolates obtained from *Carica papaya* exhibit notable binding activity against 3ZR8 is a promising outcome. This suggests that *Carica papaya* may host compounds with potential utility in the effort to combat *Phytophthora sp*. invasions.

Table 4. 1: Binding affinity of compounds with good inhibition potential on phytophthora effector protein

Compound	Binding Affinity (kcal/mo)	Compound	Binding Affinity (kcal/mol)
1. Carpaine	-7.2	9. Campesterol	-6.2
2. Benzylglucosinolate	-7.0	10. Indolylglucosinolate	-6.2
3. Quercitin	-7.0	11. Ascorbic acid	-5.7
4. Riboflavin	-6.8	12. Lycopene	-5.7
5. Catechin	-6.6	13. Resveratrol	-5.7
6. Liquiritigenin	-6.6	14. α-tocoperol	-5.4
7. Vanillic acid	-6.3	15. Ferilic acid	-5.4
8. β-sitosterol	-6.2	16. Mendelic acid	-5.4
Alliin (Reference Inhibitor)			-5.3

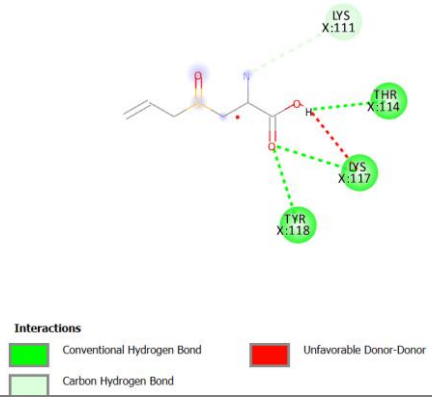
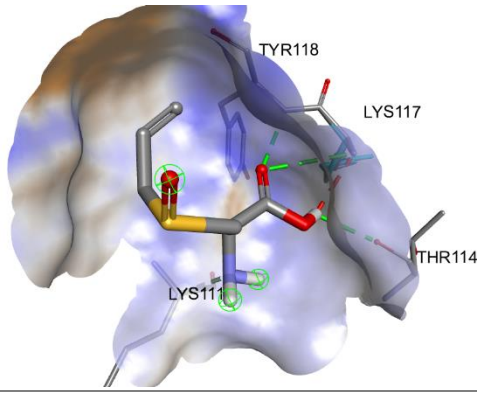
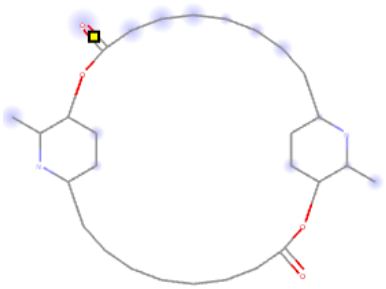
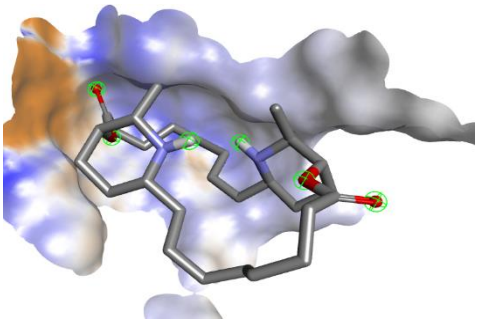
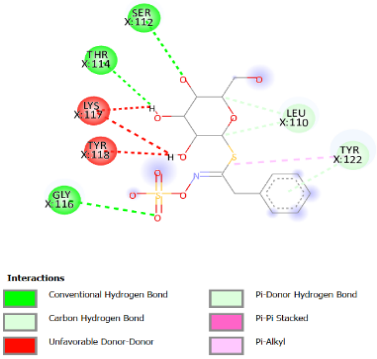
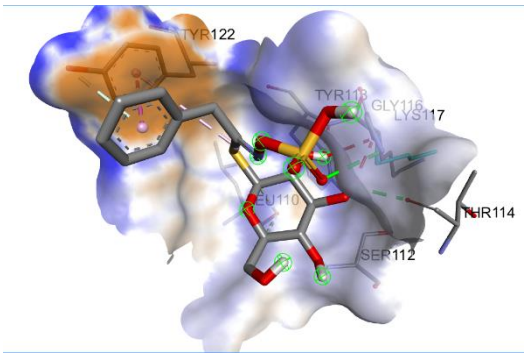
Source: *in silico* work (2022)

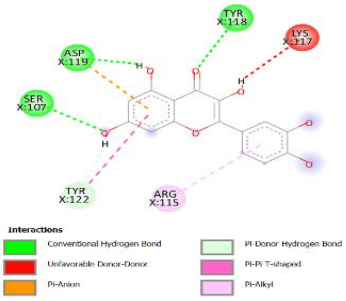
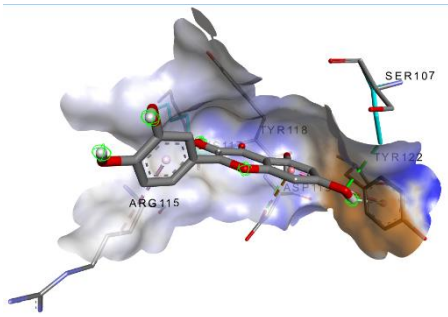
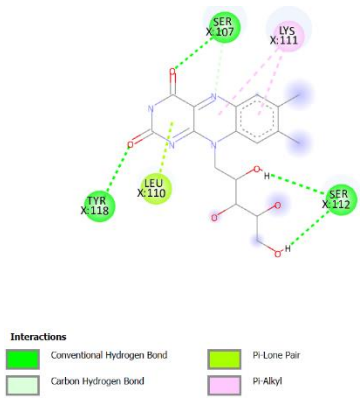
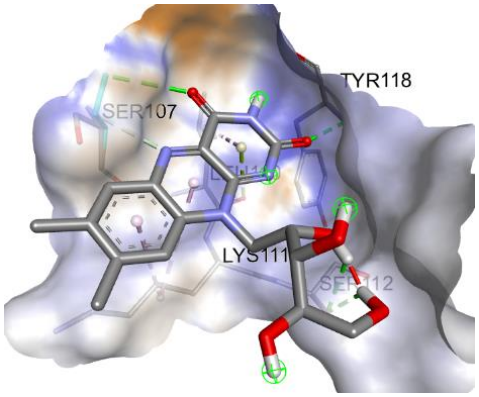
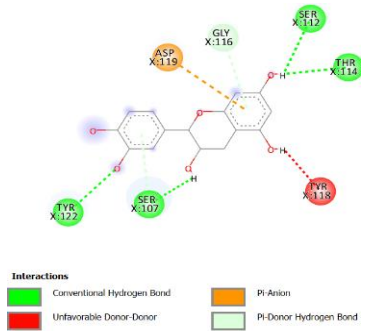
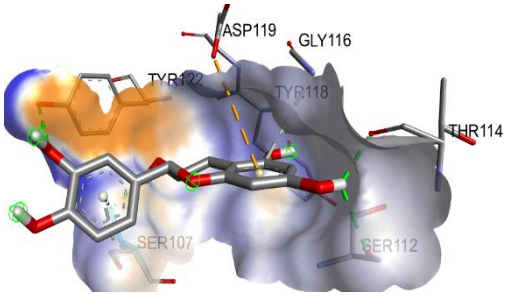
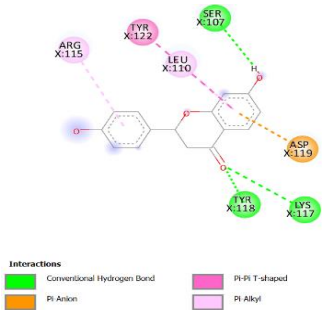
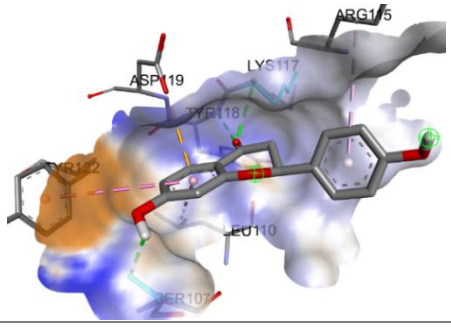
Six molecules were chosen for further analysis from the initial pool of 16 ligands that met the binding affinity requirements as potential inhibitors for 3ZR8. The decision to focus on ligands that have binding affinities similar to

the highest ligand (carpaine) is based on the principle that the higher the binding affinity, the stronger the interaction between the ligand and the receptor (Borquaye *et al*., 2020; Joshi *et al*., 2021).

This implies that ligands with similar binding affinities may have comparable structural and functional properties that can be exploited in drug development.

Table 4. 2: Interaction of ligands with 3ZR8 binding pocket residues

Alliin: 2D (reference inhibitor)	**3D**
	
Carpaine: 2D	3D
	
Benzylglucosinolate: 2D	3D
	

Quercetin: 2D	3D
	
Riboflavin: 2D	3D
	
Catechin: 2D	3D
	
Liquiritigenin: 2D	3D
	

Source: *in silico* work (2022)

In the 2D interactions (Table 4.3), it is observed that the reference inhibitor formed conventional hydrogen bonds with THR114, TYR118, and LYS117, a carbon-hydrogen bond with LYS111, and an unfavorable donor-donor interaction. Carpaine interacted with 3ZR8 through electrostatic interactions. Benzylglucosinolate formed three conventional hydrogen bonds with SER112, THR114, and GLY116, two carbon-hydrogen bonds with LEU110 and TYR122, a pi-alkyl interaction with TYR122, and an unfavorable donor-donor interaction with LYS117 and TYR118.

