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In vitro and *in vivo* effect of Mancozeb 80 WP on development of *Pestalotia heterocornis* agent of leaf blight of Cashew in Far North Cameroon

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ABSTRACT
Objective: Cashew cultivation in Cameroon is facing enormous constraints, including fungal diseases. Among these diseases, pestalotia leaf blight occupies an important place in terms of damage in field but no strategy management is available in Cameroon. The aim of the present study is to characterize and evaluate the effects Mancozeb (Mz) fungicide on the development of *Pestalotia heterocornis*, the causative agent of pestalotia leaf blight both *in vitro* and *in vivo*.
Methodology and results: Isolates of *P. heterocornis* were obtained from diseased cashew leaves collected in the field. Different morphotypes of spores were observed under an optical microscope and their biometric parameters were then measured. Growth diameter and pathogenicity test were performed. The Mancozeb concentrations C1 = 500µg /ml, C2 = 50µg / ml and C3 = 5µg /ml were used to compare their effect on the growth and germination of spores of isolates. Several spore morphotypes were identified. The percent inhibition of growth with resistant isolate was 100% and that of the most sensitive was 69.68% at dose C_1. Mancozeb completely inhibited (100%) the germination of conidia of *P heterocornis* at all concentrations tested. *In vivo*, the highest concentration (500µg /ml) of Mancozeb reduced development of disease on plant tested.
Conclusion and application of findings: Mancozeb 80WP reduced the development of *P. heterocornis in vivo* and *vitro*. Mancozeb at the concentration of 500µg /ml can be adopted by the farmers to protect cashew trees against *Pestalotia heterocornis* in nursery.
Key words: *Anacardium occidental* L., *Pestalotia heterocornis*, characterization, Mancozeb 80 WP, percent of inhibition.

INTRODUCTION

Cashew tree (*Anacardium occidentale* L.) is a tropical plant native to Brazil (Coutinho *et al.*, 2016). Its cultivation contributes to the socio-economic development of several countries in the world (Marlos *et al.*, 2007). The nut, which is the main commercial product, is used in food, cosmetics, medicine and automotive industry (Lacroix, 2003). It also participates in the conservation of biodiversity and the reconstitution of degraded and impoverished croplands. Ivory Coast has become the world's largest producer and exporter of cashew nuts with more than 849 thousand tons ahead of India, Vietnam and Brazil (FAOSTAT., 2020; Ndiaye, 2021). Cameroon is not cited among these major producing countries in Africa. Cashew was introduced through the National Fund for Rural Development (FONADER) in 1972 (Hamaya *et al.*, 2019). However, its cultivation is only conducive to the three northern regions of the country namely Adamawa, the North and the Far North. Currently, due to growing demand, many projects to extend its cultivation to other regions such as the East and Center of the country are underway (Hamawa *et al.*, 2019). Today, cashew nuts are a growing cash crop and represent a great opportunity for Africa through the export of its nuts (Dedehou *et al.*, 2015). Its global production has almost doubled in less than a decade, from 2,361,384 tons in 2002 to 4,152,315 tons in 2012 (Adeigbe *et al.*, 2015). Despite the enormous potential for financial income from cashew nut sales worldwide (Adeigbe *et al.*, 2015), cashew nut production in Cameroon is still very low (Hamawa *et al.*, 2019). Currently, there is a document on the "National Strategy for the Development of Cashew Value Chains in Cameroon 2019-2023" which highlights Cameroon's potential to produce and export cashew nuts. Unfortunately, cashew is threatened by many biotic and abiotic constraints that are accompanied by yield losses. Among the biotic constraints, diseases and pests are the most detrimental and compromise cashew yield in terms of quality (Viana *et al.*, 2007). Depending on variety, production area and season, yield losses are estimated to be in the order of 70-100% in Benin (Afouda *et al.*, 2013). In addition, lack of maintenance of agricultural fields also hinders production (Uwagboe *et al.*, 2010). The poor quality of nuts used as seed, post-harvest losses, inadequate storage places for nuts decrease the purchase price of nuts (Nugawela *et al.*, 2006). In the Far North region, particularly in the locality of Maroua and Yagoua, twelve diseases including seven fungal diseases (anthracnose, bud dryness, pestalotiosis, alternaria, rust, powdery mildew and tree dieback), one bacterial disease and three viral diseases and one algae were identified (Ngoh Dooh *et al.*, 2021). Like anthracnose, pestalotiosis appears to be the most dangerous disease with an incidence of 85.33% and 96.66% respectively in Maroua and Yagoua. The same observation was made in Benin (Afouda *et al.*, 2013). The agent responsible of pestalotiosis is *P. heterocornis*, a highly fungus that attacks a variety of crops such as guava, pecan and strawberry (Mazarotto *et. al.*, 2014; Embaby, 2007; Keith et al. 2006). The fungus infects leaves and reduces orchard yields (Silue *et al.*, 2018). Chemical control is the most effective global method against cashew nut diseases (Tonon *et al.* 2017; Silue *et al.* 2018). However, in Cameroon, despite the high incidence of pestalotiosis in all orchards (Ngoh Dooh *et al.*, 2021), no protective measures are undertaken by farmers. Thus, the aim of this study is to characterize and evaluate the efficacy of mancozeb 80 WP against *P. heterocornis* the agent responsible of leaf blight of cashew *in vitro* and *in vivo*.

