Characterisation and *in-vitro* Control of *Curvularia lunata*, the Causal Agent of Brown Leaf Spot Disease of Rice in Ghana.

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Abstract

Irrigated rice production is common in the coastal savannah zone of Ghana. Recent observations have shown the incidence of a new leaf spot disease in some rice basins in the area. Research was carried out to determine the incidence and severity of the disease in the area, identify the causal agent using its cultural and morphological features, complemented with phylogenetic studies of the internal transcribed spacer region. Also, the effect of some selected fungicides and an antagonistic *Trichoderma harzianum* on the mycelial growth of the fungus was determined *in vitro*. The disease was found in all the major rice growing districts in the coastal savannah zone with severities ranging from 22.6 to 50.9%. *Curvularia lunata* was identified as the causal agent of the disease. Carbendazim, Nordox and Mancozeb, totally depressed the mycelial growth of the fungus whiles T. *harzianum* inhibited the growth of the fungus. These fungicides and antagonistic organism have the potential for the control of the disease in the field.

Caractérisation et contrôle in-vitro de *Curvularia lunata*, l'agent causal d'un *Brown*

Résumé

La production de riz irrigué est courante dans la zone de savane côtière du Ghana. Des observations récentes ont montré l'incidence d'une nouvelle maladie des taches foliaires dans certains bassins rizicoles de la région. Des recherches ont été menées pour déterminer l'incidence et la gravité de la maladie dans la région, identifier l'agent causal en utilisant ses caractéristiques culturelles et morphologiques, complétées par des études phylogénétiques de la région d'espaceur transcrit interne. L'effet de certains fongicides sélectionnés et d'un Trichoderma harzianum antagoniste sur la croissance mycélienne du champignon a également été déterminé in vitro. La maladie a été trouvée dans tous les principaux districts rizicoles de la zone de savane côtière avec des degrés de gravité allant de 22,6 à 50,9 %. *Curvularia lunata* a été identifié comme l'agent causal de la maladie. Le Carbendazim, le Nordox et le Mancozeb ont totalement déprimé la

croissance mycélienne du champignon tandis que T. harzianum a inhibé la croissance du champignon. Ces fongicides et organismes antagonistes ont le potentiel pour le contrôle de la maladie sur le terrain.

Mots clés: *Curvularia lunata*, tache foliaire brune; *Oryza sativa*; Phylogénétique ; sensibilité aux fongicides, effet antagoniste.

Introduction

Rice has been described as the most important human food for people (Zibaee, 2013). The crop provides 21% of global human per capita energy and 15% of per capita protein (Gnanamanickam, 2009). Currently, almost half of the world's population rely on rice every day since it's the staple food across Asia where around half of the world's poorest people live. Rice is becoming increasingly important in Africa and in America. As of 2018, two countries, namely China and India, together contributed more than half of the world total rice production (FAOSTAT, 2018).

In Ghana**,** rice has become the second most important food staple after maize and its consumption keeps increasing. These have been attributed to population growth, urbanization and change in consumer habits (Essabrah-Mensah, 2018). The main rice growing areas in the country include the Volta, Northern, Upper East and Upper West Regions. In total, land cultivated to rice is around 239, 340 hectares. The country is not sufficient in rice production. Currently, consumption outstrips domestic production between 66-70%. The country therefore spends huge amounts of foreign exchange to import rice from Thailand, Vietnam and India. The government of Ghana plans to make the country self-sufficient in rice production by 2025. Under the Planting for Food and Jobs project, the government has invested heavily in the sector in some selected 122 districts of the country to increase outputs in rice production (PFJ, 2017).

One major factor that could derail the country's sufficiency in rice production is the incidence of destructive diseases. Over the years, several diseases have been reported affecting rice in Ghana (Oduro, 2000; Offei *et al*., 2008). Blast disease ascribed to *Magnaporthe oryzae*, has been identified as the major fungal disease of rice (Oduro, 2000). Nutsugah *et al* (2003) had reported that brown leaf spot caused by *Bipolaris oryzae*, is an emerging threat to lowland rainfed rice production system in the Northern Region of Ghana. In Ghana, very damaging disease epidemics of rice are rare. This could be attributed to the rapidity in which farmers change cultivars of the crop and their heavy investment into the purchase and use of fungicides. Currently, one rice cultivar gaining popularity in the Irrigated rice ecologies of the Eastern and Volta Regions of Ghana is the Legon 1, previously known as the Ex-Baika. Until recently, incidence of foliar diseases on the Legon 1 variety was rare and in few occasions, lesions, initially thought to be blast symptoms, were found in some rice basins.

Currently, observations have shown that the production of rice in general and the Legon 1 variety in particular is under threat by an unknown disease. Symptoms of this new disease were similar to those reported for brown leaf spot caused by *B . oryzae* (Nutsugah *et al* (2003). However, the disease appears to be severer and more destructive than what was known for *B. oryzae*. Information also indicated that the disease may be present in farmer fields in the irrigated

rice ecologies of the coastal savannah regions of Ghana, where most farmers have recently adopted the Legon 1 rice variety due to its intense aromatic and tastier nature. The disease is new in Ghana and there is therefore very little information about its aetiology and prevalence. However, formulation of a good control measure against any newly introduced disease will rely on useful information such as the identity of the causal agent, its susceptibility to disease control agents and epidemiology of the disease. This research work was therefore carried out to determine the extent of spread of the disease in the irrigated rice growing areas of the country, identify the causal agent of the disease and determine its sensitivity to some of the major fungicides available on the Ghanaian market.

