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In vitro Evaluation of Commercial Fungicide Othello® (Azoxystrobin and Difenoconazole) Against *Cocos nucifera* and *Elaeis guineensis* Foliar Pathogens

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Abstract

Cocos nucifera L. and *Elaeis guineensis* Jacq. are important cash crops that provide a source of livelihood for millions of farmers in Africa. The seedling stages of these crops are highly susceptible to diseases, of which fungi leaf spots and blight rank high, which negatively impacts the development. As part of efforts to minimize the development of resistance against fungicides, we evaluated the efficacy of the commercial fungicide Othello®, which is composed of two different fungicide groups, Azoxystrobin and Difenoconazole, against oil palm and coconut foliar pathogens *in vitro*. The main aim of this study was to assess Othello® against *Bipolaris bicolor*, *Curvularia* sp., *Colletotrichum* sp., and *Neopestalotiopsis* species. Each pathogen was plated on PDA amended with different doses of the fungicide in a completely randomized design, with five replicates per treatment, and data were analyzed with an R statistical tool. The results from our research revealed that all tested concentrations of Othello® (2.5 µL per mL, 5 µL per mL, and 7.5 µL per mL) caused 100 % mycelial growth inhibition in all four pathogens compared to the control (unamended PDA plates) ($P < 0.01$) after 28 days of incubation. Our subsequent study showed that even the lowest concentration of Othello® (0.5 µL per mL) caused 100 % mycelial growth inhibition. The lowest dosage effect of the fungicide was more pronounced in *Neopestalotiopsis* sp. with 100 % inhibition while ≤ 80 % inhibition was recorded for *Bipolaris bicolor* and *Curvularia* species. This research lays the foundation for the potential use of Othello® to manage *B. bicolor*, *Curvularia* sp., *Neopestalotiopsis* sp., and *Colletotrichum* sp., but may require semi- and open-field validation.

Keywords: *Colletotrichum*, *Curvularia*, efficacy, fungal pathogens, synthetic fungicide

1. Introduction

The oil palm (*Elaeis guineensis* Jacq.) also known as the African oil palm and coconut (*Cocos nucifera* L.), often referred to as the tree of life, native to West Africa, are the most important palm species. Moreover, palm species from the Arecaceae family, characteristically stemless and tree-like monocot plants are essential crops for humans, especially in the tropics (Cosiaux et al., 2018). For instance, for more than 7000 years, indigenous cultures have relied on the year-round availability of oil palm fruit as a source of semi-wild food due to its numerous nutritional and health benefits, and commercial and industrial uses (Boateng et al., 2016). Palm oil and palm kernel oil are the two types of oils obtained from the palm tree and have numerous uses such as making soups, margarine, glycerin, cooking fats, and pomade (Dislich et al., 2017; Azlan et al., 2018; Paterson et al., 2018; Maluin et al., 2020). Similarly, coconut is considered a multipurpose perennial plantation crop. It serves as a source of nutritious drinks, edible oil, other edible nutritious products, fiber, fuel, and other industrial uses (Bourke and Harwood 2009; Eyres et al., 2016; Sunpapao et al., 2022). Coconut is high in dietary fiber, vitamins (C, E, B1, B3, B5, and B6), minerals (iron, selenium, sodium, calcium, magnesium, and phosphorous), and proteins (albumins, globulins, prolamines, and glutelins) which have anti-inflammatory, antioxidant, antifungal, antihelminthic, antimicrobial, antinociceptive, and antitumor properties to prevent and fight chronic diseases (Okolo et al., 2019; Reddy et al., 2019; Afram et al., 2022).

