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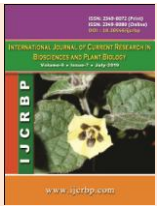

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Pathogenicity and *in vitro* control of *Lasiodiplodia theobromae* and *Fusarium sp.*, pathogens associated with cocoa dieback in Cameroon

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ABSTRACT

Cocoa orchards in different areas of Center and South-West regions of Cameroon were found to suffer from dieback disease. *Lasiodiplodia theobromae* was the most frequent pathogen isolated from plant materials followed by *Fusarium sp*. Inoculation of healthy hybrids plants stressed or not with *L. theobromae* either alone or in combination with *Fusarium sp*. produced typical symptoms whereas *Fusarium sp*. failed to produce these symptoms when plants were not stressed. On amelonado cocoa, only *Lasiodiplodia* isolates produced symptoms when plants were stressed. Symptoms were more severe in plots where plants have been stressed, and Lasiodiplodia from Ndikinimeki was the most pathogenic isolate. *In vitro* efficacy of two chemical fungicides *viz*. carbendazim + chlorotalonil and Mancozeb 80 WP and aqueous extract of *Azadirachta indica* (AEAI) evaluated against *L. theobromae* and *Fusarium sp*. by poisoned food technique at different doses. All the employed doses of the tested fungicides were effective in reducing the growth of the tested fungal species. The best effective ones were carbendazim + chlorotalonil and aqueous extract of *A. indica* seeds at low and high concentrations while, Mancozeb 80 WP was effective at high concentrations only. Carbendazim + chlorotalonil fungicide and aqueous extract of *A. indica* seeds have to evaluate *in vivo* and in natural orchards infected before to be valuate in the fight against cocoa dieback in Cameroon.

Introduction

Cameroon is the fifth largest producer of cocoa (*Theobroma cacao* L.) in the world and the crop generates 25% of foreign exchange for the economy, providing employment to nearly eight million people (CICC, 2017). However, its productivity is low because, cocoa is grown in an area of 700 000 ha, with an annual production of 269 485 tons (CICC, 2017). This low productivity is mainly induced by ravagers such as mirids (Babin, 2009) and diseases which is the main cause of black pod caused by *Phytophthora megakarya* (Mfegue, 2012).

A preliminary survey of cocoa orchards in the bimodal humid forest zone showed that, the disease is becoming epidemic and its incidence

varied according to the locality. Studies were therefore carried out to study the symptomatology, incidence and cause of the disease (Mvondo et al., 2018). Results obtained from that preliminary study showed that, dieback disease caused by *L. theobromae* and *Fusarium sp*. and it is a constraint of cocoa production in Cameroun. So, the determination of the real role of the individual specie in inducting the disease needs to be evaluated so that, control and management strategies can be implemented as means to prevent and/or reduce the incidence of cocoa dieback.

Chemical and biological control methods were usually practiced in order to prevent the plant diseases and to protect the crop plants against pathogens. After more than a century experience of controlling dieback disease, the ideal control regime is still to be found (Adu-Acheampong et al., 2012). Implementation of integrated disease management (IDM) programs which combine cultural, chemical, and biological approaches are highly recommended to control crop diseases, reduce cost, and improve production efficiency. So, use of two chemical fungicides and aqueous extract of neem (*Azadirachta indica*) might significantly reduce the incidence of cocoa dieback disease.

Despite its negative impact on the environment and human health, the use of chemicals continues to be the major strategy to lessen the menace of crop diseases. The aqueous extract of *A. indica* (AEAI) seeds seems to have a high inhibiting potential against parasitic attacks in the farm and during storage of the product. Due to this effect, so much work has been done to show some effects such as nematicides (Kosma et al., 2011), insecticides (Mboussi et al., 2018), bactericides and fungicides (Adeniyi et Abiodun, 2015; Mboussi et al., 2016; Ndogho et al., 2017). The objectives of this study were to evaluate pathogenicity of the most frequently isolated organisms *L. theobromae* and *Fusarium sp*. and to focus on development of news fungicides for disease management strategies to combat cocoa dieback in Cameroon.

Materials and methods

Materials

The biological materials consist of the pods of two varieties cocoa, seeds of *A. indica*, isolates of *L. theobromae* (L Ndiki, L Ntui, L Okola, L Kumba, L Buea) and *Fusarium* sp.. The chemical material consists of two chemical fungicides *viz.,* Mancomax and Banko plus. The trade name of chemical fungicides and their active ingredient and doses are given in Table 1.

Table 1. Trade name of chemical fungicides and their active ingredient.

Commercial name	Chemical name	Recommended dose
Banko Plus	Chlorothalonil 550 g/l Carbendazime 100 g/l	5.33 µl/ml
Mancomax 80 WP jaune	Mancozeb 80 WP.	10 mg/ml

Methods

Colony and conidial morphology

Samples of roots, fruits, stems, branches and leaves were collected from cocoa trees showing symptoms of disease in cocoa orchards of Buea and Kumba, for isolation of the pathogens. Kumba and Buea are two localities of monomodal humid forest zone in Cameroon. Isolation and identification of the pathogens were made in the same way as described by Mvondo et al. (2018), for the samples of the bimodal humid forest zone. The single spore culture from Ndikinimeki, Ntui and Okola obtained by Mvondo et al. (2018) and those from Kumba and Buea were randomly selected for pathogenicity testing and *in vitro* control.

