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**IDENTIFICATION, VARIABILITY AND CONTROL OF BROWN LEAF
SPOT DISEASE PATHOGEN(S) OF RICE**

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of the requirement for the award of a Master of Philosophy degree in Crop Science
(Plant Pathology)

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Declaration

Candidate's Declaration

I, Titus Akansin Bukari hereby declare that this thesis is the results of my own original work and that no part of it has been presented for another degree at this university or elsewhere.

Signature: Date:

Supervisor's 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 Akenten Appiah-Menka University of Skills Training and Entrepreneurial Development.

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Abstract

Brown leaf spot disease (BLSD) of rice results in germination failure, seedling mortality, yield and quality reduction. Typical BLSD with serious effect on yield was observed in rice farmers fields in Atwima Nwabiagya District of the Ashanti region. The study therefore sought to isolate, identify and control BLSD of rice pathogen(s). Diseased rice leaves were sampled and cut into pieces, disinfected, and arranged equidistantly on a PDA medium for fungal pathogen growth. The fungal pathogens were identified using morphological features and confirmed by DNA sequencing. The pathogenicity of isolated fungal pathogens were confirmed through Koch postulate. Two (2) epiphytes of rice (*Purpureocillium lilacinum* and *Aspergillus flavus*), two (2) plant extracts (*Azadirachta indica* and *Nerium oleander*), a positive control (mancozeb) were tested for their ability to control the disease *in-vitro* and in screenhouse. Molecular identification using BLAST search analysis in the NCBI specifically identified the pathogenic isolates into *Bipolaris zeicola*, *Bipolaris oryzae*, *Curvularia lunata*, *Curvularia clavata*, *Curvularia soli*, *Curvularia geniculata*, *Curvularia senegalensis*, *Curvularia subpapendorfii* and *Curvularia platzii*. All isolates showed symptoms of brown leaf spot similar to what was observed on field of rice plants after confirming through Koch postulate. The investigation on the pathogenicity of the obtained *Curvularia* isolates displayed the existence of a pathogenic variability among the isolates. Apart from *B. oryzae* and *C. lunata*, which were the known pathogen associated with rice brown leaf spot disease in Ghana, new species such as *B. zeicola*, *C. clavata*, *C. soli*, *C. geniculata*, *C. senegalensis*, *C. subpapendorfii* and *C. platzii* were found to be associated with rice in a disease complex and causing brown leaf disease individually, suggesting together they contribute to disease severity in a complex. Perhaps, it is the first report of *B. zeicola*, *C. clavata*, *C. soli*, *C. senegalensis*,

C. geniculata, *C. subpapendorfii* and *C. platzii* associated with brown leaf spot disease of rice in Ghana and have since been deposited at NCBI GenBank (with accession numbers: PQ410456, PP564497, PP564496, PP564502, PP564499, PP564501 and PP564498 respectively). Two rice epiphytes (*A. flavus* and *P. lilacinum*) and two botanicals (*A. indica* and *N. oleander*) inhibited mycelia growth of brown leaf spot pathogens in an *in-vitro* condition and subsequently reduced brown leaf spot severity of rice seedlings in screenhouse experiment.

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Dedication

I dedicate this thesis to Mrs. Mary Bukari, my mother and Miss. Lucy Atiiga.

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List of Publications (Manuscripts Submitted)

1. Pathogenic variability among *Curvularia* species causing brown leaf spot disease of rice (Published in the Journal of Plant Pathology on November 08, 2024)
2. Evidence of *Bipolaris zeicola* associated with brown leaf spot disease on rice (*Oryzae sativa*) in Ghana (Manuscript submitted to Acta Biologica Slovenica on October 21, 2024).
3. *Curvularia* species complex Associated with Brown Leaf Spot of Rice (Manuscript submitted to the European Journal of Plant Pathology on November 13, 2024)

Chapter One: Introduction

1.1 Background to the Study

Rice is the primary food source for more than half of the world population, with China and India collectively responsible for almost 50.0% of global production and consumption (Jat *et al.*, 2020). Currently, rice is grown worldwide on 167 million hectares, yielding around 769 million tons of rough rice annually (Jat *et al.*, 2023). Africa accounts for 7.8% of global rice production, South America for 6.4%, and North America for 1.4% (Anonymous, 2018). In West Africa, the rice value chain has been prioritized due to its significant role as a primary calorie source, prompting domestic producers to boost production amid the food crisis. This led to the development of National Rice Development Strategies by African policymakers (Ababa, 2020). However, the domestic rice value chain is underperforming, leading to imports accounting for 30% of consumption.

Despite increasing production and consumption, the low production-to-consumption ratio suggests Africa countries are not self-sufficient (Nasrin *et al.*, 2015 and Van Ittersum *et al.*, 2016). For example, in Ghana, despite government initiatives to boost rice production, the country continues to import rice to fulfil its local demand (Tanko *et al.*, 2019; Alidu *et al.*, 2016). Challenges such as drought, acidity, salinity, biotic stress, diminishing soil health, diseased seeds and poor income of farmers impedes rice production (Mheni, 2024; Gupta *et al.*, 2023). According to (Stukenbrock & Gurr, 2023; Satianpakiranakorn *et al.*, 2020) fungal diseases such as blast, sheath blight, bakanae, brown leaf spot, and filthy panicle caused by fungal pathogens decrease global annual rice production by 14%.

1.2 Problem Statement

Brown leaf spot disease is prevalent across rice producing regions leading to about 90% reduction in rice yield (Dhaliwal *et al.*, 2018) with the most destructive one occurring around the 1942 in India which was coined as the Bengal famine. Recently in Ghana, *Curvularia lunata* has been reported as a major fungal pathogen of the disease in certain parts of the country (Honger *et al.*, 2022) although earlier reports by Nutsuga *et al.* (2003), reported the association of *Bipolaris* spp. to the disease. This cast doubt about the main fungal pathogen responsible for the disease in Ghana and especially in the study area where no information on the disease has previously been reported.

In Ghana, general loss of rice ranges between 4.07% and 12.05% at the production level on farmer's field (Appiah *et al.*, 2011), and specifically in 2020, it was estimated by AHPLIS (2021) as 11.9%. Brown leaf spot disease of rice and other cereal crops have become extremely costly as producers battle with its control (Samberg *et al.*, 2013; Ackaah *et al.*, 2023). Different chemicals and resistant cultivars are currently being employed for this objective, brown leaf spot pathogens can rapidly develop new strains, leading to resistance breakdown. Synthetic fungicides contribute to environmental pollution, pesticide resistance, decline in soil quality and harm to ecosystems posing treats to United Nations Sustainable Development goals (UN SDG goal 2 and 3), which aims at reducing hunger and promoting sustainable agriculture and promoting good health at all stages of life (Law *et al.*, 2017; Pooja, 2014). The growing awareness of chemical fungicides' negative effects has prompted scientists to explore alternative environmentally friendly approaches, such as biocontrol agents.

1.3 Justification of the Study

Brown leaf spot disease of rice has the capacity to significantly eradicate crop yield, it caused up to 90% decline in production in the case of Bengal famine (Imran *et al.*, 2020). Honger *et al.* (2022) have also confirmed the severity and destructive nature of this disease in Ghana. It is therefore critical to seek for the control of BLSD of rice to help curb the declined production rate of rice in Ghana and Sub-Saharan Africa which is partly due to disease infection. This will match production rate to consumption. Identification and pathogenic characterization of the species is of great importance in controlling the disease as Thakur *et al.* (2023) indicated that the variability in pathogens allows for differences in their infection level and disease development which serves as a guide in developing management techniques.

The most important question is how many species are existing on a planet and which of those are pathogens of plant. Statistically, taxonomists critically identified and catalogued 1.2 million species, and still, 8.7 million species need to describe. It seem endlessly for identification of all taxa on a morphological basis. Besides, identification by morphological means has several limitations, like phenotypic plasticity, sexual dimorphism, immatures, polymorphism, cryptic behaviour and many more. For implication on which specific pathogen (taxa) causes a specific disease at a particular time and location, literature suggests a combination of morphological and molecular approach in their identification (Varadi *et al.*, 2017; Kalman *et al.*, 2020). In addition to facilitating the identification of novel pathogens, these approaches also facilitate the investigation of the epidemiology, pathogenesis, and treatment response of the newly identified diseases, thereby presenting additional evidence for the causal relationships between the organism and the disease which together aid pathogenic characterization of the species and disease control (Thakur *et al.*, 2023; Moradi *et al.*, 2019). Rahman

et al. (2020) are of the view that, PCR method's high sensitivity and specificity make it an appealing alternative to culture methods commonly used in environmental research.

Despite the usefulness of chemical control, the method is devilled with its adverse harmful effects, biological control is the alternative due to its eco-friendly as against the chemical fungicides and traditional methods in the context of fulfilling UN SDG goals 2 and 3. Epiphytic microorganisms have recently garnered considerable attention (Yan *et al.*, 2019; Bubici *et al.*, 2019). By providing nourishment and shelter, the host plant enables epiphytes to thrive in less competitive environments and safeguards the interior of the plant from pathogens. A more robust and enduring plant defense may result from the intimate association of epiphytes with the plant (Vishwakarma *et al.*, 2021; Khare *et al.*, 2018). Isaac *et al.* (2021) and Naz *et al.* (2021) reported that *Paecilomyces lilacinum* and *Aspergillus flavus* being endophytic fungi has been used as a biological control agent. Fariman *et al.* (2022) utilized *Stenotrophomonas maltophilia* to suppress the radial growth of *Pyricularia oryzae*, the causal agent of rice blast disease. Additional research on the biological management of rice blast was carried out by Chou *et al.* (2020), Chen *et al.* (2021), Safari & Mohammed (2021), and others. Despite these studies being on blast disease, the control agents used were not natives of the host (rice) plant.

Natural products such as botanicals offer antimicrobial properties against plant pathogens, but their use as biocontrol agents is uncommon in Ghana and West Africa (Bandyopadhyay *et al.*, 2016).

1.4 Objective of the Study

1.4.1 Main Objective

The study aimed to identify and control the pathogen (s) causing brown leaf spot disease of rice.

1.4.2 Specific Objectives

The specific objectives are to:

1. isolate and confirm identity of fungal pathogen(s) causing brown leaf spot disease of rice in the study area using both morphological and molecular tools
2. determine the cultural, morphological and pathogenic variations among the major fungal pathogen(s)
3. evaluate the efficacy of epiphytes (*Purpureocillium lilacinum* and *Aspergillus flavus*) and botanicals (*Nerium oleander* and *Azadirachta indica*) crude/ aqueous extracts against brown leaf spot disease causal pathogen(s).

Chapter Two: Literature Review

2.1 Rice (*Oryza sativa* L) and its Importance

Rice, originating in India and China, is part of the genus *Oryza* and family Poaceae, with 25 species, with only two being widely cultivated (Veltman *et al.*, 2019). Based on data provided by the FAO (2020), the bulk of the world's rice production and consumption is primarily concentrated in Asia, with a total harvested area of 43,789 million hectares and a yield of 38,480 hg/ha. Rice is a crucial dietary staple in numerous countries, providing protein, lipids, carbohydrates, crude fiber, minerals, and vitamins like Thiamine, Riboflavin, Niacin, and Tocopherol (Bathla *et al.*, 2019). According to Verma & Srivastav (2017) rice comprises 80% carbohydrates, 7-8% protein, 3% lipids, and 3% fiber. Antioxidant and medicinal properties of the crop contribute to its wide utilization in the pharmaceuticals, cosmetics and industries. Starch, fermentation, and byproducts from the crop is used as animal feed (Zhang *et al.*, 2021).

2.2 Status of Rice Production in Ghana

Rice is a strategic commodity and the fastest-growing food source in majority of African countries including Ghana and it is crucial for the welfare of the consumers and general populace (Prasad *et al.*, 2017). The demand for rice is growing at a rate of 6% per annum, requiring Africa to spend \$60 billion annually on rice imports to bridge the gap. Ghana's rice economy is thriving due to population growth, urbanization, and consumer preferences. Despite domestic production efforts, the country relies heavily on imported rice to sustain domestic demand which is crucial for food security albeit high impact on the country's foreign exchange savings (Dorvlo *et al.*, 2023).

2.3 Production Constraints of Rice

Population growth increases residential water requirements, reducing available land and water for agriculture, leading to slow irrigation development. This results in increased competition and adverse effects on water availability, food production, and environmental quality. Rice cultivation faces challenges due to drought and freshwater scarcity, as rice thrives in flooded soil. Water scarcity and drought limit rice yield potential, while alternate wet-and-dry conditions cause nitrogen loss and weed infestation (Fahad *et al.*, 2019).

Salinity is an abiotic stress which adversely affects crop growth and production (Yadav *et al.*, 2020). A saline soil has a higher concentration of soluble salts such as cations and anions and has an electrical conductivity (EC) equal or greater than 4 dSm⁻¹. The salinity problem is very common in arid and semiarid areas. Salinity accounts for 20% of the global cultivated area and also half of the total irrigated land of the world. Cereal crops vary in their response to salinity, from barley being the most tolerant and rice being the most sensitive. Currently, salinity is the second major abiotic constraint in rice production after drought (Rasheed *et al.*, 2020; Fahad *et al.*, 2019).

Inappropriate farming methods, deforestation, and overgrazing lead to soil degradation, including organic matter loss, nutrient depletion, acidification, salinization, chemical contamination, and physical properties like water and wind erosion (Nayakekorale, 2020). Hossain *et al.* (2020) reported that soil degradation poses a significant threat to food security, with issues like excessive tillage, low C, Zn, K, and inefficient N use hindering rice crops. Rice yield is affected by deficiencies in phosphorus, zinc, iron, salts, iron, and aluminum. Zinc deficiency is the most common issue, causing a 65% reduction in susceptible rice cultivars. Iron toxicity negatively impacts rainfed, lowland

rice in acidic soil with low P and K content. Aluminum toxicity is also a significant constraint (Faria *et al.*, 2021).

Rice is adaptable to a wide range of climatic conditions. However, temperature and radiation are two major environmental factors, which directly or indirectly, play a vital role in rice production. Rasheed *et al.* (2020) identified temperature as a significant production constraint in rice productivity, with low-altitude rice growing near temperature tolerance limits potentially subjecting them to higher stress.

According to Roling (2019), the proportion of researchers to extension workers is very low. This is a major constraint because profitable technology is not extending into the farming community. The lack of education in the rice farming community is a serious problem, because Ghimire & Suvedi (2018) reported that better-educated farmers are more willing to adopt new technologies than vice versa. Rice cultivation faces global socioeconomic and policy constraints, including unfavorable input and output pricing policies, limited access to credit, poor infrastructure, and unavailability of farmer organizations, leading to decreased profit and increased competition. Therefore, growers cannot expand production due to financial constraints, major socioeconomic constraints in rice production include poor farming communities, poor infrastructure, and high input cost and many more (Fahad *et al.*, 2019).

2.3.1 Pests and Diseases

Rice plants face insect attacks from terrestrial arthropods and non-rice pest insects, including nearly 20 insect-pests, causing economic damage and disease transfer (Bhatt *et al.*, 2018). Rice yield losses are primarily caused by the presence of important insect-pests such as stem borer, gall midge, brown plant hopper, and leaf hopper as reported by Bhatt *et al.* (2018). Insect pests cause damage to plants, disrupting grain physiology

and reducing crop yield. Despite various strategies, developing countries still rely on conventional methods to control pests.

Rice fields often contain a variety of weeds, influenced by cultural practices like continuous planting, which encourages adapted weeds to thrive in the soil (Gopal *et al.*, 2019). Rice fields contain perennial weeds, grasses, sedges, and broad-leaved weeds. Crop rotations encourage a diverse flora, with grasses including *Echinochloa colonum*, sedges including *Cyperus rotundus*, and broad-leaved weeds like *Ecliptaalba* (Acharya, 2022). Fahad *et al.* (2019) estimated that uncontrolled weed growth can lead to 44%-96% rice yield loss, increasing production costs, pest infestation, and affecting harvesting quality. Controlling weeds costs 15% of world rice production, including herbicides, cultural practices, and hand weeding.

The ability of rice to attain high production has been reduced due to the presence diseases. Disease infection is the most important among biotic constraints of rice, viz, sheath blight, bacterial blight, rice blast, brown leaf spot, narrow brown leaf spot, bacterial leaf streak, grassy stunt and many more.

Rice blast, caused by *Pyricularia grisea*, is a fungal disease that significantly reduces rice yield by 30% globally, equivalent to feeding 60 million people. It attacks all plant parts and is caused by a new pathogen, *Magnaporthe oryzae*. Actual losses can range from 5% to 85% depending on location (Fahad *et al.*, 2019).

Sheath rot is a fungal disease causing incompletely exerted panicles with empty grains, spread by stem borers. Reducing losses can be achieved through resistant rice varieties, seed eradication, and fungicide use.

Brown leaf spot is a fungal disease-causing oval symptom on leaves, infecting seedlings and mature plants. Caused by *Bipolaris oryzae*, *Curvularia* spp., and *Cercospora* spp. (Yuvarani *et al.*, 2021).

2.4 Occurrence of Brown leaf spot Disease of Rice

Basically, it is a seedling disease, but can also infect both leaves and panicles in rice (Plate 1). Infected seeds can cause 50% mortality in seedlings and blight. The Great Bengal Famine in 1942 resulted in yield losses and two million deaths due to extreme temperatures and cloudy weather. Rice brown leaf spot was first described in 1900. The highest incidence of brown leaf spot disease was observed and the primary cause was identified as being the cultivation of susceptible varieties (Ramaiah, 2000). Singh (2016) reported that, the time frame during which rice brown leaf spot disease incidence was at its peak was from bud to flower. Particular regions of Madhya Pradesh are susceptible to yield losses of up to 90 percent due to the pathogenic *Drechslera oryzae* (Sandeep, 2015). In Nigeria, rice brown leaf spot has become a significant impediment, second only to blast caused by *Pyricularia oryzae*, accounting for 40% of rice yield reduction (Agu & Ukweze, 2002).

The progression of BS severity on flag leaves in rice fields of Brazil was significantly impacted by the increasing levels of *B.oryzae* seed-borne inoculum, particularly the symptoms observed anterior to flowering (Schwanck *et al.*, 2021). According to a survey carried out in Paraguay between 2014 and 2015, the incidence of the rice brown leaf spot disease ranged from 30% to 40% (Quintana, 2019). Muhammad *et al.* (2019) documented that the field conditions in Pakistan exhibited a greater degree of severity for *B. oryzae*. Brown leaf spot is endemic in Kenya due to low-input agriculture and physiological stress experienced by cereals (Nganga *et al.*, 2022).

Farmers' investment in fungicides and rapid crop cultivation have prevented rice disease epidemics in Ghana, including the burgeoning threat of brown leaf spot, attributed to *Bipolaris oryzae*, according to Nutsugah *et al.* (2003). Ex-Baika, now Legon 1, grew significantly despite brown leaf spot infection, illustrating the

challenges faced by the rice industry in managing similar diseases (Nutsugah *et al.*, 2003 and Honger *et al.*, 2022). Both groups identified *Curvularia lunata* as the cause of brown leaf spot disease in Ghana, marking the first reported association between any *Curvularia* species and the disease.

2.5 Disease Cycle and Epidemiology of Brown Leaf Spot of Rice

Rice brown leaf spot is a destructive fungal disease affecting rice cultivation in Ghana. Symptoms appear as minute spots on leaf sheath, glumes, blades, and coleoptiles, with adolescent and juvenile spots resembling sesame seeds (Ransingh *et al.*, 2021). The leaf undergoes desiccation as multiple areas coalesce (Plate 1).

The brown leaf spot pathogen causes visible symptoms on foliage, with leaf blades and glumes showing the most prominent markings. The foliage exhibited a variety of spot sizes and shapes (Quintana *et al.*, 2019). Leaf spots are circular to oval, dark brown to reddish brown, with a light center. Eye-spots are more conspicuous. Infected seeds fail to fill, and susceptible cultivars have blotches longer than 1 cm (Plate 1). This disease is commonly referred to as the "poor rice farmer's disease" due to its predominance in deficient and poor soils (Zadoks & Schein, 1979).

Extensive research has been conducted on the disease cycle and epidemiology of *B. oryzae* in relation to rice (Plate 2). To induce sporulation in *B. oryzae*, both light and dark periods were necessary. Conversely, blue light inhibited it while near-ultraviolet light stimulated it. Thakur *et al.* (2023) determined that the ideal temperatures for conidial germination and growth of *B. oryzae* are 25⁰C to 30⁰C and 27⁰C to 30⁰C, respectively. Conidia germinate within the pH range of 6.8 to 7.0, with an optimal range of 2.6 to 10.9. Additionally, Mau *et al.* (2022) documented that the occurrence and intensity of brown leaf spot were greater in lowland irrigated areas than in upland areas. *B. oryzae* spread rapidly due to persistent rainfall, cloudy conditions, and high

temperatures. The disease's incidence fluctuates by 8.8% and 9.2% with annual precipitation. The pathogen survives and disperses year-round (Chakraborty *et al.*, 2020) (Plate 2). Conidia and mycelia are prevalent survival structures in rice seeds and crop residues. Fungal infections, such as *B. oryzae*, cause crop yield decline. Nutrient-deficient soils aid brown leaf spot disease infection, leading to epidemic proportions in low pH and inadequate nutritional profiles (Surendhar *et al.*, 2022). Higher disease intensity was positively correlated with N, P, and Mn content, whereas Imran *et al.* (2020) found negative correlations with Ca, Mg, K, Fe, and the K/N and Fe/Mn ratios of the plant. Furthermore, increase in disease severity can also occur due to a deficiency of N or K. The disease is particularly prevalent in environments characterized by limited water resources and nutritional imbalance, specifically nitrogen deficiency. The disease infection affects transpiration, ionic composition, and carbon assimilation, leading to brown patches worsening due to fungus colonization, cell damage, MDA concentrations, electrolyte leakage, and reduced photosynthesis (Fahad *et al.*, 2019).

Singh (2016) discovered that the disease prognosis methodology utilized the correlation between meteorological parameters and airborne fungal spores of the RBS pathogen extensively. Schwanck *et al.* (2021) discovered that the pathogen responsible for rice brown leaf spot disease was climate-dependent. Conversely, Chakraborty *et al.* (2020) documented that *B. oryzae* conidia are present year-round in the atmosphere and rice fields, with rice brown leaf spot pathogenic conidia identified during both dry and wet seasons, but declining post-harvest. *B. oryzae* spores enter coleoptile after rice germination, causing secondary infections and leaf blotches, which initiate the subsequent life phase (Plate 1).

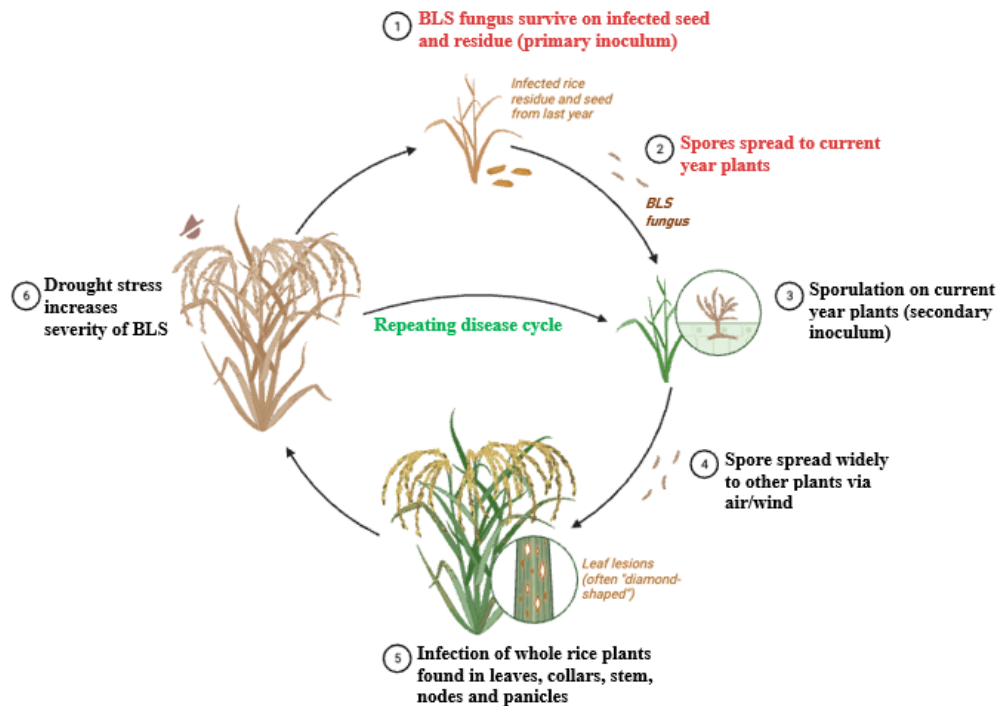


Plate 1: Disease Cycle of Brown Leaf Spot of Rice

2.6 *Bipolaris* spp. as Pathogen of Brown Leaf Spot

B. oryzae can be classified into various groupings according to its cultural and morphological characteristics. Previous studies have documented significant variability among pathogen isolates in terms of characteristics such as size, color, number of cells per conidium, and mode of infection. The sporophores as thick, erect, geniculate at the base and lighter towards the apex, and they emerge in clusters of three to five conidia through the stomata. Kumari *et al.* (2015) documented cultural characteristics of *B. oryzae*, including hairy or cottony mycelium that was black, grey, or white in color, and spores measuring between 4.10 and 5.51 μm in width and 5.34 to 7.48 μm in length (Plate 3) (Shoemaker, 1959). Furthermore, on the basis of colony morphology and growth pattern, they classified 52 isolates into the following five categories: Black with fluffy growth (16 isolates), Black with suppressed growth (10 isolates), Grey with cottony growth (9 isolates), Grey and white mix with cottony growth (12 isolates), and white with cottony growth (5 isolates). Septate mycelia of the

brown leaf spot pathogen, ranging in color from dark green to gray, were identified (Quintana *et al.*, 2019) (Plate 2). The mycelium of the pathogen *B. oryzae* is branched, light brown to black in color, septate, and aerial/submerged (Jaiganesh & Kannan, 2019). The fungus produces a mat that varies in color from greyish brown to dark brown upon contact with infected tissues. Sporophores proliferate in clusters of three to five through stomata, while conidia consist of five to ten septa (Nayak & Hiremath, 2019).