MATERIALS AND METHODS

Methods

Sampling of leaves with diseases: Sampling of diseased leaves was carried out in two orchards (Palar (14.1961°E-10.4236°N) and Djarengol (14.4814°E-10.6279°N) located in the town of Maroua. Leaves showing symptoms of pestalotia leaf spot were randomly sampled at the level of affected plants using a knife and following the diagonal in each orchard surveyed. Each sample was then placed in a plastic bag and transported to the laboratory.

Obtaining isolates: In the laboratory, after washing the samples under running water, they were disinfected by soaking them in a diluted 2% sodium hypochlorite solution for 3 minutes and then cut into 8 pieces of 0.5 cm. The fragments were then rinsed 3 times with sterile distilled water and dried on sterile filter paper. The disinfected leaf fragments were introduced into Petri dishes containing the WA medium. After the mycelium had formed around the fragments, 0.8 cm mycelium discs were removed and transferred to PDA media boxes with a handle and hermetically sealed. Incubation took place at room temperature under a 12/12 photoperiod for 4 to 7 days (Gupta *et al.* 1986). After incubation, the pure cultures were obtained by successive transplantation of the mycelium into new Petri dishes containing PDA medium.

Monospore cultures: The monospore culture technique described by Booth (1971), which allows a pure culture to be obtained from the fungal spores by spreading on a PDA medium, was used. First, the isolate to be cultured was transferred to a box containing PDA medium for 5-6 days. Then, a mycelial disc was taken from the periphery of the box and introduced into a tube containing 9 ml sterile distilled water. After shaking, a sporal suspension was obtained. Finally, dilutions to the tenth from the sporal suspension were prepared. For this purpose, 1 ml of the sporal suspension was introduced into a tube containing 9 ml of sterile distilled water and the mixture was stirred. The procedure was repeated as many times, and then three drops of the sporal suspension were introduced into a box containing the PDA medium. After 24 h incubation at room temperature and using a binocular loupe, the identification and delimitation of the germinating spores took place. Three to four (3 to 4) conidia were collected using a sterile seed loop and placed in the center of a petri dish containing PDA medium (Rappily (1968).

Characterization of isolates: The characterization of *Pestalotia heterocornis* isolates consisted of a description of the cultural characteristics of each isolate obtained, the determination of the growth rate of each isolate and the identification of the different spore morphotypes under light microscopy and the pathogenicity of the different isolates obtained.

Description and evaluation of the growth rate of the different selected isolates: The description of macroscopic characters was performed on five-day-old PDA cultures incubated at room temperature. Mycelial appearance, colour and growth diameter were evaluated. The mycelial diameter of each isolate was measured daily from 48 hours after culture, using a ruler graduated in cm on the back of the Petri dish. The mycelial growth diameter of each isolate was then calculated using the formula of Singh *et al.* (1993) which follows:

$$D = \frac{d_1 + d_2}{2} - d_0$$

Where d0 is the diameter of the mycelial disc; d1 and d2 are the perpendicular diameters of the fungus and D is the mycelial growth diameter of each isolate.

Identification of conidial morphotypes: The identification of the different spore morphotypes of *Pestalotia heterocornis* was based on the observation of spore shape, the

presence or absence of appendages and the number of flagella and partitions per conidia (Sajeewa *et al.*, 2011).

Measurement of conidial and flagellum size: Conidial size was determined by measuring the length and width of five (5) arbitrarily selected conidia in a conidial suspension of each isolate. Flagellation size was determined by measuring the length of the flagella of the individual conidia retained per isolate. All measurements were performed using an eye-micrometre installed in an optical microscope and the mean values were retained.