Pathogenicity test

Pathogenicity tests of the most frequent fungi, *L. theobromae* and *Fusarium sp*. were carried out in greenhouse of Institute of Agricultural Research for Development (IRAD). Six-month-old healthy cocoa come from several hybrid varieties of the IRAD and amelonado variety were used. For inoculation, five isolates of *L. theobromae* and one isolate of *Fusarium sp.,* representing all the species identified in the study were grown on 2% PDA for 10 days prior to inoculation. To inoculate cocoa plants, wounds were made on the stems between soil and cotyledons by removing the outer bark with a 06 mm diameter cork-borer. 06 mm diameter plug of each isolate was placed into each wound, with the mycelium facing the cambium.

The inoculated wounds were wrapped with parafilm to prevent desiccation and contamination. Control plants were inoculated with sterile PDA plugs. The cocoa plants were arranged in a split plot consisting of three blocks with the main factor variety, secondary factor isolates and experimental constraint water stress. Two weeks after inoculation, half of the plants in each block experienced a three-week water stress. After these three weeks of stress, all the plants in the block were watered after two days until the end of the experiment. The entire trial was repeated twice. For each experiment, 288 plants were used, 144 for each variety. In each sub-block, 3 plants per variety were used for inoculation of each isolate.

Observations of symptoms caused by *L. theobromae* and *Fusarium sp*. were conducted on leaves every week until twelve weeks after inoculation. Disease incidence was calculated using the formula $I = a/b \times 100\%$, where I is disease incidence by *L. theobromae* or *Fusarium sp., a* is the number of leaves with symptoms and b is the total number of leaves observed (Rosmana et al., 2014).

Disease severity was evaluated through a diagrammatic scale consisting of four scores based on stem and crown death (Adu-Acheampong, 2009). In this scale, score 1 = plants with no visual symptoms; 3 = slight distortion of apical leaves and visible browning on leaves, or those that show signs of recovery; 6 = isolated patches of dead leaves appearing half-wilted, half green and score 9 = dead plant i.e. no green tissue.

Twelve weeks after inoculation the bark lesion lengths as well as the length of cambium discoloration were measured to assess the pathogenicity of the tested. Isolate was qualified to pathogen when the length of the lesion produced in the cambium was upper to 06 mm (Correia et al., 2016).

To determine colonization of isolates in plant tissues, a small piece of necrotic tissue was cut from the edges of all lesions and placed on PDA for isolations to show that the inoculated fungus was associated with the lesions. For this purpose, tissues were disinfected in 70% ethanol for 2 minute, followed in 0.5% sodium hypochlorite, and sterilized water as mentioned above and a small portion of these plant parts were placed on PDA medium in Petri dish. The occurrence of *L. theobromae* and *Fusarium sp*. isolates was then identified and compared with the isolates used for inoculation.

In vitro evaluation

The relative efficacy of two chemical fungicides *viz.,* Banko Plus and Mancomax; and aqueous extracts of *A. indica* at four concentrations was tested against fives isolates of *L. theobromae* and one isolate of *Fusarium sp*. under laboratory condition. The *in vitro* evaluation of the fungicides was by food poisoning method. For the preparation of different concentration of chemical fungicides, the decimal dilution was used. So, four concentrations of each fungicide were prepared from the manufacturer recommended dose in mixing the tested concentrations of fungicides with PDA medium before solidification to obtain: 10^{-6} DR, 10^{-5} DR, 10^{-4} DR and 10^{-3} DR. Then, PDA mixed with fungicide was poured into five sterilized plates (90 mm).

To obtain four concentrations of AEAI: 12.5 mg/ml, 25 mg/ml, 50 mg/ml and 100 mg/ml; 2.5 ml, 5 ml, 10 ml and 20 ml were taken into mother solution (50 g of seeds grinded/100 ml distilled water) and were respectively added with 97.5 ml, 95 ml, 90 ml and 80 ml of PDA. 100 ml of each solution obtained was poured into five sterilized plates.

The plates were inoculated in the center with an equal disc (6 mm in diameter) of the test pathogenic fungi (*L. theobromae* isolates and *Fusarium* sp.) then, incubated at ambient temperature (25°C). Plates free of any one of the tested fungicides were used as control. Five Petri plates were used as replicates for each particular treatment. Two perpendicular diameters of the fungal growth were measured daily until the upper surface in control treatment was fully covered with the mycelia growth. The means of the colony diameters were calculated and the percent growth inhibition of *L. theobromae* and *Fusarium* determined using the formula adopted by Opara and Wokocha (2008) as follows:

$$\%\ \text{Growth inhibition} = \frac{dc - dt}{dc} \times 100$$

Where, dc = Colony diameter of control; dt = Colony diameter of treated plates.

Determination of minimal inhibiting concentrations

The effective fungicide concentration (µl/ml for chemical fungicides, and mg/ml for AEAI) to inhibit 50% of mycelial growth (EC50) was calculated for individual isolates and fungicides by linear regressions of the mycelial growth inhibitions versus the log10 transformation for each concentration of each fungicide (Pereira da Silva et al., 2012).

Fungicidal and fungistatic test of different aqueous extracts

After incubation, the treatment in which the growth of mycelium was completely inhibited is counted and explants are transferred into a new plate containing PDA medium. Seven days after, if there is a sign of regrowth in the medium, the extract is qualified as fungistatic; in the contrary case it is qualified as fungicide (Kishore et al., 1993).

Statistical analysis

An analysis of variance (ANOVA) was done using SPSS 20.0 (the generalized linear model). Duncan test at 5% were used to compare the averages.

Results

Colony and conidial morphology

The 20 fungal strains isolated from diseased tissues from Kumba and Buea were described and identify according to their morphological structures based on microscopic observations and literature guides. All isolates showed morphological features typical of the genus *Lasiodiplodia*, namely slowly maturing conidia with thick walls and longitudinal striations (Punithalingam, 1976 and 1980). They grew rapidly on PDA, covering the entire surface of the Petri dishes within 3 days. The aerial mycelium was initially white, turning dark greenish-grey or grayish after 4–5 days at 25°C. According to dimensions (20.2 - 26 × 9.4 – 14.36 µm) and shape (ovoid with a broad and rounded apex and tapered at the base) of conidia, all isolates are identified as *L. theobromae*.