Likewise, Valarmathi & Ladhalakshmi (2018) classified the pathogen morphologically into four distinct groups: Group I exhibited black filaments with fluffy growth, Group II grey filaments with fluffy growth and white patches, Group III grey filaments with fluffy growth, and Group IV grey filaments with inhibited growth.

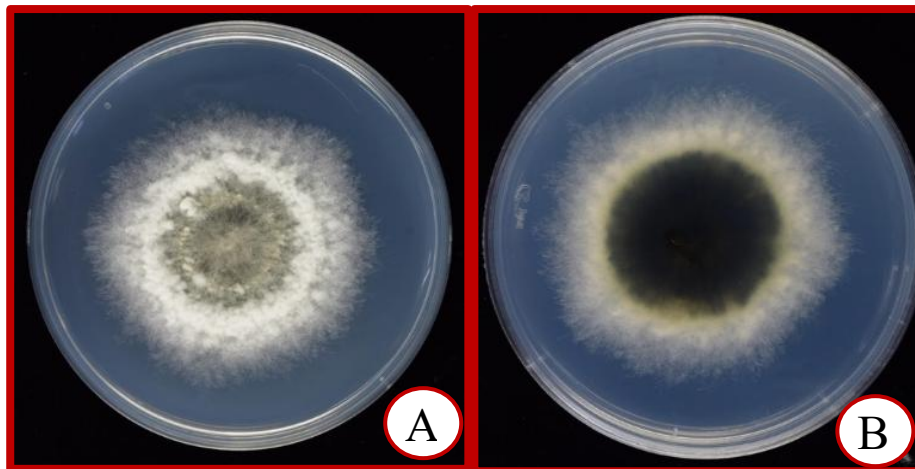


Plate 2: Colony of *Bipolaris oryzae* (A: Surface view, B: Reverse view)

Basavaraj *et al.* (2023) reported that sporulation was elevated in PDA medium, sporulation was also elevated in malt extract agar medium following a 96-hour incubation period at 28⁰C. Isolates that exhibited black, airy growth typically produced a greater quantity of conidia. According to Valarmathi & Ladhalakshmi (2018), rice polish agar medium exhibited a high level of sporulation. *B. oryzae* conidia were

typically curved, fusoid, or obclavate; on occasion, they approached a cylinder-like shape; they ranged in color from pallid to medium golden brown; they had five to six septa with a hilum; and the size of conidia produced by the isolates varied considerably (Valarmathi & Ladhakshmi, 2018). Analogous discrepancies have been documented in the septations, length, breadth, and shape of conidia produced by *B. oryzae* isolates (Kumari *et al.*, 2015; Nayak & Hiremath, 2019).

The bipolar germination pattern of the *B. oryzae* conidia led to their nomenclature as *Bipolaris* (Kumar *et al.*, 2016). The septate, solitary, or small group conidiophore of *B. oryzae* is simple, linear or flexuous, and pale to mid-brown. It is endowed with conidia on both sides. According to Quintana *et al.* (2019), the dimensions of conidiophores and conidia of *B. oryzae* isolates differ from 7.0-10.0 by 20.0-26.0 μm depending on the location of isolates. *Bipolaris oryzae* conidia were club-shaped to cylinder-shaped, generally curved, light brown to golden brown in color, and comprised of six to thirteen transverse cell walls (Quintana *et al.*, 2019) (Plate 3).



Plate 3: Typical conidia of *Bipolaris oryzae*

Based on DNA fingerprinting and variable number tandem repeat analysis, Kamal & Mia (2009) characterized 152 *B. oryzae* isolates. The analysis revealed a high degree of diversity among the isolates, which were categorized into 12 fingerprint categories

with a 75% similarity level. Archana *et al.* (2014) used UPGMA cluster analysis to analyze *B. oryzae* isolates' genetic diversity. Results showed a 50-91.6% polymorphism range, with abundant diversity within the ITS region, indicating scarce genetic diversity in *B. oryzae*. Monisha *et al.* (2019) extracted DNA from five *B. oryzae* isolates using CTAB method, amplifying a 570 bp fragment. PCR amplification confirmed the isolates as *Bipolaris* spp. Molecular characterization of 77 *Bipolaris* species using the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA), the 28S nuclear ribosomal large subunit rRNA gene (LSU), the D1/D2 domain of large-subunit (LSU) ribosomal DNA (rDNA), and the protein-coding gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This particular form of multigene analysis elucidated the correlation and association among the isolates (Manzar *et al.*, 2022).

2.7 The New Pathogen: *Curvularia* sp.

2.7.1 Taxonomic Position

Helmithosporium curvulum and *Acrothecium lunatum* Wakker (now *C. lunata*) are among the numerous species of *Curvularia* that have been reported from various regions of the world. Some of these species were previously classified under distinct genera before Boedijn (1933) identified the genus *Curvularia*. Subramanian (1954) conducted a taxonomic analysis of various taxa within this genus. Boedijn classified the genus *Curvularia* into three groups: *geniculata*, *lunata*, and *maculans* (Plate 7), based on the relative position and number of septa in their conidia. *Curvularia* is a facultative feeble pathogen of soil and plants that is widely distributed and airborne. It primarily survives as a saprophyte in tropical and subtropical regions. It is a filamentous, dematiaceous fungus. *Curvularia* spp. are fungi that are densely pigmented

and possess spores (conidia) that are well-suited for the majority of aerial dispersal. The Ascomycetes genus *Cochliobolus* contains its telomorph. *Cochliobolus drechsler* (1934) and its anamorphs *Curvularia* Boedijn (1933) are ubiquitous pathogens that primarily affect grasses (Poaceae). Devastating disease epidemics of critical food commodities, including maize, wheat, and rice, have been the result of the actions of certain species.

2.7.2 Cultural and Conidia Variability of *Curvularia*

Variability studies in *Curvularia* have not been extensively carried out barring a few references like who observed physiological differentiation in *C. lunata* isolates with regard to virulence. Cui and Sun (2012) studied that initial symptom as purple- brown tiny islands spots emerging on the leaf surfaces with diameters ranging from 0.5 to 3 mm, which later developed grayish white centers and a black-brown banding pattern on the edges (Plate 5) (Alex *et al.*, 2013).

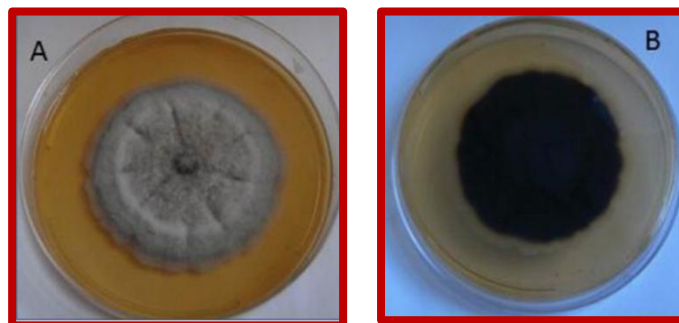


Plate 4: Culture of *Curvularia* spp. A: surface view, B: Reverse side view

According to the morphological variability studies conducted by Chinchure & Patil (2023), sixty isolates showed considerable variability with respect to colony characters, colony diameter, sporulation, size of conidia, mycelium width and dry mycelium weight. The colony was sparse to dense, light grey to dark black, fair to fast growing, Excellent sporulation was (> 50 conidia per microscope field) exhibited by some of the

isolates, while other isolates showed poor sporulation (<1-10 conidia per microscope field), and maximum size of conidia was observed in 20.30 μm long \times 10.90 μm wide). Maximum dry mycelium weight was observed as 482.22 mg. Yuvarani *et al.* (2021) also characterized *Curvularia* species and reported that, colonies of grown on PDA medium were grey to brown in colour. The conidia of all the isolates were boat shaped, swollen at third cell, grey, black to dark brown, third cell from base darkest and largest, with three septa, end cells of the conidia were sub hyaline, germinated at ends and the conidial length and breadth ranged from 22.26 to 17.36 \times 11.80 to 7.79 μm (Plate 6).

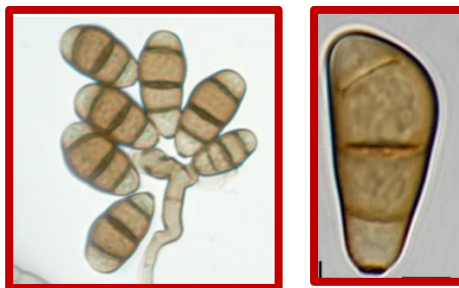


Plate 5: Conidia of *Curvularia* spp. (Marin-Felix *et al.*, 2017)

2.7.3 *Curvularia* spp. as Brown leaf spot Pathogen

Curvularia is an Anamorphic Ascomycetes found in plants and soils of tropical and temperate regions. Most species are facultative pathogens of the soil, plants and cereals. They are mostly found as saprobes or parasites on gramineous hosts (cereals and grasses). *Curvularia* species are known to cause leaf spot of several cultivated as well as wild host plants. *Curvularia* spp. have a wide host range, including humans and animals. Species of *Curvularia* that are plant pathogens include *C. lunata*, *C. geniculata*, *C. brachyspora*, *C. clavata*, *C. inaequalis*, *C. oryzae*, *C. affinis*, *C. brachyspora*. *Curvularia* leaf spot (CLS) is one of the common foliar fungal diseases of rice and other crops that is widely distributed worldwide. *Curvularia* leaf spot can be traced back to the 1921, where Miltra reported it on millet. The disease first appears

as brown to tan coloured spots on the leaves after three to four days. It attains almost 1 mm size. The spots are surrounded by a yellowish region and when those appeared closely on the leaves, formed a yellowish zone after coalescing the yellowish region which attained a size approximately from 2 mm to 4 mm diameter. The most identified specie of *Curvularia* as spot pathogen is *C. lunata*, although several species have reported to be associated with members of the grass family. For example, Khoo *et al.* (2023) reported it as the causal agent of leaf of rice in Malaysia, Zhou *et al.* (2021) in China, Limtong *et al.* (2020) also reported same from Thailand. For other crops like maize, Akonda *et al.* (2015) studied incidence and severity of different disease in maize. *Curvularia* leaf spot was recorded at all stage of crops. Higher incidence was observed 30 DAS. Where severity level was up to 0.85%.

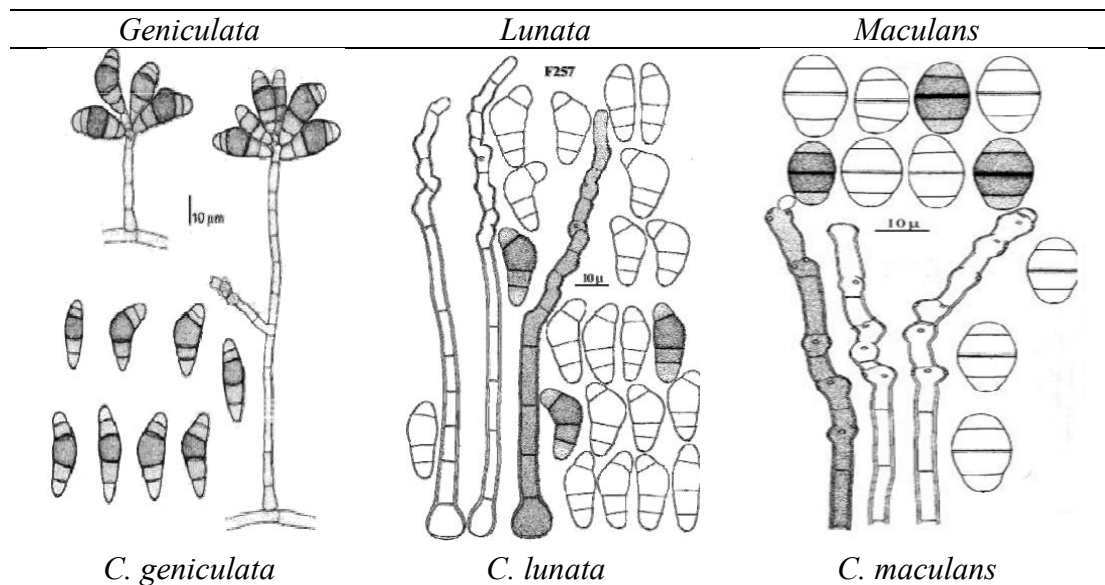


Plate 6: The three main species of *Curvularia*,

Source: www.mycobank.org, 2023

2.7.4 Symptoms

Various researchers studied the symptoms of *Curvularia* leaf spots on agricultural plants and on some tree species. Generally, symptoms were described as minute,

circular, chlorotic, pinhead-size translucent spots with brown margin on the leaf surface. Later, the mature spots turned as necrotic spots and surrounded by visible halo- lesions on leaves. Symptoms of *Curvularia* leaf spot on *P. deltoides* was described by Manzar *et al.* (2021). It had small, round to irregular, light brown leaf spots appeared on the leaf blade with frequent haloing. Sometimes, the center of the spot was gray with well-defined brown margins. Later, these spots coalesce to create large necrotic spots with a distinct centre and irregular, prominent, halo margin (Plate 1).

2.8 Detection Tools

The reliable and accurate detection of brown leaf spot pathogens underpins the ability to delimit its distribution and organize successful management strategies (Spadaro, 2023). Conventional methods for soil-based detection of pathogens uses leaf, fruit and plant material to bait the pathogen from flooded soil samples (Lewis, 2018), or sterile techniques using genera-specific media to isolate the pathogen from host root samples. For both soil bioassays and sterile isolation techniques, the steps involved are highly time consuming and it can take up to 20 days for a definitive detection (Meena *et al.*, 2020). Moreover, identification keys which came into existing along the line, are heavily based on morphological criteria. These keys are not considered to be optimal, as morphological similarities between genera like *Bipolaris* and *Curvularia* species, together with natural variations within taxa, make species determination challenging (Patsa *et al.*, 2018).

Molecular diagnostic tools which utilize PCR provide the most sensitivity for detection of brown leaf spot and other pathogens (Hariharan & Prasannath, 2021). For example, a recent study developed a TaqMan real-time PCR detection method to enable the rapid quantification of *P. agathidicida* within the soil (Byers *et al.*, 2021). Initial tests showed

the method could distinguish between brown leaf spot disease pathogens and a variety of other pathogenic species (Chen *et al.*, 2022; Temperini *et al.*, 2022). Results suggest this TaqMan real-time PCR detection method will allow rapid and accurate detection of brown leaf spot pathogens, which will facilitate delimitation of its distribution.

2.9 Management of Brown Leaf Spot Disease of Rice

2.9.1 Inorganic/Synthetic Fungicide

Use of fungicides is the most effective method of controlling brown leaf spot disease whenever there is outbreak of disease. A number of fungicides have been evaluated against brown leaf spot pathogen under both *in vitro* and *in vivo* conditions. The fungicidal applications provide an effective control of Brown leaf spot disease in a short time span and it is the most widely used control measure, according to Surendhar *et al.* (2022). Propiconazole and Hexaconazole are effective fungicides against brown leaf spot pathogens in both laboratory and field conditions. Propiconazole showed 97.89% inhibition in *in vitro* and 96.58% inhibition *in vivo*. Carbendazim also inhibited brown leaf spot pathogens. Spraying Hexaconazole and Propiconazole during early booting stages can reduce disease severity and increase grain yield. Sandeep (2015) tested various fungicide concentrations against poplar leaf spot pathogens using food poison technique. However, the use of synthetic fungicides and crop management strategies has not been successful in eradicating many pathogens, besides regular use of chemical fungicides leads to development of resistant strains of fungi and these fungi with time become tolerant to the fungicides, rendering it ineffective. Fungicides also results in environmental hazards and chronic health problems. Again, it negatively affects non-target organisms in the soil (El-Baky & Amara, 2021). Biocontrol agents

offer a viable alternative to conventional chemicals in agriculture and forestry for protecting crops against weeds, insects, and diseases.

2.9.2 Biological Management of Brown Leaf Spot Disease

Ecological methods promote sustainable agricultural practices, with biocontrol using antagonists being a promising strategy for ecofriendly and green agriculture to protect crop plants (He *et al.*, 2021).

Epiphytes are organisms that grow on living plants, independent of host plants for water and nutrition. They are found in the phyllosphere, a habitat rich in bacteria, fungi, yeasts, and algae. Epiphytes, plant pathogens, natural antagonists, and growth promoters, adapt to habitats through morphological and physiological adaptations, influenced by host tree bark size and texture (Hongsanan *et al.* 2016).

The process by which biocontrol agents rapidly and extensively colonize plant surfaces, specifically seeds, is thought to have a significant impact on the suppression of diseases (Latz *et al.*, 2018). In contrast to alternative biocontrol agents that operate through antagonistic interactions, epiphytes function as biocontrol agents by virtue of a mutualistic relationship that is inherent in nature (Gautam & Avasthi, 2019). Seeds are utilized to transmit asexual strains of epiphytes, which are asymptomatic (Schouten, 2019). When all is said and done, two bunches of phylloplane growths are there viz., casuals and occupants. Occupants create on dynamic leaves' surfaces, but casuals cannot multiply. Focus on phylloplane growths is overlooked, with endophytes, saprophytes, and foes being more significant. Extreme attacks on phylloplane organisms are common.

The epiphytic fungus *P. lilacinum* is more widely known as *Paecilomyces lilacinus*, having undergone a recent taxonomic revision (Senthilkumar *et al.*, 2020), it was nominated as *Penicillium lilacinum* by Thom in 1901, and then it was revised as

Paecilomyces lilacinus by Samson in 1974. *P. lilacinum*, an emerging pathogenic mold, causes ocular and subcutaneous infections, but also presents potential as a biocontrol agent against nematode infestation and other pathogens. Ahmad *et al.* (2019) conducted an in vitro experiment to assess the ability of regional strains of *P. lilacinus* and *P. variotii* to reduce the number of predator worm embryos of *Fasciola gigantica* and *Meloidogyne* spp. In this study, *P. lilacinus* was more effective than *P. variotii* at reducing the diverse species of worms. *Paecilomyces lilacinus* on tomato plant development and *Meloidogyne* spp. were introduced to the soil but *P. lilacinus* in the soil resulted in enhanced tomato plant growth characteristics but treatments that contained to *P. lilacinum* but only containing *Meloidogyne* spp. resulted in a progressive decline in plant development.

The greatest reduction in plant growth was observed when *Meloidogyne* spp. were introduced monoclally. Baidoo *et al.* (2017) conducted On-farm, field, and greenhouse experiments where they tested *Purpureocillium lilacinum* (*Paecilomyces lilacinus*) strain 251 were tested as nematicide, an *P. lilacinum* were moderately successful in decreasing the population density of *Meloidogyne incognita*. However, nematode decrease had no discernible effect on yield. Dahlin *et al.* (2019) also evaluated the management potential of chemical and biological control methods on *Meloidogyne incognita* infested tomatoes. The study evaluated tomato yield, gall index, soil, and root nematode populations using Velum and *Purpureocillium lilacinum* strain 251 as wettable granulate. Velum effectively reduced *M. incognita* nematode population, controlling nematode populations.

Aspergillus, a large fungi genus, comprises 250 species, classified into seven subgeneras. Some are pathogens, while others have biocontrol potential against phytopathogenic organisms. The effectiveness of *Aspergillus flavus* NRRL21882 in

mitigating aflatoxin contamination in peanut fields prior to and after harvest has been investigated by Lavkor & Var (2017) and it yielded the highest aflatoxin reduction of 76%. AflaSafe (a combination of four non-toxic strains) has been granted provisional registration because it shown high efficacy in mitigating aflatoxin levels in all treated fields of maize (Bandyopadhyay *et al.*, 2016).

Researchers are exploring the use of plant extracts to combat plant pathogenic fungi due to their effective secondary metabolic compounds, environmental benefits, and high specialization (Al-Baldawy *et al.*, 2021). In an in vitro study, Chouhan *et al.* (2022) examined the efficacy of two botanicals, neem leaf extract and neem oil. The researchers found that a 3% concentration of neem oil inhibited the growth of *Helminthosporium oryzae* the most. A study found that the aqueous leaf extract of all medicinal plants, with a concentration of 0.50%, effectively inhibited the growth of *B. oryzae*. Additionally, five plant extracts were evaluated by Hussain *et al.* (2019) against *B. oryzae*. The authors found that fungal growth was significantly inhibited at higher doses of the extracts, with minimum growth observed in ginger extract, followed by garlic, datura, neem, and *C. gigantea* leaf extract. According to a study by Channakeshava & Pankaja (2018), among the nine plant extracts that were assessed in vitro against *B. oryzae*, garlic extract inhibited mycelial growth by 100% at all concentrations tested (5%, 10%, and 15%). Using the poisoned food technique.

Kumar & Simon (2016) reported that the most effective treatment against brown leaf spot of rice was *Azadirachta indica* at 10% FS at 30, 45, 60, and 75 days. The *Azadirachta indica* treatment significantly improved plant height, tiller count, shoot weight, yield, and cost-benefit ratio in rice brown leaf spot disease prevention compared to carbendazim and control groups.

Previous studies have documented the antifungal properties of aqueous extracts derived from different components of neem, including neem oil and its principal constituents (Kumar & Simon, 2016). An investigation was conducted to assess the antifungal properties of *Azadirachta indica* L. against *Alternaria solani* Sorauer. The findings confirmed that the ethyl acetate fraction inhibited fungal growth the most effectively (minimum inhibitory concentration of 0.19 mg). Furthermore, this fraction was more effective than the fungicide (metalaxyl + mancozeb), which has a MIC of 0.78 mg.

Research shows plant products can suppress diseases through their active constituents, including proteolytic enzymes, cardenolides, alkaloids, cardioactive glycosides, tannins, flavonoids, and procerain (Sivapalan *et al.*, 2023). *Nerium oleander* leaves contain high polyphenol content, with cinnamic acid being the principal constituent and epicatechine, catechin, and chlorogenic acid as additional elements (Ayouaz *et al.*, 2020). According to the findings of Jaddoa *et al.* (2021), the methanolic extract of *Nerium oleander* showed significant antibacterial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. It also showed antifungal activity against *Fusarium oxysporum*, *Alternaria alternata*, *Fusarium solani*, and *Rizoctonia solani*, with essential oil showing antibacterial activity.

2.9.3 Efficacy of Integrated Management of Rice Brown Leaf Spot

Disease

Harish *et al.* (2008) demonstrated that two applications of neem cake extract, *N. oleander* leaf extract, and *T. viride* (Tv2) on rice plants in the field at one fifteen days after the onset of the disease and one hundred thirty-three days later, decreased the

incidence of brown leaf spot by 70%, 53%, and 48%, respectively, and increased the yield by 13%, 18%, and 15%, respectively. In a separate investigation, it was discovered that the utilization of *Pseudomonas fluorescens* RRb-11 for seed bacterization and root dip followed by foliar spray containing 15% leaf extract of *D. metel* yielded comparable results in terms of diminishing the severity of the disease and enhancing the growth parameters of the plants (Meena & Gopalakrishnan, 2013). According to Naqvi (2019), there is potential for the commercialization of formulations containing botanicals and biological agents to assist producers in their efforts to combat the disease.

In order to assess the effectiveness of various chemical, botanical, and biological therapies against the brown leaf spot disease of rice, Lamsal (2012) conducted an invitro experiment. The findings indicated that seed treatment and foliar spray utilizing neem oil-based products, such as Nico-neem and locally sourced botanicals like Simali, show potential as environmentally sustainable methods for managing brown leaf spot disease in rice. These treatments achieved comparable results to the chemical treatment. Ramachandra *et al.* (2016) found that a mixture of *Lawsonia inermis*, coconut cake extracts, and Bacillus strain BA2 reduced disease incidence in rice seed. The lowest incidence was observed in rice seed treated with *P. fluorescens*, commencing from the initial application 15 days after transplanting. In a study conducted by Mahmud (2020), it was found that the application of BAU-Biofungicide 2% (Trichoderma-based preparation), along with extracts of neem and garlic effectively inhibit *B. oryzae* mycelial growth, resulting in lower disease incidence, higher grain yield, and a favorable cost-benefit ratio, emphasizing the need for integrated disease management. (Narasimhamurthy *et al.*, 2021).