Evaluation of pathogenicity of *P. heterocornis* isolates: The pathogenicity of the different isolates was assessed by the whole leaf detached test. A total of 12 leaves at a rate of 3 leaves per isolate were collected from plants aged about 3 months.

Preparation and inoculation of leaves: The leaves were first cleaned with ethanol (70%), then with 1% sodium hypochlorite for 30 seconds. Then they were rinsed three times in sterile distilled water. They were placed in transparent polystyrene trays containing sterile filter paper soaked in sterile distilled water 12 hours before application of the spores. Calibrated using a hematimeter at 3-4,105 spores/ml, a conidial suspension of each isolate was applied to the underside of the leaves and incubated for 14 days at room temperature. To confirm the presence of *Pestalotia heterocornis*, the Koch postulate was applied by re-isolating the individual isolates of the fungus after symptoms appeared on the inoculated leaves.

Assessment of symptoms on detached leaves: Evaluation of symptoms on the detached leaves was done 10 days after inoculation. The modified sensitivity scale developed by (Nyassé *et al.*, 1995) was used. The severity index was calculated based on a sensitivity scale. Where, 0: absence of symptoms; 1: small points of penetration; 2: network points; 3: necrosis diameter between 0.5-1; 4: necrosis diameter between 1-2; 5: necrosis diameter > 2-3.

Evaluation of the effect of Mancozeb on the development of *Pestalotia heterocornis in vitro*

Preparation of the different concentrations: A stock solution of Mancozeb was prepared by solubilizing 2g of Mancozeb powder in 200ml of sterile distilled water, an initial concentration of 0.01g/ml. Different doses were obtained by adding 9, 0.9 and 0.09 ml of the stock solution to 171, 179.1 and 179.91 ml of PDA culture media, respectively, for a final volume of 180 ml. The different concentrations C_1=500µg/ml, C_2=50µg/ml and C_3=5µg/ml were obtained

Evaluation of the effect of Mancozeb on the growth of different isolates: Using a punch, 8 mm diameter mycelial discs were taken from 6-7 day old cultures and placed in the center of each petri dish containing the previously prepared media. Incubation took place at room temperature, at a photoperiod of 12/12. For each treatment, the experiment was repeated 3 times. Radial diameter measurements of each isolate were made daily starting 48 hours after the mycelial discs were cultured. At the end of the experiment, the inhibition percentages I (%) of the different concentrations of mancozeb were determined using the formula:

$$I\ (\%) = \frac{Dc(cm) - Df(cm)}{Dc(cm)} x100$$

Where I (%) = percent inhibition;

Dc = mean diameter of the culture without fungicide;

Df = average diameter of the culture with fungicide.

Evaluation of the effect of Mancozeb on spore germination: Slides containing the fungicide-free PDA culture media (control) and added at different concentrations of Mancozeb 80 WP (C1=500µg/ml, C2=50µg/ml and C3=5µg/ml) were inoculated with spore suspensions (4-5 x 105 spores/ml)

of each isolate. The slides were placed in a sterile, moistened tray and incubated at room temperature. Each treatment was repeated 3 times. The number of germinated spores was assessed 48h after incubation by microscopic observation of the germ tubes on each slide.

Evaluation of the *in vivo* effect of Mancozeb on cashew tree plants

Preparation of the inoculum: The most virulent isolate was selected for this test. The resulting spore suspension was calibrated using the Malassez cell at 3-4,105 spores/ml. A wetting agent (about 1ml soap solution) was added to the spore suspension to facilitate adhesion to cashew tree leaves.

Inoculation of plants: The cashew tree plants, each with about 5 to 6 leaves, were previously washed with running water and then disinfected with a sodium hypochlorite solution (1%). They were then rinsed three times successively with sterile distilled water. The plants were treated at concentrations C1= 500 µg/ml and C3=5µg/ml. A control treatment with sterile soapy water was developed. Each treatment was repeated three times. After treatment, the plants were covered with transparent plastic for at least 12 hours. The next day, the plants were inoculated by spraying with 6 ml of spore suspension solution per plant, i.e. about 1 ml per leaf. Immediately after inoculation, the plants were covered with transparent plastic to allow the maintenance of relative humidity to promote spore germination. The plants were watered every 2 days. Evaluation of the efficacy of Mancozeb to protect plants or reduce *P. heterocornis* infections was performed by observing symptoms 14 and 21 days after inoculation.

Data Analysis: SPSS 20.0 software was used to perform statistical analyses. The comparison of means was done by Duncan's test at the 5% threshold.