Pathogenicity test

Symptoms of disease on inoculated plants

Inoculation with five isolates of *L. theobromae* and *Fusarium sp.* resulted in symptoms on the leaves starting from the lower leaves and moving to upper leaves. In the stressed sub block, the symptoms produced by all isolates of *L. theobromae* appeared two weeks after inoculation, while for the *Fusarium sp.* appeared at sixth week after inoculation on hybrid cocoa plants. Symptoms on amelonado cocoa plants appeared six weeks after inoculation with *L. theobromae* isolates only. After, three weeks of hydric stress and five of watering, more than 95% of hybrids plants inoculated with *L. theobromae* isolates were completely wilted (Fig. 1-a) and only, some plants inoculated with *Fusarium* presented a yellowing of lower leaves (Fig. 1-b). For amelonado plants, only some inoculated with *L. theobromae* presented a yellowing of lower leaves (Fig. 1-c), the others and those inoculated with *Fusarium* were apparently healthy (Fig. 1-d). All control plants had a healthy appearance, so no symptoms of the dieback disease on that plants (Fig. 1-e).

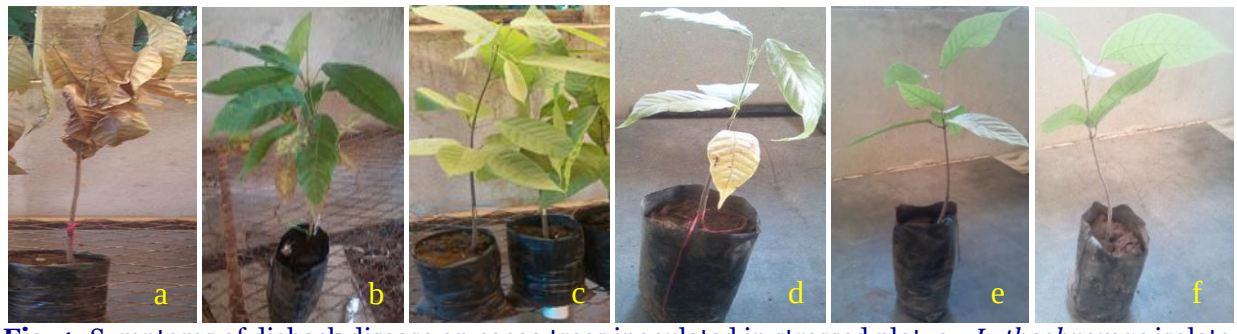

Fig. 1: Symptoms of dieback disease on cocoa trees inoculated in stressed plot; a - *L. theobromae* isolate; b - *Fusarium*; c - control (hybrid cocoa). d - L. theobromae isolate; e - Fusarium; f - control (amelonado cocoa).

In the no stressed sub block, symptoms on the leaves on hybrid plants appeared six weeks after inoculation as yellowing. It was about yellowing and wilting of the lowers leaves (Fig. 2-a). However, those symptoms were only observed on plants inoculated with *L. theobromae* isolates. No symptoms of dieback disease on plants inoculated with *Fusarium sp*. and control plants (Fig. 2-b and 2-c). For amelonado plants, no symptoms of the disease were observed irrespective of the inoculated isolate (Fig. 2-d, 2-e and 2-f). Symptoms observed in all plots were similar to those generally observed in cocoa plantations naturally infected by dieback disease.

Fig. 2: Symptoms of dieback disease on cocoa trees inoculated in no stressed plot; a - *L. theobromae* isolate; b - *Fusarium*; c - control (hybrid cocoa). d - *L. theobromae*; e - *Fusarium*; f - control (amelonado cocoa).

Disease incidence

In the stressed plots, disease incidence was higher in the hybrid plants infected with L Ndiki + Fusa and L Ndiki (100 %). Duncan test ($p<0.05$) did not show a significant difference between disease incidence recorded on plant inoculated with isolate of *L. theobromae* from Ntui and Kumba; and from Buea and Okola. The smallest disease incidence was obtained with *Fusarium sp*. No disease in the plants control. On amelonado cocoa, disease incidence was higher on plants infected by L Ndiki + Fusa and L Ndiki (12 et 11.8%), followed by those infected L Ntui (10.3%), L Kumba (8.1%), L Buea (4.2%) and L Okola (3%). No disease on amelonado plants inoculated with *Fusarium sp*. and control (Fig. 3-A).