Chapter Three: Materials And Methods

3.1 Study Location

3.1.1 Description of the Atwima Nwabiagya

Geographically located in the western portion of the Ashanti region in Ghana, the Àtwima Nwabiagya district shares boundaries with Offinso Municipal Assembly to the north; Amansie-West and Bosomtwe-Atwima Kwanwoma districts to the south; Kumasi Metropolis and Kwabre districts to the east; and Ahafo Ano South and Atwima Mponua districts to the west (Forkuo & Frimpong, 2012; Antwi-Agyei *et al.*, 2019) (Figure 1). The district is characterized by a preponderance of semi-deciduous vegetation. The Barekese Water Works Forest Reserve and the Owabi Water Works Forest Reserve function as water shed protection for the Offin and Owabi rivers, respectively. The predominant soils in the district are the Kumasi-Asuansi Compound Association, the Kobeda-Eshien Compound Association and the Nyanano-Opimu Simple Association (Appiah *et al.*, 2015), these soils are good for cultivation of a variety of crops such as Maize, rice, sugar cane, coffee, cocoa, cassava, vegetables, cocoyam, yam, citrus, ginger, oil palm and plantain (Arthurappiagyei, 2016). The district experiences bimodal rainfall with precipitation spanning from 170 cm to 185 cm. Major precipitation occurs between mid-March and mid-July, while minor precipitation occurs between mid-September and mid-November (Forkuo *et al.*, 2021). The temperature ranges relatively consistently from 27⁰C in August to 31⁰C in March. With a mean relative humidity ranging from 87% to 91%, the area is identifiable. During February and April, relative humidity levels are typically lowest, ranging from 83⁰ to 87⁰ in the morning and 48⁰ to 67⁰ in the afternoon (Danquah, 2016).



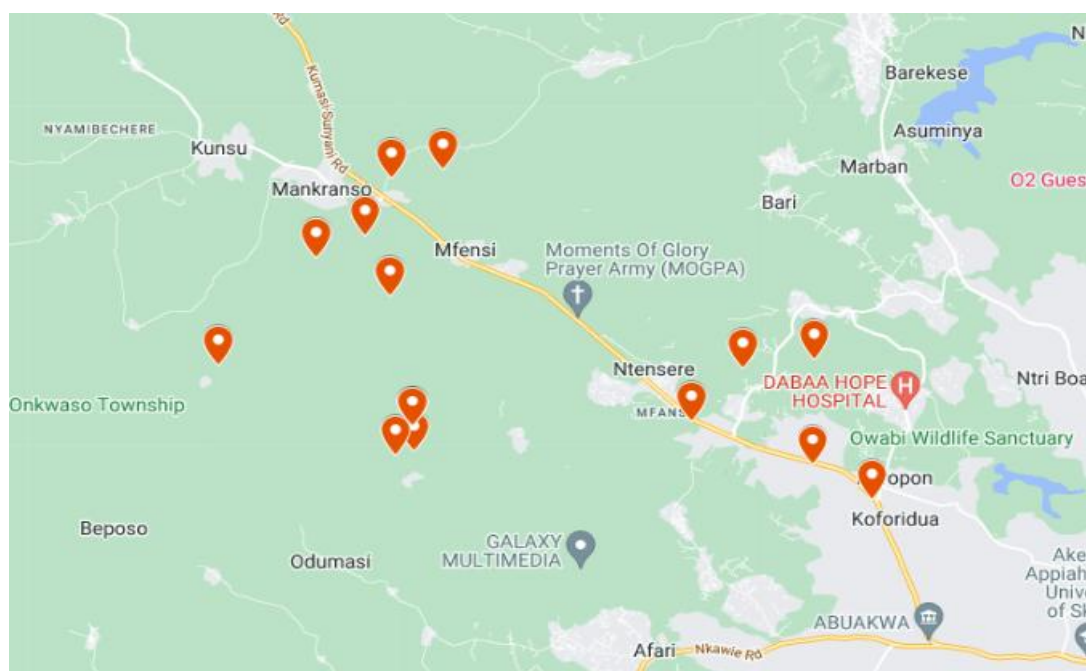
Figure 1: Map Showing Atwima Nwabiagya District

3.1.2 Collection and Labelling of Leaf Samples.

Leaves of AGRA rice variety showing symptoms of the brown leaf spot disease were collected from 15 rice farms at different geographic locations (Figure 3.2) in Atwima Nwabiagye District, Ghana in February, 2023 and transported to the Microbiology Laboratory at the Plant Health Division, Crop Research Institute, Council for Scientific and Industrial Research, Fumesua (CRI, CSIR). The collected samples were stored at 4⁰C in a refrigerator until use. The GPS address of each farm where the samples were taking was also recorded at the time of taking samples. Table 1 and Figure 2 shows samples and their GPS address and the locations for the samples on map respectively.

Table 1: Location of Samples

S/N	Code	Location	GPS Coordinates	
			Decimal Degrees (DD)	Degree, Minutes and Seconds
1	AK-A	Akropong	6.738005, -1.732809	6°44'16.8"N 1°43'58.1"W
2	KK-A	Kokobeng	6.774025, -1.746557	6°46'26.5"N 1°44'47.6"W
3	TA	Taabre	6.746726, -1.746658	6°44'48.2"N 1°44'48.0"W
4	NT-A	Ntensere	6.757892, -1.775690	6°45'28.4"N 1°46'32.5"W
5	BE-A	Besease	6.767868, -1.774408	6°46'04.3"N 1°46'27.9"W
6	AS-A	Asakra	6.772344, -1.888397	6°46'20.4"N 1°53'18.2"W
7	MF	Mfensi	6.771570, -1.763196	6°46'17.7"N 1°45'47.5"W
8	MM	Mfensi	6.799915, -1.864917	6°47'59.7"N 1°51'53.7"W
9	AD	Adankwame	6.790123, -1.847576	6°47'24.4"N 1°50'51.3"W
10	AA	Adankwame	6.785087, -1.829520	6°47'06.3"N 1°49'46.3"W
11	NN-A	Nineteen	6.820496, -1.847043	6°49'13.8"N 1°50'49.4"W
12	NN-B	Nineteen	6.822893, -1.834648	6°49'22.4"N 1°50'04.7"W
13	NK-A	Nkontomire	6.756487, -1.841929	6°45'23.4"N 1°50'30.9"W
14	NK-B	Nkontomire	6.749298, -1.845999	6°44'57.5"N 1°50'45.6"W
15	NK-C	Nkontomire	6.750285, -1.841475	6°45'01.0"N 1°50'29.3"W




Key:
 Sampled rice farms

Figure 2: Map Showing Location of Samples

3.2 Isolation and Identification of Fungal Pathogens Associated with Brown Leaf Spot Disease of Rice

3.2.1 Location of study

The study was carried out at the Plant Pathology Laboratory of the CSIR-Crops Research Institute, Kumasi, Ghana.

3.2.2 Collection of diseased Leaf Samples

Five leaves of AGRA rice variety showing symptoms of the brown leaf spot disease were collected from each rice farm in the selected locality (Fig. 3.2, Table.3.1.) in February, 2023. Leaves samples from each farm were put in separate transparent polyethene bags, coded and transported to the Laboratory. At the lab., the coded samples were stored at 4⁰C in a refrigerator until use.

3.2.3 Preparation of Culture Media

Two hundred grams (200 g) of peeled Irish potatoes were rinsed after slicing and boiled for 30 mins with distilled water in a conical flask. A cheesecloth cloth was applied to filter the boiled potatoes and the volume was increased to 1L with distilled water along with dextrose (glucose) (20 g) and agar (20 g). The suspension was dissolved with heat and shaking, then left to cool down at room temperature for two min, then sterilized in an autoclave at 121⁰C for 15 mins as used by Pourakbar *et al.* (2021). After which the media was allowed to cool and then amended with antibiotics (ampicillin) (250 mg in 0.5 L of PDA) to suppress bacteria growth (Muthomi *et al.*, 2017; Esquivel & Bell, 2021). The amended PDA is then transferred to Petri dishes and ready to be used

3.2.4 Isolation and Identification of Fungal Pathogens

Single rice leaf from each of the 15 farms surveyed was thoroughly washed under running tap water in the CSIR-Crops Research Institute Plant Pathology laboratory to remove all debris. The washed leaves were carefully cut into approximately 3-cm to contain portions of both diseased and healthy leaf tissue. The leaf pieces were surface sterilized with 3 % commercial bleach and 70% ethanol for three minutes. The sterilized leaf tissues were rinsed three times with sterile distilled water (Parzymies, 2021) and dried on sterile Whatman filter paper in a laminar flow hood. Five (5) pieces of the sterilized-dried leaf tissues were plated on the PDA agar medium and the plates were incubated at $26\pm 2^{\circ}\text{C}$ for seven days in an incubator. Hyphal tips of actively growing fungal pathogens were picked with sterilized inoculating needle and transferred unto fresh PDA media. Sub culturing was repeated until pure cultures of the fungal pathogens were obtained and maintained. Standard identification manual (Aslam *et al.*, 2021; Imran *et al.*, 2020; Simanjuntak, 2020; Zhou *et al.*, 2021; Imran *et al.*, 2022) was used to identify the isolated fungal pathogens, under a compound microscope (SWIFT, USA).

3.2.5 Production of Pure Cultures of Fungal Pathogen isolates from Single Conidia Spore

Single conidia isolate of major BLSF fungal pathogen identified was prepared as described by Appiah-Kubi *et al.* (2022) and Tamin *et al.* (2019) by flooding the surface of plates with distilled water. The surfaces were gently scraped with a sterilized glass rod to dislodge the conidia to form a conidia-mycelia-water suspension. The conidia suspension was filtered through a double-layer cheesecloth to remove excess mycelia. An aliquot (2 millimeter) of conidia suspension was picked with the aid of a pipette

and added to 8 ml of distilled water and shaken vigorously using a vortex machine. One millimeter of the final suspension was picked and inoculated on a water agar medium. The plates were incubated at 26 ± 2 °C for 24 hours in an incubator and observed under stereomicroscope. Growth from single conidia was picked with sterilised inoculating needle and placed on PDA media and incubated for use in the study.

3.2.6 Phylloplane Fungi Isolation

Phylloplane fungal isolates on surface of healthy and diseased-free rice leaves were isolated and identified using the procedure as described above (section 3.2.4)

3.2.7 Maintenance of *Curvularia* and *Bipolaris* Isolates

Isolates of identified *fungal pathogens* were maintained on PDA medium. Mycelia disc of 3-mm diameter were cut from a seven-day old culture of each isolate and placed in the center of PDA medium plate. The plates were sealed with parafilm to reduce drying and incubated at $26\pm 2^{\circ}\text{C}$. The cultures were observed and transferred monthly unto PDA medium to keep them active (Adomako, 2019).

3.2.8 Production of *Curvularia* and *Bipolaris* spp. Inoculum

Conidia suspensions were produced from *Curvularia* and *Bipolaris* isolates by adding 10 ml of distilled water to the surface of 14-day-old culture of each isolate grown on PDA medium. The surface of their mycelia were gently scrapped with the edge of a sterilized glass rod to dislodge the sporangia. The mycelia-conidia-water suspension was filtered through cheesecloth to remove mycelia fragments. The final suspension obtained per isolate was adjusted to 1×10^6 conidia/ml with the aid of a haemocytometer and a tally counter (Appiah-Kubi *et al.*, 2022) for use in the study.

3.3 Morphological Characterization of Fungal Pathogen Isolates

3.3.1 Conidia Morphology of *Curvularia* and *Bipolaris*

Curvularia and *Bipolaris* isolates successfully identified were used for the morphological characterization. Conidia morphology of the isolates were studied in 14-day-old cultures grown on PDA medium. Conidia suspension of each isolate was produced as described above (section 3.2.5). The shape of conidia, size of conidia (length x breadth), presence of septations and number of conidia per milliliter were counted under compound microscope and recorded. The experiment was arranged in a complete randomized design with three replications. Measuring of the conidia size was done with the aid of micrometer fitted on SWIFT compound microscope connected to a laptop computer.

3.3.2 Culture Characteristics and Colony Growth of Fungal Isolates Studies

Mycelia disc of 2-mm diameter were cut from the margin of the seven-day old cultures of *Curvularia* and *Bipolaris* isolates and placed in the centre of PDA medium plates. The plates were incubated for seven days in an incubator at 26 ± 2 °C and the diameter of the colony measured and recorded. To measure colony diameter, two diagonal lines were drawn on the reverse side of each plate and growth measured from the tip of the hyphae along the diagonal lines using a plastic rule. The initial inoculum diameter (2-mm) was deducted from the final measurements obtained. Colony morphology such as the number of growth zonations (rings), margin type, colony elevation, colony texture, colony opacity, colony shape, colony colour (Front Side and Reverse Pigmentation) as described by Petersen (2016) and Muresan (2017) are shown in figure 3. Colony morphology such as colony colour and colony growth of isolates were visually

observed and recorded. Three replications arranged in a complete randomized design was used for the study.

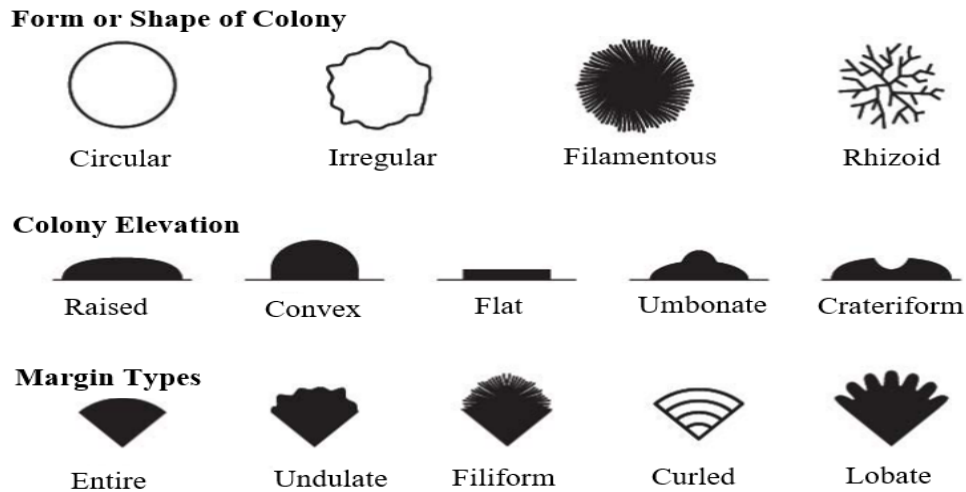


Figure 3: Manual Colony Morphology

Sourced: Petersen (2016)

3.3.3 Evaluation of Pathogenicity of *Curvularia* and *Bipolaris*

Isolates

Both detached leaf and whole plant assays were conducted to determine the virulence of the isolated fungal pathogens. For the detached leaf assay (Bhattarai *et al.*, 2020) healthy rice leaves of the AGRA variety was raised under screenhouse conditions. Fresh and diseased free rice leaves were collected and sterilized with 1% sodium hypochlorite solution then blotter dried and placed on moistened filter paper in 9 cm Petri dishes where three replicates were maintained for each isolate. Inoculation was done by placing a 2 cm mycelia disk of isolates on each leaf sample and sterile PDA for control plate, the set up was incubated for 3 days at 25⁰C. The incubated tissues were observed daily for symptom initiation. The number of days for symptom appearance was recorded for each isolate. Also, lesion size produced by each isolate was measured.

For the screenhouse assay, AGRA rice seedlings were obtained by sowing rice seeds in pot containing 1 kg sterilized soil and perforated at the bottoms, and they were left to germinate and grow. Each pot was placed in another bigger pot containing supplemented water in other to create artificial lowland condition for the rice plants. Inoculation was done with spore concentration of 1×10^6 after 21 days of planting by using the spray gun and autoclaved distilled water was used to inoculate the control treatment, three replicates were maintained for each isolate. Days to first symptom appearance, number of infected leaves, number of diseased plants, disease incidence, Severity and intensity index were evaluated for the screenhouse experiment. Incidence (%) (formular 1), severity (%) (formular 2) and area under disease progressive curve (AUDPC) (formular 3) was calculated using equation 1, 2 and 3 respectively. Disease incidence was rated as the presence or absence of the disease (percentage of infected leaves on the plant) and severity was rated as the percentage of disease damage, and it was rated on the scale of 0 to 9 as used by Sania *et al.* (2015) & Dhaliwal *et al.* (2018) according to percentage of foliage with yellowing or necrosis in acropetal progression: 0 = 0%, 1 = 1%, 2 = 3%, 3 = 5%, 4 = 12%, 5 = 25%, 6 = 40%, 7 = 65%, 8 = 75% and 9 = more than 75% leaf area infected (IRRI, 2013). Fungal colonies were re-isolated from lesions of inoculated tissues to fulfil Koch's postulate.

$$\text{Disease Incidence (DI)} = \frac{\text{Number of Infected Plants}}{\text{Total Number of Plants}} \times 100 \quad \dots\dots (1)$$

$$\text{Disease Severity (DS)} = \frac{\sum (n \times v)}{Z \times N} \times 100 \quad \dots\dots\dots (2)$$

where, n = number of leaves in each disease category/score, v = numerical value of symptoms score, N = total number of leaves observed in each plant and Z = maximum numerical value of symptoms category.

Based on the disease severity scores, the AUDPC for each fungal species was calculated using the formulae as described by Wilcoxson *et al.* (1975) which has been widely used by many researchers (Bisht *et al.*, 2020; Nainwal *et al.*, 2020).

$$AUDPC = \sum_i^{n-1} \left(\frac{Y_i + Y_{i+1}}{2} \right) (t_{i+1} - t_i) \dots\dots\dots (3)$$

where, Y_i = disease severity at the i^{th} observation, n = the last disease assessment (number of assessments), t_i = time period of the assessment at the i^{th} observation.

Experimental Design, Treatments and Data Analysis

The experimental design used in assessing both the in-vitro and greenhouse pathogenicity was Completely Randomized Design with 3 replications each. Each isolate represented a treatment and data collected was subjected to Analysis of Variance (ANOVA), where significant means were separated using Tukey’s honestly significant difference at 1% probability using Genstat statistical software version 18.

3.4 Molecular Characterization of Rice Brown Leaf Spot Disease

Pathogens

3.4.1 DNA Extraction of Isolates

CTAB-based protocol developed by Murray & Thompson (1980), which has been used by many researchers (Santos *et al.*, 2018; El-Dawy *et al.*, 2021) was followed for the extraction of DNA of isolates. The extraction process was carried out by scraping the mycelium and reproductive structures formed in monocultures of the isolates previously identified by morphological analysis and cultivated in PDA for seven days in normal room temperature. With the help of a sterile scalpel, the mycelium was transferred into well labelled tubes, and the biomass was macerated in 900 µl of pre-warmed 2% CTAB extraction buffer at 65⁰C and mix then incubated at 65⁰C for 1 hour. Afterwards, it was centrifuged at 10000 rpm for 10 minutes and the upper phase (750µl)

was taken in a fresh tube. 2µl RNase (10mg/ml) was added and the set up was incubated at 37° C for 30 minutes. 750µl of chloroform was added and quick vortex, then centrifuged again at 10,000rpm for 10mins. 500µl of the upper phase is taken in 2 ml tube then 250µl of 7.5M NH₄OAc and 1 ml of ethanol (99%) added. It was then kept on ice for over an hour and centrifuged at 12,000rpm for 15mins. The liquid was poured out and another quick centrifuge done and all liquids was removed. It was then dried in laminar flow until ethanol is no more detected. Finally, pellets were then dissolved in 25-50 µl of TE buffer.

3.4.2 DNA Amplification by Polymerase Chain Reaction (PCR) and Sequencing Analysis

To amplify the DNA extracted from isolates, primers PN3 (forward: 5' CGTTGGTGAACCA GCGGAGGGATC 3') and PN16 (reverse: 5' TCCCTT TCAACAATTCACG 3') (Sunpapao *et al.*, 2014) was used for *Curvularia* isolates (isolate 17 to 25), primers ACT512 (forward: 5' ATGTGCAAGGCCGGTTTCGC 3') and ACT783 (reverse: 5' TACGAGTCCTTCTGGCCCAT 3') (Meghvansi *et al.*, 2013) was used for *Bipolaris* isolates (isolate 3 to 16) and this was because isolates were initially suspected to be of *Cercospora* genus due to their inability to produce conidia at the early stage of the study (Djeballi *et al.*, 2010), primers ITS1 (forward: 5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (reverse: 5' TCCTCCGCTTATTGATATGC 3') was used for *Pycilomyces* (isolate 8), primers ITS-Fu (forward (5'-CAACTCCCAAACCCCTGTGA-3')) and ITS-Fu-Rev (reverse: 5'-CGACGATTACCAGTAACGA-3') was used for *Fusarium* (isolate 9) and primers Fum-F (forward: 5'-CGACCTNTATCCTCCCAAT-3') and Fum-R (reverse: 5'-ACCCTCAGTGTAGTGACC-3') (Majeed *et al.*, 2016; Jeon *et al.*, 2015; Ferdinandez

et al., 2021) was also used for *Aspergillus* (isolate 26). The volume of amplification reactions included 25 µL of Taq DNA Polymerase 2x Master Mix, 5 µL of each 10 µM primer oligonucleotide, 10 µL of ultrapure water and 5 µL DNA of the samples. The PCR reactions were cycled as 95⁰C for 2 mins as initial cycle, followed by 94⁰C for 45 sec, followed by 55⁰C for 1 min and 72⁰C for 2 mins the repeated 40 times and a final extension of 72⁰C for 5 mins and then 4⁰C hold. The products were subjected to electrophoresis in agarose gel, and then the DNA was purified and subjected to the sequencing using Sanger sequencing method at the DNA sequencing laboratory, Functional Biosciences, USA using their standard protocols.

3.4.3 Data Analysis

After the sequences were obtained, they were alignment using the ClustalW in MEGA (version 11.0) software program, then, similar sequences were identified using the GenBank BLASTn tool (Altschui *et al.*, 1990). After alignment, these sequences were processed using the MEGA software program (version 11.0) (Hajigholizadeh *et al.* 2020) to elaborate a phylogenetic tree of the isolates, using the neighbour joining tree and 1000 Bootstrap replicates.

3.5 Evaluating the Efficacy of Epiphytes and Plant Extracts to Manage Brown Leaf Spot Disease

3.5.1 Preparation of Botanicals (*Nerium oleander* and *Azadirachta indica*)

The collected plant leaves were chopped after washing under running tap water three times to remove dust. The leaves were then surface disinfected with ethanol and again washed in water for 2-3 times. It was spread and left under laminar flow for a period

of three weeks to dry. The dried leaves of each plant species were made into powder separately using a sterilized mortar and pestle and then sieved with one millimeter sieve. The extracts were filtered through cheese cloth. Serial dilutions (1:10) were made from this concentrated extract by mixing equal amount of mixture of plant extract and water on weight basis was considered to be 100% (stock solution) from which further dilutions was made (Kumar & Simon, 2016; Chouhan *et al.*, 2022).

3.5.2 *In-Vitro* Evaluation of Epiphytes, Botanicals and Mancozeb on Brown leaf spot Pathogens

Poisoned food technique was used to determine the inhibitory effect of the *Nerium oleander* and *Azadirachta indica* on pathogens. Extracts and carbendazim were added to double PDA medium to make concentration 30% (v/v) (Chouhan *et al.*, 2022) and were mixed thoroughly before solidification and poured in Petri plates under aseptic condition in laminar air flow chamber. Petri plates were inoculated with equal discs (8 mm) of pathogen taken from the mother culture. To compare the growth inhibition, a control plate containing PDA medium was also kept along and inoculated with pathogen in the similar way. Mycelial growth was recorded daily in both the control plates and treatment plates at normal room temperature and per cent growth inhibition was calculated by using equation 4 which was also used by Chouhan *et al.* (2022).

P. lilacinum and *A. flavus* were tested against *Bipolaris oryzae* and *Curvularia lunata* in dual culture plates. The experiment followed the method of Tann & Soyong (2016). The three antagonists and a virulent isolate (*Bipolaris oryzae* and *Curvularia lunata*) were cultured on potato dextrose agar (PDA), and incubated at the room temperature. The edge of actively growing colony was cut into 8 mm diameter by the sterilized cork borer and one agar plug of each fungus was transferred to the opposite sides on PDA

plates of 9 cm diameter and separately cultured each antagonist and pathogen served as controls, then incubated at room temperature for 9 days. Data were collected as colony diameter (cm) and number spore production, which were counted using a Haemocytometer under a compound microscope. The analysis of variance (ANOVA) was computed, and treatment means were compared using Tukey's Honestly Significant Difference at $\alpha = 0.01$.