Despite the economic importance of oil palm and coconut trees, their production is constrained due to pests and diseases throughout the developmental stages of these crops, the seedling stage of these crops is the most

susceptible stage to diseases, especially leaf spot disease, the most common and devastating disease in the nursery (Sharadraj and Mohanan, 2014; Obeng et al., 2023). Leaf spot disease negatively impacts the health of oil palm and coconut seedlings by restricting growth, lengthening nursery age, increasing plant mortality, extending the immature plant phase, and serving as a source of inoculum for other planting materials such as seeds (Suyanto et al., 2022). In established nurseries in Ghana, the more common diseases are *Pestalotiopsis* (Sporocadaceae) leaf spot, *Curvularia* (Pleosporaceae) leaf spot (Lekete et al., 2022; Obeng et al., 2023), *Cercospora* (Mycosphaerellaceae) leaf spot, spear rot, and anthracnose (Yawson et al., 2014; Obeng et al., 2023). A report from our previously unpublished data shows that 20 – 50 % of seedlings of both oil palm and coconut are infected, resulting in delayed development of the seedling and even death of some heavily infected seedlings. Similarly, previous studies demonstrated the negative impact of the fungi *Ceratocystis paradoxa* (Ceratocystidaceae)*, Pestalotiopsis menezesiana, and Bipolaris setariae* (Pleosporaceae) on the health of coconut (Sunpapao et al., 2014; Sunpapao et al., 2022).

Chemical control, especially the use of synthetic fungicides continues to be the mainstay of current efforts to manage leaf spot disease because of their immediate mode of action and ease of use (Susanto and Prasetyo, 2013; Wang et al., 2016). Difenoconazole is one of the active fungicides that is used to prevent the spread of leaf spot disease (Susanto and Prasetyo, 2013). Azoxystrobin has also been demonstrated to be useful in the management of a variety of diseases affecting diverse crops including palm trees (Bagi et al., 2014; Wang et al., 2016). However, in recent years, there have been several reports of antimicrobial resistance due to the use of these fungicides, especially those with only one active ingredient (Agustina et al., 2019). For instance, Luo et al. (2021) reported the resistance of *Colletotrichum* species (Glomerellaceae) which ranged from moderate to high against Azoxystrobin *in vitro*. Additionally, Alfaro-Alvarado (2019) reported the resistance of *Mycosphaerella* sp. (Mycosphaerellaceae) against Qol fungicides. Likewise, a previous in vitro study by Wang et al. (2021), reported the resistance of the pathogen *Lasiodiplodia theobromae* against the difenoconazole fungicide group. These examples demonstrate the need to incorporate the use of fungicides with multiple active ingredients in integrated disease management strategies for the management of pathogens of agricultural importance.

Recently, several newly registered fungicides containing a combination of more than one active ingredient have been utilized extensively globally for the control of some *Alternaria* species (Wang et al., 2016). The combination of two or more active ingredients/fungicides for the management of pathogens is a promising alternative since it is very difficult for pathogens to develop resistance against them (Hu, 2019). However, there is limited information on the efficacy of the combined use of two different active ingredients/fungicides such as Azoxystrobin and Difenoconazole against foliar pathogens associated with oil palm and coconut tree crops in Ghana. Therefore, the objective of this study is to assess the efficacy of the commercial fungicide Othello® which is composed of two different fungicide groups; Azoxystrobin and Difenoconazole against oil palm and coconut foliar pathogens such as *Bipolaris bicolor*, *Curvularia* species, *Neopestalotiopsis* species, and *Colletotrichum* species in vitro.

2. Materials and Methods

2.1 Experimental Site

The experiment was carried out at the Plant Pathology Laboratory of the Council for Scientific and Industrial Research (CSIR), Oil Palm Research Institute (OPRI), Kusi (Denkyeambour District) in the Eastern Region of Ghana (Fig 1). With an annual rainfall of 1,681.7 mm and an average annual temperature of 32.2°C, Kusi has a bimodal rainfall pattern (Oil Palm Research Institute Meteorological Weather Station, 2023).

Figure 1. Map of Denkyeambour District (Source: Ghana Statistical Service, GIS)

2.2 Sample Collection

Pure fungal isolates stored in a refrigerator (4 °C) were obtained from the Plant Pathology unit of the CSIR-OPRI. The isolates were revived on full-strength potato dextrose agar (PDA), and fresh cultures were established for the experiment.