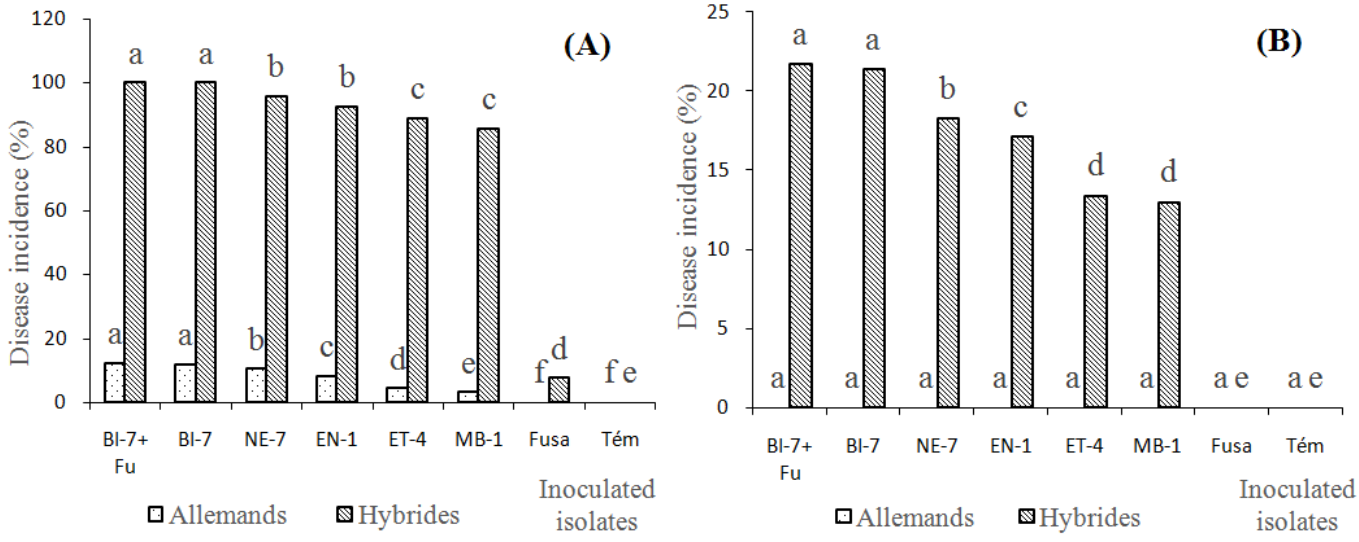

Fig. 3: Disease incidence 12 weeks after inoculation: (A) - in stressed sub block; (B) - in no stressed sub block (BI7 = L. Ndiki, NE = L. Ntui, EN1 = L. Kumba, ET4 = L. Buea, MB1 = L Okola), Fusa = *Fusarium* and Tem = control).

In the no stressed sub block, the disease incidence was higher on hybrid plants inoculated with L Ndiki + Fusa and L Ndiki (21.7 and 21.4%). The Duncan test showed a significant difference between those recorded with the others isolates. No symptoms were observed on the plants inoculated with *Fusarium* and control plants. Disease incidence was zero on amelonado plants whatever the inoculated isolate (Fig. 3-B).

Disease severity

Disease severity was higher in stressed sub-blocks; and in these, it was higher on hybrid cocoa trees. All hybrid cocoa inoculated with *Lasiodiplodia* isolates were completely necrotic or half-dead, hence the scores of 9 and 6. The hybrid cocoa trees inoculated with *Fusarium* showed wilting of the lower leaves (score 3). All amelonado cocoa inoculated with *Lasiodiplodia* isolates showed wilting of the lower leaves. No symptoms on amelonado cocoa inoculated with *Fusarium* sp. and control (Table 2). In the no stressed blocks, all hybrid cocoa inoculated with *Lasiodiplodia* isolates showed wilting of the lower leaves (score 3). No symptoms on amelonado cocoa was observed whatever the inoculated isolate hence the score 1 (Table 2).

Table 2. Disease severity on cocoa plants 12 weeks after inoculation.

Isolates inoculated	Sub block stressed		Sub block no stressed	
	Amelonado cocoa	Hybrid cocoa	Amelonado cocoa	Hybrid cocoa
L Ndiki BI-7+ Fusa	3	9	1	3
L Ndiki BI-7	3	9	1	3
L Ntui NE-7	3	9	1	3
L Kumba EN-1	3	9	1	3
L Buea ET-4	3	6-9	1	3
L Okola MB-1	1	6-9	1	3
Fusarium	1	3	1	1
Control	1	1	1	1

Virulence of isolates

All isolates (*L. theobromae* and *Fusarium sp.*) produced lesions longer than 0.6 cm on the cambium of hybrid cocoa plants. However, in the stressed plots, the measures of lesions were only made on plants inoculated with *Fusarium* because plants inoculated with *Lasiodiplodia* isolates were completely necrosed. No visible necrosis on control plants.

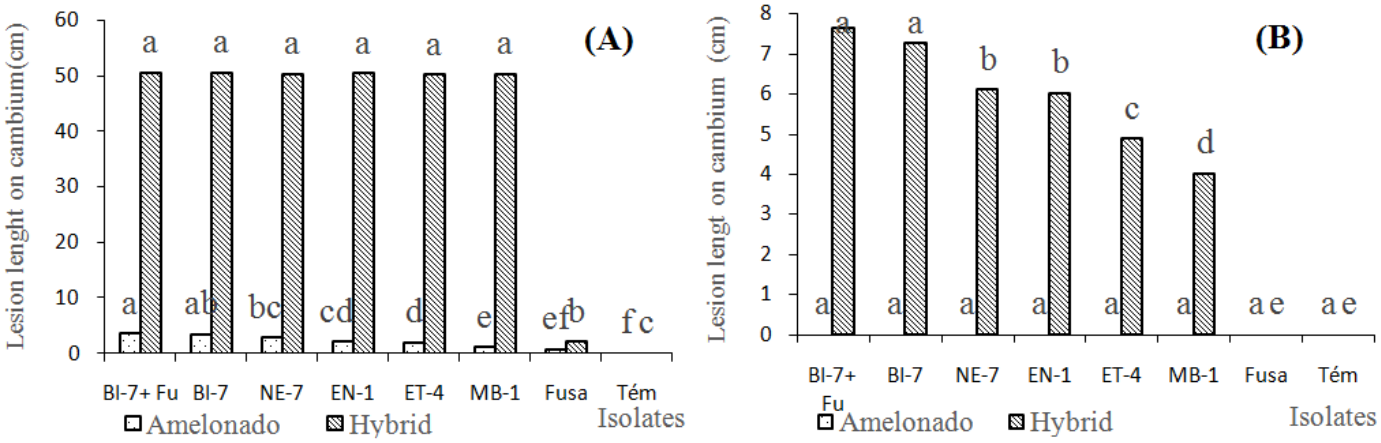

Fig. 4: Means lesion lengths (cm) on cambium of cocoa plants 12 weeks after inoculation with isolates: A- in stress block; B-in no stressed block (BI7 = L. Ndiki, NE = L. Ntui, EN1 = L. Kumba, ET4 = L. Buea, MB1 = L Okola), *Fusarium* (Fusa) and control (Tem) in no stressed sub block. Lesion lengths caused by isolates marked with the same letter are not significantly diff erent (P<0.05).