Treatments

- T1 Mancozeb
- T2 *A. flavus*
- T3 *P. lilacinum*
- T4 *Nerium oleander*
- T5 *Azadirachta indica*
- T6 Control

$$\text{Growth inhibition (\%)} = \frac{(\text{Growth in control} - \text{Growth in treatment}) \times 100\%}{\text{Growth in control}} \dots \text{eq.4}$$

3.5.3 Screenhouse Epiphytes, Botanicals and Mancozeb on Brown

Leaf Spot Pathogens

Rice seedlings were planted following similar steps for pathogenicity test. Inoculation was done with 20 g/L of water spore suspension of the epiphytes at a concentration of 1×10^6 spores per ml, chemical fungicide (Mancozeb) at 10 ml/L of water and Phytoextracts (*Nerium oleander* and *Azadirachta indica*) at 20 ml/L of water were used. For the Screenhouse pathogenicity, inoculation was done in three (3) different ways (biocontrol agent inoculated before pathogen in 5 days interval, both biocontrol agent and pathogen inoculated at the same time and pathogen inoculated before biocontrol

agent in 5 days interval). Data were collected in all the three different folds of the experiment, data taken included incubation period, disease incidence and severity, formulars used were the same as used for pathogenicity test. The set-up was laid in completely randomized design with three replications.

Chapter Four: Results

4.1 Symptoms Description of Brown leaf spot Disease of Rice in

Study Area

It was observed that the disease usually appears during both the major and minor cropping seasons as small, brown leaf spots which formed predominately on lower (older) and middle leaves of both seedlings and matured rice plants. Mostly, small, yellow brown, necrotic spots which have varying shapes appears on leaf surface first and later, these spots change to light to dark brown and often surrounded by yellow haloes (Plate 1). The nearby spots fuse and grow together as one large spot with chlorotic margins as the disease progresses with no effective control measure implemented, and will appear as numerous spots of various shapes and prominent haloes which sometimes seen as blight on whole leaf lamina in due course of time (Plate 1).

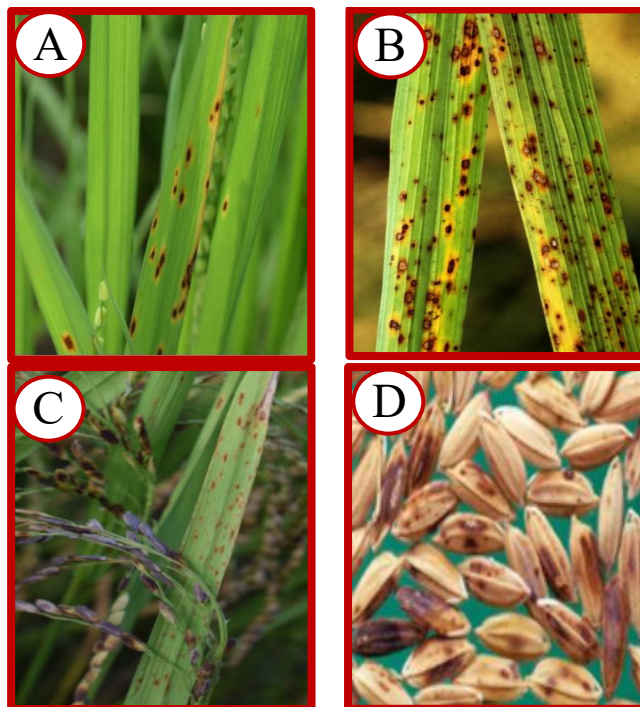


Plate 7: Symptoms of Brown leaf spot Disease of Rice (A: Early symptom, B & C: Severe Symptoms and D: Symptom on Rice Seeds) sourced: RRDII (2024)

4.2 Frequency of Occurrence of *Bipolaris* and *Curvularia* Isolates

Out of the fifteen (15) samples collected and successfully processed, eleven (11) isolates of *Bipolaris* spp., ten (10) isolates of *Curvularia* spp. and two (2) epiphytes (*Purpureocillium lilacinum* and *Aspergillus flavus*) were isolated from different rice fields in Atwima Nwabiagya District (Table 2). The maximum number of *Bipolaris* isolates (2 isolates each) were isolated from diseased leaf samples obtained at Adankwame, Asakraka, Mfensi and Nkontomire, while only 1 isolate each was obtained from Besease, Kokoben, Nineteen and Taabere (Table 2). No isolate of *Bipolaris* was obtained from Akropong and Ntensere. Maximum numbers of *Curvularia* isolates (2 isolates each) were collected from Adankwame and Mfensi, and one isolate each was obtained from Akropong, Kokoben, Nineteen, Ntensere and Taabere. No *Curvularia* isolate was collected from Asakraka, Besease and Nkontomire (Table 2). The *A. flavus* and *P. lilacinum* used as biocontrol agents were collected from Asakraka and Nkontomire respectively (Table 2).

4.3 Morphological Characterisation of *Curvularia* and *Bipolaris*

Isolates

Curvularia spp. and *Bipolaris* spp were successfully isolated from diseased rice leaves. Morphologically, the different species were identified using both macroscopic and microscopic features were observed on twenty-one (21) day old cultures. Eleven of the isolates (KK-A, TA-A, AS-A, BE-A, MM-A, MF-A, AD-A, AA-A, NN-A, NK-A, NK-A, NK-B, AK-A and KK-B) were identified as *Bipolaris*, while 10 isolates (AK-A, KK-B, TA-B, NT-A, MM-B, MF-D, AA-B, AD-B, NN-B and NK-C) were classified as *Curvularia* spp.





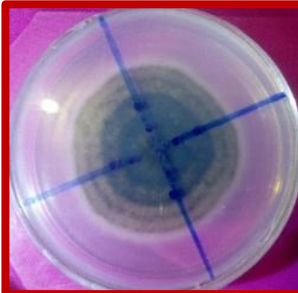


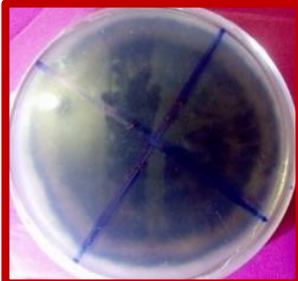

Table 2: Isolates, Codes and Locations

Isolate No.	Genus	Code	Location/Town
	<i>Purpureocillium</i>	NK-D	Nkontomire
	<i>Aspergillus</i>	AS-B	Asakraka
3	<i>Bipolaris</i>	KK-A	Kokobeng
4	<i>Bipolaris</i>	TA-A	Taabre
5	<i>Bipolaris</i>	AS-A	Asakraka
6	<i>Bipolaris</i>	BE-A	Besease
7	<i>Bipolaris</i>	MM-A	Mfensi
10	<i>Bipolaris</i>	MF-A	Mfensi
12	<i>Bipolaris</i>	AD-A	Adankwame
13	<i>Bipolaris</i>	AA-A	Adankwame
14	<i>Bipolaris</i>	NN-A	Nineteen
15	<i>Bipolaris</i>	NK-A	Nkontomire
16	<i>Bipolaris</i>	NK-B	Nkontomire
11	<i>Curvularia</i>	AK-A	Akropong
17	<i>Curvularia</i>	KK-B	kokobeng
18	<i>Curvularia</i>	TA-B	Taabre
19	<i>Curvularia</i>	NT-A	Ntensere
20	<i>Curvularia</i>	MM-B	Mfensi
21	<i>Curvularia</i>	MF-D	Mfensi
22	<i>Curvularia</i>	AA-B	Adankwame
23	<i>Curvularia</i>	AD-D	Adankwame
24	<i>Curvularia</i>	NN-B	Nineteen
25	<i>Curvularia</i>	NK-C	Nkontomire

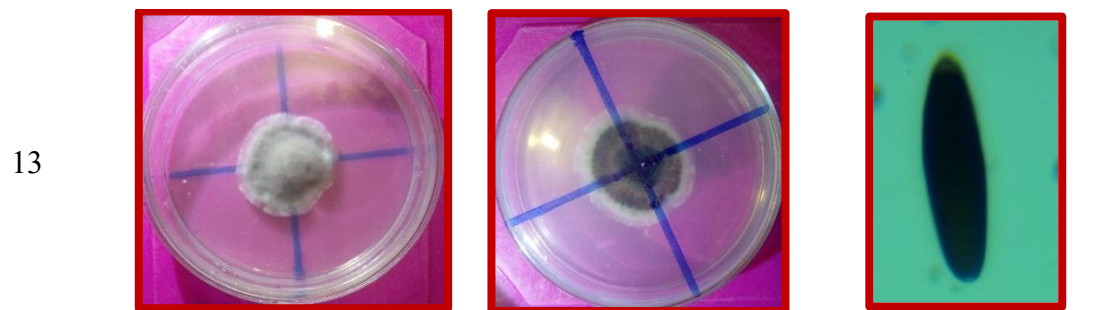
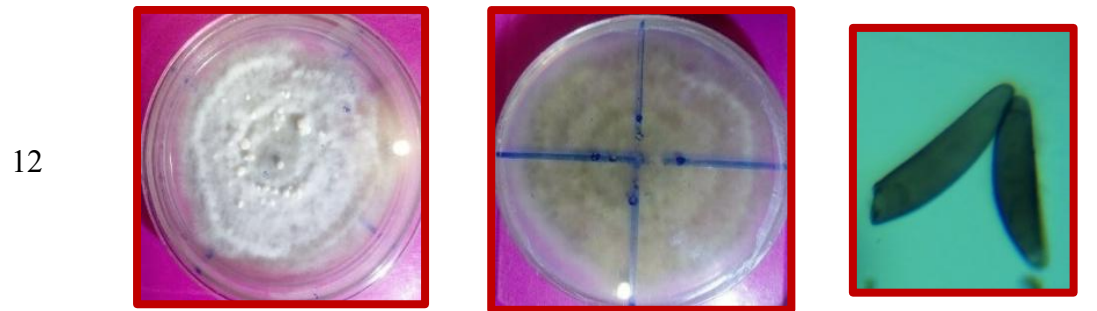
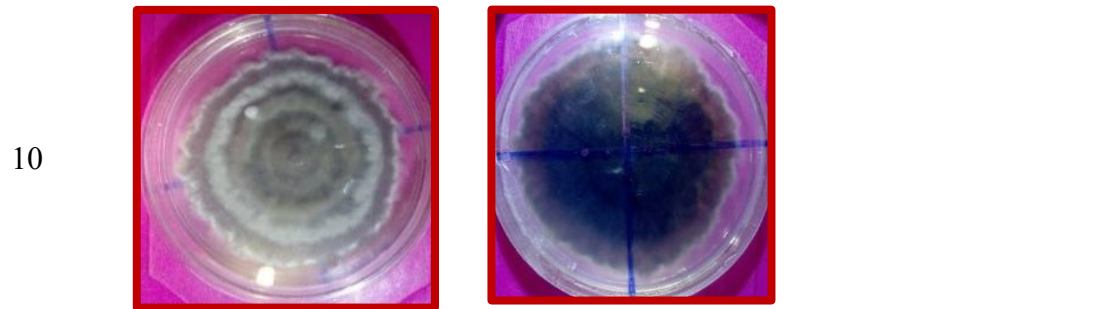
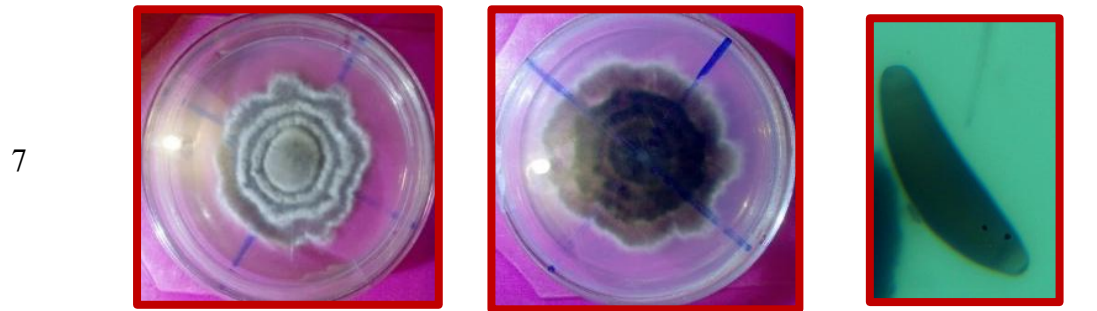
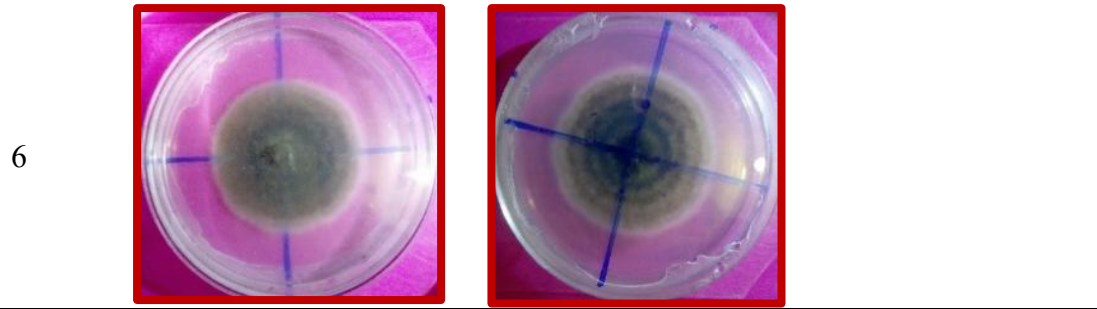
4.3.1 Cultural and Conidia Characteristics of *Curvularia* and *Bipolaris* Isolates

Variations existed among both *Bipolaris* and *Curvularia* isolates identified. With respect to the *Bipolaris* isolates, isolates exhibited convex, raised or umbonate colony elevation (Plate 8). Isolates 3 to 12, 14 and 15 of the isolates showed convex elevation, while 13 and 16 produced raised or umbonate elevations. Variations existed in colony

margin types of all *Bipolaris* isolates. Two margin types were conspicuously produced by the isolates. as filiform and some maintained or turned to undulate. Colony of all isolates were not transparent except 15 and 16 when held closely against the eye. The surface colour of most isolates was light brown and dark reverse with filamentous shape and a few being irregular. Isolate 6 and 10 had no conidia after over three months of incubation. Most conidia were fusiform in shape and had up to nine septa, sizes ranged from 52-74 μm . *Bipolaris* isolates had poor sporulation rate, the average number of conidia per teasing was 9 or below.

Isol.	Colony		Conidium
	Surface View	Reverse View	
3			
4			
5			

Continuation of Plate 8



Continuation of Plate 8

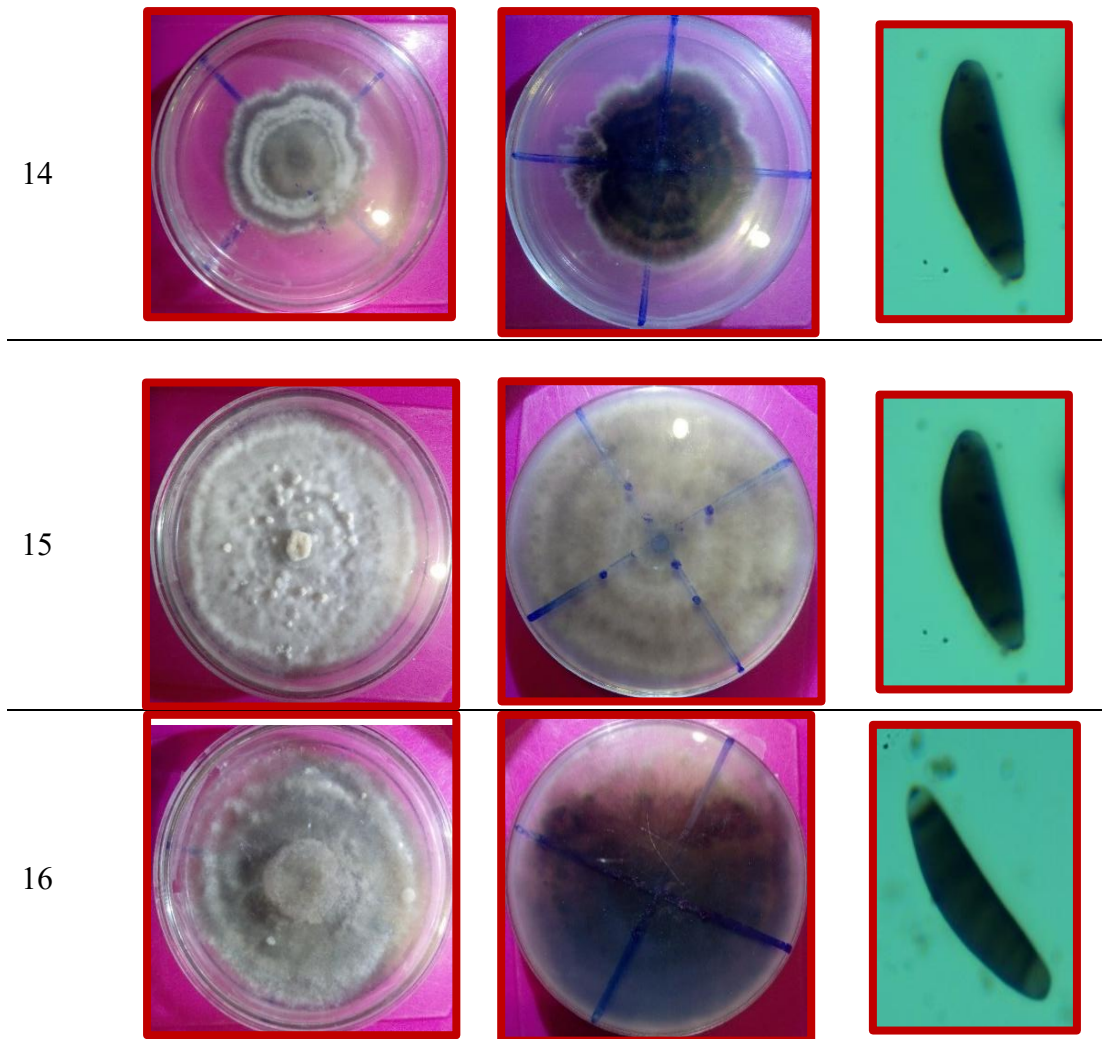



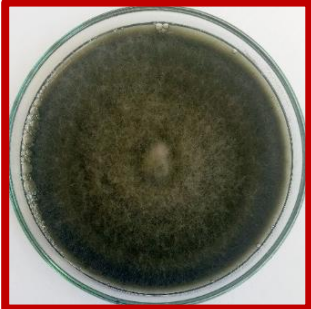


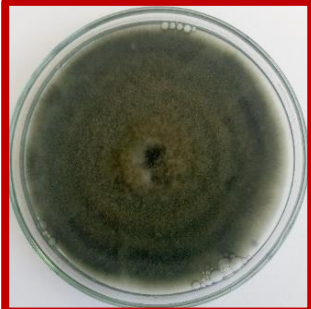
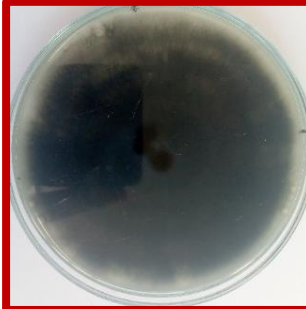

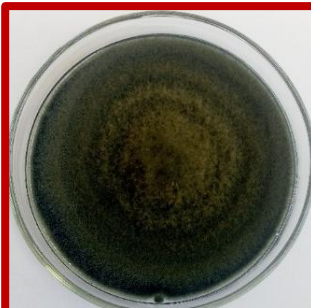
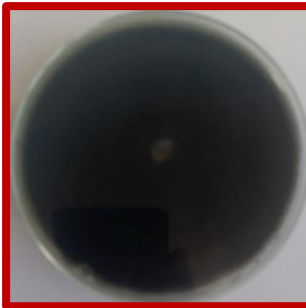



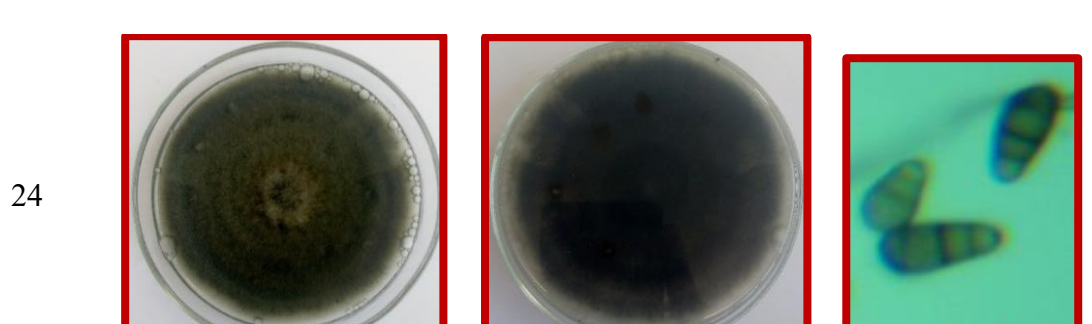
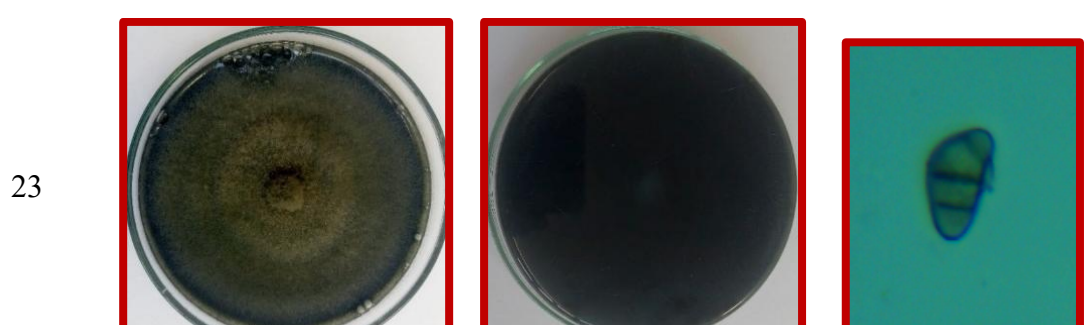
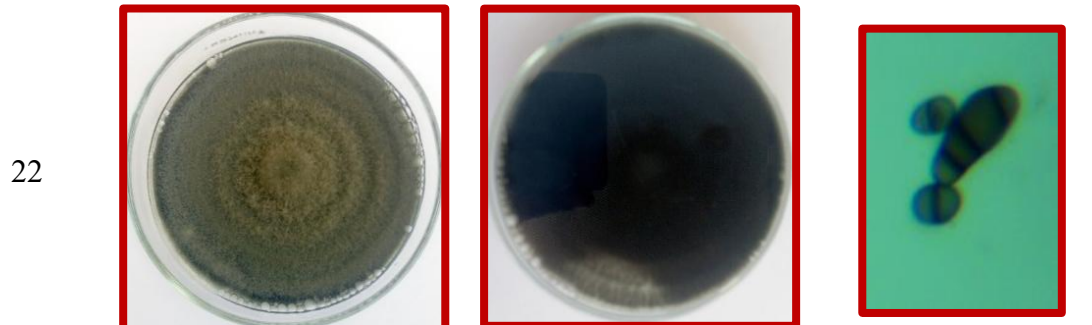
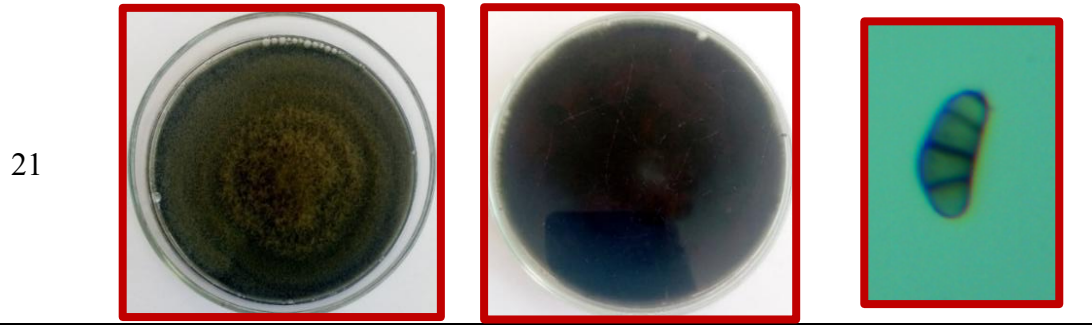
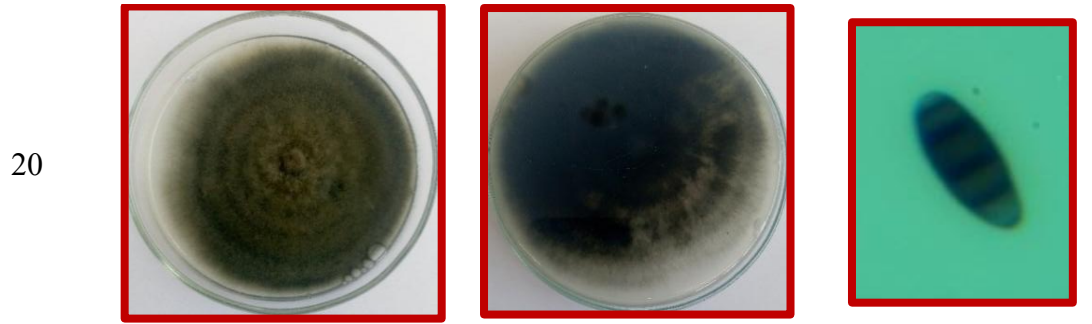
Plate 8: Colony and Conidia of *Bipolaris* isolates

Isoalate 11, and isolates 17 to isolate 25 were of *Curvularia* genus based on their cultural and conidia characteristics observed (Plate 9). When isolates were maintained on PDA, they developed colonies that varied in elevation from umbonate to flat with a few being raised. Most of the isolates, especially isolates 11 and 17 started with filiform margin type and changed to entire or undulate. Surface of some colonies (isolate 20, isolate 21 and isolates 22) were brown at early stage and that of the others were greyish brown, and the reverse side were dark or greenish dark. Most colonies were transparent when held closely against the eye except isolate 25 and isolate 18. Colony shape of the isolates at the initial stage were rhizoid-like, almost all had turned filamentous in shape. Conidia shape of most isolates belonging to *Curvularia* were fusiform and few were

straight in nature, with size ranging from 18-20 μm and number of septa ranged from 2-3. It had moderate to good sporulation rate.