RESULTS

Morphological characteristics and growth diameter of *Pestalotia heterocornis* isolates: Several aspects of the mycelium were observed in the different isolates on PDA medium. Some isolates showed mycelium with a compact and fully fluffy appearance, others were characterized by a fluffy appearance in the center with regular and irregular borders (Figure 1). Colony colour varied with age.

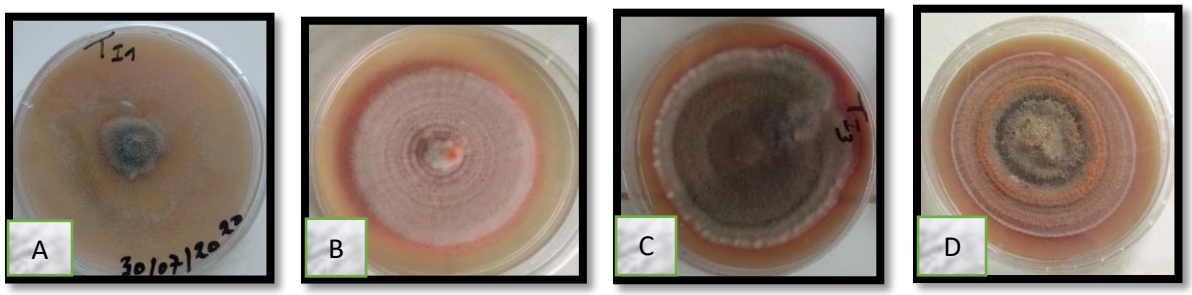

Figure 1: Macroscopic appearance of *Pestalotia heterocornis* isolates on PDA after 7 days of incubation

Statistical analysis showed that there is a significant difference (P=0.0001) between the growth diameters of the different isolates (Table 1). The largest mycelial growth diameter was observed with Pesta1 at 7.2 cm on day 4 and the smallest diameter was observed with Pesta 4 at 2.58 cm.

Table 1: Mycelial growth of *P heterocornis* isolates on PDA after 4 days of incubation

Isolate	Diameter (cm)
Pesta1	7.2 ± 0.4c
Pesta2	3.0 ± 0.3b
Pesta3	3.9± 0.3 b
Pesta4	2.6 ± 0.3a

The numbers followed by the same letter show no statistical difference according to Duncan's test at the 5% threshold.

Morphotypes and spore biometry of the different isolates: Microscopic observation showed spores with several forms. The fusiform form (Figure 2 A, B, C, I) was the most recurrent form, contrary to the globular form (Figure 2 D, E, F, G, H). The conidia were isolated or agglomerated (Figure 2 A). The number of partitions varied from 2 to 4 and the number of flagella varied from 0 to 4 with an abundance of conidia to 2 flagella (Figure 2 B, D). On the other hand, some conidia had neither flagella (Figure 2 F) nor appendix (Figure 2 E).