Duncan test showed that, the mean lengths of lesions produced by all *Lasiodiplodia* isolates on amelonado cocoa differed significantly ($P<0.05$) from the controls. The lesion length produced by *Fusarium* on amelonado plants was not significantly different of control (Fig. 4-A). In the no stressed sub blocks, all *Lasiodiplodia* isolates produced lesions significantly different from control on hybrid cocoa. However, isolate L Ndiki was most virulent and produced longer lesion (7.25 ± 0.16) than the other isolates. No visible lesions on the cambium of plants inoculated with *Fusarium sp*. and control plants. No lesion was observed on cambium of amelonado cocoa whatever the inoculated isolate (Fig. 4-B).

All the isolates of *L. theobromae* were successfully re-isolated from all the cocoa trees inoculated in stressed sub blocks. In the no stressed sub blocks, *L. theobromae* were only re-isolated from all hybrids cocoa trees. *Fusarium sp*. was exclusively re-isolated from the lesion emerging from inoculated hybrid cocoa trees in stressed sub blocks.

Effect of chemical fungicide and AEAI on the growth of different pathogens

In vitro effect of Banko plus on growth of different pathogens

Inhibition of the growth of *Lasiodiplodia* isolates and *Fusarium sp*. under the influence of chlorothalonil + carbendazim was visible 24 h after incubation. In comparison with control, all doses tested presented a significant inhibition of the mycelia growth of pathogens. For isolates of L. Ntui, L. Ndiki, and Fusa, an inhibition of 100% was obtained with C3 and C4. The smallest inhibition (60.95%) was obtained with dose C1 on the *Fusarium* strain (Fig. 5). There was total inhibition of mycelia growth of L Okola, L Buea and L Kumba isolates with all doses tested (Fig. 6).

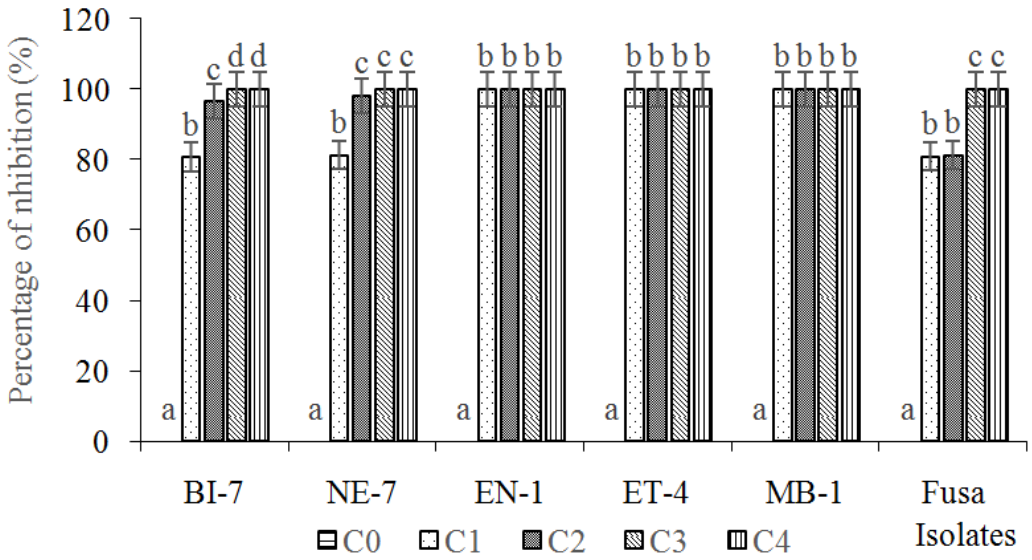

Fig. 5: Banko effect on growth of different isolates. *The values of the same isolates carrying different letter are significantly different when $P<0.05$.

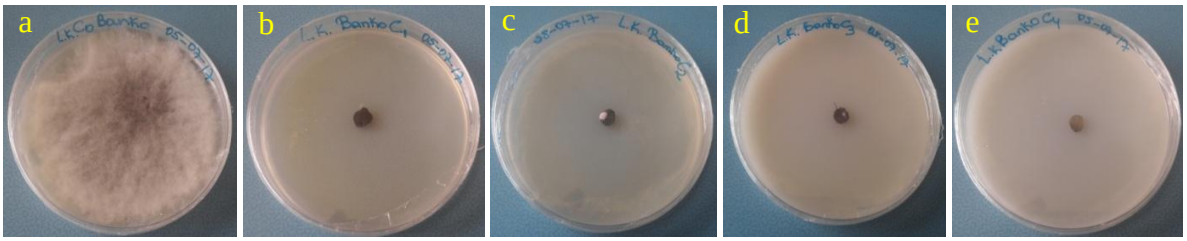

Fig. 6: Effect of Banko (chlorothalonil + carbendazim) on *in vitro* mycelial growth of *L. theobromae* from Kumba.

Effect of Mancomax on growth of different pathogens

Mancozeb 80 WP proved to be efficient against the growth of *Lasiodiplodia* isolates and *Fusarium sp*. An inhibition of 100% was obtained with the treatment C4 on all the *L. theobromae* isolates and *Fusarium sp*. (Fig. 7). C3 also showed a total inhibition (100 %) of the growth of L Okola, L Buea and L Kumba isolates (Fig. 8). C1 showed the smallest inhibition (7.16%) on L Ndiki (Fig. 7).