Quercetin formed conventional hydrogen bonds with ASP119, TYR118, and SER107, a pi-donor hydrogen bond with TYR122, and an unfavorable donor-donor interaction with LYS117, as well as pi-alkyl interactions with ARG115. Riboflavin formed conventional hydrogen bonds with TYR118, SER107, and SER112, a pi-lone pair interaction with LEU11, and both pi-alkyl and carbon-hydrogen bonds with LYS111. Catechin also formed conventional hydrogen bonds with TYR122, THR114, SER107, and SER112, interacted with ASP119 through a pi-anion bond, and had an unfavorable donor-donor interaction with TYR118. Liquiritigenin exhibited conventional hydrogen bonding with LYS117, TYR118, and SER107, pi-alkyl interactions with ARG115 and LEU110, a pi-pi T-shaped interaction with TYR112, and a pi-anion interaction with ASP119.

4.3 Phytochemical screening

Phytochemical analysis of the three crude extracts, GLE, AICDLE, and SDE, revealed the presence of several bioactive compounds. Alkaloids, saponins, tannins, flavonoids, glycosides, and terpenoids were detected in all three extracts. Phytosteroids were found in the extracts AICDLE and SDE but

were notably absent in GLE. This suggests that AICDLE and SDE may possess unique phytochemical profiles compared to GLE.

Table 4. 3: Phytochemicals Detected in Crude Extracts

Phytochemical test	Observation	GLE	AICDLE	SDE
Alkaloids (Drangendroff's)	Orange-red precipitate	+	+	+
Saponins (Foam)	Frothing of extracts>10 mins	+	+	+
Tannins (FeCl3)	Blue-black colour	+	+	+
Flavonoids (Lead acetate)	Reddish brown precipitate	+	+	+
Glycosides (Kellar-Killiani)	Reddish brown ring	+	+	+
Terpenoids	Reddish brown colour	+	+	+
Phytosteroid	Bluish green	-	+	+

+ denotes presence and – denotes absence of the specific phytochemical in tested sample.
Source: Lab work (2022)

4.4 *In vitro* studies of the effect of crude extracts on *P. palmivora*

The measured radial growths of *P. palmivora* isolate under poison food condition for different concentrations of the GLE were as follows: 1.26 cm, 1.21 cm, 1.68 cm, and 1.62 cm for concentrations of 20 mg/ml, 15 mg/ml, 10 mg/ml, and 5 mg/ml, respectively. Despite the numerical differences in radial growths among the concentrations, these differences were not found to be statistically

significant (P>0.05). This indicates that using 5mg/ml achieved an equivalent result to using 20mg/ml in pathogen control.

The measured radial growths of *P. palmivora* under the influence of AICDLE at different concentrations were as follows: 1.57 cm, 2.43 cm, 2.87 cm, and 4.20 cm for concentrations of 20 mg/ml, 15 mg/ml, 10 mg/ml, and 5 mg/ml, respectively. The results suggest that the inhibitory effect of AICDLE on the radial growth of *P. palmivora* is dose-dependent. Higher concentrations of AICDLE correspond to lower radial growth values, indicating better inhibitory performance. The average radial growth decreases as the concentration of AICDLE increases. To determine the significance of the observed differences, a one-way analysis of variance ANOVA was conducted. The ANOVA revealed a significant difference (P<0.05) between the concentrations of 20 mg/ml and 5 mg/ml. This indicates that there is a variation in the effect of concentration on the radial growth of *P. palmivora*, and the higher concentration of 20 mg/ml was more effective in inhibiting the radial growth compared to the lower concentration of 5 mg/ml.

Table 4. 4: Antifungal activity of crude extracts against *P. palmivora*

Conc mg/ml	GLE±SEM (RG/cm)	AICDLE±SEM (RG/cm)	SDE±SEM (RG/cm)	F	P
20	1.26±0.11$^{a(1)}$ (PI=83.42)	1.57±0.22$^{a(1)}$ (PI=79.34)	2.43±0.44 $^{a(1)}$ (PI=68.03)	2.45	0.14
15	1.21±0.12$^{a(1)}$ (PI=84.08)	2.43±0.41 $^{ab(1,2)}$ (PI=68.03)	2.96±0.57 $^{a(2)}$ (PI=61.05)	2.49	0.04
10	1.68±0.20$^{a(1)}$ (PI=77.89)	2.87±0.56 $^{ab(1)}$ (PI=62.24)	2.53±0.58 $^{a(1)}$ (PI=66.71)	3.07	0.25
5	1.62±0.10$^{a(1)}$ (PI=78.68)	4.20±0.67$^{b(3)}$ (PI=44.7)	2.96±0.47 $^{a(2)}$ (PI=61.05)	0.67	0.01
Delco (5)	0 (PI=100)	0 (PI=100)	0 (PI=100)		
Control (-)	7.6±0.9				
F	3	4.82	0.29		
P	0.07	0.02	0.83		

Conc=Concentration (mg/ml); GLE= Green Leave Extract, AICDLE= Aging Induced Chlorophyll Deficient Leave, SDE= Matured Seed Extract; SEM=Standard Error of Mean; PI= Percentage Inhibition; RG=Radial growth; cm= centimeters; Delco (5) = Commercial synthetic fungicide at recommended dose of 5mg/ml; Control (-) = Negative control; F= ANOVA F-value, p= ANOVA p-value.

^{ab} different superscript letters indicate a significant difference among means in each column, $p<0.05$ by Tukey's post-hoc test.

^{1,2,3} different superscript letters indicate a significant difference among means in each roll, $p<0.05$ by Tukey's post-hoc test.

Source: Lab work (2022)

The results show that the radial growth of *P. palmivora* varied slightly across the different concentrations of SDE (Table 4.4). The radial growth values were relatively similar, with no clear trend of increasing or decreasing growth as the concentration changed. The relatively consistent radial growth values across the different concentrations suggest that SDE might have a limited impact on inhibiting the growth of *P. palmivora*, or that the concentrations tested were not optimal for achieving significant inhibition.

The average radial growth of *P. palmivora* in the negative control group was 7.6 cm. In contrast, the positive control group treated with the commercially available synthetic fungicide Delco exhibited no radial growth (0 cm). Comparing the different extracts, the results indicate that GLE performed better against *P. palmivora* compared to AICDLE and SDE. Higher doses of GLE showed more effective inhibition of radial growth compared to AICDLE and SDE.

At higher doses, AICDLE exhibited better inhibitory effects on *P. palmivora* compared to SDE. Conversely, at lower doses, SDE showed better inhibitory performance than AICDLE. These findings highlight the importance of dosage and the specific extract used in achieving effective control of *P. palmivora's* radial growth. GLE, in general, demonstrated the most promising inhibitory effects against *P. palmivora*, regardless of dosage, while AICDLE and SDE showed varying levels of effectiveness depending on the concentration used. The positive control group treated with Delco, the synthetic fungicide, exhibited complete inhibition of radial growth. This indicates that Delco is a highly effective fungicide against *P. palmivora*, surpassing the inhibitory effects observed with any of the tested extracts.

4.5 *In vitro* studies of the effect of crude extract on *P. megakarya*

A one-way ANOVA, indicated a significant difference ($p<0.05$) in radial growth among the various concentrations of GLE (Table 4.4). Additionally, a post hoc test using Tukey's method confirmed a significant difference between the highest concentration (20mg/ml) and the lowest concentration (5mg/ml). These findings demonstrate that using a higher concentration of GLE resulted in greater inhibition of radial growth in *P.*

megakarya. The dose-dependent response observed in this study suggests that the inhibitory effects of GLE on *P. megakarya* are influenced by the concentration of the extract. This information can guide the application and usage of GLE as a potential control measure against *P. megakarya.*

Table 4. 5: Antifungal activity of crude extracts against *P. megakarya*

Conc mg/ml	GLE±SEM (RG/cm)	AICDLE±SEM (RG/cm)	SDE±SEM (MG/cm)	F	P
20	1.26±0.11 $^{a(1)}$ (PI=79.67)	1.47±0.17 $^{a(1)}$ (PI=76.29)	2.02±0.49 $^{a(1)}$ (PI=67.42)	1.61	0.25
15	1.39±0.82 $^{ab(1)}$ (PI=77.58)	2.77±0.70 $^{a(1)}$ (PI=55.32)	1.73±0.36 $^{a(1)}$ (PI=72.1)	2.49	0.14
10	1.66±0.44 $^{ab(1)}$ (PI=73.22)	3.17±0.71 $^{a(1)}$ (PI=48.87)	1.76±0.42 $^{a(1)}$ (PI=71.61)	3.07	0.09
5	2.34±0.29 $^{b(1)}$ (PI=62.26)	2.94±0.78 $^{a(1)}$ (PI=52.58)	2.03±0.51 $^{a(1)}$ (PI=67.26)	0.67	0.54
Delco (5)	0 (PI=100)	0 (PI=100)	0 (PI=100)		
Control (-)	6.2±1.7				
F	7.2	1.42	0.13		
P	0.004	0.42	0.94		

Conc=Concentration (mg/ml); GLE= Green Leave Extract, AICDLE= Aging Induced Chlorophyll Deficient Leave, SDE= Matured Seed Extract; SEM=Standard Error of Mean; PI= Percentage Inhibition; RG=Radial growth; cm= centimeters; Delco (5) = Commercial synthetic fungicide at recommended dose of 5mg/ml; Control (-) = Negative control; F= ANOVA F-value, p= ANOVA p-value.

