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In vitro assessment of copper oxychloride and flutriafol for the control of cashew leaf and fruit blight fungi

Avaliação in vitro do efeito do oxicloreto de cobre e flutriafol no controlo dos fungos associados a queima da folha e fruto do cajueiro

Evaluación in vitro del efecto del oxicloruro de cobre y flutriafol en el control de hongos asociados a la quema de hojas y anacardos

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Abstract
In Mozambique, cashew crop cultivation is practiced by both family and commercial sectors. However phytosanitary problems have been found to inflict production and productivity in both sectors and are mainly due to the diseases caused by fungi. Adoption of appropriate disease control measures translates into benefits to the national economy resulting from production increases and subsequent increases in export volumes. However, the knowledge of effective disease control approaches requires the testing of various control methods. Thus, the present study was intended to objectively assess the effectiveness of Coprox Super 85% WP (Copper Oxychloride 850g/kg) e Starback 25% SC (Flutriafol 250 g/l) for the control of cashew leaf and fruit blight fungi complex. The growth media PDA in Petri dishes was amended with fungicides and then centrally inoculated with mycelial discs. Mycelial growth of individual fungal isolates was assessed by measuring two perpendicular diameters and these were later used to calculate the growth rate and inhibition percentage. Flutriafol had higher control effectiveness against cashew leaf and fruit blight fungi than copper oxychloride and is therefore recommended for field trials.
Keywords: Cashew; Leaf and fruit blight; Fungi isolates; Fungicides; Flutriafol; Copper oxychloride.

Resumo
Em Moçambique, a cajucultura é praticada pelo sector familiar e comercial. Porém, problemas fitossanitários têm interferindo na produção e produtividade com grande destaque para as doenças causados por fungos. A adopção de mecanismos de controlo traduz-se em benefício para economia nacional pelo aumento da produção e, consequentemente, aumento do volume das exportações. Entretanto, o conhecimento efectivo dos mecanismos para o maneio de doenças passa, necessariamente, pela testagem dos diversos métodos de controlo. Assim, o presente estudo teve como objectivo avaliar o efeito dos fungicidas Coprox Super 85% WP (Oxicloreto de cobre 850g/kg) e Starback 25% SC (Flutriafol 250 g/l) no controlo dos fungos responsáveis pela queima da folha e do fruto do cajueiro. Os fungicidas foram incorporados em PDA e, de seguida, fez-se inoculação de discos de micélios no centro da placa. Foi avaliado o diâmetro médio dos isolados em dois sentidos perpendiculares entre si, taxa de crescimento e percentagem de inibição. Resultados mostraram que o Flutriafol tem alta capacidade de controlo dos isolados causadores da queima

da folha e do fruto do cajueiro, podendo por conseguinte, ser adoptado como potencial químico para o controlo da queima da folha e do fruto do cajueiro, depois de testado no campo.
Palavras-chave: Cajueiro; Queima da folha e do fruto do cajueiro; Isolados; Fungicidas; Flutriafol; Oxicloreto de cobre.

Resumen

En Mozambique, el cultivo de anacardos es practicado por el sector familiar y comercial. Purén, problemas los fitosanitarios han interferido en la producción y la productividad con gran énfasis en las enfermedades causadas por hongos. La adopción de mecanismos de control es un beneficio para la economía nacional al aumentar la producción y, en consecuencia, aumentar el volumen de las exportaciones. Sin embargo, el conocimiento efectivo de los mecanismos para el manejo de enfermedades implica necesariamente la prueba de los diversos métodos de control. Así, el presente estudio tuvo como objetivo evaluar el efecto de los fungicidas Coprox Super 85% WP (Oxicloruro de cobre 850g/kg) y Starback 25% SC (Flutriafol 250 g/l) sobre el control de hongos responsables de quemar la hoja y el fruto del anacardo. Los fungicidas se incorporaron a PDA y luego se inocularon discos de micelio en el centro de la placa. Se evaluó el diámetro promedio de los aislados bidireccionales perpendiculares entre sí, la tasa de crecimiento y el porcentaje de inhibición. Los resultados mostraron que flutriafol tiene una alta capacidad de control de los aislados que causan la quema de la hoja y el fruto del anacardo, y por lo tanto puede ser adoptado como un potencial químico para el control de la quema de hojas y la fruta de anacardo, después de ser probado en el campo.
Palabras clave: Anacardo; Quema de la hoja y el fruto del anacardo; Aislado; Fungicidas; Flutriafol; Oxicloruro de cobre.