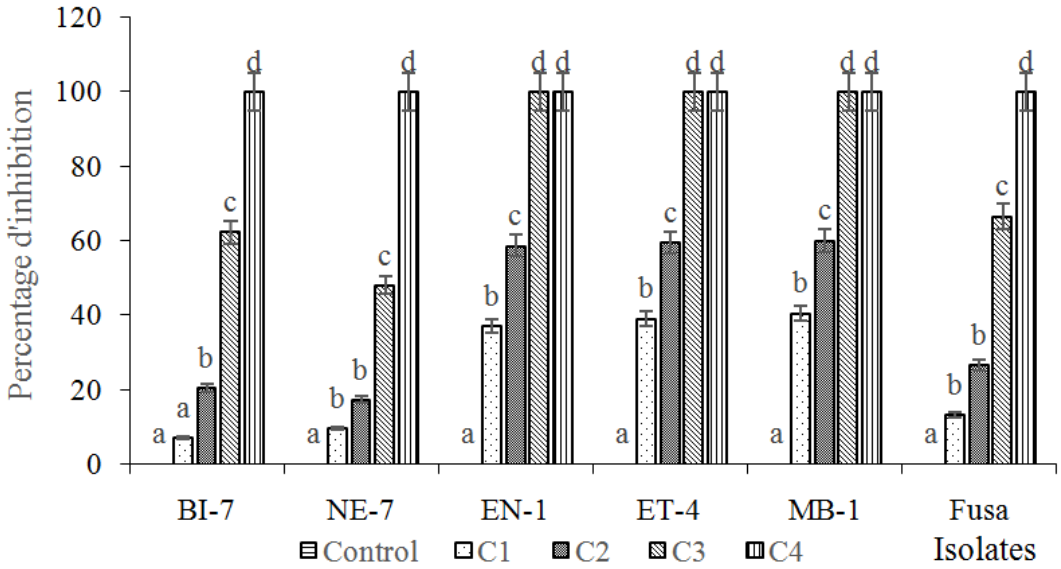

Fig. 7: Mancomax effect on growth of different isolates. *The values of the same isolates carrying different letter are significantly different when $P<0.05$.

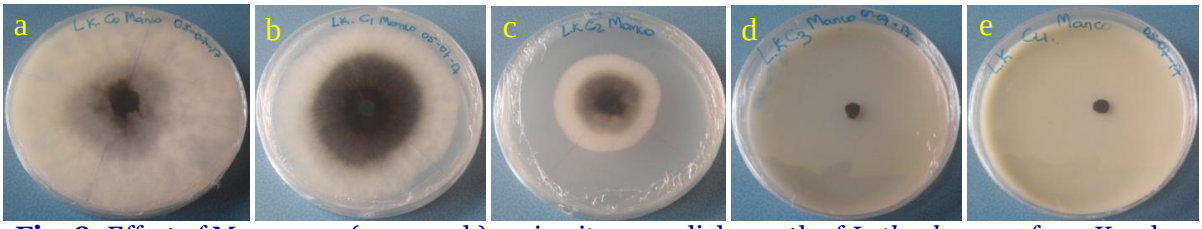

Fig. 8: Effect of Mancomax (mancozeb) on *in vitro* mycelial growth of *L. theobromae* from Kumba.

Effect of AEAI on growth of different pathogens

AEAI considerably reduced the mycelium growth of all *L. theobromae* isolates and *Fusarium*, hence the significant difference observed at 5% between control and tested doses. Here, inhibition was proportional, because it increased with the extract concentration. Hence the observation of a total inhibition of the mycelium growth of all *Lasiodiplodia* isolates and *Fusarium sp*. with C4 dose (Fig. 9). However, an inhibition of 100% was also obtained on the mycelia growth of L Kumba, L Okola, L Buea and L Ntui isolates at C3 (Fig. 10).

MIC50 of pesticides

The different regression lines of different strains helped to determine the minimum inhibitory concentration. Banko plus showed the lowest minimal inhibitory concentration on all isolates tested. LBuea strain showed the lowest minimal inhibitory concentration of the extract while the L Ndiki strain shows the highest minimum inhibitory concentration (Table 3).

Fungicidal and fungistatic activities of different pesticides

All doses which completely inhibited the mycelium

growth of isolates were used for that test. The tested doses C1, C2, C3 and C4 of Banko Plus and C3 and C4 of AEAI were fungicidal on all *Lasiodiplodia* isolates and *Fusarium*. The tested doses C3 and C4 of Mancomax were fungistatic on all *Lasiodiplodia* isolates and *Fusarium*.

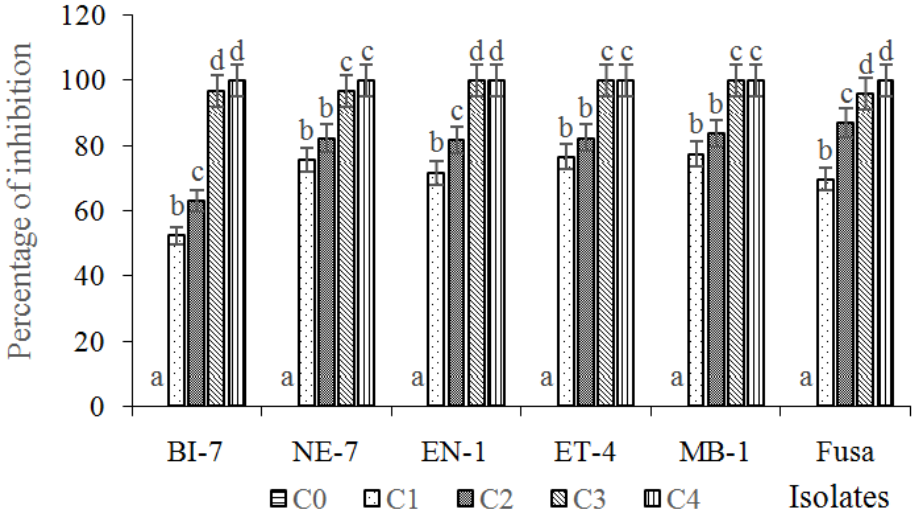

Fig. 9: AEAI effect on growth of different isolates. *The values of the same isolates carrying different letter are significantly different when $P<0.05$.