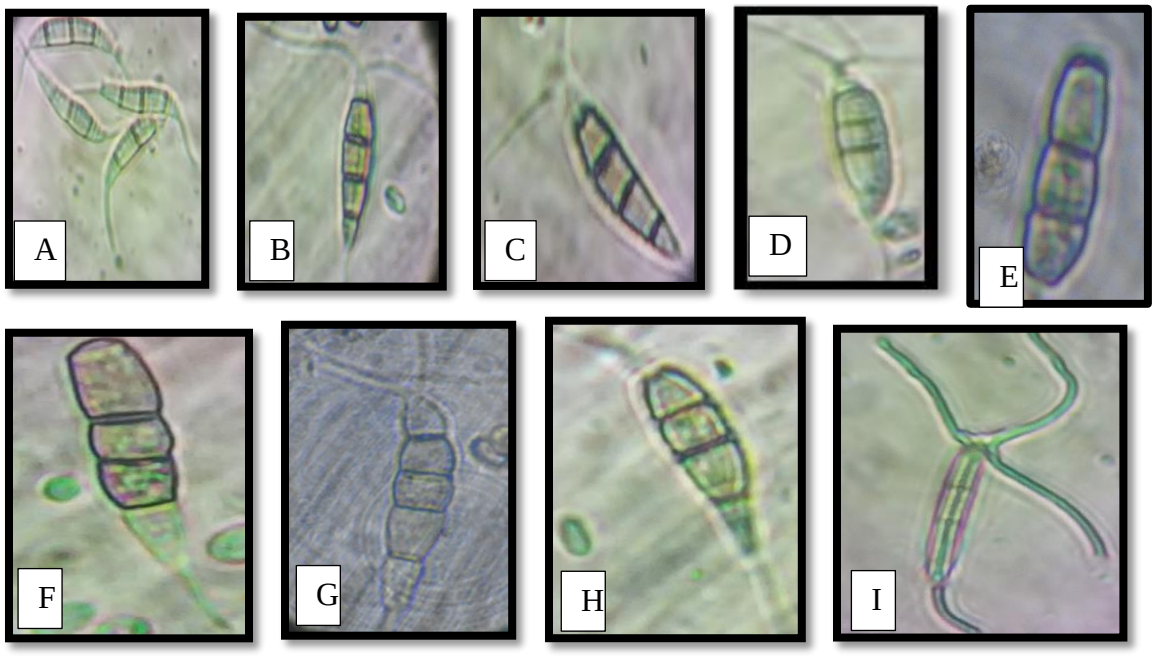

Figure 2: Microscopic characteristic of some spores of *Pestalotia heterocornis*

The conidia of the different isolates ranged in size from 10.4-18.2 µm in length to 3.9-5.2 µm in width. Pesta1 recorded the highest value, 18.2 and 5.2 µm for length and width, respectively, while Pesta3 showed the lowest size 10.4 and 3.9 µm for length and width, respectively (Table 2). Flagellum measurements (Table 2) showed variation between the different lengths obtained. The longest flagellum was observed in isolate 4 at 20.8 µm. The shortest flagellum was recorded in Pesta 2 with a mean value of 10.4 µm.

Table 2: Conidial biometry of isolates of *P. heterocornis*

Isolates	Conidia size (µm))		Average length of the flagella (µm)	Average number of the flagella
	Length	Width		
Pesta1	18.2± 0.7	5.2± 0.3	13± 0.4	0 - 3
Pesta2	13± 0.4	5.2± 0.3	10.4± 0.6	2 - 3
Pesta3	10.4± 0.6	3.9± 0.3	18.7± 0.7	1 - 4
Pesta4	15.6± 0.5	3.9± 0.3	20.8± 0.6	2 - 3

Each value is the mean ± standard deviation of the measurements performed on 5 conidia.

Pathogenicity test: Statistical analysis revealed a highly significant difference in pathogenicity testing between different isolates (P=0.0001). The severity index varied from isolate to isolate. Pesta1 was the most virulent with the highest severity index, 7.5%. However, no symptoms were observed on the leaves that received isolate 4 (Figure 3). Microscopic observation of the fungi re-isolated from the diseased leaves obtained confirmed that they were identical to the original isolates.

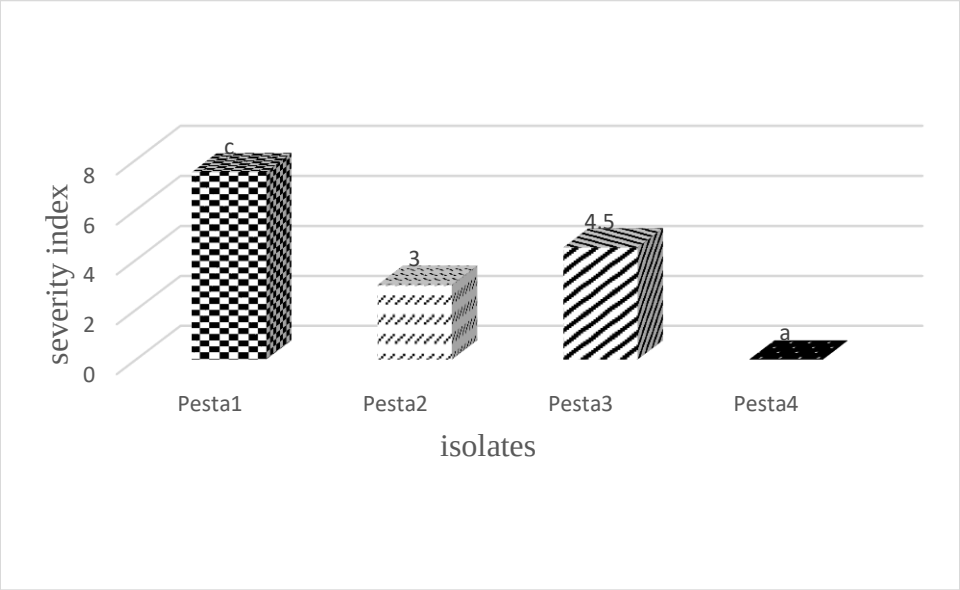

Figure 3: Severity index of the different isolates of *Pestalotia heterocornis* after the pathogenicity test on detached whole leaves

