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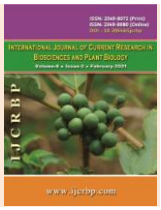

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Effectiveness of *Anonidium mannii* seed extracts against *Colletotrichum lindemuthianum* and *Fusarium solani*, agents responsible for anthracnose and fusariose of common beans (*Phaseolus vulgaris* L.) in the Centre region-Cameroon

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Abstract

Anthracnose and Fusariose of beans caused by *Colletotrichum lindemuthianum* and *Fusarium solani* respectively are two dreaded diseases that can lead to yield losses of 30 to 100% in the field. The present study was carried out to evaluate the antifungal activities of seed extracts of *Anonidium mannii* (Annonaceae) on the *in vitro* growth of *C. lindemuthianum* and *F. solani*. Extracts of *A. mannii* seeds obtained from acetone, water and methanol solvents, were used for phytochemical screening and for the preparation of solutions with concentrations of 12.5, 25 and 50 µl/ml. To evaluate radial growth, explants of 0.7 cm diameter each were obtained from two strains of *C. lindemuthianum* and two strains of *F. solani.* They were placed each at the centre of sterile Petri dishes containing the PDA medium supplemented with the different concentrations of extracts and incubated at 23°C for 6 days. The results of the phytochemical screening revealed the presence of alkaloids, phenols, saponins in the *A. mannii* seed extracts and this depended on the solvent used. The minimum inhibitory concentrations (MIC_{50} and MIC_{90}) were also calculated. At a concentration of 50µl/ml each, acetone extract inhibited the growth of strain 2 of *C. lindemuthianum* (2.33 cm) more than the methanol extract (3.26 cm) in six days of incubation. Similarly, the growth of strain 2 of *F. solani* was strongly inhibited by methanol extract (1.98 cm). The MIC_{50} of the aqueous extract gave 27.38 µl/ml with *F. solani* and 32.24 µl/ml for the acetone extract with *C. lindemuthianum*. Due to their antifungal power, after purification with bioactive compounds, the aqueous, acetone and methanol extracts revealed by the phytochemical screening could be used as synthetic fungicides for the control of anthracnose and fusariose of common bean.

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Introduction

An increase in bean production is observed in Africa due to a high demand from local and regional markets (Snapp et al., 2018; Kebede et al., 2020). In 2018, dry bean production in Central Africa was 1.08 million tons from 1.7 million hectares. In Cameroon, this production is 0.4 million tons on 0.3 million hectares. The average consumption of chemical pesticides for bean production is 0.04kg/ha and 0.18kg/ha respectively in Central Africa and Cameroon (FAO, 2020). Cryptogamic diseases, especially anthracnose and fusariose, are responsible for estimated yield losses of more than 1 million tons per year in sub-Saharan Africa, where chemicals are not readily available to smallholder farmers (PROTA, 2015). Despite the variety, beans grow in both forest and high altitude zones of Cameroon (IRAD, 2013). Diversed climatic and ecological conditions favour the proliferation of anthracnose and fusariose caused by *Colletotrichum lindemuthianum* and *Fusarium solani* f. sp. *Phaseoli*, (Meddah et *al.*, 2011; Rakotoarimanga et al., 2014; LeClair et al., 2015; Faucher et al., 2018). In the absence of appropriate phytosanitary control, *F. solani* f sp. *phaseoli* is the most predominant and damaging pathogen due to its durability and viability (Katan, 2017; Mukamuhirwa et al., 2018). Its damage in the field can result in yield losses of up to 84% of common beans (*Phaseolus vulgaris* L.) (Mukankusi, 2008; Wakesho et al., 2008; Schneider et al., 2001; Coleman, 2016; Kimberly et al., 2020). Due to *C. lindemuthianum*, production losses are estimated at about 50% in the Great Lakes region of Central Africa (Autrique et al., 1989) and 72.19% in cashew nuts in Benin (Houndahouan et al., 2018). Chemical control remains the main measure to counteract the action of pathogens responsible for cryptogamic diseases, due to the high consumption of synthetic pesticides which raised imports to 20,338 tons for 100.87 million US$ in 2018 in Cameroon (CTNBC, 2020). The increased use of chemical pesticides in the agricultural sector can be explained by the poor sensitization of research results. This is also caused by the users' ignorance of the existence of biological control measures which can eliminate or at least reduce environmental damage, investment costs and contribute to the protection of humans and the conservation of biodiversity (Ambang, 1996; CIAT, 2006).