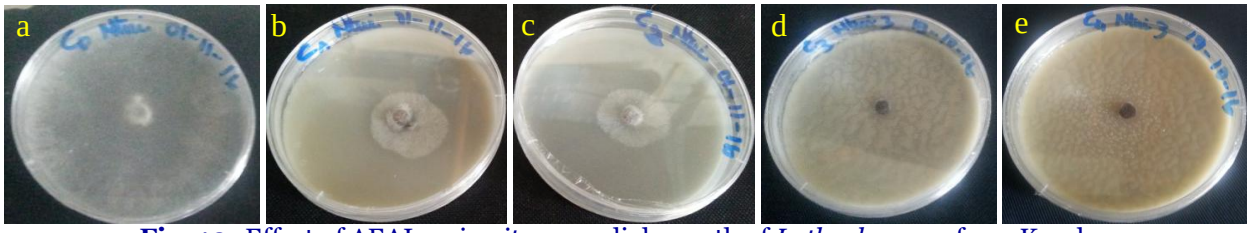

Fig. 10: Effect of AEAI on *in vitro* mycelial growth of *L. theobromae* from Kumba.

Table 3. The MIC50 of the mycelium growth of different isolates and tested fungicides.

Isolates	Banko Plus (µl/ml)	Mancomax (µl/ml)	AEAI (mg/ml)
	MIC 50	MIC 50	MIC 50
L Ndiki	0.97	1	12.59
L Ntui	0.97	1	1.64
L Kumba	10^{-6}DR#	1	2.65
L Okola	10^{-6}DR#	1	1.53
L Buea	10^{-6}DR#	1	1.25

Discussion

Fusarium sp. only caused symptoms in plants inoculated when they were stressed, while all *L. theobromae* isolates caused symptoms on stressed and unstressed plants. Severity of the symptoms of plants inoculated with *Lasiodiplodia* isolates was higher than that observed on plants inoculated with *Fusarium sp.* in stressed sub block. It clearly indicates that, cocoa dieback is caused by *L. theobromae* and *Fusarium sp.* plays few significant roles in disease development when the plants are stressed. These results are in close confirmation to those of Khanzada et al. (2004), who recorded that, inoculation of healthy mango plants with *L. theobromae* either alone or in combination with *F. solani* produced typical symptoms whereas *F. solani* failed to produce these symptoms. Similarly, Safdar et al. (2015) observed that, *L. theobromae*, *P. parasitica*, *C. glocosporioides*, *Curcularia lunata*, *F. oxysporium*, *Helminthosporium spp.*, *F. solani* and *A. niger* were pathogenic on guava.

However, *L. theobromae* produced the highest percent infection and the largest lesion size. Nevertheless, Rosmana et al. (2014) showed that, cocoa dieback only is caused by *Fusarium sp*.

There was variability in the aggressiveness among *L. theobromae* isolates. Isolate from Ndikiniméki (BI-7) was more aggressive than isolates of Ntui (NE-7), Kumba (EN-1, Buea (ET-4) and Okola (MB-1). Findings of Shah et al. (2010) on pear dieback in India, as well as those of Rodriguez et al. (2017) on mango dieback in Peru, also showed variability in the aggressiveness of *L. theobromae* isolates.

In this work, *Fusarium sp*. isolate was less virulent when plants were stressed. *Fusarium sp*. and other species can colonize plants endophytically, an insidious process as it does not lead to symptom development, but contributes to a build-up in inoculum levels. However, stress may alter the relationship between a *Fusarium sp.* endophyte and its plant host, leading to disease development (Burgess and Bryden, 2012). Both groups (*Lasiodiplodia* and *Fusarium*) of fungi required anterior injury of the stem to invade and degrade the woody tissue of the cacao tree. These results suggest that *L. theobromae* and *Fusarium sp.* infect cocoa trees through pruning wounds and mirid wounds during feeding. Thus, pruning wounds and mirid lesions provide not only an entry point for pathogens, but are also a source of stress for the host plant. This in combination with environmental stressors (drought, high temperatures) may be the main cause of the increased incidence of the disease (Rodriguez et al., 2017; Voula et al., 2018).

Symptoms in stressed sub block were more severe than those seen on unstressed sub block. Disease incidence was also higher in stressed sub blocks. The high incidences in the stressed sub blocks would simply be explained that, in times of stress (water or physiological), the species of the genus *Lasiodiplodia* act like real parasites. A particular characteristic of fungi of the *Botryosphaeriaceae* family is that, they can live as endophytes in the plant organs, in the latent phase without producing clear diseases symptoms. These results supported by those of Desperez et al. (2006) who have shown that several factors, such as water stress tending to weaken the plant can promote the advent and rapid evolution of the disease. Under non-water stress, the incidence of the disease is 0 % in all Amelonado cacao trees whatever the isolate inoculated and low in hybrid cocoa. It shows that, the favorable conditions of humidity to the development of cocoa trees would vary significantly reduce the growth of fungi in the tissues of the plant.

