Effect of Aqueous Neem Extract on Development of *Pestalotia heterocornis* Agent of Pestalotia Leaf Blight of Cashew in Far North Cameroon

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ABSTRACT

Cashew (*Anacardium occidentale* L.) trees are threatened by many fungal diseases, among which pestalotiopsis occupies an important place in terms of damage created in Cameroon. The aim of the present study is to evaluate the effects of aqueous extract of neem seed (AENS) on the development of *Pestalotia heterocornis*, the causative agent of pestalotiopsis of cashew. Isolates of *P. heterocornis* were obtained from diseased cashew leaves collected in the orchards. The concentrations of AENS were $C_1 = 500 \mu\text{g/ml}$, $C_2 = 50 \mu\text{g/ml}$ and $C_3 = 5 \mu\text{g/ml}$. Mancozeb 80 WP (Mz) was used as positive control. Effect of Mancozeb 80WP on the growth and spore germination of isolates were performed. The effect of AENS on the plants was evaluated in vivo. Four (4) isolates of *P. heterocornis* were used. AENS C_1 concentration reduced the growth of all isolates. The percentage of inhibition was ranged from 45 to 90 % with the other concentrations. AENS did not inhibited spore germination (0.0 %). But Mz totally inhibited conidia germination of all isolates. In vivo, AENS protected plants against *P. heterocornis*. AENS can be integrated in management of cashew plants against *P. heterocornis*.

Keywords: *Anacardium occidentale* L, *Pestalotia heterocornis*, neem extract, Mancozeb, inhibition.

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I. INTRODUCTION

Cashew tree (*Anacardium occidentale* L.) is a tropical plant native to Brazil [1]. Its cultivation contributes to the socio-economic development of several countries in the world [2].

The nut, which is the main commercial product, is used in food, cosmetics, medicine, and automotive industry [3]. It also participates in the conservation of biodiversity and the reconstitution of degraded and impoverished crop lands.

Ivory Coast has become the world's largest producer and exporter of cashew nuts with more than 700 thousand tons ahead of India, Vietnam, and Brazil [4]. Cameroon is not cited among these major producing countries in Africa. Cashew was introduced through the National Fund for Rural Development (FONADER) in 1972 [5]. However, its culture 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 [5].

Today, cashew nuts are a growing cash crop and represent a great opportunity for Africa through the export of its nuts [6]. 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 [7]. Despite the enormous potential for financial income from cashew nut sales worldwide [7], cashew nut production in Cameroon is still very low [5]. 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 [8]. Depending on variety, production area and season, yield losses are estimated to be in the order of 70-100% in Benin

[9]. In addition, lack of maintenance of agricultural fields also hinders production [10]. The poor quality of nuts used as seed, post-harvest losses, inadequate storage places for nuts decrease the purchase price of nuts [11].

In the Far North region, particularly in the locality of Maroua, several diseases including fungal, bacterial, algal, and viral diseases have been identified [12]. Anthracnose and pestalotiopsis are the most dangerous diseases with an incidence of 85.33% and 96.66% respectively [9], [12].

The agent responsible of pestalotiopsis is *P. heterocornis*, a highly fungus that attacks a variety of crops such as guava, pecan, and strawberry [13]. The fungus infects leaves and reduces orchard yields [14]. Necrosis appeared on leaves which can drop and cause a reduction in plant photosynthetic surface area.

Chemical control is the most effective global method against cashew nut diseases [14], [15]. However, fungicides unfortunately have consequences on the environment, human health and develop resistance of microorganisms [16].

Alternatives to chemical control, including biological control, are thus being considered. The latter comprises two approaches, including the use of antagonistic microorganisms and the use of plant extracts. Plants have already proven their effectiveness against several fungi [17]-[19].

Neem extract is known for its insecticidal [21], [22] and fungicidal [19], [22] properties. These two characteristics could open up a new avenue in the protection of crops that are generally subject to a pest complex. The general objective of this work is to evaluate the effect of aqueous neem extract against *Pestalotia heterocornis*, the agent responsible for cashew nut pestaliosis in the Far North of Cameroon.