Various research studies have shown the efficiency of plant extracts against pathogenic fungi of the genera *Colletotrichum* and *Fusarium*, and a pronounced antifungal activity has been observed against *Fusarium oxysporum* by aqueous extracts of *Lantana camara* (Adjou and Soumanou, 2013) and *Combretum racemosum* (Zirihi et al., 2008). Essential oil from *Enantia chlorantha*, an Anonaceae from Cameroon, has a significant activity against *Fusarium moniliforme* (Nyeguea et al., 2008). Aqueous extracts of *Calycotome spinosa* stems have a significant antifungal activity against *Alternaria solani* and *Alternaria altenata*, plant pathogens of stored wheat (Alleche, 2017). Ethyl acetate extracts of *L. camara*, savory and thyme oils, aqueous and ethanol extracts of *Azadiratcha indica* and *Jatropha curcas* have an antifungal activity on *Colletotrichum gloeosporioides*, which causes anthracnose in paw-paw, yam and mango (Ademe, 2013; Sarkhosh et al., 2018; Kwowura et al., 2019; Zakaria et al., 2020).

Because of their availability to farmers, non-toxicity to humans and the environment, and their biodegradability, plant extracts with pesticidal potential have been explored as sources of biological alternatives to chemical pesticides. Among these biological alternatives for controlling leguminous fungal diseases, some aqueous or organic extracts have been obtained from the plant organs with medicinal properties. *A. mannii* (Anonaceae), a native plant of tropical Africa and Central and West Africa specifically, is a dense and gallery forest fruit tree known for its therapeutic and medicinal uses (Betti, 2003; Jiofack et al., 2009; Dibong et al., 2015; Paluku et al., 2019; Konda et al., 2012; Mokale et al., 2020). Thus, the purpose of this work is to evaluate the effectiveness of aqueous and organic extracts of *A. mannii* seeds on the growth of *C. lindemuthianum* and *F. solani* f. sp. *phaseoli in vitro*.

Materials and methods

Plant material

The plant material consisted of *A. mannii* seeds whose mature fruits were collected in the forests of Mfou, Mefou et Afamba divisions in the Centre- region Cameroon and transported to the Phytopathology laboratory of the University of Yaoundé 1. These fruits were pulped to obtain seeds that were dried at room temperature for 4 to 5 weeks.

Fungal material

C. lindemuthianum and *F. solani* were isolated from leaves and stems of diseased common beans taken from

experimental fields that had not been subjected to any phytosanitary treatment at Akonolinga (N 03°48'13" and E 012°15'37", altitude 674m) and Leboudi (N 03°54'54" and E 11°26'27" altitude 795m) located in the Centre-region Cameroon.

Chemical material

The chemical material was a synthetic fungicide called Monchamp 720 WP, whose main ingredient is Cymoxanil (120 g/kg) + Mancozèbe (600 g/kg), with a formulation of 3.33g/l.

Preparation of *A. mannii* seed extracts

The seeds of *A. mannii* have been crushed using a Victoria hand mill to obtain a brown powder. The organic extracts were prepared by macerating 500g of powder in 2 liters of solvent (acetone, methanol) for 72 hours (Stoll, 1994) and then filtered. The filtrates obtained were concentrated in a rota-steamer and the extracts obtained were kept at -4°C in the refrigerator till their use. For aqueous extraction, the powder is wrapped in muslin cloth and soaked directly in water for 12 hours and then wrung out (Stoll, 1994; Ambang et *al*., 2009, 2010) and the extract obtained is used directly.