1. Introduction

Cashew (*Anacardium occidentale* L.) is a plant cultivated in almost all countries with a tropical climate and has its center of origin in Northwest Brazil (Viana *et al*., 2020; Vivek *et al.*, 2013).

Nowadays, the cashew crop is spread in various locations of the world namely South America, Central America, Africa, and Asia. These countries such as an India, Brazil, Mozambique, Tanzania, Kenya, and Ivory Coast are among the major producers of cashew in the World (Viana *et al*., 2020; Vivek *et al.*, 2013). Despite the high economic and social importance of cashew as undaughterly justified by its dissemination and adaptability worldwide, the crop still exhibits low production and productivity. This fact is attributed to various factors among which phytosanitary problems (Sousa, 2018) that play an important impediment to productivity, in-country trade, and export (Sobrinho *et al*., 2021; INCAJU, 2019).

Cashew is infected by a bit more than dozen diseases, the majority of which are caused by fungi. All plant parts though may express characteristic symptoms of infectious processes, diseases of aerial parts are the most easily noticed, because they are easy to observe and cause direct damage to yield (Veloso *et al*., 2022; Araújo, 2013).

Control of diseases in crops is the ultimate practical objective of phytopathology without which enormous losses are known to occur (Micheriff, 2001). Thus, interventions for disease control aim at preventing losses through disease incidence or severity reduction (Araújo, 2013). Among the disease management measures, spraying cashew trees with agrochemicals is the most adopted disease control strategy (Zambolim, 2010). However, disease defensives of chemical origin do not always produce satisfactory and durable results. Therefore, there is a need for integrated disease management practices including good crop husbandry (Veloso *et al*., 2022; INCAJU, 2019; Zambolim, 2010).

According to Coutinho *et al.* (2005), pesticide selection for disease management must always consider the respective strategy against the eventual development of resistance by the target organisms. Because of that, the market introduces new molecules, combinations, and/or formulations of molecules assuming the potential of resistance occurrence.

Thus, it was proposed as a candidate for the control of cashew leaf and fruit blight the commercial Starback 25% SC, a systemic and sterol biosynthesis inhibitor fungicide containing Flutriafol at a concentration of 250g/L as an active ingredient. The fungicide is also well known due to its high level of fungitoxicity, fast translaminar penetration, and plant tissue translocation playing both protective and curative action (Rodrigues, 2006). The other candidate, Coprox Super 85% WP, is a contact fungicide with general interference with cellular functions, belongs to the group of inorganic compounds and copper

oxychloride is the active component at 850 g/kg concentration (FRAC, 2021; MASA, 2015). This is a fungicide of multiple action sites, a member of the low-risk group (FRAC, 2021). The Nuts Institute of Mozambique (IAM, IP) has been using copper oxychloride for the control of various fungal diseases in cashew plantations. However, according to many cashew farmers, the results of copper applications are no longer satisfactory. Therefore, it has been admitted that eventually, resistance has developed in the targeted disease agents due to the recurrent spraying of copper oxychloride. This justifies the search for alternative fungicides for disease control in cashew plantations in Mozambique.

Meanwhile, Flutriafol is a new product in the Mozambican market of fungicides, and it is potentially recommended for the control of a diversity of fungi in cashew. Its effectiveness against leaf and fruit blight, however, is yet unknown. Therefore, it was chosen alongside copper oxychloride. The study was designed to assess the effectiveness of Coprox Super 85% WP (Copper Oxychloride 850g/kg) and Starback 25% SC (Flutriafol 250 g/l) for the control of cashew leaf and fruit blight fungi complex.

2. Methodology

The study was conducted in the Plant Pathology Laboratory of the Faculty of Agronomy and Forestry Engineering, Eduardo Mondlane University (FAEF-UEM), Maputo. Fungal isolates were recovered from the leaf samples previously collected from Inhambane and Gaza provinces. The collection process consisted of random identification of leaves with typical disease symptoms which were disinfected in a 1% solution of sodium chloride for 30 seconds followed by a double wash in distilled and sterilized water (Diniz *et al*., 2021; Lima *et al*., 2013; Serra e Coelho, 2007). The leaves were then cut into 1 cm2 pieces and transferred to the Petri dishes containing 40 ml of ready-made PDA growth media, sealed with parafilm, and incubated at 25 ± 2 °C (Diniz *et al.*, 2021; Dominic *et al*., 2014; Cordeiro *et al*., 2007).