II. MATERIALS AND METHODS

A. Plant Materials

The plant material consisted mainly of cashew tree leaves taken from diseased plants from the orchards of the commune of Maroua I, three-month-old cashew tree seedlings purchased from nurseries and neem (*Azadirachta indica*) seeds collected in the town of Maroua and neem seeds.

B. Sampling and Obtention of Isolates

Leaves showing symptoms of pestalotia leaf blight were sampled at the level of affected plants using a knife in each orchard surveyed. Each sample was then placed in a plastic bag and transported to the laboratory.

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.

The disinfected leaf fragments were introduced into Petri dishes containing the water agar (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 [23]. After incubation, the pure cultures were obtained by successive transplantation of the mycelium into new Petri dishes containing PDA medium.

Identification keys were used to identified isolates of *P. heterocornis* [24]-[26].

C. Characterization of Isolates

Growth mycelia and pathogenicity tests were performed to characterize different isolates retained for this study.

1. Evaluation of the radial growth of the different selected isolates

Three isolates were retained. The mycelial diameter of each isolate was measured daily from 48 hours after culture, using a rule 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. [27] which follows:

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

where d_0 is the diameter of the mycelial disc; d_1 and d_2 are the perpendicular diameters of the fungus and D is the mycelial growth diameter of each isolate.

2. 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.

The leaves were first washed with tap water, then with 1% sodium hypochlorite for 30 seconds and rinsed three times in sterile distilled water. They were kept during 12 h application of the spores in a room with transparent polystyrene trays containing sterile filter paper soaked with sterile distilled.

Calibrated using a hematimeter at $3-4 \times 10^5$ spores/ml, a conidial suspension of each isolate was applied to the underside of the leaves and incubated for 14 days at room temperature. Koch postulate was applied by re-isolating isolates from infected leaves.

The modified sensitivity scale developed by [28] was used to evaluate severity of each isolate. 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.

D. Assessment of the Effect of AENS on Development of *Pestalotia Heterocornis* in vitro

1. Preparation of the different concentrations

A stock AENS solution of 0.01 g/ml concentration was prepared by solubilizing 2 g of powder in 200 ml of sterile distilled water. Different concentrations 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 $C_1=500 \mu\text{g/ml}$, $C_2=50 \mu\text{g/ml}$ and $C_3=5 \mu\text{g/ml}$ were obtained. A concentration of 5 $\mu\text{g/ml}$ of Mancozeb 80 WP were also elaborated.

2. Evaluation of the effect of AENS 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 AENS were determined using the formula:

$$I(\%) = \frac{Dc(cm) - Df(cm)}{Dc(cm)} \times 100$$

where I (%) = percent inhibition;

Dc = mean diameter of the culture without AENS or Mz;

Df = average diameter of the culture with AENS and Mz.

3. Evaluation of the effect of AENS on spore germination

Slides containing the PDA culture media (control) and slides with PDA at different concentrations of AENS and Mz were inoculated with spore suspensions (4.5×10^5 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 48 h after incubation by microscopic observation of the germ tubes on each slide. Percentage of inhibition was calculated at the end of experiment.

4. Evaluation of minimum inhibitory concentrations (MIC)

From the linear regression equation between the Neperian logarithms of the abscissa concentrations and the ordinate inhibition percentages, the concentrations reducing growth and germination by 50% (MIC₅₀) and 90 % (MIC₉₀) were determined [29].

E. Assessment of the in vivo Effect of AENS on Cashew Tree Plants

The most virulent isolate was selected for this test. The resulting spore suspension was calibrated using the Malassez cell at 3.4×10^5 spores/ml. A wetting agent (about 1ml soap solution) was added to the spore suspension to facilitate adhesion to cashew tree leaves.

The cashew tree plants, each with about 5 to 6 leaves, were previously washed with tap 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 with concentrations C₁= 500 µg/ml and C₃=5 µg/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 AENS to protect plants or reduce *P. heterocornis* infections was performed by observing symptoms 14 and 21 days after inoculation.