MATERIALS AND METHODS
EXPERIMENTAL SITES

Initial work to study the nature of the disease and collect disease samples for isolation of causal agent was carried out at the Soil and Irrigation Research Centre of the University of Ghana, Kpong (6 09'N, 00 04'E) in the Eastern Region of Ghana. The soil type in the centre is classified as Calcic Vertisol, Typic Calciustert or tropical black clay of the Akuse series (FAO/UNESCO, 1999). The shores of the Centre are bathed by the Volta lake which serves as a source of fresh water for all-year round cultivation of rice. Farmer field survey was carried out at Kpong and Akuse in the Eastern, Asutuare in the Greater Accra Region and Afife in the Volta Region of Ghana (Table 1). All farms selected were engaged in Irrigated rice production.

Determination of disease incidence and severity

Field survey for the determination of the disease incidence and severity of the new rice disease was carried out between June 2021 and January 2022. In each selected district, 10 rice basins, each of at least 1 acre belonging to different farmers, were selected at random and rice plants observed visually for the presence of the disease symptoms. The number of basins with at least one rice plant showing the disease symptoms were recorded and expressed as a percentage of the total number of basins inspected to obtain the disease incidence. Disease severity was determined on 5 tillers in the middle of the plot by rating the percentage of the leaf area covered by the leaf spots on a scale of 0-9, where 0=no disease, 1=less than 1% of leaf area affected; 2=1-3% of leaf area affected; 3=4-5% of leaf area affected; 4=6-10% of leaf area affected; 5=11-15% of leaf area affected; 6=16-25% of leaf area affected; 7=26-50% of leaf area affected; 8=51-75% of leaf area affected; 9=76-100% of leaf area affected

Table 1: Administrative regions and districts of the study area

Locality	District	Region	Ecology
Afife	Ketu North	Volta	Irrigated
Akuse	Lower Manya Krobo	Eastern	Irrigated
Asutuare	Shai-Osudoku	Greater Accra	Irrigated
Kpong	Lower Manya Krobo	Eastern	Irrigated
SIREC	Lower Manya Krobo	Eastern	Irrigated

SIREC-Soil and Irrigation Research Centre

(IRRI, 2002). The ratings were converted to percentages using the formula:

$$DSI = \frac{SNR}{N+M} * 100, \text{ where:}$$

DSI=disease severity index,
SNR=sum of all numerical ratings
N=number of samples observed
M=maximum rating

Isolation and identification of causal agent
Leaves of rice plants showing symptoms of the disease were collected from rice basins and sent to the Plant Pathology laboratory of the Crop Science Department, University of Ghana at Legon, for isolation of causal agents of the disease. The disease symptoms were characterised by brown spots on the leaf blade and on the small emerging leaves. The spots were initially brown small, round to ovoid with a chlorotic halo, evenly distributed over the leaf. The size of spots varied from 0.1 cm to 1 cm. Some of the spots had merged creating larger spots (Fig. 1).

Isolation of causal agents was first done on water agar (20 g L^{-1}; Oxoid, Basingstoke, UK) and then on potato dextrose agar (PDA, 39 g L^{-1}; Oxoid). Pieces of plant tissues taken from advancing edge of the lesion on the leaves and stem were surface sterilised with sodium hypochlorite for 15 sec., washed in sterile distilled water and blotted dry using a paper towel. Sterilized tissues were plated singly on water agar plates and incubated for seven days. The growth were then sub-cultured on PDA and incubated for another 7 days to obtain pure cultures

Pathogenicity test of isolates
Pathogenicity test of isolates was carried out on the foliage of 3-week-old rice seedlings (var. Legon 1). A spore suspension (1 x 10^5 conidia/ml) of each of the selected fungus was prepared by blending mycelium of the fungus and filtered through cheese cloth. The rice seedlings were inoculated by spraying a suspension of the spores on the leaves. The control plants were sprayed with sterile distilled water. All inoculated seedlings were covered with clear plastic bags and maintained in a greenhouse overnight for 16 h at $28 \pm 2°C$ to increase humidity in the foliage for infection to take place, after which the plastic bags were removed. The seedlings were observed daily till symptoms of the disease were observed. A fungus was considered pathogenic if it was able to cause the disease. To complete Koch's postulates, the fungus was re-isolated from the artificially inoculated leaves.

Cultural, morphological and molecular identification of isolates
Five of the fungal isolates that were pathogenic to the rice plants were selected at random and a plug of mycelium of each isolate was placed in the middle of a PDA plate and incubated for 7 days. The nature of mycelium of each isolate was recorded to aid in their identification. After that, hyphae and spores were fixed on a slide and observed under the microscope. The nature of the hyphae and spores were also recorded to further aid in identification of each isolate.

Figure 1: Symptoms of the ne rice disease in a rice basin at SIREC

DNA extraction

The five isolates selected at random were cultured on PDA for 7 days after which the mycelia were harvested for DNA extraction. DNA was extracted using the Sigma's GenFlute Plant Genomic DNA Miniprep Kit (St. Louis, MO, USA), following the manufacturer's instructions.