After 48 hours of incubation, purification of fungal cultures was made through re-isolations from the growing edges and transfer of fungal mycelium into another set of Petri dishes with PDA and re-incubated for 7 days (Majune *et al*., 2019; Dominic *et al*., 2014; Figueirêdo, 2005). This process allowed the recovery of three cultural types of isolates thereafter named A (with dark colonies), B (with orange-colored colonies), and C (white colonies with black circular rings) as presented in Figure 1.

Figure 1. Pure fungal cultures of different isolates. (A) Dark and smooth coloration, spongy and abundant mycelia. (B) Creamy isolates and orange colored in base. (C) Superficially cotton white color, vigorous growth, with black rings and dark base.

Source: Adriano *et al.* (2022).

2.1 Purification of isolates from discs in the Petri dishes

For the purification of fungal isolates, the first 15.6 g of PDA were diluted in 400 ml of distilled water and gently homogenized. The PDA solution was then autoclaved at 121°C for 40 minutes (Santos *et al*., 2017) and then poured into sterile Petri dishes. In parallel, fungicide solutions were prepared as follows: 5 g/l of distilled water for copper oxychloride and 10 ml/l of distilled water for Flutriafol. By pipetting 100 µl of each fungicide solution, all the necessary plates were amended accordingly, homogenized gently, and then allowed to solidify (Camatti-Sartori *et al*., 2011). After that, 5 mm diameter discs of isolates were aseptically placed in the center of each Petri dish, sealed, and incubated in a growth chamber (Santos *et al*., 2017; Cruz *et al*., 2001).

2.2 Trial layout and treatments

A completely randomized 3x3 factorial design (Gomez e Gomez, 1984) with four (4) replicates was followed (Table 1). The individual treatment plot consisted of 3 Petri dishes. Therefore, the total number of Petri dishes in the whole trial was 108 (= 3 x 3x3 x 4).

Table 1. Trial treatments resulting from a combination of fungicides and fungi isolates.

Factor	**Fungicides**		
	Copper oxychloride (F1)	Flutriafol (F2)	Control (cont)
Isolates	5 g/l of water	10 ml/l of water	0
A	AF1	AF2	A-cont
B	BF1	BF2	B-cont
C	CF1	CF2	C-cont

AF1 – Copper oxychloride 5 g/l of water in A; BF1 –Copper oxychloride 5 g/l of water in B; 3. CF1 – Copper oxychloride 5 g/l of water in C; AF2 – Flutriafol 10 ml/ of water in A; BF2 – Flutriafol 10 ml/l of water in B; CF2 – Flutriafol 10 ml/l of water in C; A-cont – Control in A; B-cont – Control in B; C-cont – Control in C. Source: Authors.

2.3 Measured variables

In all purified isolates growing in amended media measurements of diameter (cm) in two perpendicular directions were daily taken from the base of the Petri dishes using a millimetric ruler (Ribeiro *et al*., 2016; Tavares e Souza, 2005; Cruz *et al.,* 2001). The measurements were interrupted only when the growth in the un-amended control dishes reached the edge, in general, 7 days post inoculation (Santos *et al*., 2017; Uaciquete, 2013).

The growth rate was calculated from 48 h post-inoculation and successively every 24 hours, following equation 1, and later mean comparison was presented as proposed by Ribeiro *et al.*, (2016).

$$TC = (C2 - C1)/(T2 - T1) \quad (1)$$

Where: TC = Growth rate; C1 = Mycelial growth during the first 24 hours; C2 = Mycelial growth during the first 48 hours; T1 = 24 hours interval and T2 = 48 hours interval.