F. 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.

III. RESULTS

A. Characteristics of Isolates Used

Pest 12 and 13 showed the highest diameter of growth, 6.0 and 6.9 cm respectively. ANOVA showed significant difference ($F=58.3$, $P=0.0001$) among these isolates and pest 14 isolate which diameter of growth was 4.6 cm. The same result was observed with pathogenicity test (Table I).

TABLE I: DIAMETER AND SEVERITY INDEX OF ISOLATES

Isolates	Diameter (cm) of isolates after 8 days of growth	Severity index of isolates (pathogenicity)
Pest 12	6.0 ± 0.3 b	1 ± 0.3 b
Pest 13	6.9 ± 0.3 b	1.5 ± 0.3 b
Pest 14	4.6 ± 0.3 a	$0. \pm 0.3$ b

Values follow with same letters, for the same isolate, are not significantly difference at 5 % Duncan threshold.

B. Effect of AENS on Growth of Isolates of *P. heterocornis*

Fig. 1. Impact of AENS and Mz on growth of different isolates. A) pest 12; B) pest 13; C) pest 14.

A significant difference ($P < 0.05$) was obtained between different concentrations of AENS and Mz. With all isolates, diameter of growth increase with time (day). But growth was very high with control. Concentration C_1 and C_2 reduced growth of all isolates with percentage of inhibition ranged from 53.6 to 73.5 % at day 8. Mz inhibited growth of isolate with percentage of inhibition of 62.9, 92.3 and 100 % respectively with pest 12, pest 13 and pest 14 at the end of experiment (Fig. 1 A, B, C).

1. MIC_{50} and MIC_{90} of AENS on growth of *Pestalotia heterocornis*

MIC_{50} obtained was respectively 2.79, 1.99 and 2.41 $\mu\text{g/ml}$ for pest 12, pest 13 and pest 14. MIC_{90} was ranged from 3.56-4.94 $\mu\text{g/ml}$. The means MIC_{50} and MIC_{90} were respectively 2.40 and 4.08 $\mu\text{g/ml}$ (Table II).

TABLE II: MINIMAL INHIBITORY CONCENTRATION (MIC) OF AENS ON GROWTH OF DIFFERENT ISOLATES OF *PESTALOTIA HETEROCORNIS*

	Pest 12	Pest 13	Pest 14	Mean
MIC_{50} ($\mu\text{g/ml}$)	2.79	1.99	2.41	2.40
MIC_{90} ($\mu\text{g/ml}$)	4.94	3.56	3.74	4.08

C. Effect on AENS on Germination of Spores of *P. heterocornis*

ANOVA showed significant difference on effect of AENS on germination of spores. Any concentration of AENS inhibited germination of spores of any isolate. However, Mz inhibited totally (100 %) germination of spores of all isolates (Table III). In control and with AENS, spores developed germ tubes (Fig. 2 A, C) which were absent with Mz (Fig. 2 B).

TABLE III: PERCENTAGE OF INHIBITION (%) OF AENS ON SPORE GERMINATION

Isolate	Co	C_1	C_2	C_3	Mz
Pest 12	0 a	0 a	0 a	0 a	100 b
Pest 13	0 a	0 a	0 a	0 a	100 b
Pest 14	0 a	0 a	0 a	0 a	100 b

Values follow with same letters, for the same isolate, are not significantly difference at 5 % Duncan threshold.

Fig. 2. Germination of spores of *P. heterocornis* on PDA media supplemented with AENS (a), with Mz (b) and in control (c).

D. In vivo effects of AENS on *Pestalotia heterocornis*

The results showed a clear protection (absence of symptoms) of the plants treated with Mz (Fig. 3 c). However, At C_1 concentration of AENS, 14 days after inoculation, symptoms of pestalotia leaf blight were observed on plants (Fig. 3 b), but less than in control (Fig. 3 a).

Fig. 3. Cashew plants treated with water (A), AENS (B) and Mz (C) and inoculated with spores of *P. heterocornis*.