Polymerase chain reaction (PCR)

DNA extracted was used as templates in PCR. The PCR were carried out using the primer pair, ITS1/ITS4, to amplify the entire internal transcribed spacer region (White et al., 1990). The PCR reaction mixture was made up of 2 µl target DNA, 5 µl of 10X PCR buffer (Invitrogen, Carlsbad, CA), 2.5 µl of deoxynucleoside-triphosphate mix (2.5 mM each), 0.25 µl bovine serum albumin (20 mg/ml), 2 µl each of the forward and reverse primer, 1.8 µl of magnesium chloride (50 mM) and 0.2 µl of taq polymerase (Invitrogen, Carlsbad, CA) added to 34.25 µl of double distilled water. Each PCR was performed in a total reaction volume of 50 µl. The reaction was carried out in a Thermo Hybaid PXE Thermal Cycler (Thermo Electron Corporation, USA). The reaction cycles were denaturing for 2 min at 94°C followed by 35 cycles of 1min at 94°C, 1 min at 55°C, 2 min at 72°C and a final extension of 10 min at 72°C. Amplification products were separated by 1.5% w/v agarose gel (Invitrogen, Carlsbad, CA), stained with Ethidium bromide or gel red alongside 1.0 kb marker at 100 V for about 1.5 hours. Bands were observed under UV light and Polaroid photographs taken using the Gene Flash Documentation System (Snygene Bio Imaging).

Sequencing of amplified products

The PCR amplified product of the ITS region of isolates were sent to Inqaba Biotech Laboratory at South Africa for purification and sequencing. Ten picomole of each primer was used to sequence the product from both directions. Sequences were entered into the BIOEDIT software and edited. The sequences of the forward and reverse strands of each isolate were built into a consensus strand.

Phylogenetic analysis of the ITS region.

The ITS sequences of the 5 isolates obtained from the rice plants in the study (Table 2) and 25 sequences of isolates of different *Curvularia* species retrieved from European Molecular Biological Laboratory (EMBL) nucleotide sequence database (Table 3) were used in a phylogenetic study. Among the retrieved sequences were those of *Colletotricum siamense* which was used as the out-group. Multiple sequence alignment of the selected isolates was through ClustalW, and phylogenetic analysis was performed

Table 2: Isolates obtained from rice plants and used in the phylogenetic study and their GenBank accession numbers

Fungal isolates	Host	Country	Accession numbers (ITS)
Amankwa1	*Oryza sativa*	Ghana	Mt259783
Amankwa2	*Oryza sativa*	Ghana	MT259784
Amankwa3	*Oryza sativa*	Ghana	MT259785
Amankwa4	*Oryza sativa*	Ghana	MT259786
Amankwa5	*Oryza sativa*	Ghana	MT259787

Table 3: Isolates of *Curvularia* species downloaded from the EMBL database and used in the study and their GenBank accession numbers

Species	Strain identification	Host	Country	Accession numbers (ITS)
C. aeria	CBS 294.61	Air	Brazil	He861850
C. aeria	FMR 11667	Blood	USA	KP131931.1
C. aeria	AS4	-	Sri-Lankan	MN999546.1
C. americana	UTHSC	Homo sapien	Spain	NR_146239.1
C. americana	UTHSC 10-1276	Homo sapien	Spain	HG779020.1
C. americana	UTHSC 09-2863	Homo sapiens	Spain	HG779019.1
C. australiensis	BRIP 19588a	*Chloris gayana*	Australia	Kc424613
C. australiensis	IMI 53994	*Oryza sativa*	Australia	Kc424595
C. australiensis	FC2AP	*Aegle marmelos*	India	Kr363626
C. coicis	CBS 192.29	-	Japan	NR_147457.1
C. coicis	ZJ13	Coix Lachryma-jobi	China	KJ572136.1
C. coicis	CBS 126978	-	Netherlands	MH864368.1
C. geniculata	CDKVR02	*Cynodon dactylon*	India	KP666183.1
C. geniculata	EAN403	-	Malaysia	MK518444.1
C. lunata	CBS 730.96	*Bouteloua dactyloides*	Ex-type	NR_138223.1
C. lunata	B2836	Soil	Malaysia	MK204512.1
C. oryzae	CBS 169.53	Ex-type	-	NR_138221.1
C. oryzae	2715	*Espeletia sp.*	Colombia	EU272519.1
C. oryzae	C104	*Glycine max*	Brazil	JQ936251.1
C. pseudobrachyspora	CPC 28808	*Eleucine indica*	Thailand	NR_164423.1
C. pseudobrachyspora	HNWN001	*Arecha catechu*	China	MH516132.1
C. senegalensis	PE-39	*Passiflora edulis*	Brazil	MK804485.1
Curvularia senegalensis	JUF0019	*Aloe Vera*	Bangladesh	MH368101.1
Curvularia senegalensis	SC5.2	*Saccharum sp.*	India	MH087110.1
C. siamense	MAN-GH19	*Mangifera indica*	Ghana	KJ019351.1

using MEGA5 (Tamura *et al*., 2011). The Neighbor-Joining method (Saitou and Nei, 1987) was used to infer the evolutionary history. The percentage of replicate trees in which the associated taxa clustered together was evaluated with a bootstrap analysis with 1000 replicates. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Clustering of isolates was used to infer the species of the isolates obtained in this study.

Fungicide sensitivity test
Single recommended rates of four fungicides (Table 4) commonly used by rice farmers in the study area were evaluated for their *in-vitro* effect on the mycelial growth of the causal agent of the disease. The poisoned food technique was used for the evaluation. Each fungicide was weighed into conical flasks containing molten PDA (39 g L^{-1}). The mixture was swirled carefully to ensure

thorough mixing of the powder and liquid medium after which the amended media were poured into sterilised petri dishes to set. Mycelial plug of each isolate selected was picked and placed in the middle of each plate.The inoculated plates were labeled and incubated under ambient conditions of 23-25ºC and 65±5% R.H for 7 days. Unamended PDA served as control. The diameter of the mycelium of the organism was measured daily, beginning from the second day after incubation. The diameter obtained was used to calculate percentage inhibition of the mycelial growth of the organism using the formula;

$$\text{Percentage inhibition} = \frac{T_0 - T_i}{T_0} * 100, \text{ where:}$$

T_0 = diameter of mycelium on unamended PDA
T_i = diameter of mycelium on amended plates
Data on percentage inhibition was arcsine transformed and subjected to analysis of variance and means separated using LSD at 5%.