^{ab} different superscript letters indicate a significant difference among means in each column, $p<0.05$ by Tukey's post-hoc test.

^{1,2} different superscript letters indicate a significant difference among means in each roll, $p<0.05$ by Tukey's post-hoc test.

Source: Lab work (2022)

While the concentrations of AICDLE did not consistently correlate with increasing growth inhibition, it was observed that the highest inhibition was achieved at the concentration of 20mg/ml. This result indicates that there may

be other factors influencing the inhibitory effects of AICDLE on the radial growth of *P. megakarya*, beyond the concentration alone. The composition and interactions of the extract's components could be contributing to the observed variations in growth inhibition. Despite the lack of a clear dose-dependent relationship, the highest inhibitory effect was observed at the highest concentration of AICDLE (20mg/ml). This suggests that using a higher concentration of AICDLE may be more effective in suppressing the radial growth of *P. megakarya* compared to lower concentrations.

The average radial growth rates of *P. megakarya* under the influence of SDE were 2.02 cm to 1.73 cm for concentrations between 5 mg/ml to 20 mg/ml. The concentration of 15 mg/ml demonstrated the best growth retarding effect on *P. megakarya*. This concentration resulted in the lowest average radial growth rate among the tested concentrations, indicating a higher inhibitory efficacy compared to the other concentrations.

The average radial growth of the negative control group (no treatment) was 6.2 cm, while the positive control group treated with the commercially available synthetic fungicide Delco exhibited no radial growth (0 cm).

Comparing the extracts, it is evident that GLE exhibited the most effective inhibitory effects against Pm, followed by SDE and AICDLE.

4.6 *In vitro* modulation studies

Considering the superior activity demonstrated by GLE compared to other extracts, it was selected for optimization through modulation studies. A modulation studies was designed to improve the effectiveness of *Carica papaya* as a control for black pod disease. To facilitate these studies, it was crucial to determine the Minimum Fungicidal Concentration (MFC) of the Delco and the

MFC for GLE. The MFC of the Delco was determined to be 0.6mg/ml (Table 4.6).

This finding indicates that Delco exhibits a strong inhibitory activity against the target pathogen at a low concentration. The determination of the MFC for Delco serves as a benchmark for its effective use as a control measure, providing valuable information regarding the concentration needed to inhibit the growth of the pathogen effectively.

Table 4. 6: Determination of the minimum fungicidal concentration (MFC) for Delco.

Treatment: Conc mg/ml	Pp (MG cm)	Pm (MG cm)
5	0	0
2.5	0	0
1.25	0	0
0.62	0	0
0.31	0.4	0.3

Conc= concentration in mg/ml, Pp= *P. palmivora*, Pm= *P. megakarya* MG=Mycelial growth, cm; centimeters.

Source: Lab work (2022)

Modulation studies were conducted using different ratios of Delco to GLE in order to assess their combined inhibitory effects against *P. palmivora* and *P. megakarya*. The Delco: GLE ratios were tested by measuring masses in mg dissolved in ml of carrot agar. Of particular interest were the following combinations: A (0.3:0.3), B (0.3:0.9), C (0.3:0.15), and D (0.3:30). The results showed that there was no radial growth observed for any of these tested combinations, indicating 100% inhibition *in vitro* against both *P. palmivora* and

P. megakarya (Figure 4. 2). The fractional inhibition concentration index (FICI) values were calculated for each combination. The FICIs for combinations A, B, C, and D were determined to be 0.51, 0.54, 0.57, and 0.65, respectively (Table 4.7).

These FICI values suggest a partially synergistic effect for the tested combinations. The interpretation of the FICIs indicates that the combinations of Delco and GLE demonstrate a cooperative interaction in inhibiting the growth of *P. palmivora* and *P. megakarya*. The FICI values falling within the range of 0.5 to 1.0 suggest a degree of synergy, indicating that the combined inhibitory effects are greater than what would be expected from each component alone.

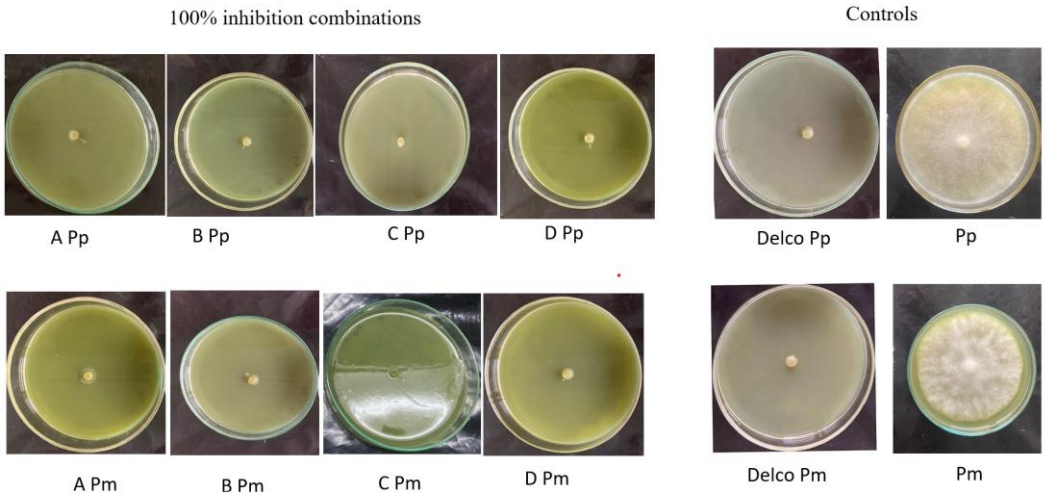


Figure 4. 2: Complete in vitro inhibition against *P. palmivora (Pp)* and *P. megakarya* (Pm)

Source: Lab work (2022)

These formulations reduced the required amount of Delco by about 90%, while the GLE quantities remained within reasonable limits. This approach has the potential for substantial cost savings. The formulations will

limit the negative environmental impact associated with synthetic fungicide use, making this modulated fungicide a more environmentally friendly alternative.

The combinations of Delco and GLE in E (0.01:5), F (0.03:5), and G (0.07:5), allowed growth of *P. palmivora*. The radial growth rates for *P. palmivora* were 3.2 cm, 2.8 cm, and 3.30 cm, corresponding to 36.3%, 44.3%, and 33.3% inhibition, respectively.

The combinations of Delco and GLE in different ratios, H (0.15:0.5), I (0.02:0.4), and J (0.02:0.5), demonstrated complete inhibition of *P. palmivora* growth *in vitro*, resulting in 100% inhibition. The fractional inhibition index (FICI) values for combinations H, I, and J were 0.50, 0.53, and 0.55, respectively, indicating a synergistic effect for H, and partially synergistic effects for I and J. The same combinations were not able to achieve 100% inhibition of *P. megakarya* growth *in vitro*. The radial growth rates for *P. megakarya* were 0.5 cm for H, 0.6 cm for I, and 0.2 cm for J, corresponding to inhibition percentages of 87.5%, 87.1%, and 96.3%, respectively. The differential response observed between the two pathogens may be attributed to variations in their susceptibility or inherent differences in their growth patterns. It is possible that *P. megakarya* exhibits a higher level of resistance or requires a different concentration or combination of control measures to achieve complete inhibition.

Table 4. 7: *In vitro* activity of GLE-Delco modulated combinations

	Fungicide (Combinations)	Pp			Pm			RMK
No.	Ratio (mg/ml) (Delco-GLE)	FICI	Pp MG	PI	FICI	Pm MG	PI	RMK
1	A(0.3:0.3)	0.51	0.00±0.00	100	0.51	0.00±0.00	100	PS
2	B(0.3:0.9)	0.54	0.00±0.00	100	0.54	0.00±0.00	100	PS
3	C(0.3:1.5)	0.57	0.00±0.00	100	0.57	0.00±0.00	100	PS
4	D(0.3:3)	0.65	0.00±0.00	100	0.65	0.00±0.00	100	PS
5	E(0.01:5)	0.26	3.20±0.09	36.3	0.26	3.40±0.05	15.8	S
6	F(0.03:5)	0.3	2.8±0.11	44.3	0.3	3.0±0.04	24.2	S
7	G(0.07:5)	0.36	3.30±0.11	33.3	0.36	1.1±0.00	73	S
8	H(0.15:5)	0.5	0.00±0.00	100	0.5	0.5±0.00	87.5	S
9	I(0.2:4)	0.53	0.00±0.00	100	0.53	0.6±0.05	87.1	PS
10	J(0.2:5)	0.58	0.00±0.00	100	0.58	0.2±0.11	96.3	PS

Combinations (A-J)= mass of GLE-Delco per ml. Fractional Inhibitory Concentration Index (FICI)Value interpretation: ≤0.5, (S)Synergy; >0.5–1.0,(PS)Partial synergy; >1–4.0, Indifference; (A)antagonism; >4.0 (Valderrama *et al*., 2020). PI= % Inhibition; RMK=Remark; MG=Mycelial growth; *Phytophthora Palmivora* (Pp), *Phytophthora megakarya* (Pm)

Source: Lab work (2022)

4.7 *In vivo* assessment of modulated extracts (Pod work)

The purpose of the *in vivo* evaluation was to investigate the real-world efficacy of the modulated fungicide combinations A, B, C, and D and determine their potential as anti-Phytophthora agents. This step is crucial for validating the results obtained from the modulation studies conducted *in vitro*. During the *in vivo* evaluation, cocoa pods were inoculated with Phytophthora and treated with the selected modulated fungicides. Lesions if produced on the pods were examined and scored based on their severity. The scoring system provided both qualitative and quantitative measure of the anti-Phytophthora activity of each fungicide.