For each extract, the extraction yield was calculated using the formula below quoted by Ngoh Dooh et al. (2014).

$$\text{Yield}(\%) = \frac{\text{Mass of extract (g)}}{\text{Mass powder (g)}} \times 100$$

Phytochemical screening

Secondary metabolites present in the aqueous and organic extracts of *A. mannii* seeds were determined by adapting the standard procedures described by Harbone (1973), and Edeoga et al. (2005). These techniques are based on the appearance of color, precipitation and foam in the presence of the different reagents characterizing each class of secondary metabolites.

Thus, their presence has been indicated by the appearance of: persistent and thick foam for saponins; white precipitates for phenols, pale yellow for alkaloids, purple red for terpenes, dark blue for tannins and greenish blue precipitates for sterols.

Isolation and purification of *C. lindemuthianum* and *F. solani*

The pathogens (2 strains for each pathogen) were isolated from the leaves and stems of diseased common beans showing symptoms of anthracnose or fusariose. Fragments from plant organs of about $2cm^2$ were disinfected with 70% ethyl alcohol for 2 minutes and then with 5% sodium hypochlorite solution for 5 minutes. These fragments were then rinsed three times with sterile distilled water and dried under a hood on hydrophilic paper; then four fragments were placed in a Petri dish containing the PDA (Potatoes Dextrose Agar) culture medium supplemented with antibiotics including ampicillin 250mg/l and nystatin 20mg/l (Djeugap et *al*., 2009; Tsopmbeng et *al*., 2012). Pathogen colonies visible around the fragments after three days of incubation in the laboratory at 23°C, were collected and transferred to Petri dishes containing the previously prepared PDA culture medium. Morphologically pure cultures of the mycelium and fruiting bodies were obtained after several replicates using the slightly modified Brooks (2005) and Scot et *al*., (2011) method. Morphological criterion such as growth rate was used to characterize the strain obtained (Ondo, 2006).

In vitro evaluation of the antifungal activity of crude extracts

The *in vitro* evaluation of the antifungal activity of the extracts was carried out on two strains of each phytopathogen at concentrations of 12.5, 25 and 50µg/ml for the organic and aqueous extracts from stock solutions of 500µg/ml. A synthetic fungicide, Monchamp 720 WP based on Cymoxanil (120 g/kg) + Mancozèbe (600 g/kg), was used as a positive control by taking 1g of the powder for 5ml of distilled water from a 50g sachet.

Mycelial explants of *C. lindemuthianum* and *F. solani*, 0.7cm in diameter, were punched from a seven-day old pure fruit culture and placed at the centre of the Petri dish containing the media with the various extracts or chemical fungicide. A negative control not supplemented with extract was developed. Each treatment was repeated three times. Incubation was carried out at 23°C under a photoperiod of 12/12 for one week. Every two days, the radial growth diameter of each cultured explants was measured and this continued until the mycelium filled the control boxes. The radial growth of the pathogen was assessed by measuring two

perpendicular diameters plotted on the back of the Petri dish. The radial growth of the fungus was obtained by subtracting the diameter of explant from the average of the two perpendicular diameters using the formula proposed by Dohou et al. (2004):

$$D = \frac{D1 + D2}{2} - D0$$

Where,
D0 is the diameter explant; D1 and D2 are the diameters of culture measured in the two perpendicular directions.

The inhibition rate (I %) due to each extract is evaluated in relation to the mycelial growth in the control boxes according to the formula proposed by Dohou et al. (2004).

$$I(\%) = \frac{Dco\ (mm) - Dxi\ (mm)}{Dco\ (mm)} \times 100$$

Where,
I (%): percentage of inhibition; Dco is the average diameter of the control batch and Dxi the average diameter of the batches in the presence of the extract.