Antagonostic effect of *Trichoderma harzianum* on *C. lunata*.
An isolate of *Trichoderma harzianum* isolated from a fallow field in Ghana, was

Table 4: Fungicides and their recommended rates used in the in-vitro control of *C. lunata* obtained from rice plants

Name	Active ingredient	Use	Rate of application
Nordox	750 g/kg Cuprous oxide	Synthetic fungicide	1.25 g/l
Mancozeb	800 g/kg Mancozeb	Synthetic fungicide	2 g/l
Carbendazim	500 g/kg Carbendazim	Synthetic fungicide	2 g/l
Ex-icute	51% plant oils	Organic fungcide	5 ml/l
Yellow gold	Bamboo-vinegar distillate	Organic fungicide	3 ml/l
Kobe	Chrysophenol parietin	Bio-fungicide	15 ml/l
Levo	50% Sophora flavescent plant extract	Botanical insecticide	15 ml/l

obtained from the Plant Pathology laboratory of the Crop Science Department, University of Ghana and maintained on PDA plates. The dual culture method was employed to determine effect of the fungal antagonist against the mycelial growth of *C. lunata.* Mycelial plugs (5 mm diameter) of *T. harzianum and C. lunata* were placed on opposite directions in 9 mm Petri dish at 6 cm from each other. The paired cultures were incubated at 25 °C. Petri dishes inoculated only with the test pathogen served as control (Zivkovic *et al.*, 2010). Each treatment was replicated three times and the set up was laid out using a completely randomized design (CRD).

The percent growth inhibition (PGI) of the pathogen was calculated using the formula:

$$PGI = \frac{CR - CT}{CR} * 100$$

where *CR* represents the distance (measured in mm) from the point of inoculation to the colony margin on the control dishes, and CT, the distance of fungal growth from the point of inoculation to the colony margin on the treated dishes in the direction of the antagonist (Korsten and De Jager, 1995).

Data Analysis
Data on the percentage growth inhibition of the pathogen were arcsine transformed and subjected to analysis of variance using the Genstat software. The means were separated using LSD at 5%.

Results
Disease incidence and severity
The disease was found in all the areas surveyed during the period of the study. At least one rice plant per basin was found with the disease symptom, resulting in a 100% disease incidence in all the areas included in the survey (Table 5). Also, the disease severity ranged from 22.6% at Afife to 55.3% in

Table 5. Disease incidence and severity of the new disease in the study area

Locality	Incidence (%)	Severity (%)($\pi\pm$se)
Afife	100.0	22.6±2.0
Akuse	100.0	54.4±0.7
Asutuare	100.0	47.1±0.7
Kpong	100.0	55.3±0.5
SIREC	100.0	50.9±0.7

Kpong (Table 5).

Cultural and morphological features of the causal agent
A fungus was consistently isolated and the fungal colonies grew up to 7 cm (diameter) when the tissue was incubated on PDA at 25°C in the dark for 7 days. The colonies were black with velvety texture and smooth margins (Fig, 2A). Hyphae were dark in colour (2B). Conidiophores were brown, unbranched, septate, and geniculate at the apical region. Conidia were straight to pyriform in shape, smooth-walled, transversely septate with mostly three septa. Conidia, 15.4 to 22 × 8.8 to 11 (average 18.8 × 9.9 μm, n = 50) in size, were produced apically in a sympodial mode and pale brown to brown in colour with the middle two cells being darker than the end cells (Fig. 2C). The conidia were of the dimensions 15.5 to 25.5 × 8.0 to 13.0 μm. The cultural and morphological characteristics of the isolated fungus suggest it belonged to the genus *Curvularia*.

Molecular characterisation of isolates.
Sequences and phylogenetic studies of the ITS region.
An approximately 600 bp product of the ITS region was amplified using the primer pair ITS1/ITS4 from the isolates. The assembled sequences were 568 bp long. An approximately 600 bp product of the ITS
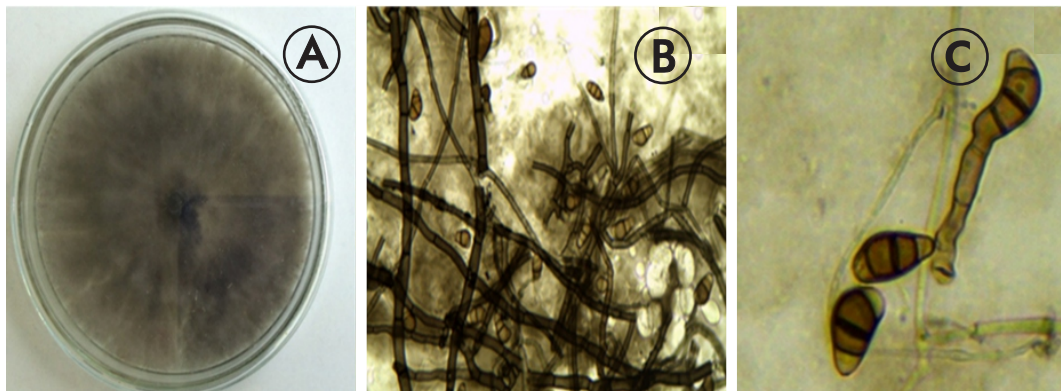
Figure 2: Cultural and morphological characteristics of *Curvularia lunata* isolated from the diseased rice leaves. A=mycelial growth on PDA, B=dark hyphae with few conidia, C=conidia showing septations (Mgx400)