2.3 In-vitro Fungicide Sensitivity Study

Food poisoning technique (Kumar et al, 2013) was used to screen the fungicide product against the fungal strains. In each plate, 5 mm of actively growing mycelia plug of a seven-day-old *Curvularia* species (KC-21-2), *Neopestalotiopsis* species (KOP-21-20), *Colletotrichum* (KOP-21-14), and *Bipolaris bicolor* (KC-21-1) were plated inversely in the middle of each plate (9 cm) containing medium and each was replicated five times. The plates were then incubated at 30 ± 2 °C in a humidified incubation bowl under diffused sunlight during the day and in the dark (night). A preliminary experiment (Experiment 1) was conducted to test different concentrations (0 µL per mL, 2.5 µL per mL, 5 µL per mL, and 7.5 µL per mL) of the fungicide (Othello®) against *B. bicolor*, *Curvularia* sp., *Neopestalotiopsis* sp., and *Colletotrichum* sp. *in vitro* and the results are shown in Table 1. A further study (Experiment 2) was conducted testing relatively lower concentrations (0 µL per mL, 0.5 µL per mL, and 1 µL per mL) of the fungicide against the same fungi strains tested in Experiment 1 and the results are shown in Table 2.

Table 1. Percentage inhibition of the mycelia growth of some selected fungi species tested against varying concentrations of the commercial synthetic fungicide Othello® after 28 days

	*Percent inhibition (%) of mycelia growth			
Pathogen	0 µL per mL (Control)	2.5 µL per mL	5 µL per mL	7.5 µL per mL
Bipolaris bicolor	0 [a]	100 [b]	100 [b]	100 [b]
Curvularia sp.	0 [a]	100 [b]	100 [b]	100 [b]
Neopestalotiopsis sp.	0 [a]	100 [b]	100 [b]	100 [b]
Colletotrichum sp.	0 [a]	100 [b]	100 [b]	100 [b]

*Percentage inhibition was calculated from mean values of colony diameter obtained from the various concentrations (0 µL per mL, 5 µL per mL, 5 µL per mL, and 7.5 µL per mL), five replicates per treatment for seven-day intervals for 28 days and after incubation.

Daily colony diameter measurements were taken with a ruler from the backside of the plates (an average of two diagonal measures per plate). The experiments were maintained for a total of 28 days, and the efficacy of the fungicide was determined using the Chaurasia et al. (26) formula described below:

$$I = \frac{C-T}{C} \times 100 \quad (1)$$

Where:

I = Inhibition percentage

C =Average growth of control plate

T = Average growth of fungicide-treated plate.

Also, the average daily growth rate was calculated with the formula below;

$$\text{Fungal daily growth rate} = \frac{\sum(A-B)}{\text{Total number of culture plates per treatment}} \quad (2)$$

Where A is the colony diameter for the current day, B is the colony diameter for the previous day.

2.4 Statistical Analysis

A Shapiro-Wilk test was used to check for normality of the data from the mycelia growth of the various fungal strains in both experiments in this study. Further, since the data was not normally distributed, a non-parametric Kruskal-Wallis test was performed followed by post-hoc comparisons using Dunn's test with Bonferroni's adjustment. Means were separated at a 1 % probability level. All the analyses were done in R software using the R Studio user interface. A generalized linear model (GLM) using a binomial distribution was used to calculate the minimum inhibitory concentration and time (MIC and MIT).