Isol.	Colony		Conidium
	Surface View	Reverse View	
11			
17			
18			
19			

Continuation of Plate 9



25

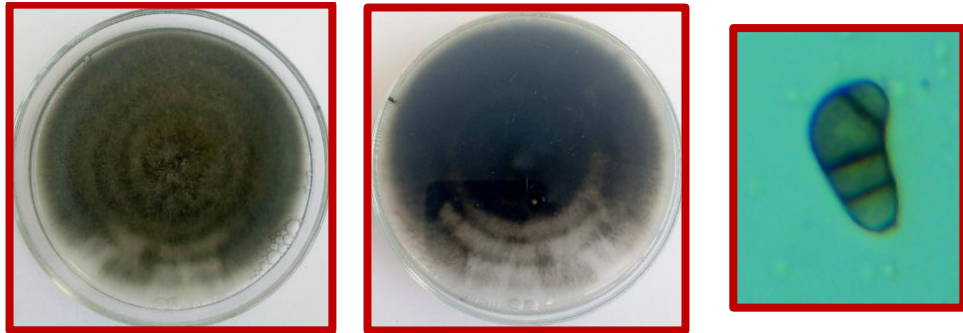


Plate 9: Colony and Conidia of *Curvularia* Isolates

4.3.2 Number of Growth Rings or Zones of Isolates

After the analysis, there were statistically significant difference in the number of growth rings form on the colony of isolates that were categorized as *Bipolaris* at 72, 120 and 168 HAI. At 72 HAI, isolate 2 and isolate 10 recorded the highest number of growth rings, which was at least 2 rings, followed by isolate 3, isolate 4, isolate 6, isolate 7, isolate 12 and isolate 15, which had at least 1 ring formed. Three (3) isolates (isolate 13, isolate 14 and isolate 16) had no rings on its colony at 72 HAI (Table 3).

At 120 HAI, all isolates that were characterized as *Bipolaris* had rings on its colony surface of which few were transparent and could be observed from the reverse side. Isolate 3, isolate 5, isolate 10 and isolate 14 had two (2) or more rings, while isolate 13, isolate 14 and isolate 16, which had no rings at 72 HAI now had at least one (1) ring each. At 168 HAI, some isolates (isolate 3, isolate 10 and isolate 14) had as more as four (4) or more rings and three (3) isolates (isolate 4, isolate 6 and isolate 13) still had as few as one (1) ring and the rest had less than four, but more than one ring (Table 3).

The number of growth rings of *Curvularia* isolates are shown in table 4.2. After the analysis, there were no significant difference in growth rings among the isolates at 72 HAI and at 120 HAI.

There was significant difference in number of rings among the isolates at 168 HAI, although isolate 19 and isolate 25 had the highest number of rings as four. Six isolates (isolate 17, isolate 18, isolate 20, isolate 21, isolate 22, isolate 23 and isolate 24) had at least three (3) rings at 168 HAI, while the fewest rings were recorded on isolate 11 as two (2) rings.

Table 3: Number of Growth Rings of *Bipolaris* and *Curvularia* Isolates

<i>Bipolaris</i> Isolate	72 HAI	120 HAI	168 HAI	<i>Curvularia</i> Isolate	72 HAI	120 HAI	168 HAI
3	1.3	2.3	4.7	11	0.0	1.0	1.3
4	1.0	1.3	1.3	17	0.7	1.3	2.7
5	2.0	2.7	3.3	18	1.0	1.3	3.3
6	0.7	1.3	1.3	19	0.7	1.7	3.7
7	1.0	1.7	3.3	20	0.3	1.7	3.3
10	1.7	2.0	3.7	21	1.0	1.7	3.3
12	0.7	1.7	2.3	22	0.7	2.3	3.3
13	0.0	0.7	1.0	23	1.0	1.7	3.0
14	0.0	2.7	4.0	24	0.7	1.3	3.0
15	0.7	1.3	2.0	25	1.0	2.0	4.0
16	0.0	1.0	2.3				
HSD (0.01)	1.4	2.4	2.0	HSD (0.01)	1.4	2.1	2.3
CV (%)	47.6	39.7	21.7	CV (%)	58.3	37.9	21.2

HSD= Tukey's honestly significant difference, CV= coefficient of variation, HAI= Hours after incubation

4.3.3 Variations in Colony Diameter of *Bipolaris* and *Curvularia*

Isolates

At 24 HAI, all isolates had more than 1 cm diameter growth. The highest growth was recorded on isolate 6 (1.83 cm), followed by isolate 5, which also recorded 1.73 cm, three isolates (isolate 4, isolate 15 and isolate 16) had between 1.50-1.55 cm, three

isolates (isolate 10, isolate 12 and isolate 13) again recorded between 1.42-1.47 cm while isolate 3 and isolate 14 recorded 1.32 cm and 1.35 cm, respectively. Isolate 7 recorded the smallest growth (1.28 cm) (Figure 4)

At 96 HAI, all isolates except isolate 13 had 5 cm colony diameter growth and the highest was on isolate 5 (8.00 cm), which was followed by isolates 16 and isolate 15, which had 7.70 cm and 7.50 cm growth, respectively. Three isolates (isolate 3, isolate 10 and isolate 12) recorded between 6.55 cm and 6.83 cm growth, while three (isolates 14, 6 and 4) also recorded between 5.00 cm and 5.50 cm and then isolate 13 had 2.77 cm as the smallest growth (Figure 4).

At 144 HAI, no isolate with the exception of isolate 13 had below 6.50 cm colony diameter growth. While isolate 13 recorded 3.82 growth as the smallest, four isolates (isolates 3, 5, 15 and 16) had full growth (9 cm). Isolates 10 and 12 recorded 8.92 cm and 8.98 cm growth, respectively. Isolates 6 and 7 also had 6.70 cm and 6.63 cm growth, respectively and then isolates 4 and 14 recorded 7.58 cm and 7.63 cm, respectively (Figure 4).

The colony diameter of *Curvularia* isolates was measured every 24 hrs until at least one of the isolates attained full growth (9 cm). Data were analysed and presented in Figure 5. There were high significant differences in the colony diameter of the *Curvularia* isolates at 24 to 144 HAI (Figure 5). At 24 HAI, there were at least more than 0.60 cm diameter growth on all the isolates. Four isolates had less than 1.00 cm growth although (isolate 18 had 0.78 cm, isolate 18 had 0.93 cm, isolate 22 also had 0.82 cm and isolate 24 had 0.65 as the smallest growth). Isolates 17, 20, 21, 23 and 25 recorded 1.08 cm, 1.17 cm, 1.25 cm, 1.20 cm and 1.00 cm, respectively, while isolate 11 had the largest growth of 1.48 cm (Figure 5).

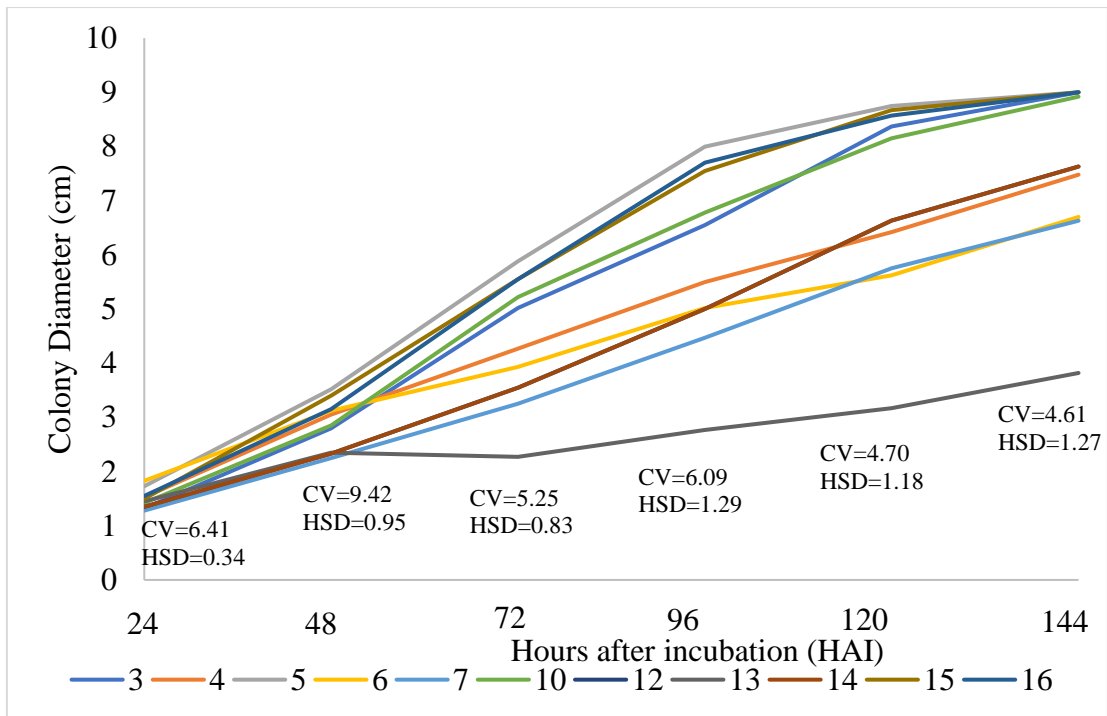


Figure 4: Colony Diameter of *Bipolaris* Isolates

At 96 HAI, all *Curvularia* isolates had more than 4.90 cm colony diameter growth, the largest growth was recorded on isolate 20 as 6.35 cm. Isolate 23 recorded the next largest of 5.90 cm followed by isolate 19 (5.87 cm, 21 (5.78 cm), but 17 and 25 which recorded equal growth of 5.75 cm with the smallest growth recorded by on isolate 22 (4.95 cm).

All *Curvularia* isolates had more than 8.00 cm colony diameter growth at 144 HAI except isolate 11. Isolate 11 lagged and grew slowly after 96 HAI, only 0.88 cm growth was achieved by it between 96 HAI and 120 HAI, and only 0.67 cm was achieved between 120 HAI and 144 HAI, meanwhile it achieved more than 1.00 cm growth between 24 HAI and 48 HAI and again between 48 HAI and 72 HAI. Regardless of the fast growth by the other isolates apart from isolate 11, only isolate 20 had full growth (9.00 cm) (Figure 5).

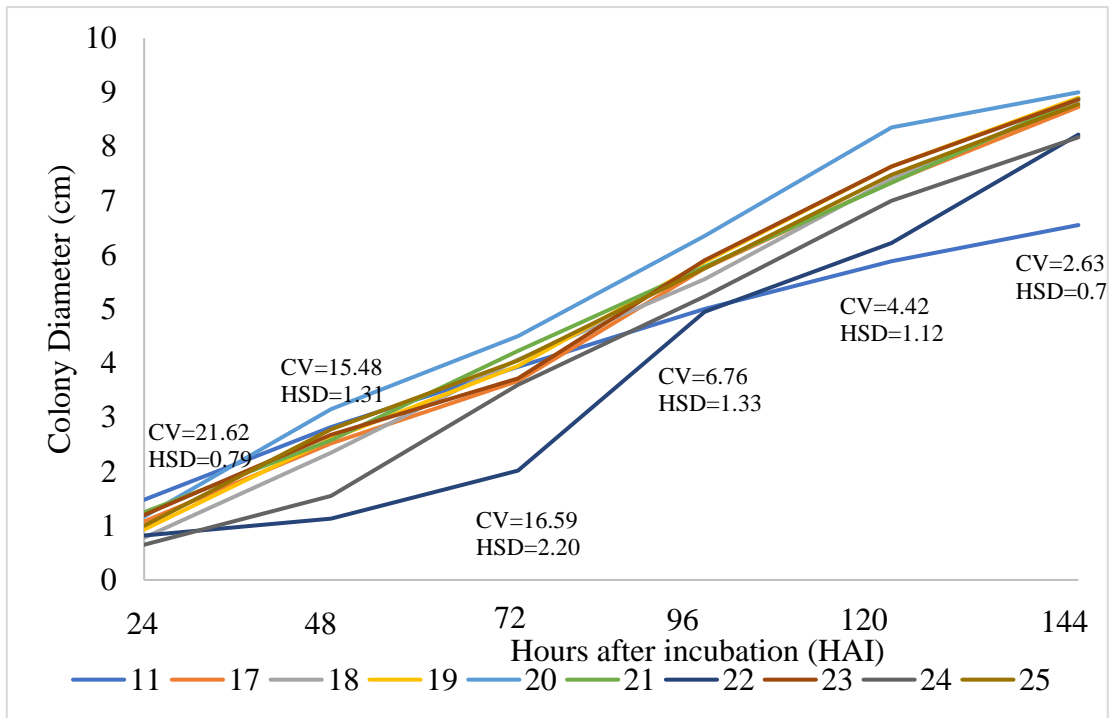


Figure 5: Colony Diameter of Curvularia Isolates

4.3.4 Conidia Morphology of *Bipolaris* and *Curvularia* Isolates

Isolates that were characterized as *Bipolaris* had a very poor sporulation rate; conidia were produced after 3 months of incubation. For isolate 6 and isolate 10, no conidia were observed at all during the study period. All conidia were tapered at both ends and straight or curved (Plate 8) (Table 4). Size of conidia ranged from 50.00 to 75.00 μm (Table 4) in isolates 3 to 7, 10, 12 and 16.

There were conidia in all the isolates grouped as *Curvularia* with varying sporulation rates. Only isolate 19 had poor sporulation rate (less than 9 conidia per observation). Isolates 11, 17, 20 and 23 had moderate sporulation rates (10-49 conidia per observation) and the rest of the isolates had good sporulation rates (more than 50 conidia per observation). Some conidia were tapered at both end or at one end and oval or straight. Conidia had 3 to 4 septa with an average size of 17.50-20.07 μm (Table 5) (Plate 9).

Table 4: Conidia Features of *Bipolaris* Isolates

Isolate	Sporulation Rate	Size of Conidia (µm)	Shape of Conidia	Number of Septa
3	Poor	56.08	Fusiform	6
4	Poor	53.34	Fusiform	7
5	Poor	54.46	Fusiform	9
6	<i>No Spore</i>	-	-	-
7	Poor	54.92	Fusiform	9
10	<i>No spore</i>	-	-	-
12	Poor	54.56	Fusiform	10
13	Poor	36.03	Fusiform	10
14	Poor	59.82	Fusiform	8
15	Poor	53.27	Fusiform	9
16	Poor	72.23	Fusiform	7

Table 5: Conidia Features of *Curvularia* Isolates

Isolate	Sporulation Rate	Size of Conidia (µm)	Shape of Conidia	Number of Septa
11	Moderate	20.07	Fusiform	2
17	Moderate	19.77	Fusiform	3
18	Good	17.56	Fusiform	2
19	Moderate	18.49	Fusiform	3
20	Moderate	17.99	Fusiform	2
21	Moderate	18.63	Fusiform	3
22	Moderate	18.64	Fusiform	3
23	Moderate	19.25	Fusiform	3
24	Good	17.82	Fusiform	3
25	Good	17.99	Fusiform	3

4.4 Pathogenicity of Isolates

4.4.1 Variations in Pathogenicity of *Bipolaris* and *Curvularia* Isolates on Detached Rice Leaves (In-vitro)

All the isolates evaluated were found to be pathogenic, producing brown leaf disease symptoms (Plate 10). For *Bipolaris* isolates, disease symptoms on detached; leaves were observed on 2-4 days of inoculation, the yellowing (chlorosis) started and developed into lesions then extending from the place of inoculation and along the edges and affecting even midribs of the rice leaves. Brownish spots appeared on both the abaxial and adaxial side of the leaf (Plate 10). The effect of all the eleven (11) *Bipolaris* and ten (10) *Curvularia* isolates ultimately caused necrosis and led to the rotting of leaves in a period of 6 days of which the leave treated as control had no lesion. Fine yellow patches were seen on the inoculated leaves and whole leaves subsequently dried up (Table 7) (Plate 10).

Table 6: Lesion Size (cm) Caused by Isolates

Isolate	48 HAI	72 HAI	96 HAI	120 HAI
<i>Bipolaris</i> Isolates				
3	0.60	0.93	2.03	4.17
4	0.50	1.57	1.83	2.10
5	0.93	1.37	2.93	3.30
6	0.17	0.43	0.57	1.60
7	0.60	1.23	1.53	1.97
10	1.27	1.90	3.27	3.83
12	0.33	0.60	0.67	1.13
13	0.70	1.27	1.53	1.73
14	0.57	1.37	1.83	2.50
15	1.27	2.40	3.20	4.40
16	1.10	1.73	2.00	2.43
<i>Curvularia</i> Isolates				
11	0.50	0.67	0.90	1.27
17	0.93	3.10	4.37	5.10
18	1.40	2.67	5.20	5.47
19	1.30	1.53	3.43	4.07
20	0.17	3.37	4.10	4.37
21	0.63	2.17	4.83	5.13
22	0.93	1.17	1.53	1.93
23	0.33	1.23	2.73	3.13
24	0.57	1.20	2.20	4.37
25	0.37	1.47	1.80	2.73
HSD (0.01)	2.21	1.00	1.84	2.04
CV (%)	19.47	38.85	32.39	22.84

HSD= Tukey's honestly significant difference, CV= coefficient of variation,
HAI= Hours after incubation


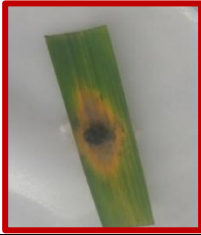















	HAI	<i>Bipolaris zeicola</i>	<i>Bipolaris aryzae</i>	Control Treatment	
96					
	HAI	<i>Curvularia clavata</i>	<i>Curvularia soli</i>	<i>Curvularia senegalensis</i>	Control Treatment
96					
	HAI	<i>Curvularia clavata</i>	<i>Curvularia soli</i>	<i>Curvularia senegalensis</i>	Control Treatment
96					
	HAI	<i>Curvularia geniculata</i>	<i>Curvularia lunata</i>	<i>Curvularia warraberensis</i>	Control Treatment
96					
	HAI	<i>Curvularia platzii</i>	Control Treatment		
96					

Plate 10: *In-Vitro* Pathogenicity of *Curvularia* and *Bipolaris* spp.

4.4.2 Screenhouse Pathogenicity on Rice Plants

The screenhouse (in-vivo) pathogenicity experiment was performed using all isolates of *Bipolaris* sp. and *Curvularia* sp. Symptoms of BLSD (Plate 11) were observed on rice leaves from 5 DAI to 20 DAI.

Incubation Period of *Bipolaris* and *Curvularia* Isolates

Generally, isolates of *Curvularia* produced BLSD symptoms earlier than isolates of *Bipolaris* spp.

Incubation period for all the isolates was determined. After the analysis, there was a significant difference among the treatments. A statistically significant difference existed between isolate 17 (*Curvularia soli*) and all other isolates except isolate 18 (*Curvularia lunata*), isolate 24 (*Curvularia subpapendorfii*) and isolate 25 (*Curvularia geniculata*). There was no statistically significant difference among the *Bipolaris* isolates and between the *Bipolaris* isolates and *Curvularia* isolates except with isolate 17 (Table 8). Although isolate 17 recorded the earliest symptom (5 DAI), most of the isolates took up to 10 DAI in producing visible symptoms.

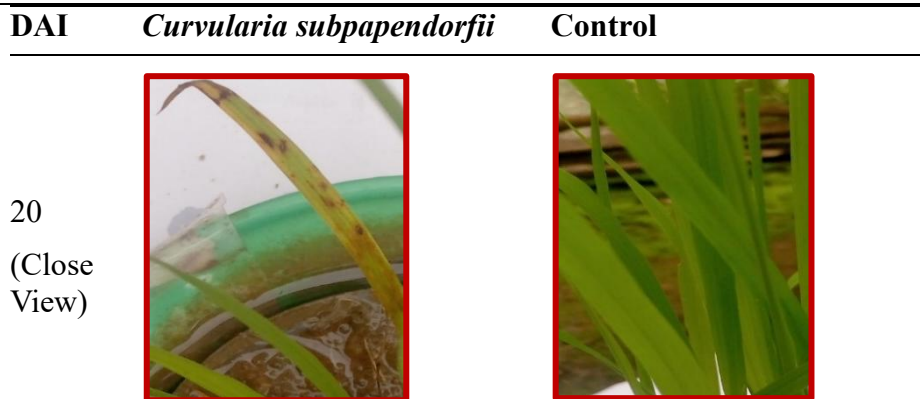
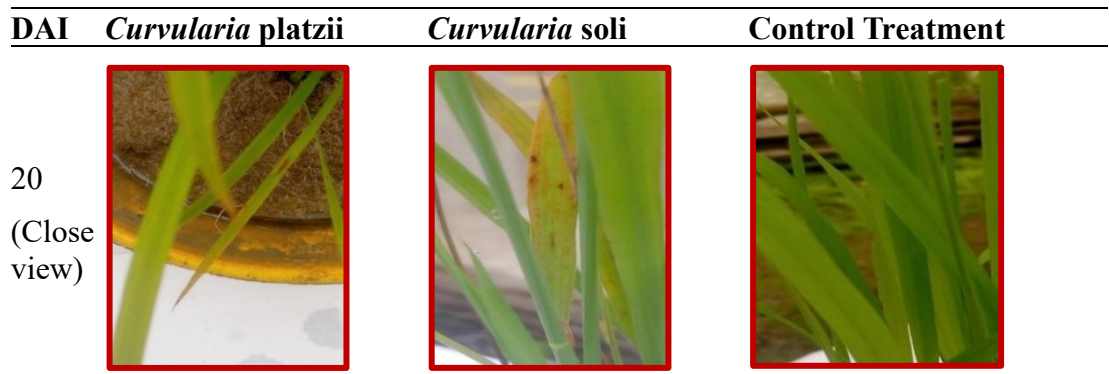
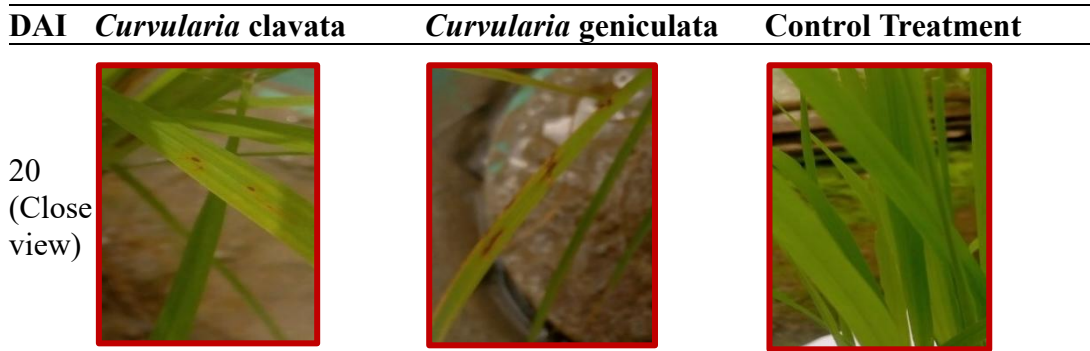
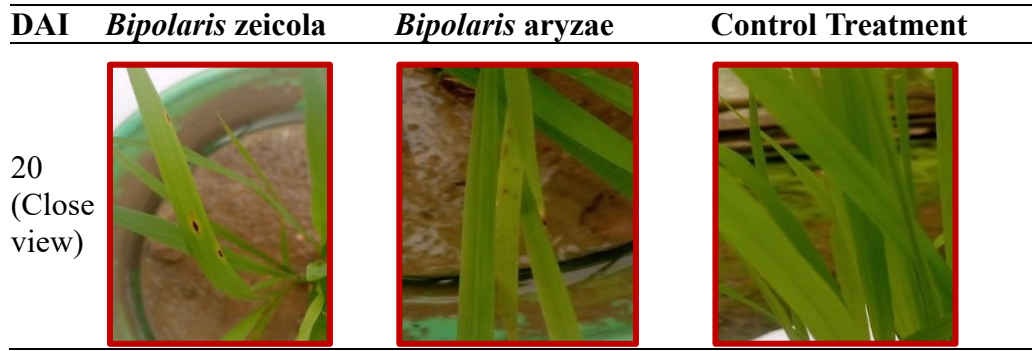


Plate 11: Screenhouse Pathogenicity of *Curvularia* and *Bipolaris* spp.