region from isolates collected in the study and those retrieved from the GenBank (Table 2) were aligned and used in phylogenetic study. The optimal tree with the sum of branch length of 0.60982473 (Fig/ 3). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). There were a total of 419 positions in the final dataset. Out of the 30 Curvularia species used in the study, all 5 isolates obtained in this study clustered together in the *Curvularia lunata* clade. The clade includes the type strain of *C. lunata* and another *C. lunata* strain whose identity has been confirmed. The clade is supported by a high bootstrap support of 99% (Fig. 3). This showed that the isolates obtained from the diseased rice belong to the *Curvularia lunata* species. The other isolates clustered in clades corresponding to their original species.

Pathogenicity test

All isolates used in the pathogenicity test were able to induce the brown leaf spot disease symptoms on rice seedlings. The symptoms initially began as tiny transparent spots which later became transparent. As the symptoms aged, they enlarged and became dark in colour. These spots were absent on the control seedlings. The isolates were re-isolated from the symptoms on the artificially inoculated seedlings.

Fungicide sensitivity test

Significant difference ($p>0.05$) in the percentage inhibition of the mycelial growth of the fungus by the different fungicides, was recorded from day 1 to day 7 after incubation (Table 6 and Fig. 4). A day after incubation, the highest percentage inhibition of 100% was recorded on media amended with Nordox, Mancozeb, Carbendazim, Ex-icute and Levo, while the lowest was recorded on media amended with Bamboo distillate. The percentage inhibition on media amended with Kobe was higher than what was obtained on media amended with bamboo distillate. At 7 days after inoculation, the highest inhibition (100 %) was obtained on media amended with Nordox, Mancozeb, Carbendazim while the lowest was obtained on media bamboo distillate amended media. During this period, Ex-icute amended media resulted in the highest inhibition of the fungus, among the different organic fungicides followed by Levo and Kobe (Table 6 and Fig. 4)

Antagonistic effect of *Trichoderma harzianum* against *C. lunata*

Antagonism between *T. harzianum* and *C. lunata* was observed on the third day of incubation (Fig. 4). During this period, mycelium of *T. harzianum,* which was initially white, grew rapidly toward the mycelium of *C. lunata*. A zone of inhibition, in which neither of the two mycelia grew, was formed just before the two mycelia touched each other (Fig.4C). From day 4 after incubation, the mycelium of *T. harzianum* continued to grow almost around the *C.*

lunata, eventually, preventing further expansion of the mycelium of the pathogen by the 7th day of incubation (Fig. 4F).

Data obtained showed that there was no inhibition during the first three days of incubation when mycelia of the two fungi had not interacted. The inhibition of mycelial growth of *C. lunata* by the fungal antagonist as recorded from day 4 to 7 were 21%, 30%, 41% and 50% respectively (Fig. 5) Similar results were obtained after repeating the experiments.
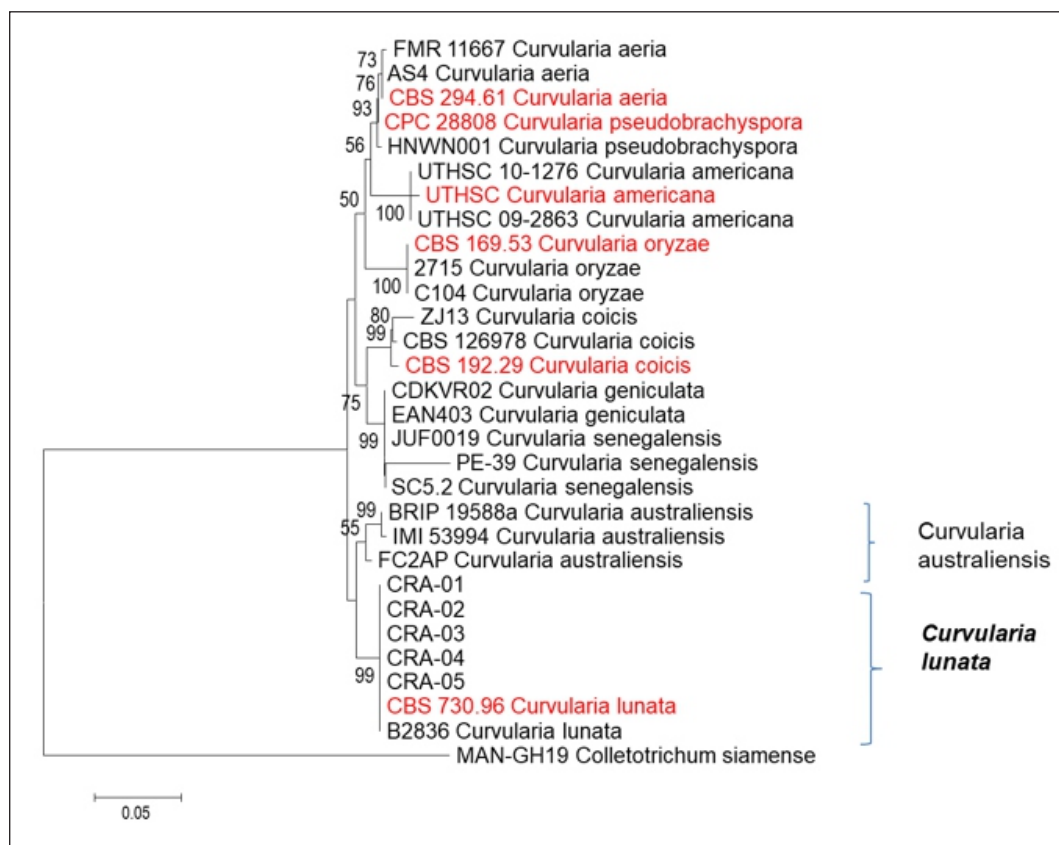