The percentage of growth inhibition for the colonies was estimated at the end of the trial (7 days later), based on equation 2 proposed by Menten (1976).

$$\% \text{ of inhibition} = \frac{\text{Growth in control Plates} - \text{Growth in treated plates}}{\text{Growth in control plates}} \times 100 \quad (2)$$

2.4 Statistical analysis

The data were grouped per treatment and the means were calculated concerning the mean diameter of the isolates and mycelial growth rate of the different isolates, all in a Microsoft Office Excel 2019 spreadsheet. In the same software, graphs were plotted, and tables were constructed for subsequent export to the STATA 16 statistical package for the analysis of variance (ANOVA) procedure. The normality of residues was verified by the Shapiro-Wilks test and the heteroscedasticity of variances by the Breusch Pagan test (Torman *et al*., 2012; Gomez e Gomez, 1984).

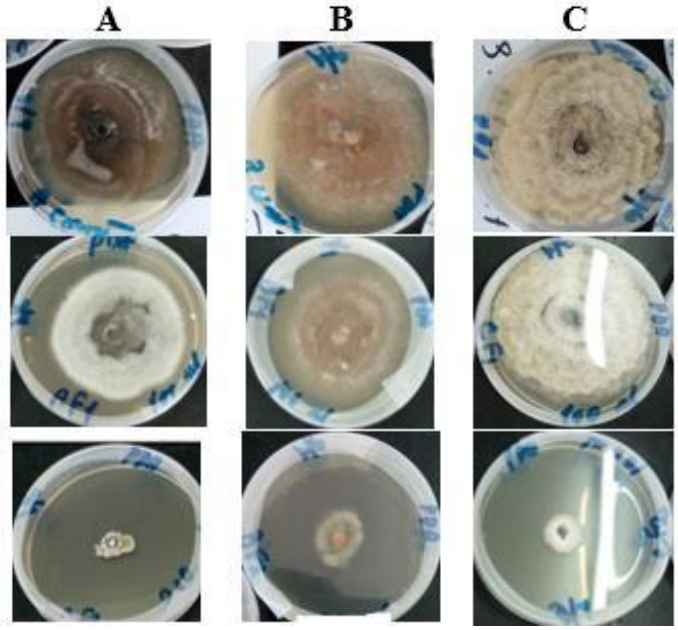
For means comparison, the Tukey test was performed at 5 % probability (Gomez e Gomez, 1984; Ribeiro *et al*., 2016). To fulfill the ANOVA assumptions, data on growth rate were log-transformed for periods of 24, 72, and 120 hours (Gomez e Gomez, 1984; Torman *et al*., 2012).

3. Results and Discussion

3.1 Isolates' identity and colony diameter

The identity of fungi from individual colony types was established through molecular techniques by Adriano *et al*. (2022). Thus, from colony type A, plate coded as A04 the isolate was identified as *Neofusicoccum* spp.; from colony type B, plate code A01, the isolate was identified as *Colletotrichum* spp. and finally from colony type C, plated as A02 was identified as *Pestalotiopsis* spp. The average growth diameter of colonies' morpho-types A, B, and C, on PDA, amended with copper oxychloride and Flutriafol, demonstrated variations in radial growth from the first to the seventh day of incubation when the growth in control plates reached the edges of the Petri dishes.