Table 7: Incubation Period (Days) of All the Isolates

Isolate	Incubation Period (DAI)	Isolate	Incubation Period (DAI)
<i>Curvularia Isolates</i>		<i>Bipolaris Isolates</i>	
11	9.7	3	9.7
17	5.0	4	10.0
18	8.3	5	10.0
19	10.0	6	11.3
20	10.0	7	10.0
21	10.0	10	10.0
22	10.0	12	10.0
23	10.0	13	10.0
24	7.3	14	11.3
25	7.3	15	9.7
		16	10.0
HSD (0.01)	4.42	HSD (0.01)	4.32
CV	12.7	CV	12.7

CV= coefficient of variation, HSD (0.01) = Tukey's honestly significant difference at 1% probability, DAI= Days after inoculation

Incidence of Brown leaf spot Caused by Isolates

The disease incidence produced by all isolates is shown in Table 8. There was no statistically significant difference ($p < 0.56$) among the isolates in disease incidence at 10 DAI to 25 DAI, not even between *Bipolaris* and *Curvularia* isolates (Table 8).

Severity of Brown leaf spot Caused by Isolates

There was no statistically significant difference among isolates in disease severity at 10 DAI and 25 DAI, but there was a significant difference among the *Curvularia* isolates at 25 DAI. Statistically significant difference was obtained among the isolates at 15 DAI and 20 DAI. Considering only *Bipolaris* isolates, no significant difference in disease severity were obtained throughout (10).

Table 8: Disease Incidence (%) of All Isolates

Isolate	10 DAI	15 DAI	20 DAI	25 DAI
<i>Curvularia Isolates</i>				
11	26.07	37.14	38.86	48.93
17	23.36	36.93	38.86	50.85
18	20.12	27.63	35.84	49.94
19	18.43	21.14	28.78	41.07
20	17.75	27.36	36.27	51.19
21	15.10	17.81	26.12	38.74
22	18.43	23.86	37.22	48.93
23	21.14	28.78	37.14	52.86
24	23.86	33.00	45.00	50.85
25	12.29	21.14	36.93	52.86
<i>Bipolaris Isolates</i>				
3	12.29	23.86	34.93	36.85
4	15.00	31.00	45.00	48.93
5	21.14	31.00	34.93	46.92
6	23.86	35.22	43.08	59.21
7	28.78	37.22	43.08	52.78
10	15.00	22.14	31.00	45.00
12	17.71	26.07	35.22	46.92
13	15.00	21.14	36.93	45.00
14	26.07	34.93	39.06	55.07
15	15.00	29.89	31.92	45.08
16	21.14	28.28	39.15	48.93
HSD (0.01)	30.50	28.34	28.34	29.97
CV	44.00	24.93	21.48	17.32

CV= Coefficient of variation, *HSD (0.01)* = Tukey's honestly significant difference at 1% probability, *DAI*= Days after Inoculation

At 15 DAI, the highest severity was recorded on plants inoculated with isolate 23, which is among the *Curvularia* and the lowest was also recorded on isolate 10, which is among the *Bipolaris* isolates. All *Curvularia* isolates recorded higher severity value more than the most virulent *Bipolaris* isolate (isolate 6) (10).

At 20 DAI, the trend of disease severity was similar to what was observed at 15 DAI. All *Curvularia* isolates recorded higher severity than the most pathogenic among the *Bipolaris* isolates and the highest was recorded on isolate 22 and the lowest was again recorded on isolate 10 (Table 9).

Table 9: Disease Severity (%) of All Isolates

Isolate	10 DAI	15 DAI	20 DAI	25 DAI
<i>Curvularia Isolates</i>				
11	19.18	48.15	46.99	52.04
17	40.00	65.00	71.14	83.51
18	33.43	56.02	64.79	87.60
19	30.59	35.01	52.41	62.59
20	34.21	56.31	64.45	87.82
21	32.37	32.05	50.15	64.95
22	30.00	60.78	71.83	75.52
23	34.93	69.38	67.04	90.00
24	25.00	66.92	63.11	71.78
25	30.00	41.75	50.00	54.31
<i>Bipolaris Isolate</i>				
3	21.68	29.10	44.04	71.99
4	13.22	17.67	36.05	71.59
5	23.31	25.23	39.47	69.43
6	19.34	31.62	46.74	63.45
7	18.27	28.89	46.16	44.87
10	0.00	12.95	31.45	70.17
12	17.21	26.69	40.81	66.82
13	18.74	23.05	37.65	63.61
14	24.93	28.81	40.56	85.17
15	13.62	15.71	39.93	64.06
16	25.13	29.51	44.39	60.81
HSD (0.01)	41.69	40.64	29.54	51.97
CV	48.48	29.83	16.55	20.89

CV= Coefficient of variation, HSD (0.01) = Tukey's honestly significant difference at 1% probability, DAI= Days after Inoculation

Area Under Disease Progressive Curve (AUDPC)

There was significant difference among the isolates in Total Area under Disease Progressive Curve (AUDPC) (Table 11). There was no significant difference among only *Bipolaris* isolates. The trend was similar to the severity results, *Curvularia* isolates had higher AUDPC values than the highest among the *Bipolaris* isolates. Isolate 18 recorded the largest AUDPC followed by isolate 23, followed by isolate 17 and the lowest among the *Curvularia* isolates was recorded on isolate 25, followed by the highest among the *Bipolaris* isolates (isolate 3).

Table 10: AUDPC for All the Isolates

Isolate	Total AUDPC (Severity)	Isolate	Total AUDPC (Severity)
<i>Curvularia Isolates</i>		<i>Bipolaris Isolates</i>	
11	853.37	3	889.96
17	1376.10	4	749.73
18	1437.20	5	780.18
19	957.56	6	874.29
20	1229.40	7	760.68
21	1001.50	10	683.78
22	1295.20	12	816.71
23	1387.00	13	762.50
24	1229.40	14	936.44
25	930.31	15	930.31
		16	847.44
HSD (0.01)	574.40	HSD (0.01)	574.40
CV	16.39	CV	16.39

CV= Coefficient of variation, HSD (0.01) = Tukey's honestly significant difference at 1% probability, AUDPC= Area Under Disease Progressive Curve

4.5 Molecular Identification

4.5.1 PCR Amplification of Isolates

All the isolates produced were successfully amplified by the different primers evaluated. Nine *Bipolaris* isolates were amplified with primer ACT512 and ACT783 (250 bp). With respect to *Curvularia*, all isolates that were amplified by PN3 and PN16 resulted in amplicon of different bp in sizes (900 to 3000 bp) but isolate 14 and isolate 16 did not amplify (Figure 6). Four (4) *Curvularia* isolates that were not amplified by the respective primers were successfully amplified using the ITS1 and ITS4 primers (500 bp).

Molecularly, ten isolates (3,4,5,6,7,10,12,15) of fungal pathogen morphologically identified as *Bipolaris* spp were confirmed to be *Bipolaris zeicola* with only two (isolate 14 &16) identified as *B. oryzae* (Table 6). The ten (10) isolates morphologically identified as *Curvularia* spp were also genetically confirmed to belong to different species. Three isolates (18, 20, 23) were confirmed to be *Curvularia lunata*, while two isolates (19, 21), isolates 11, 17, 22, 25, and 24 were identified as *C. senegalensis*, *C. soli*, *C. platzii*, *C. geniculata* and *C. subpappendorffii* respectively. All the isolates were identified to the species level based on the high similarity with those retrieved from Gene bank of the NCBI (Table 11). The phylogram for all the pathogenic isolates (*Bipolaris* and *Curvularia* spp.) is presented in Figure 7, it was observed that the different isolates are classified into different clades

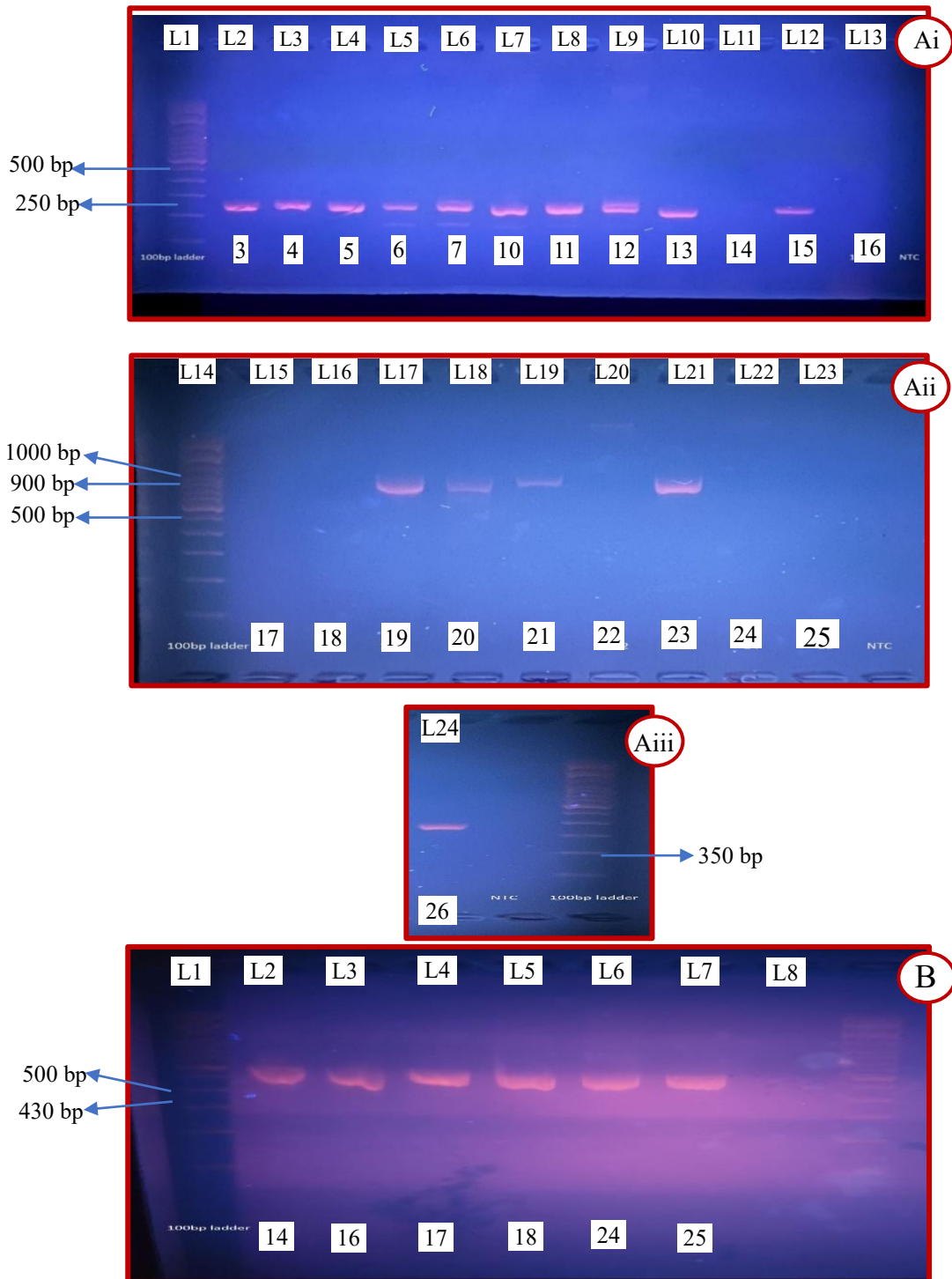


Figure 6: Gel Image of Amplification

Ai-Aiii = Initial Runs, L1 & L14: 100 bp ladder, L2-L6: isolate 3-7, L7: isolate 10, L9-L13: isolate 12-16, B = Re-runs, L2-L5: isolate 14 & 18, L6: isolate 24 and L7: isolate 25. All *Bipolaris* isolates amplified with ACT512/ACT578 primer except isolates 14 and 16. Only isolates 17, 18, 24 and 25 of the *Curvularia* spp. did not amplify with PN3/PN16 primer. Isolates 14, 16, 17, 18, 24 and 25 was later amplified with ITS1/ITS4 primer.

Table 11: BLAST Results for All Isolates

Isolates	Primer	Product size (bp)	Gen. bank acc no	Obtained Genbank march	S-Score	E-value	% similarity
3	ACT-512-F_ACT-783-R	250	CP111179.1	<i>Bipolaris zeicola</i>	388	3e-103	98.21
4	ACT-512-F_ACT-783-R	250	CP111179.1	<i>Bipolaris zeicola</i>	375	2e-99	98.15
5	ACT-512-F_ACT-783-R	250	CP111179.1	<i>Bipolaris zeicola</i>	385	3e-102	98.62
6	ACT-512-F_ACT-783-R	250	CP111179.1	<i>Bipolaris zeicola</i>	379	2e-100	97.32
7	ACT-512-F_ACT-783-R	250	CP111179.1	<i>Bipolaris zeicola</i>	377	6e-100	97.31
8	ITS1 ITS4	500	PP384770.1	<i>Purpureocillium lilacinum*</i>	917	0.0	94.75
9	ITS-Fu, ITS-Fu-Rev	500	KU715168.1	<i>Fusarium equiseti*</i>	673	0.0	100.00
10	ACT-512-F_ACT-783-R	250	CP111179.1	<i>Bipolaris zeicola</i>	387	1e-102	97.78
11	ACT-512-F_ACT-783-R	250	CP089279.1	<i>Curvularia clavata</i>	398	4.00E-106	99.1
12	ACT-512-F_ACT-783-R	250	CP111179.1	<i>Bipolaris zeicola</i>	381	4e-101	97.33
13	ACT-512-F_ACT-783-R	250	CP111179.1	<i>Bipolaris zeicola</i>	357	6e-94	98.99
14	ITS1 ITS4	435	OR727460.1	<i>Bipolaris oryzae</i>	665	0.0	100.00
15	ACT-512-F_ACT-783-R	250	CP111179.1	<i>Bipolaris zeicola</i>	374	7e-99	97.29
16	ITS1 ITS4	500	KU715274.1	<i>Bipolaris oryzae</i>	804	0.0	100.00
17	ITS1 ITS4	500	MT565489.1	<i>Curvularia soli</i>	857	0.0	100.00
18	ITS1 ITS4	500	MK733196.1	<i>Curvularia lunata</i>	732	0.00	99.00
19	PN3_PN16	900	MT476857.1	<i>Curvularia senegalensis</i>	1445	0.0	95.00
20	PN3_PN16	900	MT524328.1	<i>Curvularia lunata</i>	750	0.0	100.00
21	PN3_PN16	1000	MT476857.1	<i>Curvularia senegalensis</i>	1570	0.0	99.00
22	PN3_PN16	3000	MH414906.1	<i>Curvularia platzii</i>	1960	0.0	100.00
23	PN3_PN16	900	MG865982.1	<i>Curvularia lunata</i>	1535	0.0	98.39
24	ITS1 ITS4	500	MT229254.1	<i>Curvularia subpapendorfii</i>	828	0.0	98.91
25	ITS1 ITS4	500	KX447535.1	<i>Curvularia geniculata</i>	1053	0.0	100.00
26	Fum-F Fum-R	350	ON229052.1	<i>Aspergillus flavus*</i>	586	1e-162	97.94

4.5.2 Phylogenetic Tree for Brown leaf spot Pathogens

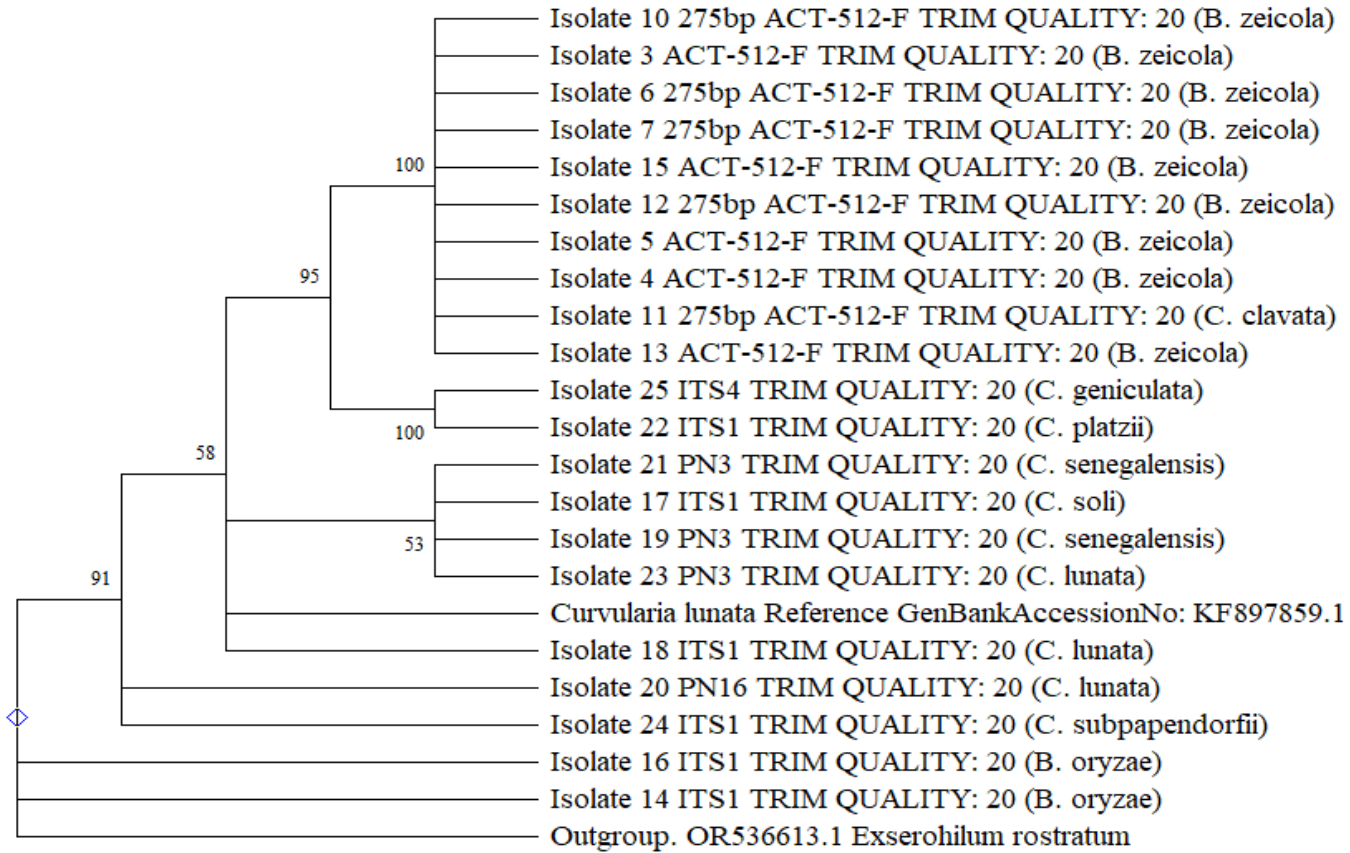


Figure 7: Phylogenetic Tree of All Pathogenetic Isolates

4.6 Management of *Curvularia* and *Bipolaris* isolates causing Brown leaf spot Disease of Rice with Botanicals

4.6.1 *In-Vitro* Control of Brown leaf spot Disease Pathogens

Mycelia Growth of Isolates as Affected by Epiphytes, Botanical and Mancozeb and their Inhibition Rates.

Mycelia growth of *Curvularia lunata* as affected by the epiphytes (Plate 13), botanicals (Plate 14) and mancozeb (Plate 15) is shown in Table 12. There were significant differences among the treatments in affecting mycelia growth and inhibition at 24 to 144 HAI (Table 12).

At 24 hrs, *Curvularia lunata* as affected by *Purpureocillium lilacinum* grew bigger than *C. lunata* in the control, which also grew bigger than *C. lunata* affected by *Aspergillus flavus*, but no significant difference was obtained between these three growth of *C. lunata*. the growth of *C. lunata* in the control and against the two antagonists were significantly different from its growth as affected by the two botanicals (*Azadirachta indica* and *Nerium oleander*) and mancozeb, but there was no significant difference between its growth in the botanicals and mancoze *B*. Its lowest growth was recorded against *A. indica*. in other words, *A. indica* greatly inhibited the growth of *C. lunata*, followed by *N. oleander*, mancozeb and then by the two antagonists, *P. lilacinum* had the minimum inhibition.

Similar trend of *C. lunata* growth at 24 hrs was also observed at 72 hrs, but this time significant difference was observed between its growth as affected by *P. lilacinum* and control and between *A. aspergillus* and the control. The highest growth was on the control, followed by the setup with *A. flavus* and the lowest was on *C. lunata* affected by *N. oleander*. This means that inhibition was higher with *N. oleander* and lower with *A. flavus*.

At 96 hrs, there were statistically significant difference among the control agents in affecting the mycelia growth of *C. lunata*. as usual, no significant difference was observed in the growth among the two botanical and mancozeb, and no difference were again observed on *C. lunata* growth as affected by the two epiphytes. The significant difference were observed between the epiphytes and botanicals, between botanical and control and between control and epiphytes. The highest mycelia growth was however recorded on *C. lunata* in the control, followed by *C. lunata* as affected by *P. lilacinum* and the minimum growth was recorded on *C. lunata* as affected by *N. oleander*. so therefore, inhibition was higher with *N. oleander* and lower with *P. lilacinum*. the significant differences in the inhibition followed similar trend as observed in mycelia growth.

The highest *C. lunata* growth at 120 hrs was obtained on the setup with *A. flavus*, followed by the set up with *P. lilacinum*, then by *A. indica*, mancozeb and *N. oleander* again has highly affected *C. lunata*, thereby causing its less growth. The trend of significant differences observed among the control agents in affecting mycelia growth of *C. lunata* and among their inhibition, and also the rate of inhibition by each agent on *C. lunata* was similar to what was observed at 48 hrs and 96 hrs.

At 144 hrs, the trend continued, but this time, mancozeb recorded the lowest mycelia growth effect on *C. lunata*, but not *N. oleander*. The highest inhibition rate was therefore observed on mancozeb and *A. flavus* had the least among the control agents.