Lesion development on cocoa pods post application of fungicides is presented in (Figure 4. 3)

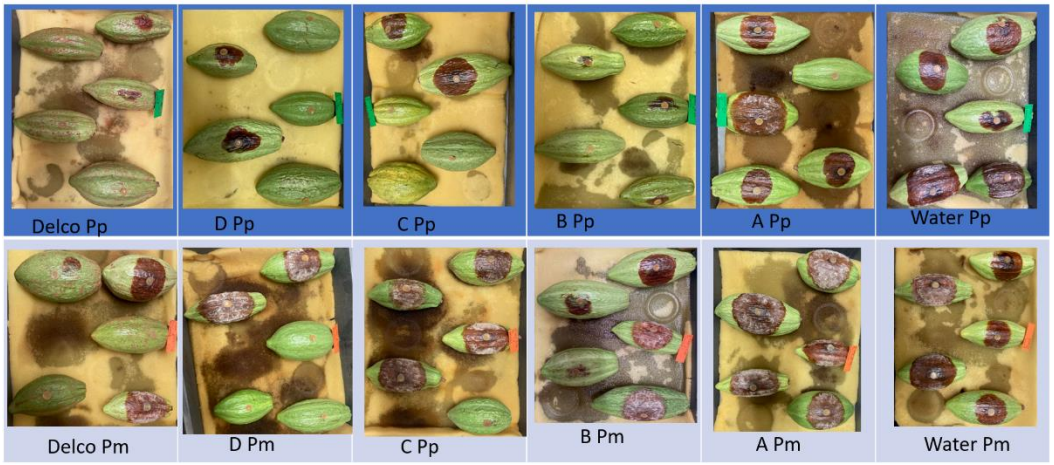


Figure 4. 3: Lesion development on detached Cocoa pods

Source: Lab work (2022)

The *in vivo* evaluation of combination A, targeting *P. palmivora* and *P. megakarya*, revealed lesions on all five cocoa replicates (Figure 4. 3,Table 4.8). This indicates that the fungicide used in the combination was not able to prevent infection *in vivo*. The inability of combination A to inhibit the growth of both pathogens on cocoa pods raises concerns about its efficacy as an anti-Phytophthora treatment. The results of this evaluation highlight the limitations of combination A in providing protection against Phytophthora in a real-world setting.

Table 4. 8: Incidence of cocoa pod infection post application of fungicide

Fungicide	Pp	pm
A	5/5	5/5
B	3/5	5/5
C	3/5	5/5
D	2/5	3/5
Delco	2/5	3/5
SDW	5/5	5/5

Occurrence of lesions on cocoa pods after treatment with A-D Modulated combinations, Delco: commercial fungicide and sterile distilled water (SDW): negative control

Source: Lab work (2022)

The *in vivo* evaluation of combination B for controlling *P. palmivora* and *P. megakarya* on cocoa pods yielded mixed results (Figure 4. *3*, Table 4.8). Out of the five replicates tested against *P. palmivora*, three were infected, indicating that the fungicide in combination B prevented infection in two out of the five pods. However, when tested against *P. megakarya*, the fungicide was not able to offer protection to any of the pods, resulting in all five pods being infected. The varying response of combination B to different pathogens highlights the complexity of developing effective control strategies against multiple *Phytophthora sp*.

In vivo evaluation of combination C for controlling *P. palmivora* and *P. megakarya* on cocoa pods yielded mixed results. Out of the five replicates tested

against *P. palmivora*, three replicates were infected, indicating that the combination was able to protect two out of the five pods (40% inhibition) from *P. palmivora* infection (Figure 4. *3*, Table 4.8). However, when tested against *P. megakarya*, combination C failed to offer protection to any of the pods, resulting in all five pods being infected.

The *in vivo* evaluation of combination D for controlling *P. palmivora* and *P. megakarya* on cocoa pods demonstrated moderate efficacy (Figure 4. 3,Table 4.8). Out of the five replicates tested against P. palmivora, two replicates were infected, indicating that the fungicide in combination D prevented infection in three out of the five pods (60% inhibition). The fact that three out of the five pods were protected from *P. palmivora* infection demonstrates the effectiveness of the fungicide in preventing infection by this pathogen. Similarly, the protection of two pods against *P. megakarya* indicates some level of control against this specific pathogen.

The *in vivo* evaluation of Delco, a commercially available synthetic fungicide, for controlling *P. palmivora* and *P. megakarya* on cocoa pods demonstrated moderate efficacy (Figure 4. *3*,Table 4.8). Out of the five replicates tested against P. palmivora, two replicates were infe

cted, indicating that the fungicide prevented infection in three out of the five pods (60% inhibition). When tested against *P. megakarya*, the fungicide offered protection to two pods, resulting in 40% inhibition. The partial efficacy of Delco against both *P. palmivora* and *P. megakarya* suggests its potential as an anti-Phytophthora treatment.

The negative control, in which SDW was used as a treatment, resulted in the infection of all the tested pods by both *P. palmivora* and *P. megakarya*

(Figure 4. 3,Table 4.8). This indicates that the SDW treatment did not provide any protection against the pathogens and allowed for the successful infection of all the pods. The complete infection of the pods in the negative control validates the reliability of the experimental setup and confirms the pathogenicity of both *P. palmivora* and *P. megakarya* on cocoa pods.

The average lesion sizes provide valuable insights into the inhibition capacity of the tested treatments against *P. palmivora* and *P. megakarya* on cocoa pods (Figure 4. 4). The results indicate differences in the effectiveness of the treatments in controlling the growth and development of the pathogens.

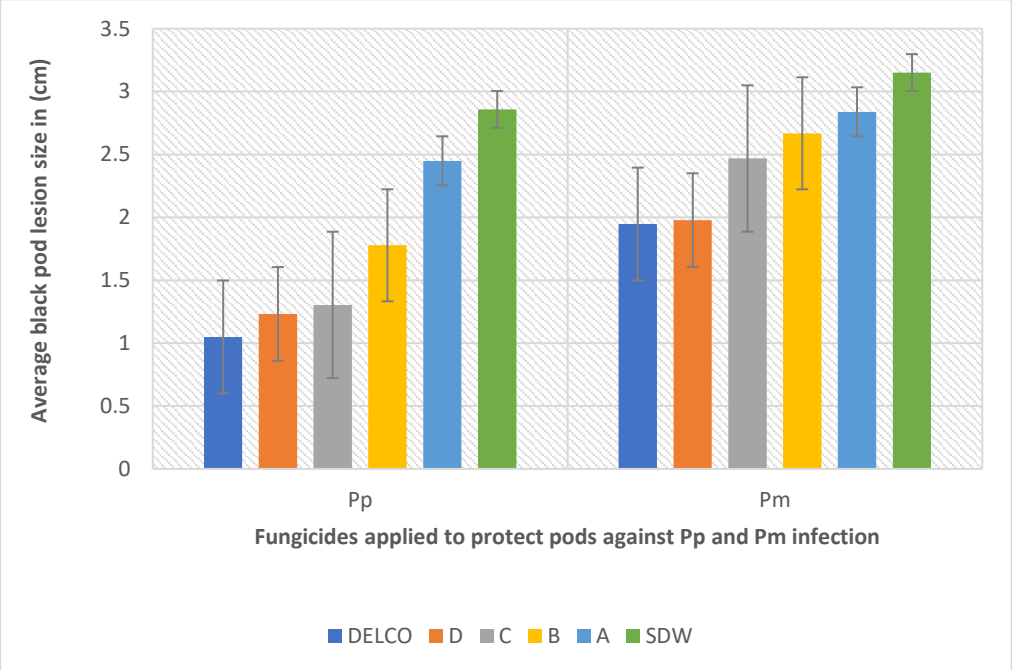


Figure 4. 4: Quantitative average lesion size of fungicides in order of efficacy in inhibiting *P. palmivora and P. megakarya* on detached cocoa pods (The values used in Figure 4.4 are square root-transformed means of the experimental data)

Source: Lab work (2022)

For *P. palmivora*, Delco exhibited the smallest average lesion size of 1.05 cm, indicating superior inhibition capacity against this pathogen.