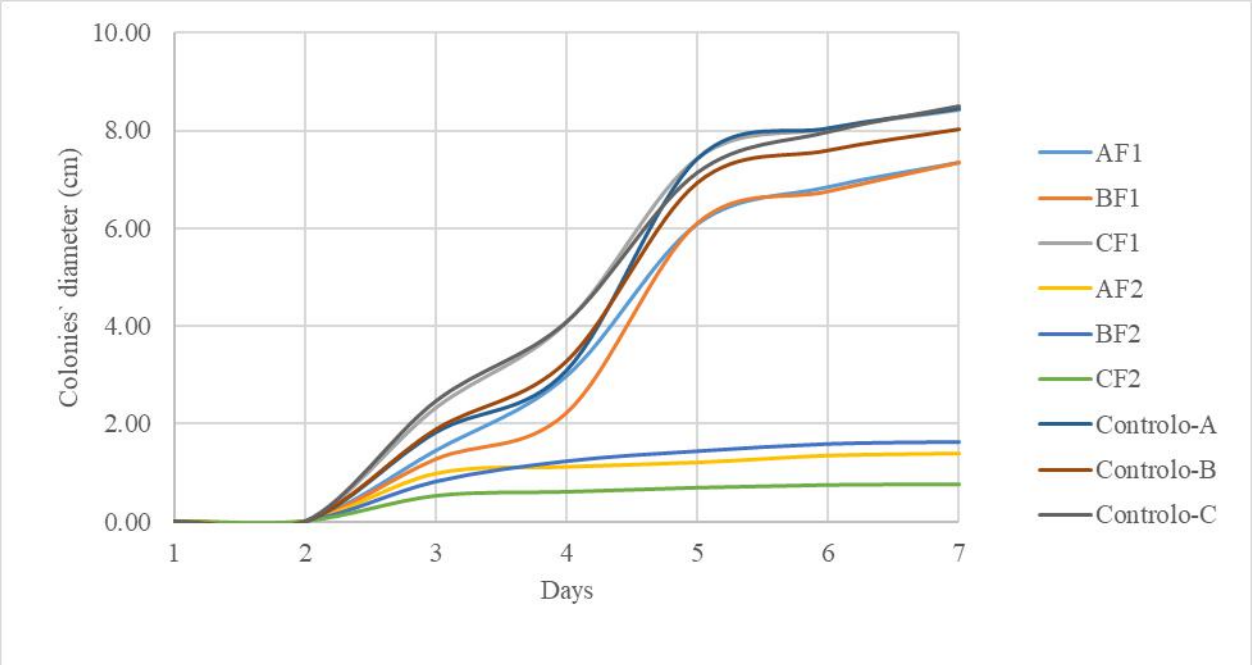
Figure 2. Growth of colonies' morpho-types A, B, and C (vertically) inoculated in fungicide-free PDA, cooper oxychloride amended PDA, and Flutriafol (horizontally), seven days after inoculation and incubation at 28ºC. (A) Dark colored colonies, (B) orange-colored colonies, (C) Cotton whitish colored and dark base with black rings.



Source: Authors.

The isolates demonstrated evidence of high sensitivity to Flutriafol. By the seventh day, the mean diameter was only 1.38 cm, 1.64 cm, and 0.78 cm in each morpho-type A, B, and C, respectively. In negative control plates, the average diameter varied between 7 and 8 cm (Figure 3).

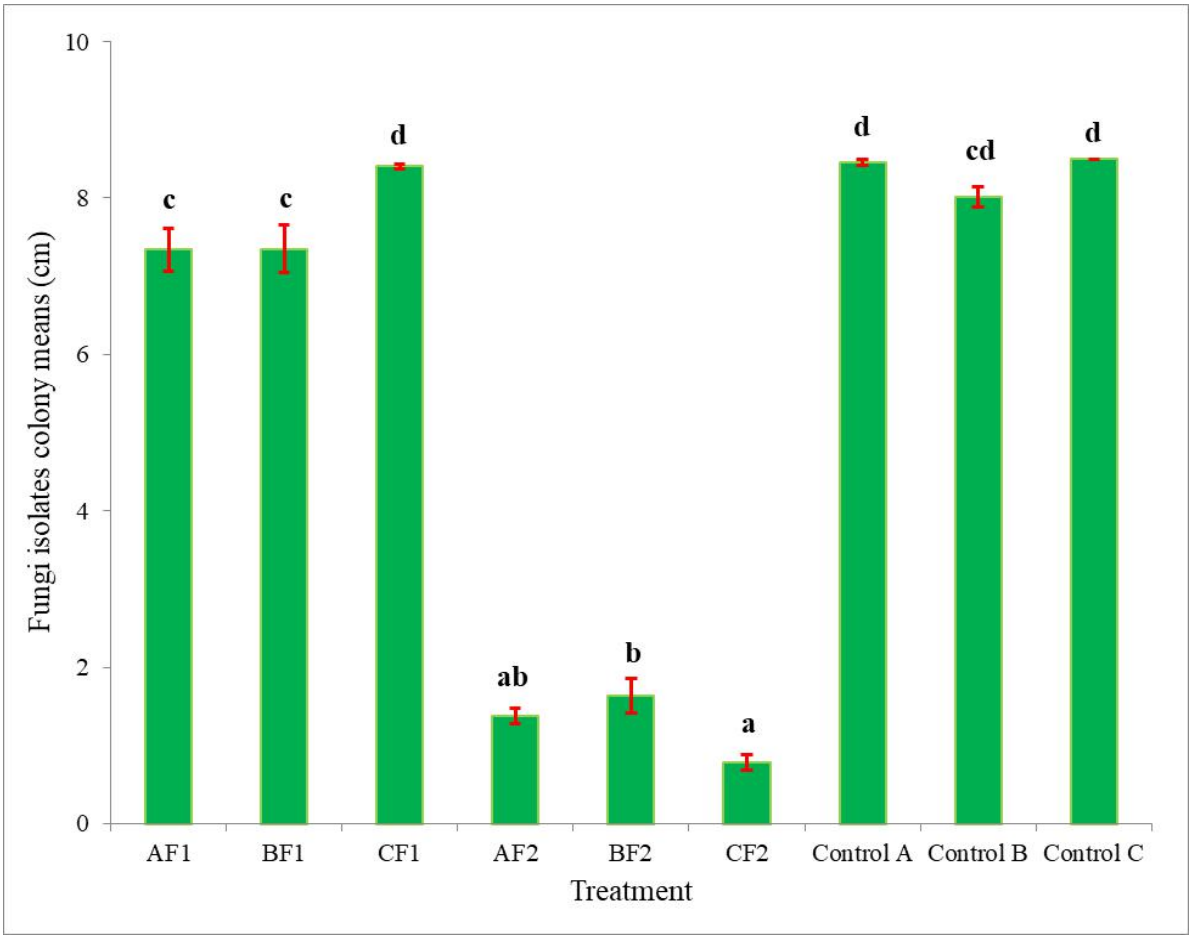
Figure 3. Progress of colonies' diameter (cm) over the number of days from inoculation.