3. Results

3.1 Inhibition of Mycelia Growth

In experiment 1, there was a significant inhibition of mycelia growth of *B. bicolor* (χ^2 = 19, df = 3, P < 0.001), *Curvularia* species (χ^2 = 19, df = 3, P < 0.001), *Neopestalotiopsis* species (χ^2 = 19, df = 3, P < 0.001), and *Colletotrichum* species (χ^2 = 19, df = 3, P < 0.001) in all the concentrations (2.5 µL per mL, 5 µL per mL, and 7.5 µL per mL) of Othello® tested compared to the control treatments (Table 1). Whereas there was a complete (100 %) inhibition of mycelial growth of all tested concentrations of Othello®, there was no mycelial growth inhibition (0 %) in all the control treatments (Table 1).

Based on the results obtained in Experiment 1, a second experiment (Experiment 2) was conducted testing relatively lower concentrations of Othello® as 0.5 µL per mL and 1 µL per mL, and control (0 µL per mL) against the fungi *B. bicolor*, *Curvularia* species, *Neopestalotiopsis* species, and *Colletotrichum* species. The results from experiment 2 indicate that the highest concentration (1 µL per mL) of Othello® significantly inhibited (χ^2 = 12.9, df = 2, P < 0.05) the highest *B. bicolor* mycelia growth (84 %), whereas the least was recorded in the control treatment (0 %) (Table 2). Similarly, in *Curvularia* species (χ^2 = 13.5, df = 2, P < 0.05) and *Neopestalotiopsis* species (χ^2 = 14, df = 2, P < 0.001) the highest concentration (1 µL per mL) of Othello® significantly inhibited (100 %) the mycelia growth of the fungi and the least recorded in the control treatments (0 %) (Table 2). Likewise, 1 ml/L of Othello® recorded significantly (χ^2 = 12.9, df = 2, P < 0.05) the highest mycelial growth inhibition (98.7 %) of *Colletotrichum* species with the least inhibition found in the control treatments (0 %) after 28 days

(Table 2).

Table 2. Percent (%) inhibition of the mycelia growth of some selected fungi species tested against varying concentrations of the commercial synthetic fungicide Othello® after 28 days

	*Percent inhibition (%) of mycelia growth		
Pathogen	0 µL per mL (Control)	0.5 µL per mL	1 µL per mL
Bipolaris bicolor	0.00 [a]	76.44 [ab]	84.04 [b]
Curvularia sp.	0.00 [a]	77.75 [ab]	100.0 [b]
Neopestalotiopsis sp.	0.00 [a]	100 [b]	100.0 [b]
Colletotrichum sp.	0.00 [a]	82.30 [ab]	98.74 [b]

*Percentage inhibition was calculated from mean values of colony diameter obtained from the various concentrations (0 µL per mL, 0.5 µL per mL, and 1 µL per mL), five replicates per treatment for seven-day intervals for 28 days and after incubation.

3.2 Mycelia Growth Dynamics and Minimum Inhibitory Effect

Mycelia growth of *B. bicolor*, *Curvularia* sp., *Neopestalotiopsis* sp., and *Colletotrichum* sp. in the control plates followed a linear growth pattern from days 1 – 7 (Figure 2), covering all the surface of the plates after day 7 (Figure 3). However, no fungal colony growth of all strains was observed in all the various concentrations of the fungicide (Othello®) tested in the first seven days of this study.