Combination D followed with an average lesion size of 1.23 cm, while combination C and B showed larger lesion sizes of 1.30 cm and 1.78 cm, respectively (Figure 4. 4). Combination A and the negative SDW treatment exhibited the largest lesion sizes of 2.45 cm and 2.86 cm, respectively, indicating their limited effectiveness in inhibiting the growth of P. *palmivora* (Figure 4. 4). With respect to *P. megakarya*, Delco again displayed the smallest average lesion size of 1.94 cm, suggesting its stronger inhibitory effect against this pathogen. Combination D showed a slightly larger average lesion size of 1.98 cm.

Combination C and B exhibited further increases in average lesion sizes of 2.47 cm and 2.67 cm, respectively. Combination A and the negative SDW treatment displayed the largest average lesion sizes of 2.84 cm and 3.15 cm, respectively, indicating their reduced ability to control the growth of *P. megakarya* (Figure 4. *4*). These results highlight the superior inhibitory capacity of Delco against both *P. palmivora* and *P. megakarya*, as it consistently displayed the smallest average lesion sizes for both pathogens.

The differences in lesion sizes among the treatments suggest variations in their ability to impede the growth and spread of the pathogens on cocoa pods.

4.8 Discussion

4.9 Molecular Docking

Alliin, an L-alpha amino acid, was identified as an effective inhibitor of 3ZR8 (Rani *et al*., 2017). The docking results from PyRx revealed that alliin exhibited a maximum binding affinity of -5.3 kcal/mol. 16 Ligands produced a binding affinity equal to or greater than alliin's. Among the ligands tested, carpaine displayed the highest binding activity with a binding affinity of -7.2

kcal/mol. On the other hand, a-tocopherol, ferulic acid, and mendelic acid showed a binding affinity of -5.4 kcal/mol (Table 4. 1). Out of the initial pool of 16 ligands that met the binding affinity requirements, six specific molecules were selected for further analysis as potential inhibitors for 3ZR8.

The rationale behind this approach is rooted in the understanding that ligands with higher binding affinities exhibit stronger interactions with their target receptors, potentially leading to more potent and effective inhibition (Singh *et al.*, 2020; Liddament et al., 2019). By prioritizing ligands with similar binding affinities to carpaine, we aim to identify compounds that could mimic or surpass the inhibitory properties of this reference ligand. This strategy enables us to narrow down the pool of ligands to a subset that holds promise for further investigation.

By focusing on ligands with similar binding affinities, we can potentially uncover structural motifs or functional characteristics that contribute to strong binding interactions with the receptor (Maurya *et al*., 2020). Such insights can aid in the design and optimization of novel drug candidates by leveraging the favorable properties shared by these ligands.

The molecules that have been selected have previously exhibited therapeutic efficacy in prior investigations.

Carpaine, an alkaloid found in the leaves of *Carica papaya*, has been documented to exhibit diverse pharmacological effects. Studies have shown that carpaine exhibits antimicrobial properties (Hastuty, 2019; Masfufatun *et al*., 2019; Poh & Jien, 2017). Benzylglucosinolate has been found to possess antifungal and antibacterial properties (Calzada *et al*., 2003; Castro-Vargas *et al*., 2016; Vierheilig *et al*., 2000).

Quercetin is a flavonoid that has been acknowledged to have a broad range of pharmacological activities. Studies have shown that quercetin exhibits antimicrobial activity and antiviral activity (Nguyen & Bhattacharya, 2022). Riboflavin is a vitamin B2 that has demonstrated various pharmacological activities in previous studies. Riboflavin exhibits inhibition of pathogens including bacteria, viruses, fungi, and parasites (Martins *et al*., 2008). Catechin is a flavonoid medicinal potency particularly as it exhibits antimicrobial activity (Bai *et al*., 2016; Wu *et al*., 2010). Liquiritigenin is a flavonoid that can reverse drug resistance in bacteria (Gaur *et al*., 2016).

The absence of any interaction between carpaine compared to the other ligands distinguishes it from the rest of the ligands in the analysis. This observation mirrors the report of (Kumaree *et al*., 2023) and underscores carpaine's unique characteristics in terms of potential molecular interactions. The fact that the docked carpaine molecule was not attached to any protein residue from 3ZR8 (Table 4.) yet produced the highest binding affinity of -7.2 (kcal/mol) with the protein could be due to several reasons: Carpaine may have a shape that is complementary to the binding pocket of 3ZR8, allowing it to interact with the protein and form stable non-covalent interactions (Kumaree *et al*., 2023). This can increase the binding affinity of carpaine with 3ZR8. Carpaine contains functional groups that can form electrostatic interactions with the amino acid residues of 3ZR8. These interactions can help to stabilize the complex and increase the binding affinity.

Carpaine may be a flexible molecule that can adopt different conformations when interacting with 3ZR8. This flexibility can allow carpaine to form stable interactions with the protein. The high binding affinity of carpaine

with 3ZR8 could be due to a favorable balance of free energy contributions, including van der Waals interactions, electrostatic interactions, and solvation energy. Even though carpaine is not attached to any specific residue, these free energy contributions can help to stabilize the complex and increase the binding affinity.

The similarities between the binding interactions of these five ligands (benzylglucosinolate, quercetin, riboflavin, catechin, and liquiritigenin) with 3ZR8 include the presence of conventional hydrogen bonds with amino acid residues such as TYR 118, SER 107, and SER 112 (Table 4. 2).

Conventional hydrogen bond formation between a ligand and a target protein is important for several reasons: Hydrogen bonding is a highly specific interaction, meaning that it allows for selective recognition and binding between a ligand and its target protein. This specificity is critical for ensuring that the ligand binds only to its intended target, and not to other proteins in the cell (Coulocheri *et al*., 2007; Craik *et al*., 2013; Fedorova *et al*., 2023). The strength of the hydrogen bond depends on several factors, including the distance between the donor and acceptor atoms, the angle between the donor-acceptor pair, and the polarity of the molecules involved. Strong hydrogen bonding interactions can increase the overall affinity of the ligand for its target, making it more likely to bind and remain bound to the protein (Bulusu & Desiraju, 2020; Chen *et al*., 2016; Yunta, 2017).

Hydrogen bonds can also contribute to the structural stability of the ligand-protein complex. These interactions can help to hold the ligand in a specific conformation or orientation within the protein binding site, preventing it from being displaced or undergoing conformational changes that could affect

its activity (Melandri, 2011; Ucisik *et al*., 2016). Apart from riboflavin and liquiritigenin, each compound also had unfavorable donor-donor interactions with at least one amino acid residue.

There are also differences in their binding interactions. For example, benzylglucosinolate had a carbon-hydrogen bond and pi donor hydrogen bond with LEU 110 and TYR 122, while quercetin had a pi-alkyl interaction with ARG 115. Riboflavin had a pi-alkyl interaction with LYS 111, while catechin had conventional hydrogen bonds with SER 102 in addition to the other residues. Liquiritigenin had a pi-anion interaction with ASP 119, which was not seen in the other compounds. In terms of binding affinity, benzylglucosinolate, and quercetin had the higher affinity at -7 (kcal/mol), followed closely by riboflavin at -6.8 (kcal/mol), and then catechin and liquiritigenin at -6.6 (kcal/mol).

The similarities and differences in the binding interactions of these compounds with 3ZR8 suggest that each compound uniquely interacts with the protein and that small structural differences can result in different binding affinities. These findings could be useful in the development of new drugs or therapeutic agents that target 3ZR8 or similar proteins.

4.10 Phytochemical screening

The phytochemical analysis of the three crude extracts, GLE, AICDLE, and SDE, demonstrated the presence of major classes of bioactive compounds. Alkaloids, saponins, tannins, flavonoids, glycosides, and terpenoids were detected in all three extracts, indicating their wide distribution and abundance in the studied plant material (Lohidas *et al*., 2015; Silva *et al*., 2007). These compounds are well-known for their potential medicinal properties and have

been extensively studied for their pharmacological activities. An observation from the analysis is the absence of phytosterols in GLE while their presence in AICDLE and SDE.

The differential presence of phytosteroids suggests that AICDLE and SDE possess distinct phytochemical profiles compared to GLE, indicating possible variations in their chemical composition and potential biological activities. The similar phytochemical profile involving alkaloids, saponins, tannins, flavonoids, glycosides, and terpenoids, in all three extracts highlights (Table 4. 1) show their universal occurrence in the plant material under investigation (Callixte *et al*., 2020). Their presence suggests that the studied plant material possesses a rich repository of bioactive compounds that could be explored for potential therapeutic applications.

4.11 *In vitro* effect of crude extracts on *P. palmivora*

The results of the experiment, evaluating the effects of different concentrations of GLE on the radial growth of *P. palmivora* under poisoned food conditions, are discussed below.

Among the tested concentrations (5-20 mg/ml), the differences in radial growth were not statistically significant ($P > 0.05$) based on a one-way ANOVA. The data indicates that using a concentration of 5 mg/ml achieved comparable results to using 20 mg/ml in terms of inhibiting *P. palmivora*'s radial growth. This suggests the potential for using lower GLE concentrations, resulting in cost savings without compromising effectiveness. (El-Sayed & Ali, 2020) conducted a study and made an observation regarding the efficacy of *Aspergillus flavipes* extract in controlling *P. parasitica*. While the higher concentration of 2% provided complete protection, the study revealed that the lower concentration

of 0.5% offered an equally potent alternative for protection. The 0.5% concentration achieved a suppression rate of over 50% in the progression of *P. parasitica*, demonstrating its efficacy despite being lower in concentration. This highlights the potential of harnessing the power of lower concentrations, offering a promising approach for effective disease control while minimizing the need for higher concentrations.