Effect of Mancozeb 80 WP on the growth of *P. heterocornis* isolates: The development of *P. heterocornis* in Mancozeb supplemented Petri dishes was reduced in proportion to the concentrations applied. A significant difference (P<0.05) was obtained between doses C_1 and C_2 and the control with Pesta1 and Pesta 2 (Figure 4 A, B). However, no significant difference (P>0.05) was observed between the control (Co) and dose C_3 with Pesta 3 and Pesta 4 isolates (Figure 4 C, D). With isolate 1, the percentages of inhibition ranged from 73.2% at C_3 to 100% at C1. Slight growth stimulation was observed in Pesta 2 and Pesta 4 isolates at C_3 concentration.

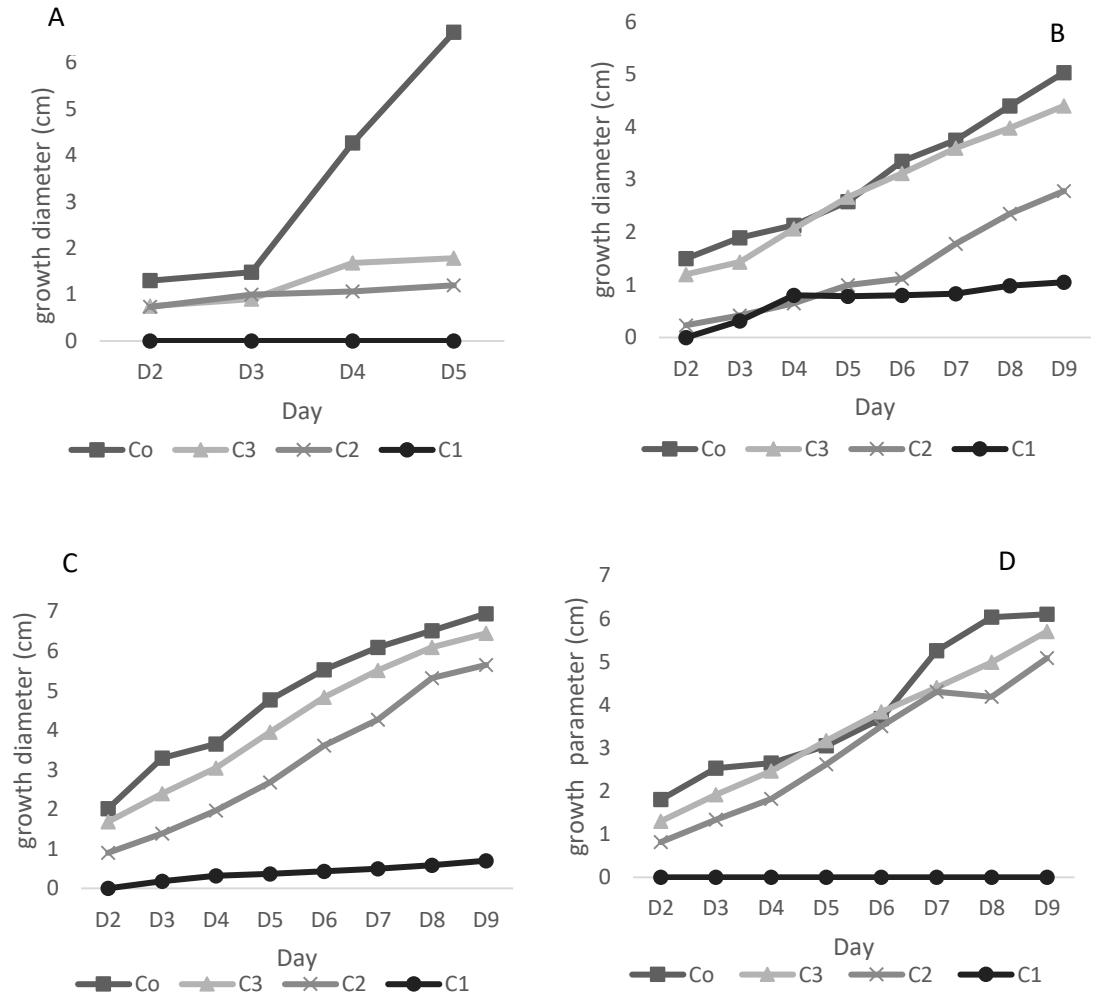

Figure 4: Evolution of mycelial growth of Pesta 1 (A), Pesta 2 (B), Pesta 3 (C) and Pesta 4 (D) isolates in Mancozeb supplemented media.