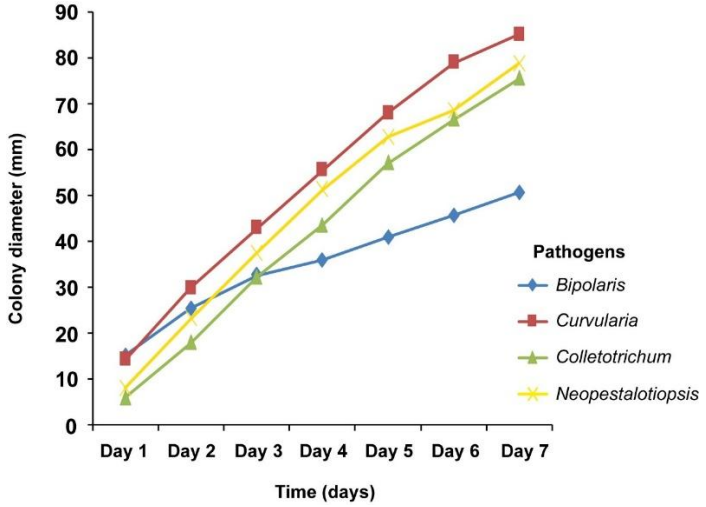

Figure 2. Daily fungal colony growth on control plates after 7 days

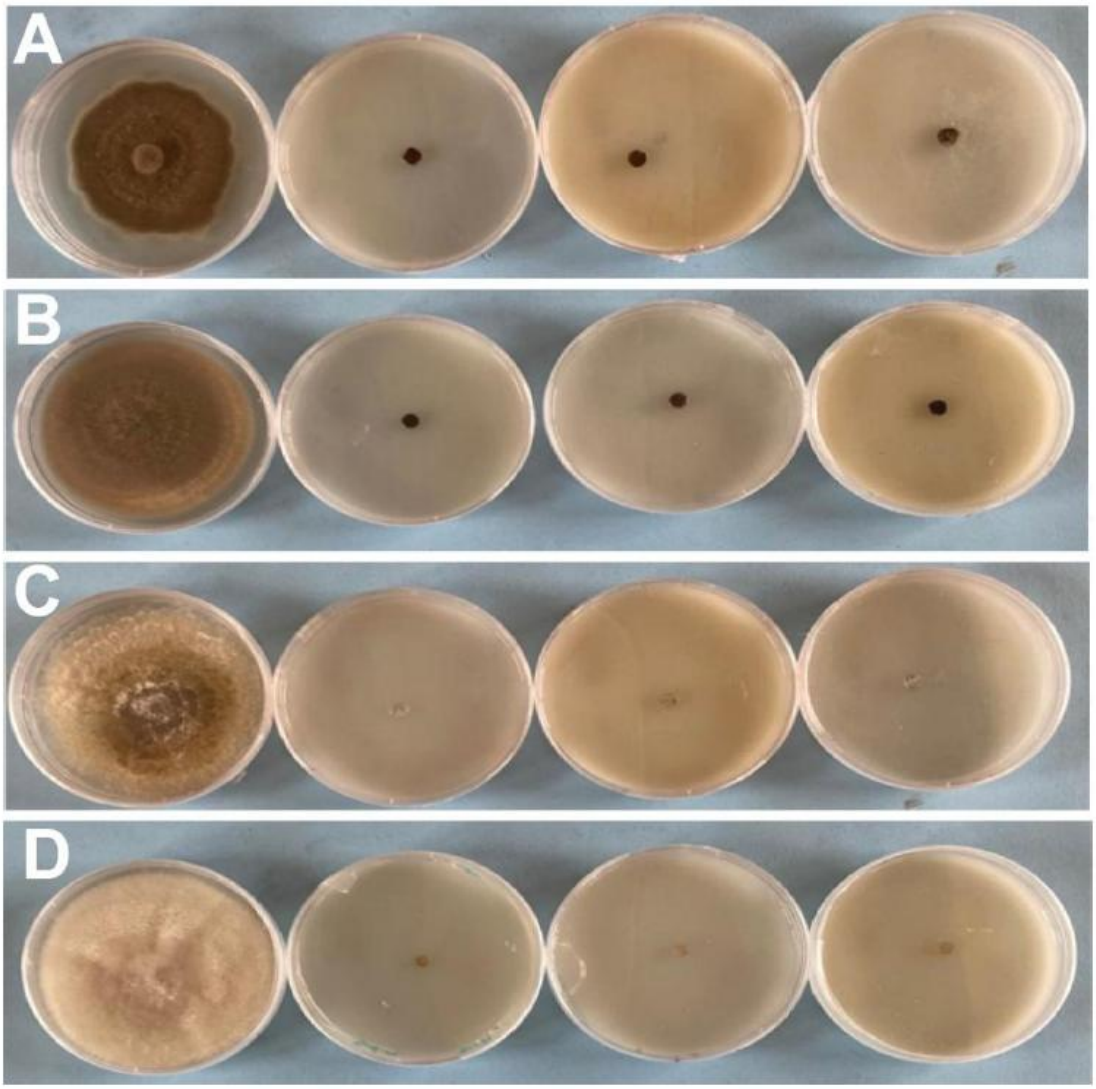

Figure 3. Fungal pathogens growing on the PDA plates amended with different concentrations of the fungicide (From left to right: Control (0), 2.5 µL per mL, 5.0 µL per mL and 7.5 µL per mL (A) Bipolaris bicolor, (B) Curvularia species, (C) Neopestalotiopsis species, and (D) Colletotrichum species) at day 28 of incubation

Results from Table 3 show the minimum inhibitory concentration (MIC) and minimum inhibitory time (MIT) which is in days. MIC is the minimum concentration of the fungicide required to cause 50 % inhibition of fungal colony/mycelial growth on assay plates whereas MIT is the minimum time required for 50 % of mycelial growth to be inhibited. The minimum inhibitory effect of the fungicide on amended culture plates varied with the fungal strains, *B. bicolor* (9 µL per mL in 41 days), *Curvularia* sp. (10 µL per mL in 75 days), *Neopestalotiopsis* sp. (5 µL per mL to infinity), and *Colletotrichum* sp. (8 µL per mL in 39 days) (Table 3).

Table 3. Minimum inhibitory concentrations (MIC50) and minimum inhibitory time of the representative isolates

Pathogen	**Minimum inhibitory effect**	
	MIC_{50} (µL per mL)	**MIT_{50}** (Days)
Bipolaris bicolor	8.9 ± 0.7	41.0 ± 6.5
Curvularia sp.	9.6 ± 0	75.3 ± 32.3
Neopestalotiopsis sp.	5.2 ± 0	~
Colletotrichum sp.	7.6± 0.5	39.4 ± 4.13

(~) means infinity, where no mycelial growth can be observed, no matter the length of time