Figure 3: A phylogram drawn with the multiple sequence alignment generated with the rDNA-ITS sequences of the 30 isolates of *Curvularia* species used in the study. *C. siamense* was used as an out group. Isolate designation and species name has been listed. Type strains have been indicated in red

Table 6: Effect of different fungicides on radial mycelial growth of *Curvularia lunata* on amended PDA incubated at 24-28C and 52-65% RH. (Values are % reduction in radial growth compared to the control)

| Treatment	Days after incubation/Inhibition (%)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Nordox	100.0[c]	100.0[d]	100.0[d]	100.0[d]	100.0[d]	100.0[e]	100.0[d]
Mancozeb	100.0[c]	100.0[d]	100.0[d]	100.0[d]	100.0[d]	100.0[e]	100.0[d]
Carbendazim	100.0[c]	100.0[d]	100.0[d]	100.0[d]	100.0[d]	100.0[e]	100.0[d]
Bamboo (3.0ml/L)	28.3[a]	26.7[a]	25.0[a]	11.7[a]	11.7[a]	10.0[a]	10.0[a]
Ex-icute (5.0ml/L)	100.0[c]	100.0[d]	100.0[d]	100.0[d]	100.0[d]	100.0[d]	100.0[d]
Kobe (15.0ml/L)	40.7[b]	50.0[b]	55.0[b]	53.3[b]	55.0[b]	53.0[b]	50.0[b]
Levo (15.0ml/L)	100.0[c]	90.0[c]	85.0[c]	71.7[c]	73.7[c]	70.0[c]	60.0[c]
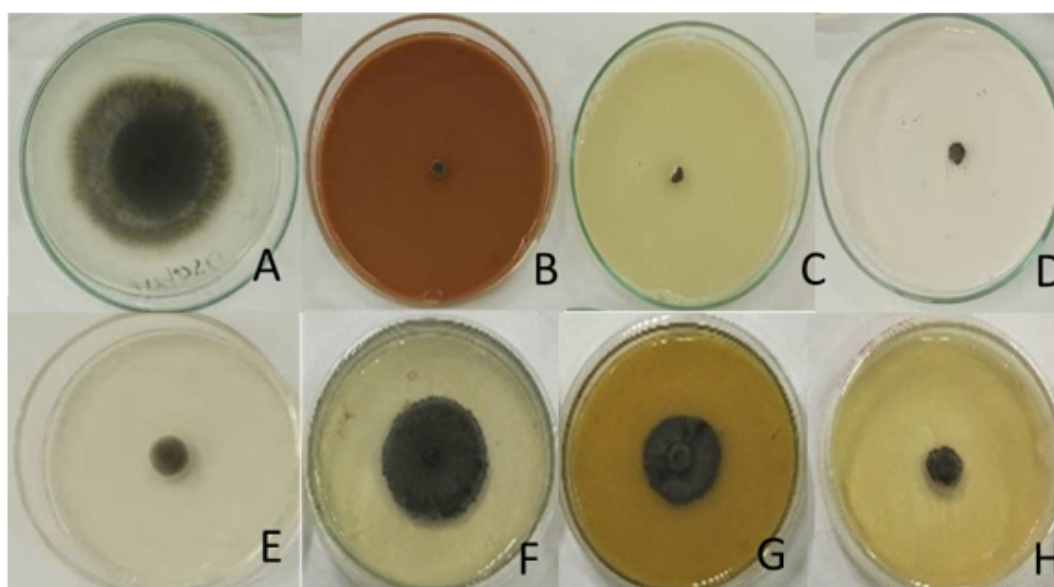
Figure 4: Diameter of mycelial growth of *C. lunata* on amended PDA amended with different fungicides. A=unamended PDA, B=Nordox, C=Mancozeb. D=Carbendazim, E=Ex-icute, F=Bamboo distillate, G=Kobe; and H=Levo
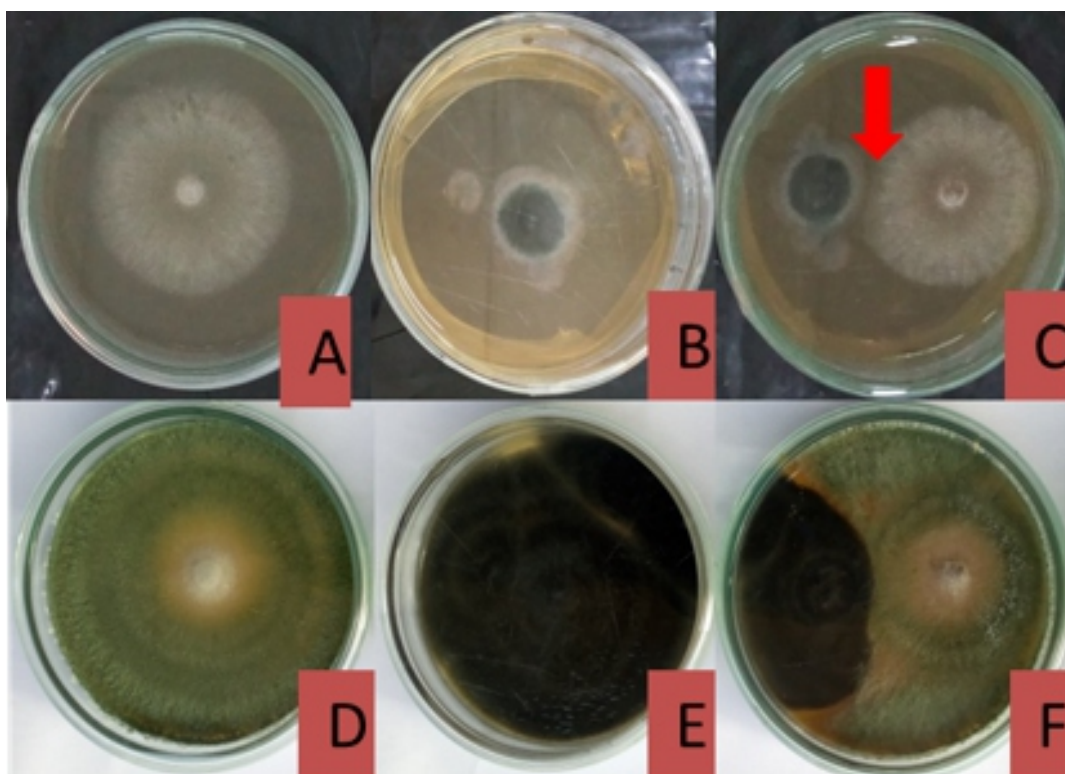
Figure 4: Antagonistic effect of *T. harzianum* on *C. lunata*, the causal agent of brown leaf spot of rice in Ghana. A and B=mycelial growth of *T. harzianum* and *C. lunata* at 3 days after incubation separately, C=mycelial growth of *C. lunata* (left) and *T. harzianum* (right) at 3 days after incubation in the same plate, C and D=mycelial growth of *T. harzianum* and C. lunata respectively at 7 days after incubation separately, F=mycelial growth of *T. harzianum* and *C. lunata* at 7 days after incubation in the same plate**.** Arrow shows point of antagonism between the two fungal species at 3 days of incubation
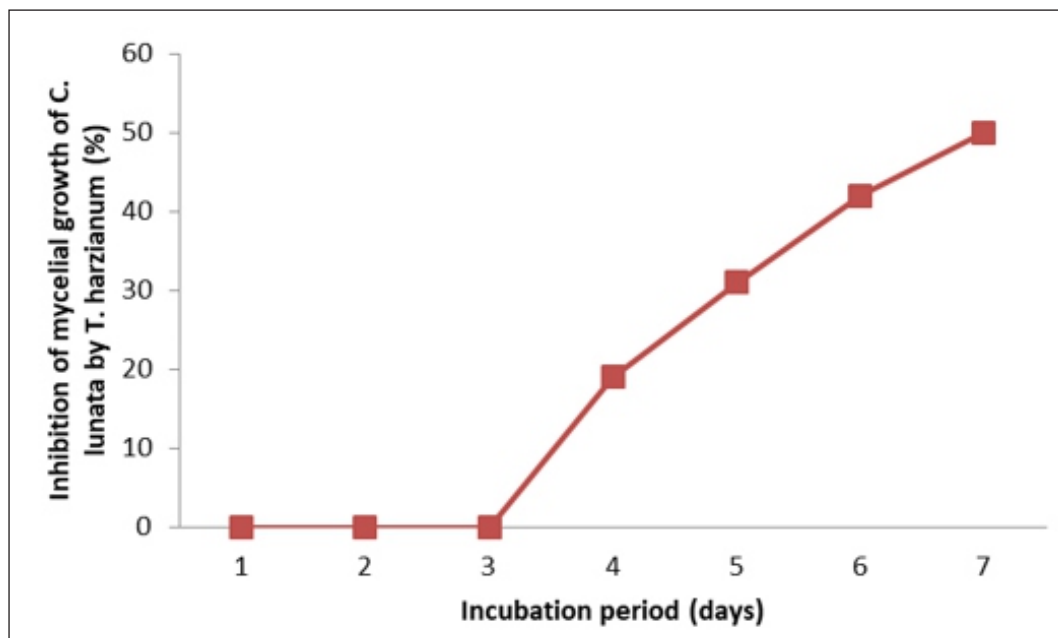
Figure 5: Inhibitory effect of *Trichoderma harzianum* against mycelial growth of *Curvularia lunata* cultured on potato dextrose agar of incubation at 23 to 31°C and 60 to 70% RH