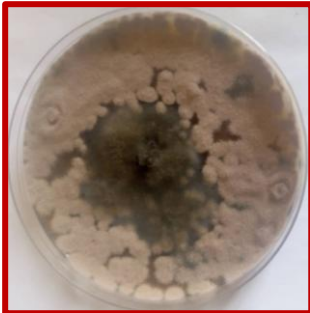
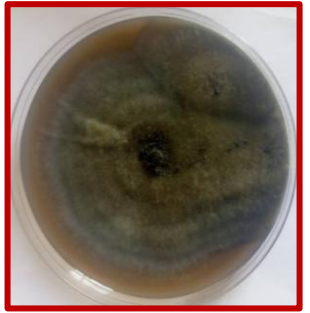
DAI	<i>Aspergillus flavus</i> against <i>Curvularia lunata</i>	<i>Purpureocillium lilacinum</i> against <i>Curvularia lunata</i>	Control (Only <i>Curvularia lunata</i>)
7 Days (168 hours)			

Plate 12: Epiphytes (*Aspergillus flavus* and *Purpureocillium lilacinum*) against *Curvularia lunata* as Biocontrol Agent on PDA (Dual Culture Technique)

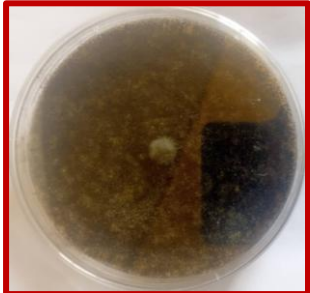
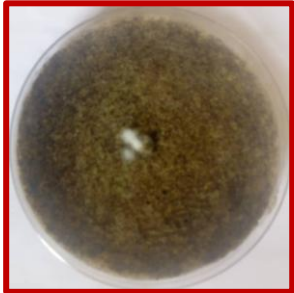
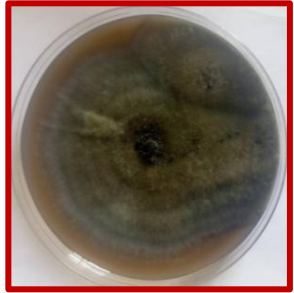
DAI	<i>Azadirachta indica</i> against <i>Curvularia lunata</i>	<i>Nerium oleander</i> against <i>Curvularia lunata</i>	Control (Only <i>Curvularia lunata</i>)
7 Days (168 hours)			

Plate 13: Botanicals (*Azadirachta indica* and *Nerium oleander*) against *Curvularia lunata* as Biocontrol Agent on PDA (Dual Culture Technique)

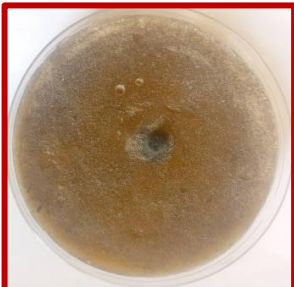
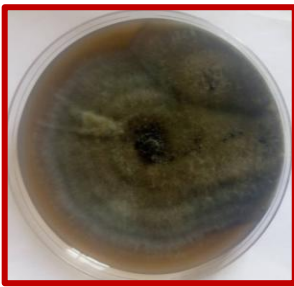
DAI	Mancozeb against <i>Curvularia lunata</i>	Control (Only <i>Curvularia lunata</i>)
7 Days (168 hours)		

Plate 14: Mancozeb against *Curvularia lunata* as Biocontrol Agent on PDA (Dual Culture Technique)

Table 12: Mycelia Growth of *Curvularia lunata* as affected by Epyphytes, Botanicals and Mancozeb and their Inhibition

Control Agent	Mycelia Growth (cm)						Percent Inhibition (%)						Mean Inhibition
	24 HAI	48 HAI	72 HAI	96 HAI	120 HAI	144 HAI	24 HAI	48 HAI	72 HAI	96 HAI	120 HAI	144 HAI	24-144 HAI
Control (<i>C. lunata</i> only)	1.50	3.00	3.73	5.50	6.33	8.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>A. indica</i>	0.70	1.03	0.83	0.87	0.87	0.90	52.90	65.38	77.74	84.16	86.33	89.43	75.99
<i>N. oleander</i>	0.73	0.60	0.63	0.70	0.73	0.77	50.83	80.02	83.02	87.22	88.42	90.99	80.08
<i>A. flavus</i>	1.30	2.63	3.50	3.73	3.90	3.90	21.85	12.08	6.02	32.06	38.37	54.11	27.42
<i>P. lilacinum</i>	1.57	2.67	3.10	4.03	3.80	3.80	4.46	11.08	16.43	26.56	39.94	55.29	25.63
Mancozeb	0.77	0.77	0.67	0.70	0.73	0.73	48.45	74.42	81.89	87.31	88.41	91.37	78.64
Mean	1.09	1.78	2.08	2.59	2.73	3.10	29.75	40.50	44.19	52.88	56.91	63.53	47.96
HSD (0.01)	0.56	0.44	0.60	0.56	0.55	0.57	30.95	16.83	17.70	11.27	9.76	6.46	15.50
CV (%)	14.45	6.99	8.18	6.11	5.67	5.21	29.52	11.80	11.37	6.05	4.87	2.89	11.08

CV= Coefficient of variation, HSD (0.01) = Tukey's honestly significant difference at 1% probability, HAI= Hours after Incubation

As shown in Plate 16 and Table 13, the highest mycelia growth of *B. zeicola* at 24 HAI was recorded on the set with *P. lilacinum*, greater than that of *A. flavus*, which was also greater than that of *N. oleander*, also greater than *A. indica* and then mancozeb recorded the least. There was no significant difference between growth of *B. zeicola* as affected by the epiphytes and the control, but there was significant difference between the botanical or mancozeb and the control, and also between the epiphytes and botanical or mancozeb. The inhibition rate of *B. zeicola* by mancozeb was higher than *A. indica*, which was higher than *N. oleander* which was also higher than *P. lilacinum*, then *A. flavus* was the least. The trend of significant difference among the treatments was similar to that of mycelia growth.

At 48 HAI, the control plate had the highest growth of *B. zeicola* and then the plate with *A. flavus* followed, then that of *P. lilacinum*, which was greater than the highest among botanicals (*N. oleander*) and then mancozeb had the least. But no statistically significant difference was obtained among the botanicals and between the botanicals and mancozeb in influencing mycelia growth of *B. zeicola*, but statistical difference was obtained between the botanical or mancozeb and the epiphytes or the control, no statistical difference was also obtained among the epiphytes. The trend of the inhibition rate of the treatments on *B. zeicola* was similar to what was obtained at 24 HAI, except that *A. indica* had the highest inhibition rate and unlike 24 HAI, there was a significant difference between the control and *P. lilacinum* (Plate 17) and Table 13.

At 72 HAI, no significant differences were observed among the treatment effect on mycelia growth of *B. zeicola*, but *A. indica* had the least influence on mycelia growth of *B. zeicola* not mancoze *B.* Similar trend of inhibition rate of *B. zeicola* at 48 HAI was also observed. Mycelia growth of *B. zeicola* in the control plate at 96 HAI was highest and significantly different ($P < 0.05$) from growth in the treatment plates. No

significant difference in mycelia growth was observed among the botanicals, but significant difference was obtained between control, botanicals, epiphytes and mancozeb as *A. indica* had the least influence on the growth of *B. zeicola*. The inhibition rate also followed similar trend in vice versa, *A. indica* had the highest inhibition rate and *P. lilacinum* had the least (Plate 18 and Table 13).

Each treatment (among and between epiphytes, botanicals, mancozeb and control) except between *A. indica* and mancozeb significantly influenced the growth of *B. zeicola* at 120 HAI and at 144 HAI, *A. indica* however had the greatest influence on the mycelia growth of *B. zeicola* thereby recording the highest inhibition rate, followed by Mancozeb, followed *N. oleander* and by *A. flavus* and *P. lilacinum*.

4.6.2 Screenhouse Control of Brown leaf spot Disease of Rice

Effect of Epiphytes, Botanicals and Mancozeb on the Incubation Period of *Curvularia* and *Bipolaris* Isolates.

There was no significant difference among the treatment effects in the incubation period of *C. lunata* in producing spot symptoms on the rice seedlings when the control agents were inoculated first before *C. lunata* at five days interval and when both were inoculated instantly, but there was significant difference when *C. lunata* was inoculated five days period before control agents were applied (Figure 8).


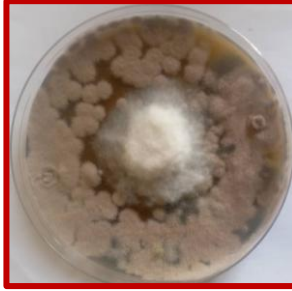

DAI	<i>Aspergillus flavus</i> against <i>Bipolaris zeicola</i>	<i>Purpureocillium lilacinum</i> against <i>Bipolaris zeicola</i>	Control (Only <i>Bipolaris zeicola</i>)
7 Days (168 hours)			

Plate 15: Epiphytes (*Aspergillus fumigatus* and *Purpureocillium lilacinum*) against *Bipolaris zeicola* as Biocontrol Agent on PDA (Dual Culture Technique)


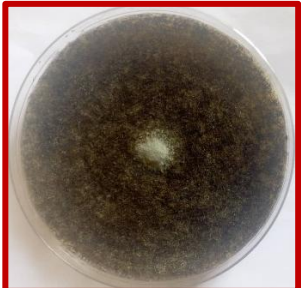

DAI	<i>Azadirachta indica</i> against <i>Bipolaris zeicola</i>	<i>Nerium oleander</i> against <i>Bipolaris zeicola</i>	Control (Only <i>Bipolaris zeicola</i>)
7 Days (168 hours)			

Plate 16: Botanicals (*Azadirachta indica* and *Nerium oleander*) against *Bipolaris zeicola* as Biocontrol Agent on PDA (Dual Culture Technique)



DAI	Mancozeb against <i>Bipolaris zeicola</i>	Control (Only <i>Bipolaris zeicola</i>)
7 Days (168 hours)		

Plate 4. 1: Mancozeb against *Bipolaris zeicola* as Biocontrol Agent on PDA (Dual Culture Technique)

Table 13: Mycelia Growth of *Bipolaris zeicola* as affected by Epiphytes, Botanicals and Mancozeb and their Inhibition

Control Agent	Mycelia Growth (cm)						Percent Inhibition (%)						Mean Inhibition
	24 HAI	48 HAI	72 HAI	96 HAI	120 HAI	144 HAI	24 HAI	48 HAI	72 HAI	96 HAI	120 HAI	144 HAI	24-144 HAI
Control (<i>C. lunata</i> only)	1.5	2.9	3.8	5.2	6.6	8.8	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>A. indica</i>	0.6	0.7	0.6	0.6	0.6	0.6	58.63	77.28	85.01	89.02	91.38	93.16	82.41
<i>N. oleander</i>	0.8	0.8	1.0	1.1	1.1	1.2	47.62	72.64	72.84	78.72	83.23	86.75	73.63
<i>A. flavus</i>	1.6	2.5	2.8	3.2	3.1	3.1	6.85	14.55	26.09	38.68	52.78	64.77	33.95
<i>P. lilacinum</i>	1.7	2.4	2.9	3.5	3.5	3.6	9.23	19.23	22.48	31.55	46.18	59.46	31.36
Mancozeb	0.6	0.7	0.7	0.8	0.7	0.7	61.01	77.09	80.76	84.53	88.80	91.69	80.65
Mean	1.1	1.7	2.0	2.4	2.6	3.0	30.56	43.46	47.86	53.75	60.39	65.97	50.33
HSD (0.01)	0.33	0.44	0.53	0.40	0.40	0.43	24.80	17.12	14.90	7.53	5.52	3.68	12.26
CV (%)	8.24	7.53	7.54	4.63	4.25	4.09	23.03	11.18	8.83	3.98	2.60	1.58	8.53

CV= Coefficient of variation, HSD (0.01) = Tukey's honestly significant difference at 1% probability, HAI= Hours after Incubation

When *C. lunata* was inoculated first, incubation period was shorter on plants applied with *N. oleander*, spot symptoms appeared 8.7 days after inoculation, followed by the control seedling, which was only inoculated with *C. lunata* (9.3 days), *A. indica* had the longest incubation period (15.3 days) and then *mancozeb* followed (11.3 days)

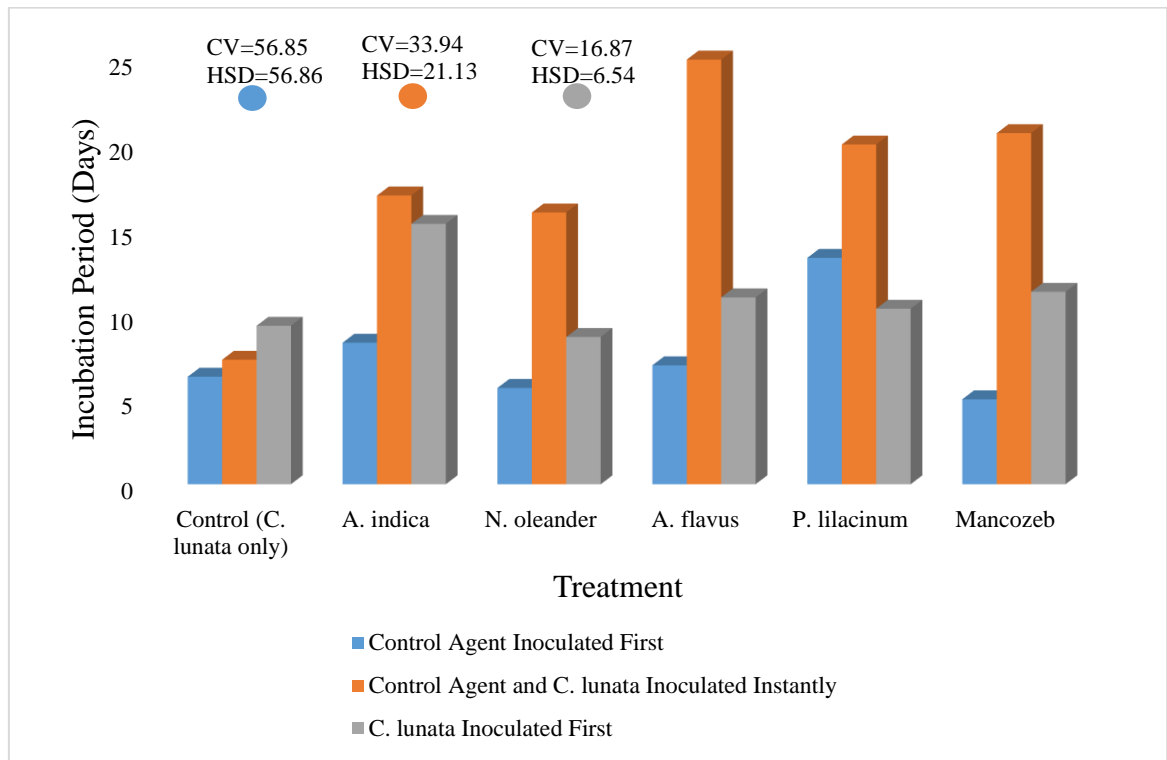


Figure 8: Incubation Period (Days) of *Cuvularia lunata* as Influenced by Epiphytes, Botanicals and Mancozeb

There was no statistically significant difference among the treatment effects in the incubation period of *B. zeicola* in producing spot symptoms on the rice seedlings when the control agents were inoculated first before *B. zeicola* at five days interval, when both were inoculated instantly and when *B. zeicola* was inoculated five days period before control agents were applied (Figure 9).

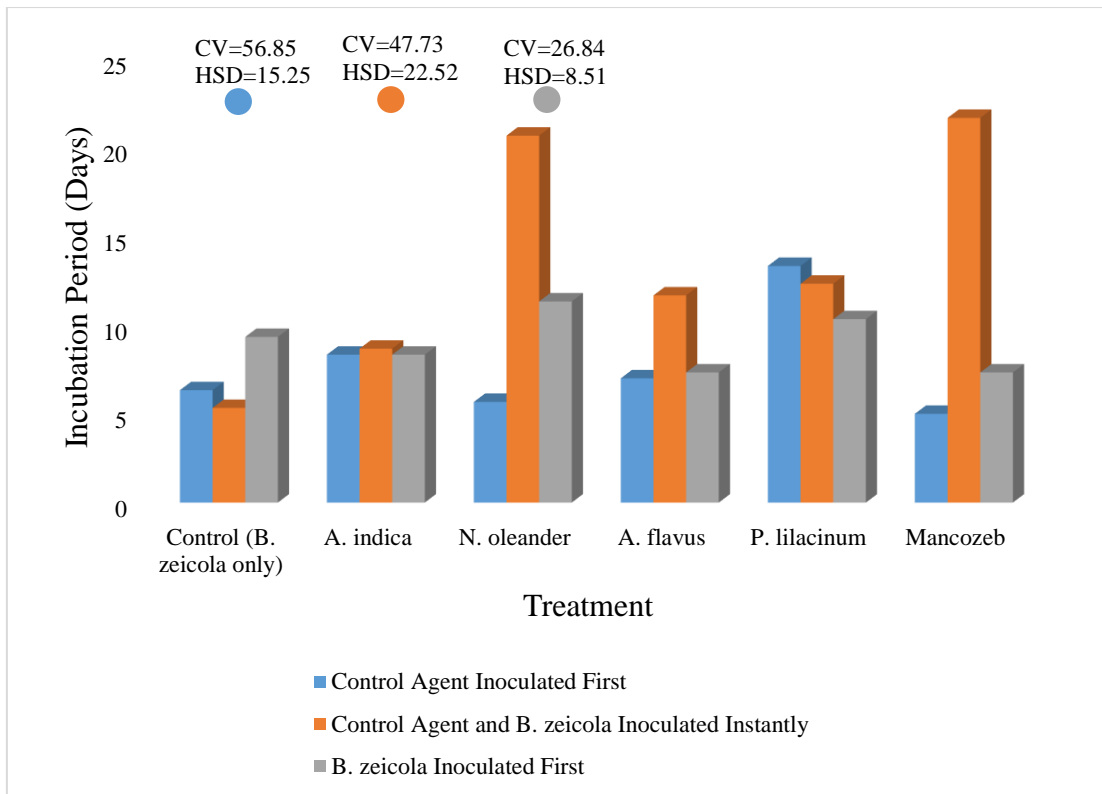


Figure 9: Incubation Period (Days) of *Bipolaris zeicola* as Influenced by Epiphytes, Botanicals and Mancozeb

Effect of Epiphytes, Botanicals and Mancozeb on Brown leaf spot Disease Incidence Caused *Curvularia* and *Bipolaris* Isolates

There was no statistically significant difference among the treatment effects in the spot disease incidence of *C. lunata* on the rice seedlings when the control agents were inoculated first before *C. lunata* at five days interval at 10 DAI to 20 DAI, but there was significance difference at 25 DAI. At 25 DAI, there was significance difference between the control treatment and all the control agent treatments except mancozeb, there was no significant difference between the mancozeb and the other control agent treatments. The highest incidence was recorded on the control treatment and then mancozeb recorded the next highest and seedlings treated with *P. lilacinum* recorded the lowest incidence (Table 16).

When both *C. lunata* and the control agents were inoculated instantly, there was no significant difference in disease incidence at 10 DAI and 20 DAI, but significant difference was observed at 15 DAI and 25 DAI. At 15 DAI, there was no significant difference in the incidence recorded on control treatment and all seedlings treated with the different control agents except those treated with mancoze *B*. Seedlings treated with mancozeb recorded no incidence, whilst the control treated recorded the highest incidence followed by seedlings treated with *A. indica*. At 25 DAI, there was significant difference in the disease incidence recorded on seedlings treated as control and those applied with control agents, but no significant difference among the seedlings treated with the different control agents.

There were significant differences in spot disease incidence at 15 DAI to 25 DAI when *C. lunata* was inoculated first before the control agents were applied at an interval of five days, but no difference was obtained at 10 DAI. At 15 DAI to 25 DAI, there was significant difference in the incidence recorded on the control treatment and all the treatment that were applied with control agents. The highest incidence was constantly recorded on the control treatment and the lowest was on seedlings treated with *A. indica*. Among the seedlings treated with the control agents, mancozeb had the highest at 15 DAI while *N. oleander* had the highest at 20 DAI and 25 DAI.

Table 14: Disease Incidence (%) of *Curvularia lunata* as influenced by Epiphytes, Botanicals and Mancozeb

Control Agent	Control agent Inoculated first					Control agent and <i>C. lunata</i> Inoculated instantly					<i>C. lunata</i> Inoculated first				
	10 DAI	15 DAI	20 DAI	25 DAI	Mean	10 DAI	15 DAI	20 DAI	25 DAI	Mean	10 DAI	15 DAI	20 DAI	25 DAI	Mean
Control (No Control Agent)	13.33	23.33	36.67	63.33	34.17	20.00	26.67	36.67	70.00	38.34	20.00	40.00	53.33	63.33	44.2
<i>A. indica</i>	6.67	6.67	13.33	13.33	10.00	6.67	6.67	6.67	10.00	7.50	0.00	3.33	6.67	10.00	5.0
<i>N. oleander</i>	10.00	10.00	10.00	16.67	11.67	3.33	6.67	13.33	13.33	9.17	10.00	10.00	13.33	16.67	12.5
<i>A. flavus</i>	13.33	16.67	20.00	23.33	18.33	0.00	3.33	6.67	6.67	4.17	3.33	6.67	10.00	10.00	7.5
<i>P. lilacinum</i>	6.67	10.00	10.00	10.00	9.17	6.67	6.67	16.67	16.67	11.67	6.67	10.00	10.00	13.33	10.0
Mancozeb	13.33	16.67	16.67	36.67	20.84	0.00	0.00	3.33	6.67	2.50	3.33	10.00	10.00	13.33	9.2
Mean	10.56	13.89	17.78	27.22	17.36	6.11	8.33	13.89	20.56	12.22	7.22	13.33	17.22	21.11	14.7
HSD (0.01)	18.57	21.98	28.77	34.25	25.89	20.34	23.49	35.24	29.95	27.26	32.17	27.55	38.06	43.95	35.4
CV (%)	49.93	44.90	45.93	35.70	44.12	94.48	80.00	72.00	41.34	71.96	126.40	58.63	62.72	59.08	76.7

CV= Coefficient of variation, HSD (0.01) = Tukey's honestly significant difference at 1% probability.

There were statistically significant difference in spot disease incidence produced by *Bipolaris zeicola* at 10 DAI to 25 DAI when the control agents were inoculated first before *B. zeicola* at five days interval. At 10 DAI to 25 DAI, the control treatment recorded the highest incidence and seedlings treated with *P. lilacinum* also recorded the lowest incidence except at 10 DAI where seedlings inoculated with *A. flavus* recorded the lowest incidence. There was no statistically significance difference in the incidence recorded among seedlings treated with the control agents, except at 25 DAI that there was difference between mancozeb and the other control agents (Table 17).

When both *B. zeicola* and the control agents were inoculated instantly, there was significant difference in disease incidence between the control treatment and the seedling applied with control agents at 10 DAI and 25 DAI. There was no statistically significance difference in the incidence recorded among seedlings treated with the control agents, except at 15 DAI where difference incidence was observed between seedlings treated with *A. flavus* or *P. lilacinum* and *A. indica* or *N. oleander* or mancozeb.

There were statistically significant difference in the incidence at 15 DAI to 25 DAI, but no difference observed in incidence at 10 DAI, when *B. zeicola* was inoculated before the control agents. At 15 DAI to 25 DAI, the significant difference was obtained between the control treatment and the treatment with the control agents, no difference was obtained among the treatment with control agents. The control treatment recorded the highest incidence throughout and *P. lilacinum* also recorded the lowest.

Table 15: Disease Incidence (%) of *Bipolaris zeicola* as influenced by Epiphytes, Botanicals and Mancozeb

Control Agent	Control agent Inoculated first					Control agent and <i>B. zeicola</i> Inoculated instantly					<i>B. zeicola</i> Inoculated first				
	10 DAI	15 DAI	20 DAI	25 DAI	Mean	10 DAI	15 DAI	20 DAI	25 DAI	Mean	10 DAI	15 DAI	20 DAI	25 DAI	Mean
Control (No Control Agent)	36.67	43.33	60.00	80.00	55.00	36.67	36.67	56.67	80.00	52.50	20.00	43.33	50.00	70.00	45.83
<i>A. indica</i>	6.67	10.00	23.33	20.00	15.00	6.67	3.33	10.00	6.67	6.67	13.33	13.33	13.33	20.00	15.00
<i>N. oleander</i>	13.33	16.67	16.67	23.33	17.50	0.00	3.33	3.33	6.67	3.33	10.00	10.00	10.00	10.00	10.00
<i>A. flavus</i>	3.33	6.67	10.00	16.67	9.17	6.67	6.67	6.67	6.67	6.67	13.33	16.67	20.00	16.67	16.67
<i>P. lilacinum</i>	10.00	6.67	3.33	3.33	5.83	6.67	6.67	13.33	16.67	10.84	6.67	10.00	10.00	13.33	10.00
Mancozeb	20.00	33.33	40.00	46.67	35.00	0.00	0.00	3.33	6.67	2.50	20.00	16.67	10.00	3.33	12.50
Mean	15.00	19.44	25.56	31.67	22.92	9.44	9.44	15.56	20.56	13.75	13.89	18.33	18.89	22.22	18.33
HSD	29.95	31.08	45.49	29.95	34.12	29.95	31.08	23.49	23.49	27.00	28.77	21.98	16.61	35.24	25.65
CV	56.66	45.36	50.52	26.84	44.85	89.98	93.38	42.86	32.43	64.66	58.79	34.02	24.96	45.00	40.69

CV= Coefficient of variation, HSD (0.01) = Tukey's honestly significant difference at 1% probability, DAI= Days after Inoculation

Effect of Epiphytes, Botanicals and Mancozeb on Brown leaf spot Disease Severity Caused by *Curvularia* and *Bipolaris* Isolates

There were statistically significant difference in brown leaf spot disease severity produced by *Curvularia lunata* at 10 DAI to 25 DAI when control agents were inoculated before *C. lunata* at five days interval. Severity was higher in the control treatment which had no control agent inoculated. Among the seedlings with control agents, those treated with mancozeb had the highest severity at 15 DAI to 25 DAI and those with *A. indica* had the lowest throughout (Table 18).

When both control agent and *C. lunata* were inoculated instantly, there was also significant difference in the disease severity. Severity was higher on seedlings treated with *A. indica* at 10 DAI to 20 DAI, but there was no severity on seedlings treated with mancozeb at 10 and 15 DAI, but recorded severity at 20 DAI. At 25 DAI, severity on seedlings treated with mancozeb was rather higher than all the other control agents.

When *C. lunata* was inoculated first before the control agents at five days interval, there was significant difference in the disease severity between the control and the treatments that control agents from 10 DAI to 25 DAI. Seedlings with only *C. lunata* had the highest severity throughout and among the control agents, seedlings treated with *P. lilacinum* had the highest severity at 10 DAI and 20 DAI, but mancozeb had the highest at 15 DAI and *N. oleander* at 25 DAI.