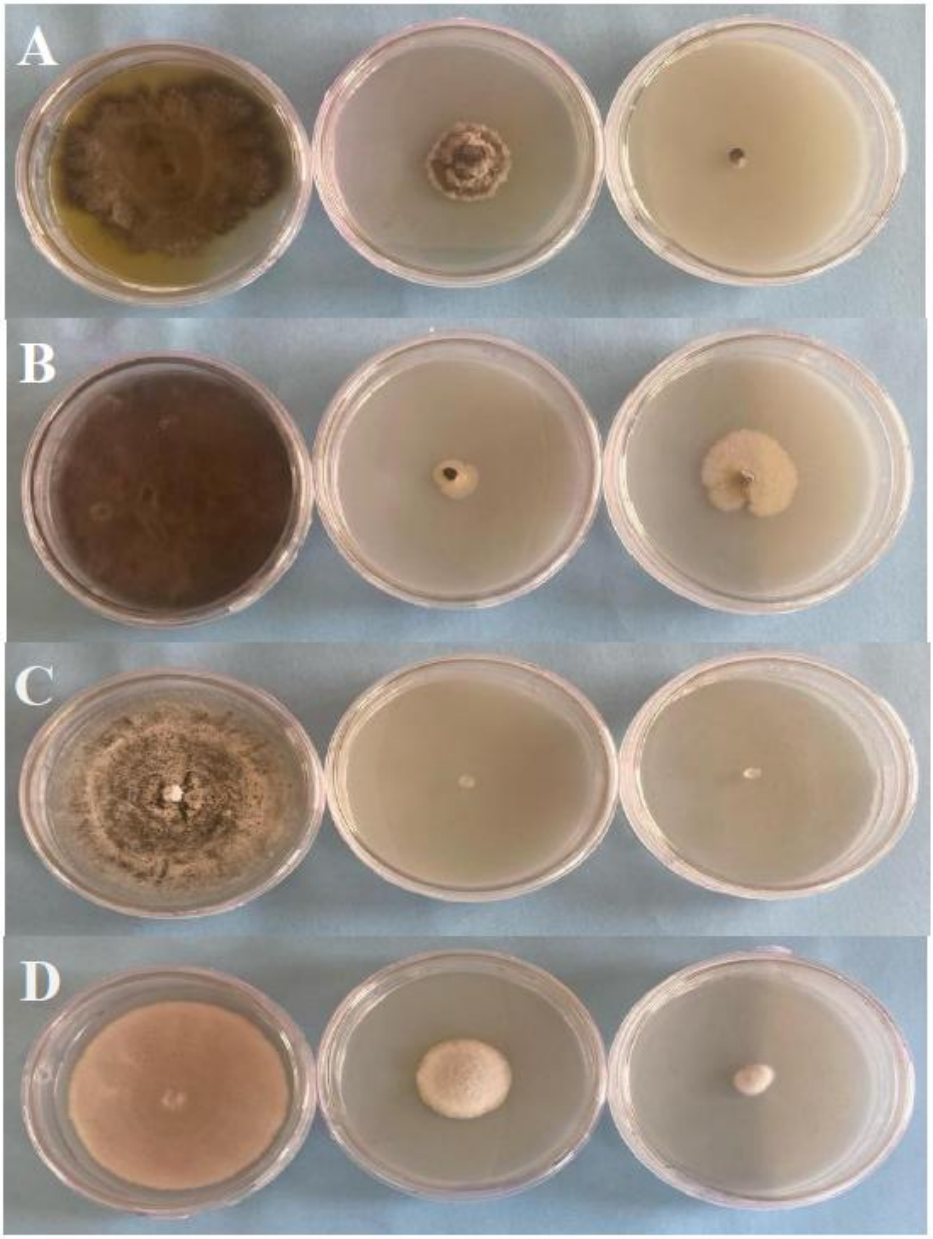

Figure 4. Fungal pathogens growing on the PDA plates amended with different concentrations of the fungicide (From left to right: Control (0), 0.5 µL per mL and 1.0 µL per mL A: Bipolaris bicolor, B: Curvularia species, Neopestalotiopsis species, and D: Colletotrichum species) at day 28 of incubation

4. Discussion

In this study, we demonstrated the efficacy of the commercial chemical product Othello®, a systemic fungicide that is composed of two distinct fungicide groups (Azoxystrobin and Difenoconazole) against oil palm and coconut foliar pathogens such as *Bipolaris bicolor*, *Curvularia* species, *Neopestalotiopsis* species, and *Colletotrichum* species in vitro. Our results indicate that all the various concentrations of Othello® tested inhibited a significant mycelial growth of the fungi *B. bicolor*, *Curvularia* sp., *Neopestalotiopsis* sp., and *Colletotrichum* sp. The effectiveness of every synthetic fungicide is measured by the mode of action of the various active ingredients. The mode of action describes what process in the epidemiology of the fungi is impacted, and it is denoted with a letter code (FRAC code) which is further categorized into groups. The group describes the target site and the specific site of activity (fungal mode of action) of the fungicide. Azoxystrobin belongs to the group of mitochondrial respiration inhibitor fungicides. It possesses broad-spectrum systemic activity against *B. bicolor*, *Curvularia* sp.,

Neopestalotiopsis sp., and *Colletotrichum* sp. (Wang et al., 2016; Wang et al., 2022). In contrast, Difenoconazole is also a systemic, broad-spectrum, and mainstream triazole fungicide that inhibits the ergosterol biosynthesis in pathogenic fungal cell membranes (Dong et al., 2019; Kumar & Kudachikar, 2019).