Higher concentrations of AICDLE have a stronger inhibitory effect on the growth of *P. palmivora*, resulting in lower radial growth values. The ANOVA revealed a significant difference ($P< 0.05$) between the concentrations of 20mg/ml and 5mg/ml. This finding indicates that the higher concentration of 20mg/ml demonstrated a greater inhibitory effect compared to the lower concentration of 5mg/ml. These results agree with Stephan *et al*. (2005) who worked on the dose-response effect of *R. rhabarbarum* extract against *P. parasitica*. Stephan observed that higher concentrations of the extract yielded better performance compared to lower concentrations, indicating a positive relationship between concentration and effectiveness.

The percentage inhibition observed for SDE was found to exceed 60%. Another observation was made during the experiment, where the concentration of 5mg/ml performed better than the concentration of 20mg/ml. This finding highlights the non-linear relationship between concentration and performance in this particular case.

This outcome suggests that the optimal effectiveness of the solution may lie within a narrower range of concentration. A similar observation was reported by (Awurum & Enyiukwu, 2013). Their research revealed that a 30% concentration of *C. papaya* SDE exhibited notable inhibitory effects against

seed-borne fungal diseases, surpassing the performance of a 50% extract concentration. In the present study, although perfect inhibition was not achieved, the tested concentration of SDE demonstrated inhibitory effects of over 60% on *P. palmivora*'s radial growth. To enhance the effectiveness of SDE, alternative concentrations or additional components can be explored. It is possible that the concentrations used in this experiment were not optimal for achieving advanced inhibition.

By investigating a broader range of concentrations or adjusting the formulation of SDE, valuable insights can be gained to improve its inhibitory properties. Expanding the range of concentrations in future experiments poses sustainability challenges. It is essential to balance between achieving higher inhibition rates and maintaining eco-friendly practices, particularly in environments where minimizing inputs and optimizing outputs are prioritized.

The average radial growth of 7.6 cm in the negative control group indicates the natural growth rate of *P. palmivora* without any treatment (Akrofi *et al*., 2015). In contrast, the complete inhibition of radial growth observed in the positive control group treated with Delco highlights its potent antifungal properties.

Comparing the different extracts, the results indicate that GLE performed better against *P. palmivora* compared to AICDLE and SDE. This suggests that GLE contains components with stronger inhibitory potential on the radial growth of *P. palmivora*. The consistently better performance of GLE across different dosages suggests its potential as an effective natural control method against *P. palmivora.* The effectiveness of AICDLE and SDE appeared to be dependent on the dosage. At higher doses, AICDLE exhibited better

inhibitory effects on *P. palmivora* compared to SDE. This may be due to higher concentrations of active compounds in AICDLE that are more effective at inhibiting radial growth.

Conversely, at lower doses, SDE showed better inhibitory performance than AICDLE. This could indicate that SDE contains specific compounds that are more effective at lower concentrations or have a synergistic effect on inhibiting radial growth. The results also highlight the importance of dosage optimization when using natural extracts. Different extracts may have varying levels of efficacy at different concentrations, and finding the optimal dosage for maximum inhibitory effects is crucial. While the extracts showed inhibitory effects on radial growth, the complete inhibition achieved by the synthetic fungicide Delco demonstrates its superiority in controlling *P. palmivora*.

4.12 *In vitro* studies of the effect of crude extract on *P. megakarya*

The significant difference between the highest concentration (20mg/ml) and the lowest concentration (5mg/ml) suggests that using a higher concentration of GLE may lead to greater inhibition of radial growth in *P. megakarya*. This suggests that the concentration of GLE plays a crucial role in achieving effective control and suppression of *P. megakarya*. The finding agrees with earlier reports by Stephan *et al*. (2005) who reported similar trends in plant-derived extracts they were studying for pathogen-inhibiting effects on *P. parasitica*. Beyond plant pathogens, Kovendan *et al.* (2012) reported, the ethanol extract of *C. papaya* leaf exhibited both larvicidal and pupicidal activity against the malaria vector *A. stephensi*. The study revealed that a 2% leaf extract treatment resulted in a mortality rate of 41% among the mosquitoes. A

significantly higher mortality rate of 96% was achieved when using a 10% leaf extract treatment.

Apart from the 10mg/ml treatment, the AICDLE showed a connection between concentration and its ability to control *P. megakarya*. This indicates that higher concentrations of AICDLE were more effective in controlling the growth of *P. megakarya*. It is possible that the composition and interactions of the extract's components play a role in the observed variations in growth inhibition. In a related study conducted by Séka and colleagues, they made an observation of concentration-dependent inhibition not only for *Azadirachta indica* but also for *Ricinus communis* and *Jatropha curcas* extracts (Séka *et al*., 2017). These extracts were tested against Fusarium, Phytophthora, and Colletotrichum, which are known to contribute to postharvest losses in *Carica papaya*. The results implied that increasing the concentration of the extracts from *Azadirachta indica*, *Ricinus communis*, and *Jatropha curcas* led to a stronger inhibitory effect on the growth or activity of Fusarium, Phytophthora, and Colletotrichum.

Among the tested concentrations, it was observed that the concentration of 15 mg/ml exhibited the best growth retarding effect on *P. megakarya*. This concentration resulted in the lowest average radial growth rate, indicating the highest inhibitory efficacy compared to the other concentrations. It was also noted that the inhibition produced by the concentrations of 20 mg/ml and 5 mg/ml were essentially the same. This suggests that there may be a threshold or saturation point in the inhibitory effect, beyond which increasing the concentration further does not provide any additional benefit in terms of inhibition (Wang *et al*., 2008). In other words, the inhibitory activity reaches a

plateau, and altering the concentration within a certain range does not significantly affect the overall inhibition achieved. In a related study conducted by Ladislava and coworkers, they discovered a broad-range inhibitory plateau exhibited by Propolis extract against *M. gypseum* and *E. faecalis* (Netıkova *et al*., 2013).

The average radial growth of the negative control group (no treatment) was 6.2 cm, indicating the natural growth rate of *P. m*egakarya in the absence of any treatment. In contrast, the positive control group treated with the synthetic fungicide Delco exhibited complete inhibition of radial growth, reinforcing the effectiveness of synthetic fungicides in controlling *P. m*egakarya. Among the tested extracts, GLE emerged as the most effective extract against *P. m*egakarya.

The dose-dependent response observed in the inhibitory effects of GLE suggests that higher concentrations of GLE lead to greater growth inhibition of *P. m*egakarya. The differences in radial growth rates between the concentrations indicates the importance of concentration optimization when using GLE as a control measure. The results suggests GLE as a potential natural alternative for managing *P. megakarya* in agricultural settings. SDE showed slightly lower inhibitory effects compared to GLE, but still demonstrated growth retardation of *P. megakarya*. Although not strictly dose-dependent, the concentration of 15mg/ml appeared to have the best growth retarding effect. This indicates that SDE contains components or compounds that have inhibitory properties against *P. megakarya*, and the concentration of 15mg/ml is particularly effective in achieving growth suppression.

Further research is required to discover the specific active compounds within SDE and their mode of action. AICDLE exhibited lower inhibitory effects compared to GLE and SDE, suggesting that its composition may not be as potent in inhibiting the radial growth of *P. megakarya*. The concentration of 20mg/ml demonstrated the best growth retarding effect among the tested concentrations, indicating the potential of AICDLE when used at higher concentrations.

Further research is needed to explore the active compounds in AICDLE and investigate their interactions with *P. megakarya* to optimize its inhibitory efficacy. The comparison of the tested extracts indicates that GLE, followed by SDE and AICDLE, shows the most promising inhibitory effects against *P. megakarya*. The inhibitory effects observed in this study were specific to the tested concentrations and further investigation is required to determine the optimal concentration ranges for each extract. The positive control group treated with the Delco exhibited complete inhibition of radial growth, surpassing the inhibitory effects observed with any of the tested extracts.

4.13 *In vitro* modulation studies

Four of the tested combinations of Delco and GLE exhibited 100% inhibition of the pathogens *in vitro*, indicating a strong potential for controlling these fungal pathogens. The FICI values of A, B, C, and D were 0.51, 0.54, 0.57, and 0.65, respectively (Table 4.7). These FICI values fall within the range of 0.5 to 1.0, indicating a partially synergistic effect (Valderrama *et al*., 2020).

The interpretation of the FICI values suggests that the combinations of Delco and GLE produce a cooperative interaction, resulting in enhanced inhibitory effects compared to using each component alone (Hassan *et al*., 2016;

Valderrama *et al*., 2020). The partial synergistic effect may enhance the overall inhibitory efficacy, reducing the required concentration of each component and potentially minimizing the negative environmental impacts associated with high chemical inputs (El-Sharkawy *et al*., 2021).