AF1 – isolate A, in PDA amended with copper oxychloride 5 g/l of water; BF1 – Isolate Bin PDA amended with copper oxychloride 5 g/l of water; CF1 – isolate C, in PDA amended with copper oxychloride 5 g/l of water; AF2 – Isolate A, in PDA amended with Flutriafol 10 ml/l of water; BF2 – Isolate B, in PDA amended with Flutriafol 10 ml/l of water; CF2 – Isolate C, in PDA amended with Flutriafol 10 ml/l of water; Control - A = Control for isolate A; Control – B = Control for isolate B and Control – C= Control for isolate C. Source: Authors.

The mean diameter results in the growth of isolates A, B, and C, inoculated in PDA and amended with copper oxychloride and flutriafol by the seventh day of daily measurements are presented in Figure 4. Flutriafol had significant effect ($F_{(8, 27)}$ = 403.92, p = 0.0000) when compared to copper oxychloride. Therefore, it can be stated that flutriafol had statistically minor mean diameters for all isolates (7.34 cm, 7.35 cm e 8.41 cm in treatments AF1, BF1 e CF1, respectively) than copper oxychloride and negative control. These last showed no significant difference suggesting the ineffectiveness of copper oxychloride.

Figure 4. Mean diameter of colonies on day seven after inoculation and incubation at 25ºC.



For the means followed by the same letter on top of the graphs' bars statistically, no differences were detected by Tukey's test at 5% de probability. AF1 – isolate A, in PDA amended with copper oxychloride 5 g/l of water; BF1 – Isolate Bin PDA amended with copper oxychloride 5 g/l of water; CF1 – isolate C, in PDA amended with copper oxychloride 5 g/l of water; AF2 – Isolate A, in PDA amended with Flutriafol 10 ml/l of water; BF2 – Isolate B, in PDA amended with Flutriafol 10 ml/l of water; CF2 – Isolate C, in PDA amended with Flutriafol 10 ml/l of water; Control - A = Control for isolate A; Control – B = Control for isolate B and Control – C= Control for isolate C. Source: Authors.

The apparent reduction of growth rate observed after day 5 (Figure 3) is probably associated with average growth, plate size, and eventually reduction in the cellular division rate of the isolates (Azevedo, 1998).