Discussion

Blast caused by *Magnaporthe oyzae* is the most important disease of rice in irrigated rice producing areas of the Coastal Savannah Zone of Ghana. Farmers in these areas appeared to have the disease under control using fairly resistant varieties and seed treatment. Currently, a new foliar disease, with brown leaf spot symptoms, is devastating rice fields in the area. Brown leaf spot, ascribed to *Bipolaris oryzae*, had been reported in Ghana and described as a potential threat to rice cultivation in the upland rice production in other parts of the country (Nutsugah *et al.*, 2003). The disease, however, has not been reported in the irrigated rice production areas. Questions have been raised as to whether the unknown disease, destroying rice fields in the irrigated rice production areas, currently, was the same as the brown leaf spot, reported in the upland rice production areas of the country.

The new disease appeared to present in most of the irrigated rice ecologies of the coastal savannah zone of Ghana and were found in all the areas surveyed in the study. The disease severity in the different localities of the rice production was almost equal. One reason which could be ascribed to the prevalence of the disease in the study area, is the inclusion of the Legon1 (Ex-Baika) rice. This variety appeared to be very susceptible to the disease and everywhere the variety was cultivated it was found with the disease. The sudden appearance of the disease within farmers' fields coincided with the period during which the variety became widespread and popular in these areas. Therefore, the suggestion that the variety could be responsible for the spread of the disease in the survey areas could have some substance.