The results obtained from the combinations of Delco and GLE in different ratios, E (0.01:5), F (0.03:5), and G (0.07:5), reveal varied findings regarding their inhibitory effects on *P. palmivora* and *P. megakarya*. In the case of *P. palmivora*, the tested combinations allowed growth, with radial growth rates ranging from 2.8 cm to 3.30 cm, corresponding to inhibition percentages ranging from 33.3% to 44.3%. These results suggest that the tested combinations were not effective in providing complete inhibition of *P. palmivora* growth *in vitro*. Similarly, for *P. megakarya*, the tested combinations also allowed some growth, with radial growth rates ranging from 1.1 cm to 3.4 cm, corresponding to inhibition percentages ranging from 15.8% to 73%. This suggests that the tested combinations showed weak to moderate inhibition of *P. megakarya*.

In the case of *P. palmivora*, the combinations J (0.15:5), I (0.2:4), and J (0.2:5) demonstrated complete inhibition of radial growth *in vitro*, resulting in 100% inhibition. These results suggest that the tested combinations were highly effective in controlling *P. palmivora*, providing a promising avenue for disease management. The FICI values of 0.50, 0.53, and 0.55 for combinations H, I, and J, respectively, indicate a synergistic effect for H and partially synergistic effects for I and J (Valderrama *et al*., 2020). For *P. megakarya*, the same combinations were not able to achieve complete inhibition. Although an

impressive inhibition was observed, with inhibition percentages ranging from 87.1% to 96.3%, complete control of *P. megakarya* was not achieved.

The differential response between the two pathogens suggests variations in their susceptibility or inherent differences in their growth patterns (Pakora *et al*., 2018). It is possible that *P. megakarya* exhibits a higher level of resistance or requires a different concentration or combination of control measures to achieve complete inhibition. The results emphasize the need for further investigation and optimization of the concentration ratios and formulation of the Delco-GLE combinations to enhance their inhibitory effects on *P. megakarya*.

4.14 *In vivo* assessment of modulated extracts (pod work)

The evaluation of modulated extracts *in vivo* to assess their anti-Phytophthora activity on cocoa pods is a crucial step in determining their practical effectiveness. *In vitro*, studies have consistently demonstrated that flavonoids possess antifungal effects. However, these effects may not always translate to *in vivo* conditions. This was reported by Nguyen *et al*. (2021) in their study on the methanol extract of *Simaba ferruginea*. Nguyen and colleagues found that despite the presence of flavonoids in the methanol extract of *Simaba ferruginea*, the *in vivo* antifungal effects were not as significant as anticipated.

The *in vivo* evaluation provides a more realistic and comprehensive assessment of the modulated extracts' ability to control Phytophthora on cocoa pods. Unlike *in vitro* studies, which focus on controlled laboratory conditions, the *in vivo* evaluation takes into account the complex interactions between the pathogen, the host plant, and the environment (Rongai *et al*., 2017). This approach allows investigators to gauge the true efficacy of the extracts under

conditions that closely mimic real-world scenarios (O' Keeffe *et al*., 2019). By observing and scoring the lesions produced on cocoa pods following inoculation with Phytophthora, researchers can quantitatively measure the anti-Phytophthora activity of each extract (Larbi-Koranteng *et al*., 2020).

The severity and extent of the lesions provide valuable information about the level of protection offered by the extracts (Larbi-Koranteng *et al*., 2020). Comparing the scores obtained from the different modulated extracts allows for a direct comparison of their effectiveness and the identification of the most promising candidates (Simo *et al*., 2019).

Extracts that demonstrate anti-Phytophthora activity, as evidenced by lower lesion scores, can be considered potential candidates for further optimization and formulation (Simo *et al*., 2019). These extracts may serve as a foundation for the development of effective control measures against Phytophthora in cocoa crops (Dassanayake *et al*., 2021). The *in vivo* evaluation can provide insights into the practical feasibility of implementing the modulated extracts as anti-Phytophthora agents in agricultural practices (Dassanayake *et al*., 2021). Understanding how the extracts perform in real-world conditions is essential for determining their potential for commercialization and widespread adoption (Rongai *et al*., 2017). The *in vivo* evaluation may reveal variations in the performance of the modulated extracts compared to the results obtained from the earlier modulation studies conducted *in vitro*. Factors such as the complexity of the host-pathogen interactions, environmental influences, and application methods impact the effectiveness of the extracts (He *et al*., 2019).

These variations highlight the importance of conducting *in vivo* studies to validate and refine the findings from *in vitro* experiments (Rongai *et al*.,

2017). The outcomes of the *in vivo* evaluation contribute to the development of sustainable and effective strategies for controlling Phytophthora on cocoa crops (Larbi-Koranteng *et al*., 2020). By identifying the most promising extracts and optimizing their formulations, researchers can work towards providing farmers with practical and reliable solutions for managing Phytophthora-related issues, potentially reducing crop losses and improving cocoa yields.

4.15 *In vivo* qualitative and quantitative output

The negative control, which used SDW as a treatment, highlights the necessity of effective disease management strategies for *P. palmivora* and *P. megakarya* in cocoa cultivation. In this group, both pathogens infected all tested pods, indicating that SDW alone provided no protection against these diseases. A quantitative measure of the lesion sizes produces the highest average lesion size of 2.86 cm for *P. palmivora* and 3.15 cm for *P. megakarya*. These results align with existing knowledge and establish the credibility of the study, reinforcing the understanding that *P. palmivora* and *P. megakarya* are indeed destructive pathogens capable of infecting cocoa pods (Akrofi 2015; Bailey & Meinhardt, 2016).

The *in vivo* evaluation of combination A against *P. palmivora* and *P. megakarya* on cocoa pods yielded concerning results. Lesions were observed on all five cocoa replicates. The average lesion size was 6 cm for *P. palmivora* and 8 cm for *P. megakarya*. Combination A's failure to stop both pathogens raises doubts about its effectiveness as an anti-Phytophthora treatment this is consistent with the arguments of (Larbi-Koranteng *et al*., 2020) on characteristics of anti-Phytophthora agents.

This outcome emphasizes the need for alternative strategies or modifications to improve the effectiveness of combination A against these pathogens. Several factors could contribute to these results, including pathogen resistance, inadequate application methods, or limitations of the fungicide formulation (Martins, 2008). It is essential to identify and address these factors to develop more efficient and reliable anti-Phytophthora treatments. The disappointing result of combination A highlights the need for *in vivo* studies to confirm the effectiveness of anti-Phytophthora treatments observed *in vitro*, with guidance from previous studies (Nguyen *et al*., 2021; Rongai *et al*., 2017).

Evaluation of combination B for controlling *P. palmivora* and *P. megakarya* on cocoa pods revealed mixed outcomes. Combination B demonstrated partial efficacy against *P. palmivora* by preventing infection in two out of the five tested pods. However, it was unable to offer any protection against *P. megakarya*, with all five pods becoming infected. The average lesion size for the pathogens was 1.78 cm for *P. palmivora* and 2.67 cm for *P. megakarya*. The differential response of combination B to the two pathogens raises questions about its effectiveness in providing broad-spectrum control (Martins, 2022; Rongai *et al*., 2017).

The evaluation of combination C for controlling *P. palmivora* and *P. megakarya* on cocoa pods revealed mixed outcomes. The combination showed partial efficacy against *P. palmivora*, protecting two out of the five tested pods (40% inhibition). However, it also failed to offer any protection against *P. megakarya*, resulting in all five pods becoming infected. The average lesion size for the pathogens was 1.30 cm for *P. palmivora* and 2.47 cm for *P. megakarya*. The susceptibility of cocoa pods to *P. megakarya* highlights the challenges

associated with developing effective control strategies for this particular pathogen (Larbi-Koranteng *et al*., 2020). The results indicate that combination C, in its current formulation or concentration, is ineffective in preventing infection by *P. megakarya* (Simo *et al*., 2019). This finding emphasizes the need for alternate enhanced combinations with desirable efficacy against *P. megakarya*.

The evaluation of combination D for controlling *P. palmivora* and *P. megakarya* on cocoa pods demonstrated moderate efficacy. The combination showed promising results by preventing infection in three out of the five tested pods (60% inhibition) against *P. palmivora*. Additionally, it protected two pods, resulting in 40% inhibition against *P. megakarya*. The average lesion size for the pathogens was 1.23 cm for *P. palmivora* and 1.98 cm for *P. megakarya*. The partial efficacy of combination D against both *P. palmivora* and *P. megakarya* suggests its potential as an anti-Phytophthora treatment.

The *in vivo* evaluation of Delco for controlling *P. palmivora* and *P. megakarya* on cocoa pods demonstrated moderate efficacy. While the fungicide prevented infection in three out of the five pods (60% inhibition) against *P. palmivora* and two out of the five pods (40% inhibition) against *P. megakarya*. The average lesion size for the pathogens was 1.05 cm for *P. palmivora* and 1.94 cm for *P. megakarya*. The observed efficacy may appear lower than expected for a commercially available fungicide. In general, commercially available fungicides are expected to exhibit higher efficacy in controlling plant pathogens (da Cruz Cabral *et al*., 2019). The moderate efficacy observed with Delco suggests that there is room for improvement in terms of its effectiveness against both *P. palmivora* and *P. megakarya*.

The fungicide's ability to prevent infection in a subset of the tested pods indicates its potential for providing partial protection against these pathogens (Lal *et al*., 2021). One possible explanation for the observed moderate efficacy is the development of resistance in the Phytophthora pathogens to the active ingredients in Delco (Lal *et al*., 2021; Shuping & Eloff, 2017). Pathogens can trigger molecular and metabolic activities to reduce their susceptibility to fungicides over time (Bailey & Meinhardt, 2016).