The symptoms obtained from the test were identical to those at baseline and the pathogen reisolated was *P. heterocornis*.

IV. DISCUSSION

Pathogenicity test revealed that the causal agent of pestalotia leaf blight is *Pestalotia heterocornis*. This result is in agree with those of [9] and [25] who showed that pestalotia leaf blight is due to pestalotia spp. This fungus has been isolated from many other plants such as olive trees [30], tee trees [25] and guava tree [31].

The morphological characterization of *P. heterocornis* allowed to highlight different isolates in terms of growth in PDA medium. This result indicated genetic diversity in *P. heterocornis*. [32] showed that growth rate is a variable in a single *Pestalotia* isolate.

AENS inhibited growth of isolates up than 60 % at all concentration used. This result can be explained by the active ingredient azadirachtin from neem which has already showed antifungal property against many fungi. Moreover, plant extract contains more than one antifungal compound such as flavonol and phenol [33]. Plant neem extracts have already showed efficiency against mainly fungi diseases. [19] demonstrated the efficacy of Neem oil aqueous extract in reducing *Phytophthora sp.* attacks on cocoa pods. Similarly, [34] have demonstrated the efficacy of neem extract on diseases in cherry production. [22] showed that aqueous neem seed extract inhibited growth of *Phytophthora megakarya* in vitro. Mancozeb 80 WP had inhibited, almost totally, the growth of *P. heterocornis* isolates in vitro. This efficacy of mancozeb on the growth is mainly due to its active ingredient mancozeb and its mode of action on cell division. These results are in agreement with those of [14] who showed the efficacy of carbendazine and propiconazole on the growth of *P. heterocornis* isolates in vitro. In addition, Mancozeb has demonstrated its efficacy on reducing in vitro and in vivo the growth of several other fungi such as *Colletotrichum gloeosporioides* [15].

AENS did not have any action on spore germination. This result disagree that obtained by [14] who have showed that, essential oil of *Ocimum gratissimum* totally inhibited spore germination of *P. heterocornis*. Antifungal properties of some plant extracts depend on composition and structure of

his compounds. Thus, these compounds act directly on pathogens and on spores [35], [36].

However, 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. Mancozeb 80 WP is a multi-site fungicide [37] which acts on enzymatic mechanisms involved in energy production. Contact fungicide are known for their action on respiration through the thiol (sulfur) function, which associates with oxygen and thus limits respiration, essential process of germination. In addition, Mancozeb 80 WP has already shown its effectiveness in inhibiting the germination of spores of many fungi [15], [38].

AENS protected cashew plants against *P. heterocornis* *in vivo*. Azadirachtin and the others antifungal compounds of aqueous neem extract have reduced development of *P. heterocornis* on the leaves area. Moreover, AENS is known to stimulate production of phytoalexins and activity of enzymes in plant [39], [40]. This result can also be explained by production of phenols and stimulation of peroxidase (POX) and glucanase enzymes activities which enhanced resistance of plant. This result is in agreement with that of [14] who showed, efficacy of *Ocimum gratissimum* essential oil against *P. heterocornis* *in vivo*.

The results of the *in vivo* effect of Mancozeb 80 WP, on *P. heterocornis* showed the efficacy of the fungicide against *P. heterocornis*. Plants. The result confirms the preventive effect of Mancozeb 80 WP which is a fungicide of contact against fungal diseases. These results corroborate those of [14] who showed that the Propiconazole protected cashew tree plants against pestalotia leaf blight *in vivo*.

Several studies have shown that plant extracts or their derived compounds have been used to control fungal diseases *in vitro*, *in vivo* and in field instead of chemical compounds [33]. The advantage of plants is the non-toxicity to humans and the environment no-development of fungal resistance and low cost of these extracts

V. CONCLUSION

Aqueous extract of neem seed (AENS) has inhibited growth of *P. heterocornis* isolates *in vitro* and protected cashew plants *in vivo*. Neem extract can be integrated in managing program of cashew plants in nurseries.

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