Results from this study showed that *C. lunata* is the causal agent of the new brown leaf spot disease in the irrigated rice production areas. In fact, this is the first report of any *Curvularia* species being associated with a plant disease in Ghana. However, several species of *Curvularia* have been associated with members of the grass family worldwide. For example, *C. lunata* had been associated with disease of rice in India (Kamaludden and Abhilasha, 2013) and Pakistan (Majeed *et al.*, 2015) while *C. australiensis* had been reported on maize in China (Chang *et al.*, 2013). In this study, the fungus was consistently associated with the disease symptoms observed on the rice leaves. Cultural and spore morphology of *C. lunata* obtained in this study, particularly, the larger, darker, curved, second cell, of the conidium has been described as a characteristic of the fungus. The fungus was able to cause the same disease symptoms on artificially inoculated rice seedlings, confirming its pathogenicity. The pathogen, *C. lunata*, has been associated with the disease in other rice growing areas of the world (Majeed *et al.*, 2015 and Kamaludden and Abhilasha, 2013), thereby giving credence to the findings in this work.

Though cultural and morphological characteristics such as conidial morphology are not entirely unreliable, they can be affected by the environment, and may not be able to distinguish among cryptic species (Bickford *et al.*, 2007; Slepecky and Starmer, 2009). Therefore they need to be complemented with molecular methods for proper identification of unknowns (Honger *et al.*, 2014; Honger *et al.*, 2017). In this study, the sequence analysis of the ITS region was included in the study to identify the *Curvularia* isolates obtained. The ITS region has been used severally as a bar code for the identification of fungal species from different taxonomic groupings and has been found to be reliable (Honger *et al.*, 2015). The

phylogram generated with the sequences clearly placed all the isolates from Ghana in the *C. lunata* clade. The clade contains the type strain of *C. lunata* and is supported by a high bootstrap value. These show beyond all reasonable doubt that the isolates were *C. lunata*. The disease could therefore be described as *Curvularia* brown leaf spot to distinguish it from the brown leaf spot caused by *Bipolaris oryzae.* The identification of the disease in Ghana was an indication that the disease which was first identified in France (Bugnicourt, 1950) is now in the West Coast of Africa

The disease was very destructive in the study area. Affected plant parts were necrotic and in severe cases, resulted in death of the entire leaf. Affected plants remained stunted and in most basins, entire tillers showed the symptom. The disease was not restricted to the rice basins as it was also found in the nursery, where in some cases the entire nursery seedlings became yellow and weak. In such instances, heavily affected seedlings will perform poorly and may not even survive in the field. The observed symptoms were similar to that of blast, the most destructive rice seedlings wherever the crop is grown (TeeBeest *et al.*, 2012). Secondly, brown leaf spot of rice caused by *B. oryzae*, whose symptoms are similar to those of the disease under the current study, is also known to be destructive (IRRI, 1996; Motlagh & Kaviani, 2008; Harish *et al.*, 2008).Therefore, the potential of this new disease to also cause massive destruction cannot be overemphasised

The pathogen was found to be highly susceptible to fungicides evaluated. The screened inorganic fungicides *viz*, Mancozeb, Nordox and Carbendazim are readily available to farmers for the control of a wide range of diseases in crops in Ghana (EPA, 2015). These offer options for the control of

the Curvularia brown leaf spot disease, if chemical control is desired. Other fungicides, derived from plant sources such as Levo and Ex-icute, evaluated in the study also offer options for the control of the disease, in situations where synthetic fungicides are undesirable. Among these organic fungicides, Ex-icute was the best option. The fungicide, though from plant sources, was able to inhibit growth of the pathogen at higher percentages for longer period and matches the performance of the synthetic ones, more appreciably, than the other organic fung-icides. Ex-icute is available in commercial quantities and therefore, if field trials confirm its efficacy as well, then it can be an alternative to the inorganic fungicides for the control of the disease.

The use of fungal antagonistics, particularly, members of the *Trichoderma* genus, is gaining popularity in many parts of the world (Shahid *et al*., 2014). In this study, *T. harzianum* showed antagonistic effect to the pathogen, *C. lunata* and was able to inhibit the further growth and expansion of the pathogen. In a similar experiment, *T. asperellium* , inhibited the growth of *C. gloeosporioides* in vitro and the incidence of onion twister disease in the field in Ghana (Gyempeh, 2013). *Trichoderma harzianum* is therefore, a potential for the formulation of biofungicides for the control of the brown leaf spot disease caused by *C. lunata* in Ghana.

Conclusion
The results from this study have shown that the new rice disease is caused *by Curvularia lunata*, a pathogen which was being reported for the first time on rice in Ghana. The disease was prevalent in the irrigated rice ecologies of the Greater Accra, Eastern and Volta regions of Ghana. The disease could be very devastating as it stunts the growth of the rice plants and could lead to yield losses if left uncontrolled. Inorganic fungicides, namely

Nordox, Mancozeb and Carbendazim and an organic fungicide, Ex-icute which were highly toxic to the causal agent of the disease are potential chemicals for the control of the disease. *Trichoderma harzianum*, which was able to inhibit the growth of the pathogen, was also found to be a potential for the formulation of a biofungicide against the pathogen. There is however, the need for field work to be done to ascertain the effect of these fungicides and fungal antagonist on the disease incidence in the field.

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