Effect of mancozeb on spore germination: Statistical analysis on spore germination showed a highly significant difference (P=0.0001). Mancozeb completely inhibited spore germination at all concentrations (Table 4). On the control slides, all spores emitted germ tubes (Fig.5 A), in contrast to media supplemented with Mancozeb 80 WP where no germ tubes were observed after 72 h (Figure 5 B).

Table 4: Percentage inhibition (%) of spore germination of isolates at different concentrations of Mancozeb

	Inhibition percentage (%)			
Isolate	Co	C_1	C_2	C_3
Pesta1	0 a	100 b	100 b	100 b
Pesta2	0 a	100 b	100 b	100 b
Pesta3	0 a	100 b	100 b	100 b
Pesta4	0 a	100 b	100 b	100 b

Values followed by the same letters in the same row are not statistically different at the 5% threshold according to Duncan's test.

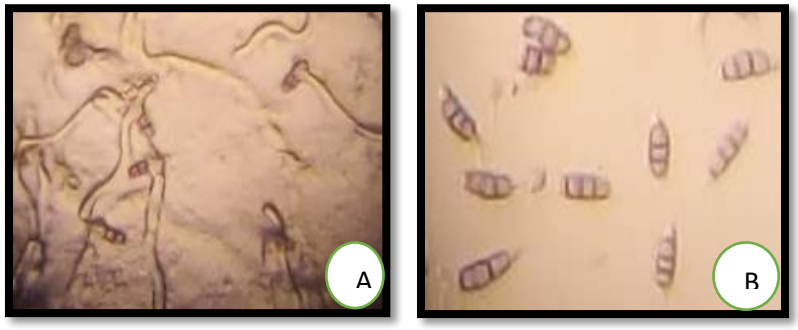

Figure 5: illustration of the spore germination test: spores with germ tube length in control media (A) and spore not having geminated in mancozeb supplemented media (B)

In vivo* effect of mancozeb on *Pestalotia heterocornis: The attack rate was slightly elevated in the control plants (Figure 6 A) and those receiving the C3 dose (Figure 6 B) of Mancozeb. However, very few symptoms were observed in the plants treated with the C1 concentration ((Figure 6 C).

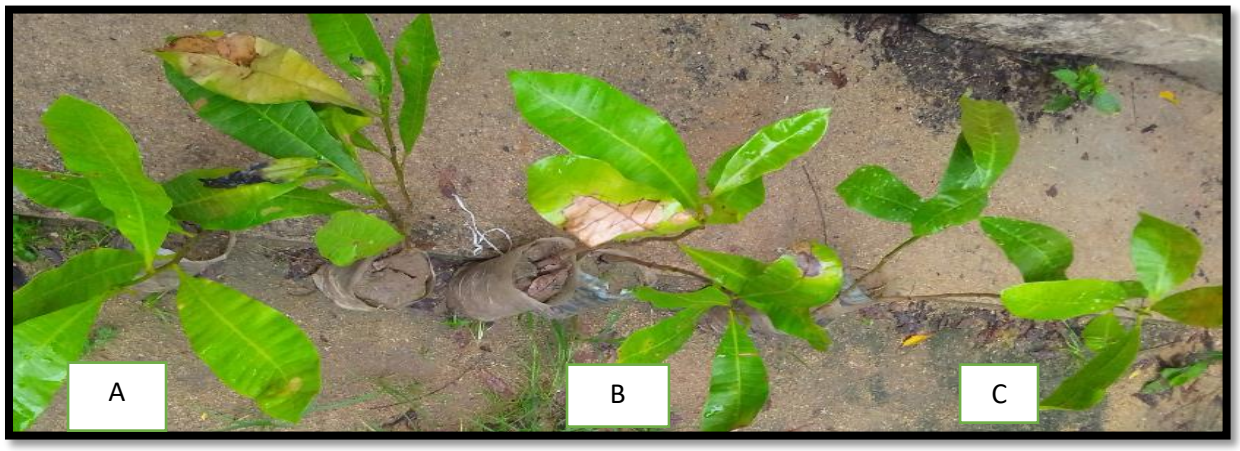

Figure 6: Symptoms of leaf blight on cashew plant inoculated with Mancozeb 80 WP and inoculated with Pesta 1, two weeks after application. A (control plant), B (plant treated with mancozeb at C_3 concentration) et C (plant treated with mancozeb at C_1 concentration)