The fact that in Experiment 1 the percent inhibition of mycelial growth (100 % inhibition) of *B. bicolor*, *Curvularia* sp., *Neopestalotiopsis* sp., and *Colletotrichum* sp. was not significantly different in all concentrations (2.5 µL per mL, 5 µL per mL, and 7.5 µL per mL) of Othello® tested compared to the control suggests that this fungicide has very high efficacy against the four fungi strains used in this study. This assertion was confirmed by our follow-up study (Experiment 2) using relatively lower concentrations (0.5 µL per mL and 1 µL per mL) of Othello® where we found as high as 100 % mycelial growth inhibition in *Neopestalotiopsis* sp. (Fig. 4c) even at the lowest concentration (0.5 µL per mL) of the fungicide tested. This may be attributed to the activities of Azoxystrobin and difenoconazole which might have inhibited cellular respiration and energy production in *B. bicolor*, *Curvularia* sp., *Neopestalotiopsis* sp., and *Colletotrichum* sp. to prevent spore germination and disruption of the membrane cells and organelles of the fungi. These results are in line with a previous study Wang et al. (2016), which reported a similar mycelial growth inhibition of the fungus *Alternaria alternata* (causal agent of the Tobacco brown spot disease) due to the combined effect of Azoxystrobin and Difenoconazole. Additionally, previous in vitro and field studies have demonstrated the efficacy of Azoxystrobin and Difenoconazole either solely or in combination to suppress pathogenic fungi of agricultural importance (Ashour et al., 2012; Billah et al., 2021; Cosseboom and Hu, 2021). Moreover, Dubos et al., (2013) and Marczewska et al. (2020) have also reported the preventive, therapeutic, eradicative, translaminar, and systemic properties of Azoxystrobin. However, it will be interesting for future studies to assess the bio-efficacy of Azoxystrobin and difenoconazole individually against *B. bicolor*, *Curvularia* sp., *Neopestalotiopsis* sp., and *Colletotrichum* sp. to enhance our understanding of the disease management potential of these fungicide groups against disease-causing pathogens associated with oil palm and coconut trees.

Our results further indicate that mycelia growth of the fungi isolates in the control plates from days 1-7 followed a linear growth pattern whereas no growth was observed in the fungicide treatment plates (Fig. 3). Mycelia growth in the control plates was expected since the media (PDA) used in this study supports the growth of fungi due to its rich nutritional composition. The progressive mycelia growth observed could be attributed to the production of energy through cellular respiration of the mycelia to induce growth. The results of this study further show that considering the minimum inhibitory concentration and time, it appears that the fungicide Othello® has the most lethal effect against *Neopestalotiopsis* sp. with the least effect on *Curvularia* sp. This suggests that a fungicide may exert a differential toxicity effect against different fungi isolates.

This study has shown that the commercial fungicide Othello® has a fungicidal effect against disease-causing agents *B. bicolor*, *Curvularia* sp., *Neopestalotiopsis* sp., and *Colletotrichum* sp. associated with oil palm and coconut trees at higher dosages. The product suppressed the four fungal isolates even at low concentrations and was persistent for over 28 days (Fig 4). The chemical composition of Othello® (Azoxystrobin and Difenoconazole) presents very minimal chances of the development of antimicrobial resistance against it and therefore makes it a promising product for the suppression of disease-causing fungi associated with palm trees. This study lays the foundation for the potential integration of the fungicide Othello® in integrated disease management programs for the sustainable management of *B. bicolor*, *Curvularia* sp., *Neopestalotiopsis* sp., and *Colletotrichum* sp. on oil palm and coconut trees, but this may require semi- and open-field validation. Due to antimicrobial resistance (AMR), it is appropriate to use fungicides of such a combination to reduce or avert antimicrobial resistance of fungi.

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Author contributions

J. O conceptualized the idea, and J. O and S. A. designed the experiments. J.O., S.A., and O.A.O. collected the data. J.O. and B.A. analyzed and interpreted the results. J.O. wrote the paper. All authors reviewed the manuscript.

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Informed consent

Obtained.

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Data sharing statement

No additional data are available.

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