3.2 Growth rate

The means of the isolates' growth rates (TC) are presented in Table 2. Growth rate differences were observed from day 1 to day 5 of incubation. During the initial 4 days, Flutriafol induced a reduced growth rate and was thus statistically different from other experimental treatments. Similar trends in fungal growth rates were reported by Medeiros (2006) while investigating the effect of fungicides in vitro, for the control of *Monosporascus cannonballus.* The growth rate means in treatments AF1, BF1, and CF1 were statistically higher than that in Flutriafol amended plates. Therefore, the efficacy of Flutriafol caused a reduction in the isolates' growth rate. Mycelial growth means in plates amended with copper oxychloride were not statistically different from those in negative controls. Similar results were reported by Andrade (2016). This finding supports the inefficacy reported by farmers upon the application of this fungicide in the field.

Table 2. Pure cultures of fungi isolates and their growth rates in PDA amended with copper oxychloride (F1) and Flutriafol (F2).

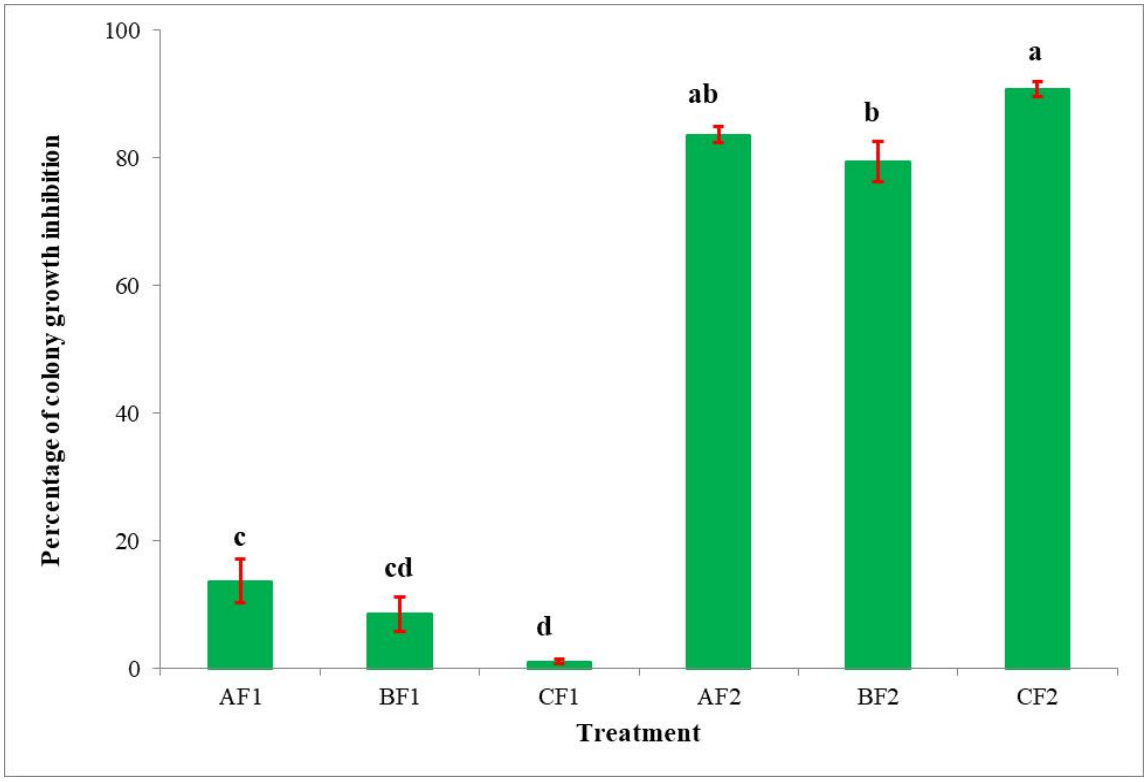
Treatments	Growth rate (TC)				
	24h	48h	72h	96h	120h
AF1	0.06 **BE**	0.07 **AB**	0.13 **B**	0.03 **A**	0.02
BF1	0.05 **DE**	0.04 **A**	0.16 **BC**	0.03 **ABC**	0.03
CF1	0.10 **A**	0.08 **B**	0.14 **BC**	0.02 **ABCD**	0.02
AF2	0.04 **CD**	0.01 **C**	0.00 **A**	0.00 **BD**	0.00
BF2	0.03 **C**	0.02 **C**	0.01 **A**	0.01 **BCD**	0.00
CF2	0.02 **F**	0.00 **C**	0.00 **A**	0.00 **D**	0.00
Control in A	0.08 **AB**	0.06 **AB**	0.18 **C**	0.03 **ABC**	0.02
Control in B	0.08 **AB**	0.06 **AB**	0.15 **BC**	0.03 **AC**	0.02
Control in C	0.10 **A**	0.07 **AB**	0.13 **BC**	0.04 **A**	0.02
Fcal	60.72	24.01	214.04	6.50	1.51
p-value	0.0000	0.0000	0.0000	0.0001	0.2315
CV (%)	45.78	65.74	71.55	74.41	83.54