DISCUSSION

Identification and pathogenicity test revealed that the causal agent of pestalotia leaf blight is *Pestalotia heterocornis*. These results agreed with those of Silue *et al.* (2017) and Afouda *et al.* (2013) who showed that pestalotia leaf blight is due to pestalotia spp. This fungus has been isolated from many other plant such as olive trees (Moustafa *et al.,* 2014) and pecan tree (Mazarotto *et al.*, 2014). Moreover, different types of spores were observed with many flagella and appendages. Sajeewa *et al.* (2011) observed in their study many spores with more than 2 flagella. The morphological characterization of *P. heterocornis* allowed to highlight different isolates in terms of colour, appearance of the mycelium in PDA medium, length (10.4 -18.2 µm), and average width (3.9 - 5.2 µm) of the conidia. These results indicate genetic diversity in *P. heterocornis*. These results are similar to those developed by

Sajeewa *et al.* (2011), Wei *et al.* (2007); Liu *et al.* (2007) who found morphological diversity in *P. heterocornis* isolated from cashew tree leaves. In addition, Hu *et al.* (2007) showed that colony morphology (colour, growth rate and texture) is highly variable in a single Pestalotia isolate. The differences observed at the level of morphotypes and more specifically at the level of each isolate of *P, heterocornis* might therefore justify the different levels of resistance presented by these isolates to the host during the different tests performed. The pathogenicity test was negative in Pesta4 where no symptoms were observed on the leaves inoculated with it. This result can be explained by the physiological condition of the leaf and the age of the spore. Armstrong *et al.* (1940) showed that the pathogenicity of some fungi decreases after a long cultivation period and is not always recovered after passage through the host. However, some studies have shown a high stability of pathogenicity during spore ageing (Messiaen & Cassini, 1968). Mancozeb 80 WP has shown efficacy in reducing the growth of *P. heterocornis* in vitro. At the concentration C1=0.5µg/ml, a total inhibition (100%) of the Pesta1 and Pesta4 isolates and an inhibition rate of more than 60% for the Pesta 2 and Pesta3 isolates were obtained. The efficacy of mancozeb (multi-site fungicide) on the growth of isolates is mainly due to its active ingredient and its mode of action on cell division. They act on enzymatic mechanisms involved in energy production. These results are in agreement with those of Silue *et al.* (2018) who showed the efficacy of carbendazine, a synthetic fungicide commonly used in cashew tree orchards, on the growth of *P. heterocornis* isolates in vitro. In addition, Mancozeb has demonstrated its efficacy on reducing the growth of several other fungi such as *Colletotrichum gloeosporioides*, *Phytophtora infestans* and *Botrytis cinerea* (Tonon *et al.*, 2017; Randriansalma *et al.*, 2014; Hamdache *et al.*, 2010). Mancozeb totally inhibited (100%) the germination of *P. heterocornis* spores at all concentrations tested. This inhibition would be explained by the action of Mancozeb on respiration. Ngoh Dooh *et al.* (2014), showed that contact and multi-site fungicides are known for their action on respiration through the thiol (sulphur) function, which associates with oxygen and thus limits respiration, which is an essential process of germination. In addition, Mancozeb 80 WP has already shown its effectiveness in inhibiting the germination of spores of many fungi (Tonon *et al.*, 2017). The results of the *in vivo* effect of Mancozeb 80 WP, on *P. heterocornis* showed the efficacy of the fungicide as a function of concentration. Plants receiving the highest concentration of Mancozeb were protected compared to those receiving the low concentrations as well as control plants. The results confirm the preventive effect of Mancozeb 80 WP, which is a fungicide of contact against fungal diseases. These results corroborate those of Silué *et al.* (2018) who showed that the Propiconazole protected cashew tree plants against *Pestalotia heterocornis* L. *in vivo*.

CONCLUSION AND APPLICATION OF RESULTS

Mancozeb 80 WP showed efficacy on *P. heterocornis,* agent of pestalotia leaf blight of cashew, according to different concentrations used (C1 = 500µg /ml, C2 = 50µg / ml and C3 = 5µg /ml), *in vitro* and *vivo*. Mancozeb 80 WP provides a basis for integrated control of cashew leaf blight. Mancozeb 80 WP can be applied on nurseries cashew plants, at the concentration of 500µg /ml and on cashew orchards to protect cashew against pestalotia leaf blight.

CONFLICTS OF INTEREST: The authors have not declared any conflict of interest.

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