Disease symptoms on Amelonado cocoa trees only observed when they stressed. Symptoms observed on hybrid cocoa when they stressed or no. Several factors such as the length of the lesion in cambium, wilting and yellowing of the leaves can be used to determine the resistance of the cocoa genotypes to dieback. Adu Acheampong et al. (2012) showed the existence of a direct correlation between visible symptoms of dieback and the lengths of necrosis in the cambium. Indeed, cocoa varieties with a large lesion length are more susceptible to dieback and those with a small length of lesion tolerant. The results thus obtained show that, hybrid varieties were susceptible to dieback and amelonado varieties were tolerant. This result seems to be in disagreement with to those of Adu Acheampong et al. (2012), who showed that the amelonado variety was more susceptible to cocoa dieback in Ghana than three hybrid cocoa genotypes (CATIE 1000, T85 / 799 and MXC 67). This discordance on sensibility of amelonado variety to dieback could be due to environmental condition in the two countries. A disease is in fact determined by the tripartite interaction between host, pathogen and environment. In both cases, host and pathogens are the same.

Aqueous extracts of *A. indica* seeds (AEAI), a contact fungicide mancomax (mancozeb80 WP) and a systemic fungicide banko plus (chlorothalonil and carbendazim) were used to evaluate their effects on the *in vitro* development of the pathogens responsible for cocoa dieback. Results show that all the pesticides used significantly reduce the mycelial growth of isolates of *L. theobromae* and *Fusarium sp*. This could be due to the sensitivity of the cell membrane in contact with biological and chemical pesticides.

The chemical fungicides used, *viz*., chlorothalonil + carbendazime (Banko plus) and mancozeb (Mancomax) inhibit the growth of all isolates at all tested concentrations. However, Banko plus was

more effective in completely inhibiting mycelial growth of all isolates at C3 and C4 and isolates of kumba, Buea and Okola at all concentrations. It also showed a fungicidal effect on all isolates, compared with Mancomax which showed very low inhibition at C1 and which also had a fungistatic effect on all isolates tested. The effectiveness of Banko Plus may possible due to its active ingredients chlorothalonil and carbendazim, which respectively gives it a contact and systemic mode of action, unlike mancomax, which the mancozeb only gives a contact mode of action. Several studies have also shown the efficacy of these two fungicides on *L. theobromae* and *Fusarium sp.* (Adeneyi et al., 2014; Safdar et al., 2015). The efficacy of systemic fungicides, like banko plus, by inhibiting mycelial growth of pathogens, is due to their ability to induce morphological anomalies on pathogens. Saeed et al. (2017) have indeed shown that the use of Score, Cidely Top and Pentiopyrad, three systemic fungicides, resulted in a significant inhibition of the mycelial growth of *L. theobromae,* inducing anomalies such as septal malformation, cytoplasmic coagulation and thickening of the hyphal extremities.

AEAI also significantly reduced mycelial growth of all isolates. Total inhibition of mycelial growth was observed at 100 mg / ml on all isolates and at 50 mg / ml on L Kumba, L Buea and L Okola isolates. This extract showed a fungicidal effect on all isolates. The efficacy of aqueous neem extracts in the control of plant pathogens has already been demonstrated by several authors. Singh et *al.* (1993) evaluated 11 plant extracts on the pathogens responsible for banana diseases, extracts of *Azadirachta indica* and *Ocimum sanctum* were the most effective in inhibiting the mycelial growth *of L. theobromae, Fusarium oxysporum, Helminthosporium speciferum, Curvularia lunata Aspergillus flavus* and *Trichothecium roseum.*

Mboussi et al. (2016), as well as Ndogho et al. (2017), achieved total inhibition of mycelial growth of *P. megakarya* and *Phakopsora pachirhizi* at 50 mg / ml, the causal agents of cocoa black pod and Asian soybean rust, respectively. The efficacy of different plant extracts on pathogens is due to the presence of compounds such as tannins, anthocyanins, saponins, sterols and several other secondary metabolites that contain high antimicrobial and antifungal activity (Scalbert, 1991). However, the effectiveness of aqueous neem extracts can also be attributed to azadirachtin, the main component of neem seeds.

Chemical fungicides showed the lowest MIC50 compared to EAAI. However, the chlorothalonil + carbendazim fungicide was a total inhibitor for some isolates (Kumba L, Buea L and Okola L) and showed very low MIC50 for other isolates (L Ndiki, L Ntui and *Fusarium*), hence its high efficiency compared to the fungicide based on mancozeb. This result is similar to those of Adeniyi et *al.* (2014) who showed that carbendazim was the most effective fungicide against *L. theobromae*, because it had the smallest MIC50 (0.068 mg/ml).

Conclusion

This study was aimed to show the implication of pathogens isolated on apparition of cocoa dieback and to evaluate the effect of aqueous extract of *A. indica* (AEAI) and two chemical fungicides Banko Plus and Mancomax in the *in vitro* development of some strains of pathogenic fungi causing cocoa dieback in Cameroon. Results showed that, all strains of *L. theobromae* inoculated caused dieback disease on cocoa plants. *Fusarium sp*. only caused dieback disease when the plant stressed. Results also put into evidence the antifungal effect of aqueous extract *A. indica* grains as well as the two chemical fungicides on the mycelia growth of the tested strains. The aqueous extract of *A. indica* grains and Banko Plus (chlorothalonil + carbendazim) showed the highest percentage of inhibition and were fungicidal of all strains compared to Mancomax (mancozeb). Bankoplus and aqueous extract of *A. indica* have to use *in vivo* condition and in cocoa orchards naturally infected by dieback disease, before being popularized in the fight against cocoa dieback in Cameroon.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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