For the means followed by the same letter vertically, no statistical differences were detected by Tukey's test at 5% de probability. AF1 – isolate A, in PDA amended with copper oxychloride 5 g/l of water; BF1 – Isolate Bin PDA amended with copper oxychloride 5 g/l of water; CF1 – isolate C, in PDA amended with copper oxychloride 5 g/l of water; AF2 – Isolate A, in PDA amended with Flutriafol 10 ml/l of water; BF2 – Isolate B, in PDA amended with Flutriafol 10 ml/l of water; CF2 – Isolate C, in PDA amended with Flutriafol 10 ml/l of water; Control - A = Control for isolate A; Control – B = Control for isolate B and Control – C= Control for isolate C. TC= Growth rate. Source: Authors.

3.3 Percentage of inhibition

The percentage of inhibition demonstrated that plates amended with Flutriafol had the highest reduction in colony diameter and thus the highest inhibition of isolates growth (Figure 5). Treatments AF2, BF2, and CF2 had inhibition percentages of 83.63, 79.43, and 90.88%, respectively after 7 days of incubation in PDA amended with fungicides. Seven days was the period for the isolates' growth in un-amended plates reaches the edges. These results were statistically higher than all other experimental treatments.

Figure 5. Percentage of colony growth inhibition in PDA amended with copper oxychloride (F1) and Flutriafol (F2).



For the means followed by the same letter on top of the graphs' bars statistically, no differences were detected by Tukey's test at 5% de probability. A= dark; B=Orange isolate and C= Cotton whitish isolate with black rings. F1 = copper oxychloride 5 g/l of water; F2 = Flutriafol 10 ml/l of water. Little bars in the middle of the big bars represent the standard error. Source: Authors.

In PDA plates amended with Flutriafol (F2) the percentage of growth inhibition was significantly lower than that in copper oxychloride treated (F1) (Figure 5). Copper oxychloride amended plates had the lowest inhibition ($F_{(5, 18)} = 336.76$, $p = 0.0000$). These findings indicate that Flutriafol contrary to copper oxychloride, was effective in inhibiting the growth of the isolates from cashew leaf and fruit blight.

All PDA plates amended with copper oxychloride showed no statistical differences in inhibition percentage among themselves nor with un-amended negative controls. Likewise, in Flutriafol-treated plates, the inhibition percentages were not significantly different among themselves. However, inhibition in copper oxychloride-treated isolates had only 15% inhibition which was significantly lower than the 79% observed in Flutriafol amended plates. Silva (2001) and Tavares & Souza (2005) studying mycelial growth inhibition of *Colletotrichum gloeosporioides* from papaya, observed similar results.

Microscopic observations of the isolates from Flutriafol-treated plates indicated no conidia development. The same finding was also reported by Andrade (2016) while investigating the effect of essential oils in vitro, against papaya anthracnose. Contrarily, isolates grown in PDA plates amended with copper oxychloride or in negative controls showed production, germination, and development of mycelium. This again confirms the effectiveness of flutriafol in inhibiting the production of conidia a cutting the life cycle of the fungi while the tested concentration, proportional to the one in use in farmers' fields, for copper oxychloride, was not effective in interrupting the isolates' life cycle.

4. Conclusion

The fungicide-amended PDA test was used for in vitro assessment of Flutriafol and Copper oxychloride against Neofusicoccum, Colletotrichum, and Pestalotiopsis a complex of fungi genera isolated from the cashew leaf and nut blight

symptoms. Flutriafol significantly reduced the colony diameter, the colony growth rate caused the highest inhibition percentage, and production of conidia was interrupted in the Petri dishes. Therefore, the study allows concluding that Flutriafol fungicide is effective in controlling isolates causing cashew leaf and nut blight symptoms and thus the product is recommended for field trials.

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