If the pathogens have developed resistance, it could explain the reduced efficacy observed in the *in vivo* evaluation (Bailey & Meinhardt, 2016). This highlights the importance of monitoring and managing resistance to maintain the long-term efficacy of fungicides. An alternative explanation for the moderate efficacy observed with Delco could be that fungicides are typically used as part of integrated pest management protocols rather than in isolation (Adu-Acheampong *et al*., 2015; Akrofi *et al*., 2015; Akutse *et al*., 2020). In this context, Delco could be considered a practical option for farmers until more advanced versions become available. Manufacturers are continuously seeking to improve their formulations and develop better products to meet the evolving needs of farmers. While Delco may have demonstrated only moderate efficacy in this study, there is potential for future advancements and enhancements in fungicide development to address the challenges posed by pathogens such as *P. palmivora* and *P. megakarya*.

4.16 Chapter Summary

The outcomes of the molecular docking studies present convincing evidence regarding the anti-Phytophthora activity of compounds obtained *from Carica papaya*. These findings strongly motivated our interest in extracting the

phytoconstituents of the plant for *in vitro* and *in vivo* evaluation. Among the tested crude extracts, the most effective one was identified as GLE. It was further optimized, resulting in 100% inhibition *in vitro* experiments when combined with Delco, a commercially available synthetic fungicide. To validate the *in vitro* results, four of the most successful combinations were subsequently tested on cocoa pods *in vivo* experiments.

The *in vivo* data revealed a distinct variation in the pathogen's response to different fungicides. This disparity underscores the potential for misleading and ill-informed conclusions regarding fungicide efficacy when relying solely on *in vitro* work. The *in vivo* data not only shed light on the pathogen's response to fungicides but also provided valuable insights into the progressive activity of modulated fungicide combinations. This information serves as a guide for the development of more effective and high-performing modulated combinations.

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.0 Overview

The general objective of this research was to assess the anti-Phytophthora activity of *Carica papaya* Linn against black pod disease. The objectives were then subcategorized into four specific objectives which are presented below.

i. Perform an initial *in silico* studies to assess the potential interactions between *Carica papaya* isolated compounds reported in literature and a well characterized target protein of black pod inducing Phytophthora species. Molecular docking was used to achieve this result.

ii. Conduct *in vitro* evaluations of *Carica papaya* crude extracts to determine their antifungal activity against Phytophthora. A completely randomized trial in conjunction with poison food technique was used to achieve this objective.

iii. Modulate the *Carica papaya* extract with synthetic fungicide to optimize the performance and enhance the antifungal efficacy *in vitro*. Completely randomized trial in conjunction with Poison food technique was a gain used to achieve this objective.

iv. Perform *in vivo* evaluations to assess the effectiveness of the best performing *Carica papaya* extract and synthetic fungicide combination against Phytophthora infections. A completely randomized trial was used for labelling detached cocoa pods to achieve this objective.

5.1 Summary

The summary of results is presented in order of stated objectives guiding the work.

Molecular docking showed that *Carica papaya* compounds strongly bind to the effector protein AVR3A11(3ZR8). This effector protein is associated with a classical black pod disease-inducing Phytophthora species. Carpaine displayed the highest docking score at -7.2 kcal/mol. Additionally, benzylglucosinolate, quercetin, riboflavin, catechin, and liquiritigenin showed promise as 3ZR8 inhibitors. These results demonstrate the carica papaya's potential as a natural source of inhibitors against black pod inducing pathogens.

The three crude extracts (GLE, AICDLE, and SDE) obtained from *Carica papaya* contained various phytochemical compounds. All three samples contained alkaloids, saponins, tannins, flavonoids, glycosides, and terpenoids. Phytosteroids was detected in AICDLE and SDE but not GLE.

In vitro, the results consistently show that increasing the concentration of crude extracts almost always leads to increased growth inhibition in both *P. palmivora* and *P. megakarya*. The result also indicated that none of the tested crude samples achieved complete inhibition (fungistatic inhibition) against the pathogens. For example, GLE displayed inhibitory effects, reaching 84.08% inhibition against *P. palmivora* (Table 4.) and 79.67% inhibition against *P. megakarya* at a concentration of 20 mg/ml (Table 4.). However, the synthetic fungicide Delco achieved 100% inhibition(fungicidal) at the recommended dose of 5 mg/ml, demonstrating its superior activity (Table 4.).

In modulation studies conducted under *in vitro* conditions, we tested a combination of Delco and GLE at different concentrations. When we kept the

proportion of Delco at 0.3mg/ml while varying GLE from 3 to 5mg/ml, this led to complete inhibition (fungicidal) of both *P. palmivora* and *P. megakarya* (Table 4.). We observe a partially synergistic effect within this range indicated by the FICI values. These findings show that the effectiveness of the Delco and GLE combination varies with specific concentrations.

In vivo experiment was conducted for pod infection scoring and leaching size determination. Combination A and the negative control showed no protection against both *P. palmivora* and *P. megakarya* (100% infection). Combinations B and C provided 40% protection against P. palmivora but were ineffective against *P. megakarya* (100% infection). Both Combination D and Delco alone (at recommended dose) achieved 60% protection against *P. palmivora* and 40% protection against *P. megakarya*. Quantitatively, Combination D and Delco alone had the average lesion sizes (less than 2cm for P. *palmivora* and less than 4cm for *P. megakarya*), indicating effective control. Combination C showed good protection against *P. palmivora* (lesion size less than 2cm) but less control against *P. megakarya* (lesion size slightly greater than 6cm). Combination B resulted in smaller lesion sizes (less than 4cm for *P. palmivora* and less than 8cm for *P. megakarya*). The negative control had the largest lesion sizes, close to 10cm for both pathogens, indicating minimal protection.

5.2 Conclusions

From the results obtained the following conclusions were made:

1. *In silico* evidence suggests that *Carica papaya* possesses potential anti-Phytophthora activity, particularly against effector proteins.
2. There was variation in the inhibitory effect of the crude leaf extracts (GLE and AICDLE) among the tested samples. Comparing the crude leaf extracts with the seed extract also revealed differences in the inhibition effect. Generally, higher concentrations of the extracts demonstrated relatively high inhibitory activity, although complete inhibition was not achieved (fungistatic).
3. The GLE extract exhibited the highest effectiveness among the tested extracts and could be readily enhanced by incorporating a small quantity of synthetic fungicide.
4. Modulation of GLE gave it fungicidal characteristics *in vitro*, resulting in 100% inhibition over a range of combinations.
5. The research findings indicated that while good anti-Phytophthora activity was observed *in vitro*, it did not fully and directly translate to the same level of effectiveness in *in vivo* conditions.
6. Combination D comprising Delco and GLE ratio of 0.3mg:3mg per ml, demonstrated comparable effectiveness to Delco alone at the recommended dosage of 5mg/ml.
7. By utilizing approximately 10% of the synthetic fungicide in the modulated extract combinations, the current study successfully reduced synthetic fungicide usage by approximately 90%. This significant

reduction in synthetic fungicide usage helps minimize potential environmental hazards and health risks associated with its application.

5.3 Recommendations

1. It will be prudent to acquire the identified *Carica papaya* compounds reported in the literature for further *in vitro* and *in vivo* evaluations.
2. The GLE extract from *Carica papaya* displayed the most effective inhibitory activity against Phytophthora sp. Therefore, it would be prudent to pursue additional characterization studies to identify the specific active compounds responsible for its anti-Phytophthora potential. This information can contribute to the development of targeted treatments.
3. Further optimization may be required to explore different formulations and dosage levels of GLE extract in combination with other natural products to maximize their inhibitory effect against Phytophthora.
4. Another recommendation would be to conduct further *in vivo* studies in field settings. While the current experiments used detached pods, it is important to understand the efficacy of the treatments within the full plant architecture. The disconnected pods may not have benefited from the complementary activity of the plant's immune system, which could have limited the infection potential of the pathogen in a natural environment.
5. The Ghana government, through the appropriate agencies such as the ministry of food and agriculture and COCOBOD, among others, should provide the necessary stimulus package for the farming of *Carica papaya* to make the raw material (leaves) available for processing into

fungicides. This has the potential to create thousands of direct and indirect jobs.

5.4 Suggestions for further research

1. Future studies could consider the investigation of different extraction solvents, including methanol, dichloromethane (DCM), hexane, or others, to assess their efficacy in extracting bioactive compounds from *Carica papaya*. Comparing the extraction yields and the resulting bioactivity of the extracts obtained using different solvents can offer insights into the most suitable solvent for obtaining potent biofungicidal extracts.

2. Future studies could consider conducting phase-specific activity assessments of the extracts, focusing on specific compound classes such as alkaloids or essential oils. This approach can help identify the major bioactive components responsible for the observed antifungal effects and provide a deeper understanding of their mechanisms of action.

3. Future work could consider exploring the economics of producing the biofungicide derived from *Carica papaya*. Investigate the cost-effectiveness of large-scale extraction, formulation, and application processes. Assess factors such as resource availability, production scalability, and market demand to determine the economic feasibility and potential commercialization prospects of the biofungicide.

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