Table 16: Disease Severity (%) of *Curvularia lunata* as influenced by Epiphytes, Botanicals and Mancozeb

Control Agent	Control agent Inoculated first					Control agent and <i>C. lunata</i> Inoculated instantly					<i>C. lunata</i> Inoculated first				
	10 DAI	15 DAI	20 DAI	25 DAI	Mean	10 DAI	15 DAI	20 DAI	25 DAI	Mean	10 DAI	15 DAI	20 DAI	25 DAI	Mean
Control (No Control Agent)	62.67	84.67	90.67	93.00	82.75	52.67	71.67	85.67	91.00	75.25	33.33	60.00	70.00	86.67	56.67
<i>A. indica</i>	5.33	8.33	9.67	10.67	8.50	8.00	8.00	11.33	18.00	11.33	0.00	3.43	10.23	12.17	5.17
<i>N. oleander</i>	6.33	13.67	12.00	12.33	11.08	2.67	7.33	7.33	8.00	6.33	6.38	7.60	18.77	37.03	15.23
<i>A. flavus</i>	20.00	25.33	29.33	33.67	27.08	0.00	2.00	6.67	9.00	4.42	6.67	8.33	20.00	28.57	14.05
<i>P. lilacinum</i>	6.67	10.67	12.67	13.00	10.75	5.67	7.00	11.00	13.00	9.17	10.00	11.57	21.50	28.58	16.33
Mancozeb	19.33	35.33	33.00	41.67	32.33	0.00	0.00	7.00	19.00	6.50	3.15	12.37	19.03	35.97	14.73
Mean	20.06	29.67	31.22	34.06	28.75	11.50	16.00	21.50	26.33	18.83	9.92	17.22	26.59	38.16	20.36
HSD (0.01)	23.71	29.31	36.86	43.69	33.39	22.47	31.93	31.33	39.11	31.21	16.70	36.89	56.81	50.47	35.51
CV (%)	33.55	28.03	33.50	36.41	32.87	55.45	56.63	41.35	42.14	48.89	47.76	60.81	60.63	37.53	50.90

CV= Coefficient of variation, HSD (0.01) = Tukey's honestly significant difference at 1% probability.

When the control agents were inoculated before *B. zeicola*, no significant difference obtained in disease severity at 10 DAI. Significant difference were observed in disease severity at 15 DAI to 25 DAI. At 15 DAI, the difference existed between the control and *A. flavus* only. At 20 DAI, the difference existed between the control and all the other control agents except mancozeb, also no difference between mancozeb and the other control agents except *P. lilacinum*. Among the control agents, highest severity was recorded on seedlings inoculated with mancozeb throughout and the lowest was on rice seedlings inoculated with *P. lilacinum* (Table 19).

When both were inoculated instantly, there were significant difference and the difference was between the control and all the control agents. Among the control agents, *A. indica* recorded the highest at 10 DAI and 15 DAI but *P. lilacinum* recorded the highest severity at 20 DAI and 25 DAI, the lowest was interchangeably recorded on seedlings treated with *N. oleander* (at 10 DAI and 20 DAI), *A. indica* at 25 DAI and mancozeb and 15 DAI.,

When *B. zeicola* was inoculated earlier, there were significant difference in the spot severity at 10 DAI, 15 DAI and 25 DAI. At 10 DAI, *A. indica* reduced severity than the other control agents and *P. lilacinum* had the lowest severity reduction. At 15 DAI, *N. oleander* had the highest reduction, whilst mancozeb had the lowest. At 25 DAI, *A. indica* had the highest severity reduction followed by mancozeb, *P. lilacinum*. *N. oleander* and *A. flavus* recorded the lowest reduction.

Table 17: Disease Severity (%) of *Bipolaris zeicola* as influenced by Epiphytes, Botanicals and Mancozeb

Control Agent	Control agent Inoculated first					Control agent and <i>B. zeicola</i> Inoculated instantly					<i>B. zeicola</i> Inoculated first				
	10 DAI	15 DAI	20 DAI	25 DAI	Mean	10 DAI	15 DAI	20 DAI	25 DAI	Mean	10 DAI	15 DAI	20 DAI	25 DAI	Mean
Control (No Control Agent)	21.67	45.67	70.33	85.67	55.84	51.33	70.33	76.67	89.33	71.92	16.00	26.86	27.08	89.31	39.81
<i>A. indica</i>	11.00	19.67	17.33	19.33	16.83	6.33	5.67	10.67	6.67	7.34	0.33	12.00	12.33	3.33	7.00
<i>N. oleander</i>	8.33	13.67	19.33	23.00	16.08	0.00	3.00	2.33	9.67	3.75	6.04	7.60	13.53	15.40	10.64
<i>A. flavus</i>	0.00	6.00	20.00	38.00	16.00	5.67	4.67	10.00	12.33	8.17	13.67	16.67	24.67	33.23	22.06
<i>P. lilacinum</i>	11.67	13.33	5.00	3.33	8.33	2.00	2.00	12.00	12.67	7.17	23.97	19.00	7.00	6.33	14.08
Mancozeb	27.67	38.67	51.00	55.33	43.17	0.00	0.00	4.67	26.00	7.67	23.33	25.00	18.33	4.00	17.67
Mean	13.39	22.83	30.50	37.44	26.04	10.89	14.28	19.39	26.11	17.67	13.89	17.85	17.16	25.27	18.54
HSD	16.08	18.13	39.07	28.13	25.35	14.31	26.25	25.51	49.81	28.97	16.08	18.13	39.07	28.13	25.35
CV	32.84	28.81	64.61	31.59	39.46	37.30	52.18	37.33	54.13	45.24	32.84	28.81	64.61	31.59	39.46

CV= Coefficient of variation, HSD (0.01) = Tukey's honestly significant difference at 1% probability, DAI= Days after Inoculation

Chapter Five: Discussion

5.1 Isolates

Field surveys across the selected rice fields in the Atwima Nwabiagya District confirmed presence of rice brown leaf spot disease. The observed symptoms on rice such as circular to oval or dark brown leaf spot with a light center, confirms the findings of Quintana *et al.* (2019), who reported that BLSD affect rice leaves and produced spot size symptoms with circular or oval dark brown leaf spots. Sun *et al.* (2020) reported that, BLSD is more prevalent in areas with low temperature and relatively high humidity. The high humidity in the study area might have contributed to the spread/ incidence of the disease in the area. In earlier studies, Asamoah *et al.* (2023) reported high incidence of *Bipolaris* brown leaf spot disease on rice seeds. Although the current study did not assess seed health/ microbial load of the rice seeds used by the farmers, it is possible that seed source and health may have contributed the presence of the disease in the area.

Twenty-one (21) pathogenic fungal isolates consisting of eleven (11) *Bipolaris* spp. and ten (10) *Curvularia* spp. were obtained from rice leaves infected with brown leaf spot disease. Obtaining *Bipolaris* spp. and *Curvularia* spp. from spot infected plant samples is not surprising, according to Surendhar *et al.* (2022), these two genera of the pleosporaceae has been the most abundant group of organisms found to be associated with brown leaf spot disease. More specifically, many researches have isolated *Bipolaris* and *Curvularia* species from brown leaf spot diseased samples of rice internationally (Zhou *et al.*, 2021; Imran *et al.*, 2022; Surendhar *et al.*, 2022) and locally (Honger *et al.*, 2022; Oppong *et al.*, 2022; Ackaah *et al.*, 2023).

5.2 Morphological and Molecular Identification

Cultural and conidia characteristics of both *Bipolaris* and *Curvularia* isolates were analysed on PDA medium. The results indicated that isolates exhibited variation with respect to diameter of colony, number of concentric rings, colony elevation, margin type, surface and reverse side pigmentation, colony shape, sporulation rate, conidia size and number of septa. These observations imply more insight about the pathogens, the causal organisms of the brown leaf spot disease. In terms of colony diameter, many isolates (both *Bipolaris* and *Curvularia* isolates) had good growth, recorded above 7 cm after 168 HAI. All *Bipolaris* isolates had varied number of growths which was statistically different where *Curvularia* isolates also exhibited varied significant difference at 168 HAI with entire or undulate margin types having brown or grey with brown surface colour and a few of the *Bipolaris* isolates being white. All isolates except a few of the *Bipolaris* isolates with brown had black reverse side pigmentation at 168 HAI. These findings on *Bipolaris* isolates are in line with Devi *et al.* (2021) who studied *Bipolaris* colony growth and reported that, colonies grew with the presence of concentric rings in some isolates at the early stage which disappears later in their growth stage.

Nayak & Hiremath (2019) as well as Marin-Felix *et al.* (2017) reported that isolates of *Bipolaris* showed significant differences in colony morphology and also the colour of the colony ranged from light greyish, greyish with white cottony, greyish with black and dark greyish. Valarmathi & Ladhalakshmi (2018) also grouped 17 *Bipolaris* isolates into four, these were group one: which was black with fluffy growth, group two: was also grey with fluffy growth and white spots, group three: was grey with fluffy growth and group four was also grey with suppressed growth. Likewise, Brindhadevi *et al.* (2021) characterised *Curvularia* spp. after growing isolates on PDA

medium and reported that it had grey to brown colour. Notwithstanding, Yuvarani *et al.* (2021) reported similar results, they isolated *Curvularia* spp and described their surface colour as brown, dark reverse side pigment with mycelia having rings formed on the surface.

Bipolaris isolates had very low sporulation rate where conidia were only produced in some plates after 3 months of incubation and some even had no conidia for the entire period of the study. Conidia were tapered at both ends, straight or curved and size ranged from 50.00 to 70.00 μm and with septations that could barely be seen. This finding agrees with that of Devi *et al.* (2021) who found the conidia of the *Bipolaris* isolates to be ovoid and fusiform (tapered at both ends). Marin-Felix *et al.* (2017), Nayak & Hiremath (2019) and Kaur *et al.* (2024) further reported, significant variation in the colony size.

The sporulation rate of *Curvularia* isolates varied, plates had many conidia per observation with only one plate with few conidia, septations were visible with an average of 2 to 4 and swollen at second or third cell. Some conidia were straight and others were ovoid and tapered at either one end or both ends. Size of conidia varied ranging from 17.50 to 20.07 μm . In the report of Honger *et al.* (2022), they characterized *Curvularia* isolates associated with brown leaf spot disease of rice and described its conidia to be smaller, geniculate and swollen at the second or third cell, some conidia were also described as curved or straight. Majumdar & Mandal (2019) also explained that, the size of *Curvularia* isolates varied significantly. Similar to the present reports, the variation in conidial morphology of an individual isolates of *Curvularia* were reported by Yuvarani *et al.* (2021). Madrid *et al.* (2014) also reported of the *Curvularia* isolates having large number of conidia in PDA plates, colonies

having black reverse side and greyish green at center and grey towards the periphery at the surface.

Morphological variations among *Bipolaris* spp. and *Curvularia* spp. as observed in this current study affirms that there is a diverse conidial morphology in *Curvularia* and *Bipolaris* genera and this might be the reason that Manzar *et al.* (2022) concluded that, it is difficult to differentiate *Bipolaris* spp. and *Curvularia* spp. solely based on morphological examination, because many of their morphological features overlap. PCR amplification and BLAST search analysis confirmed that among the twenty-three (23) fungal isolates, isolate 3-7, 10, 12-16 were all *Bipolaris* isolates, all were molecularly proved to be *Bipolaris zeicola* with the exception isolate 16 which represent *Bipolaris oryzae*. Isolate 11 and 17-25 were also confirmed to be *Curvularia*. Specifically, isolate 11 was *Curvularia clavata*, 17 was *Curvularia soli*. Isolate 18, 20 and 23 were all *Curvularia lunata*. Isolate 19 and 21 were *Curvularia senegalensis*, isolate 22 was *Curvularia platzii*, isolate 24 was *Curvularia subpapendorfii* and isolate 25 was *Curvularia geniculata*. The two epiphytes, which were isolate 8 and isolate 26 were also confirmed to be *Purpureocillium lilacinum* and *Aspergillus flavus* respectively. All these isolates were molecularly placed in different clusters depicting the significant variability among the rice brown leaf spot isolates.

Specifically identifying *B. zeicola* and *B.oryzae* is not surprising as these two have been identified to be associated with the disease by many researchers. Kang *et al.* (2018) identified *B. zeicola* to cause leaf spot of rice and maize, its first report to cause brown spot of rice in Pakistan was done by Aslam *et al.* (2021). *B. oryzae* has also been reported by Boka *et al.* (2018) on this same discussion. Similar to the present study, Singh *et al.* (2021) characterized thirteen *Bipolaris* isolates into eight species using the molecular tools RAPD, ISSR and URP data combined which showed relatively low

genetic similarity coefficient. These results are in accordance with the present observations. *Curvularia* and *Bipolaris* spp. have been reported as the most pathogens of brown leaf spot of rice with similar symptoms difficult to distinguish. Zhou *et al.* (2021) identified *C. lunata* as the cause of leaf spot on wild rice in China. *C. lunata* has also been reported to cause leaf spot of banana in Bengal (Chowhan & Chakraborty, 2023). *C. senegalensis* has also been reported to cause leaf spot on tomatoes (Huang *et al.*, 2023) and on zinnia (Bandara *et al.*, 2022). Xie *et al.* (2023) and Manzar *et al.* (2021) reported of *C. geniculata* causing brown leaf spot disease on *Bletilla striata* and maize in China and India, respectively. *C. soli*, according to Marin-Felix *et al.* (2017), is closely related to *C. geniculata* and this might explain why it is also able to induce brown leaf spot on rice in the current study.

The pathogenicity of *Bipolaris* spp. and a *Curvularia* spp were proved in both the in-vitro and screenhouse studies. In this study, variations in pathogenicity was confirmed with *Curvularia* spp being highly pathogenic compared to *Bipolaris* spp. The findings of the current study agrees with Sun *et al.* (2020), after studying the etiology and symptoms of maize leaf spot caused by *Bipolaris* spp, they reported that *B. zeicola* showed moderate or weak in virulent in the pathogenicity test. Similar to the results obtained from the in-vitro test, pathogenicity test in the screenhouse demonstrated that, virulence differentiation existed among isolates evaluated on whole rice seedlings, all isolates produced disease symptoms of the brown leaf spot disease (BLSD) with variation in their incubation period (IP), incidence, severity and AUDPC.

Specifically, for *Bipolaris* isolates, incubation period (IP) ranged from 10 to 11 days. *Bipolaris zeicola* (isolate 6 and 14) had a longer IP with the rest recording the same IP with *B. oryzae*. This indicates that, some strains of *B. zeicola* exhibit similar ability as *B. oryzae* in producing brown leaf spot on rice, in terms of incubation period. Incidence,

severity and AUDPC also followed similar trend. This result agrees with the findings of Singh *et al.* (2021), they studied the incubation period of *B. zeicola* and regarded it as a weak pathogen, although this study was conducted on maize, but not on rice. The finding is basically consistent with the findings of Sun *et al.* (2020), they reported that, their pathogenicity tests demonstrated virulence differentiation among isolates of *B. zeicola*, and that of *B. zeicola* were moderately or weakly virulent in the pathogenicity tests, but the maize leaf spot diseases caused by *B. zeicola* were relatively serious in some locations.

The IP of *Curvularia* isolates also ranged from 5 to 10 days, and the incidence, severity and AUDPC varied significantly. Specifically, incidence, severity and AUDPC were higher with *C. lunata*. All *C. lunata* isolates had faster infection rate and these results agree with previous studies (Khoo *et al.*, 2023), which they observed variations in virulence of *Curvularia* isolates and reported that *C. lunata* caused severe spot symptoms on rice leaves. Contrary to Fabriyanto *et al.* (2022) and Ribeiro *et al.* (2020), who found connections between incubation periods (IP) and total disease measured (AUDPC), this current study could not establish relationship between IP and AUDPC. Differences in pathogenicity of fungal pathogenic species could be attributed to sexual reproduction which can cause the gain or loss of genes leading ultimately variability in their virulence. Also, variations in disease parameters among *Curvularia* species as have been established in this study was previously reported by many researchers (Zhou *et al.*, 2021; Oppong *et al.*, 2022).

The disease was initially assumed to be responsible by *Bipolaris oryzae* as a worldwide pathogen of brown leaf spot disease (Liu *et al.*, 2021; Surendhar *et al.*, 2022), and later *Curvularia lunata* was also reported by many researchers as pathogen causing this same disease (Majeed *et al.*, 2016; Garcia-Aroca *et al.*, 2018). More recently, different

species of *Curvularia* genera aside *C. lunata* have also been reported on this same mission. A few of those are *C. affinis* (Halder & Kundu, 2017), *C. verruculosa* (Bawa *et al.*, 2021), *C. spicifera* (Gouda *et al.*, 2020). Other species have been reported but on different crops, like *C. asianensis* and *C. eragrostidis* causing leaf spot on *Sansevieria trifasciata* (Kee *et al.*, 2020), *C. clavate* has also caused leaf spot on tobacco (Cao *et al.*, 2023), *C. geniculata* was also reported as pathogen of leaf spot of coffee (Nam *et al.*, 2024).

In the case of Ghana, attention was given to the disease when Akan *et al.* (2015) listed it as one of the serious constraints to rice production in the country. Nutsugah *et al.* (2003) also did a survey on rice diseases and their report affirmed that of Akan *et al.* (2015). Honger *et al.* (2022), reported of an emerging foliar disease of rice with blown leaf symptoms similar to that ascribed to *Bipolaris oryzae* in developing rice fields and described it as a threat to rice production. There was a dilemma as to whether this emerging disease was the same as the brown leaf spot diseases already known in Ghana. The curiosity of the authors emerged that *C. lunata* is the causal agent of the new brown leaf spot disease of rice in Ghana. It was then the first report of *Curvularia* species associated with the disease in Ghana.

This current findings however, is the first report of *C. clavate*, *C. soli*, *C. senegalensis*, *C. platzii*, *C. subpapedorfi* and *C. geniculata* associated with brown leaf spot in Ghana. Since Honger *et al.* (2022) suggested the pathogen is a clade, it confirms that there were more species involve in the disease complex. The result of this study has therefore confirmed that there were other species involved in the disease complex and possible reason why the disease could cause massive destruction as emphasized by Honger *et al.* (2022). *C. clavata*, *C. soli*, *C. senegalensis*, *C. platzii*, *C. subpapedorfi* and *C.*

geniculata are therefore, newly reported species of *Curvularia* causing brown leaf spot of rice in Ghana.

5.3 Management of *Brown leaf spot Disease of Rice*

Both *Aspergillus flavus* and *Purpureocillium lilacinum* effectively inhibited *C. lunata* and *B. zeicola* in dual culture technique in the in-vitro test. Likewise on whole plant, *A. flavus* and *P. lilacinum* reduced incidence and subsequently reduced severity of brown leaf spot disease on rice seedlings. Incubation period of isolates (*C. lunata* and *B. zeicola*) was longer when both control agents and pathogens were inoculated together instantly. Likewise, the growth of *Curvularia* spp and *Bipolaris* spp were inhibited when the epiphytes and pathogens were inoculated instantly. The results of the present study have been previously confirmed by Elsherbiny *et al.* (2021), where *P. lilacinum* controlled orange green mould, which was caused by *P. digitatum*. There are studies of *P. lilacinus* demonstrating as a fungus causing biocontrol control effect on plant pathogenic fungi, although it has been widely exploited as cosmopolitan fungus mainly known for its nematophagous capacity (Moreno-Gavira *et al.*, 2020; Constantin *et al.*, 2022).

Non-pathogenic fungi exhibit various mechanisms in achieving its biocontrol control efficacy against phytopathogenic fungi. It was observed in the set-up that, both *A. flavus* and *P. lilacinum* inhibited the growth of *C. lunata* and *B. zeicola* in dual culture test with physical contact between both and this inhibition suggests that there is competition for nutrient as reported by Khan *et al.* (2023) and Ghorbanpour *et al.* (2018). *P. lilacinum* have been also reported to exhibit its biocontrol potential by producing fungistatic metabolites (Elsherbiny *et al.*, 2021), antagonism can occur between fungi without physical contact when one of the opponents produces diffusible

organic compounds, including bioactive metabolites, toxins and enzymes as well as the emission of volatile organic compounds such as alcohols, aldehydes, alkenes, acids, esters, ketones, terpenes, benzenoids and pyrazines. Chemical signaling by these volatile organic compounds (VOCs) plays a major role in fungal recognition systems and antagonistic effects (Elsherbiny *et al.* 2020; Roy & Banerjee, 2019; Tiwari *et al.*, 2020). Not forgetting, *Aspergillus* species produces many life-threatening biotoxins which include aflatoxins. Aflatoxins are the most poisonous mycotoxins, and mainly produced by *A. flavus* and *A. aspergillus* making these species opportunistic pathogens in humans and animals by causing invasive and non-invasive aspergillosis (Kondampati & Srinu, 2023; Navale *et al.*, 2021). For this reason, *A. flavus* should be used with caution.

Both *Azadirachta indica* and *Nerium oleander* effectively inhibited *Curvularia* spp. and *B. zeicola*. *A. indica* and *N. oleander* inhibited the mycelia growth of *Curvularia* spp. and *B. zeicola* and subsequently on whole plant, *A. indica* and *N. oleander* reduced *Curvularia* spp. and *B. zeicola* brown leaf spot incidence and severity on rice seedlings. As observed in the present study, earlier workers have reported about the efficacy of different plant products for the disease management and enhancement of crop yields (Borges *et al.*, 2018; Varma & Saran, 2019), and more specifically, *A. indica* (Rana *et al.*, 2023; Maleki *et al.*, 2017) and *N. oleander* (Mouhcine *et al.*, 2019; Dardona & Shahabuddin, 2022) have also been reported. Present findings are in agreement with these earlier studies. According to Marchiosi *et al.* (2020) plants synthesize aromatic secondary metabolites, like phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins which are fungitoxic and inhibits growth of fungal pathogens. Extracts from *A. indica* (neem plant) have been shown to be toxic to fungal pathogens (Makhuvele *et al.*, 2020), such as *Poria monticolad* causing heart rot of

Picea sitchensis (Broda, 2020), *P. oryzae* infecting rice plant in field and the harvested rice (Agbowuro *et al.*, 2020), against *Curvularia lunata* (Ameh *et al.*, 2021), and against *B. zeicola* (Singh *et al.*, 2016). *Nerium oleander* was reported for its antibacterial, antifungal, antidiabetic, antioxidant, antitumor and hepatoprotective activities (Hase *et al.*, 2017; Bakir-Cilesizoglu *et al.*, 2022; Dey & Chaudhuri, 2016). Investigations on disease suppression by plant products suggest that the bioactive compounds or chemicals present in them either act on the pathogen directly, or induce systemic resistance in host plants (Liu *et al.*, 2018).

Chapter Six: Conclusion And Recommendation

6.1 Conclusion

A total of twenty-one pathogenic fungal isolates including eleven *Bipolaris* spp. (one *B. oryzae* and ten *B. zeicola*) and ten *Curvularia* spp. (including three *C. lunata*, two *C. senegalensis*, one each of *C. platzii*, *C. tribuli*, *C. soli*, *C. subpapendorfi* and *C. clavata*) were obtained from brown leaf spot infected rice leaves sampled from some low land farms at atwima Nwabiagya District of Ghana through the combination of and molecular technique.

After the artificial inoculation, Koch's postulate proved that *B. oryzae*, *B. zeicola*, *C. clavata*, *C. lunata*, *C. soli*, *C. senegalensis*, *C. platzii*, *C. subpapendorfi* and *C. geniculata* were able to develop similar brown leaf spot symptoms observed on rice in the field conditions. The pathogenicity also proved variability among the isolates, in the sense that *C. lunata* was highly virulent and *C. clavata* was less aggressive among the *Curvularia* spp. and among the *Bipolaris* spp., some isolates of *B. zeicola* was as virulent as *B. oryzae*, while some shown less aggressiveness.

The result indicated also that *Curvularia lunata* and *Bipolaris zeicola* were effectively controlled by two each of botanicals (*A. indica* and *N. oleander*) and epiphytes (*P. lilacinum* and *A. flavus*). The incubation period, incidence and severity of brown leaf spot of rice caused by *B. zeicola* and *C. lunata* was affected when the epiphytes or the botanicals was inoculated before the pathogen, together with the pathogen and after the pathogen in 5 days intervals.

6.2 Recommendations

Molecular identification used in this study helped in unveiling the different species of *Biplaris* and *Curvularia* genera working in complexity in causing brown leaf spot disease on rice plants, and therefore molecular identification is recommended to be used in terms of phytopathogenic identification.

So far as extracts of *Azadirachta indica* and *Nerium oleander* as well as application of *Purpureocillium lilacinum* and *Aspergillus flavus* proved excellence efficacy against *B.oryzae* and *C. lunata* brown leaf spot disease of rice, future studies should focus on repeating this study in different locations in other to ascertain the current findings and subsequently, the involved biological controlled agents should be recommended for use.

The existence of pathogenic variations among *Curvularia* species causing brown leaf spot disease in rice is worrisome and therefore the information generated and presented in this study should guide future development and selection of any management strategy.