Statistical analysis

The radial inhibition growth percentages of the pathogens were transformed into probits. The effectiveness of the extracts was evaluated on the basis of the minimum inhibition concentration value of 50% (MIC_{50}) and 90% (MIC_{90}) determined after 6 days of growth according to the formula proposed by Finney (1971). The radial growth data was subjected to variance analysis using R software version 3.5.1 and the means were separated by Tukey's multiple test (P> 0.05).

Results

Extraction yield

The yield and characteristics of the different extracts obtained depended on the solvent used for extraction (Table 1). Extraction with methanol gave the highest yield of 32.89% compared to acetone (14.67%) and an average yield of 25.40% was obtained for extraction with water. All three extracts are brown in color; those with acetone and methanol are viscous while those with water are fluid.

Phytochemical screening of extracts

The presence of compounds belonging to the five families of alkaloids, phenols, saponins, tannins, sterols and triterpenes were revealed. Alkaloids are abundant in extracts with acetone, water and methanol. Phenols and saponins are abundant in the aqueous extract, present in the acetone extract and absent in the methanol extract. Sterols and triterpenes are present in the aqueous extract, abundant in the acetone extract and very abundant in the methanol extract. Tannins are present in the aqueous and acetone extracts, but absent in the methanol extract. Anthraquinones and flavonoids are completely absent in all *A. mannii* seed extracts (Table 2).

Table 1. Extraction yield and characteristics of the extracts obtained with 500 g of seeds.

Extract	Yield (%)	Characteristic
Acetone (AcE)	14.67	Clear brown and viscous
Methanol (ME)	32.89	Brown and very viscous
Water (AqE)	25.40	Brown and liquid

AcE: Acetone extract; ME: Methanol extract; AqE: Aqueous extract.

Table 2. Phytochemical composition of different extracts of *A. mannii*.

Phytochemicals	AcE	ME	AqE
Alkaloids	++	++	++
Anthraquinones	-	-	-
Flavonoids	-	-	-
Phenols	+	-	++
Saponines	+	-	++
Tannins	+	-	+
Sterols and triterpenes	++	+++	+

(-): absent; (+): present; (++): abundant; (+++): very abundant. AcE: Acetone extract; ME: Methanol extract; AqE: Aqueous extract.

Effect of *A. mannii* extracts on the radial growth of *C. lindemuthianum*

Acetone extract inhibited the radial growth of strain 2 more than strain 1 of *C. lindemuthianum* after 6 days. Regardless of the strain, after the same duration, the controls showed the same level of growth of the two strains of *C. lindemuthianum* which completely filled the Petri dishes at 7.8 cm (Fig. 1). Generally, the inhibition of mycelial growth of *C. lindemuthianum* varies from one treatment to another. Seed extracts significantly inhibited the radial growth of *C. lindemuthianum*. At 6 days of incubation, at a concentration of 50 µl/ml, acetone (2.71 and 2.33 cm) and aqueous (2.9 and 2.56 cm) extracts inhibited mycelial growth of strains 1 and 2 the most (Fig. 2A, 2B and 2C).

Effect of *A. mannii* extracts on the radial growth of *F. solani*

Inhibition of the radial growth of *F. solani* strains vary from one treatment to another. The different seed extracts significantly inhibited the radial growth of *F. solani*. At 6 of days incubation, at a concentration of 50 µl/ml, the methanol extract (3.15 and 1.98 cm) inhibited strains 1 and 2 respectively, followed by the aqueous extract (4.21 and 2.01 cm) and the acetone extract (4.35 and 4.36 cm) (Fig. 3A, 3B and 3C).

On the other hand, at the same date, the control dishes show the same level of radial growth of the mycelia of the two strains of *F. solani*, which completely filled the Petri dishes at 7.8 cm (Figs. 3A, 3B and 3C).

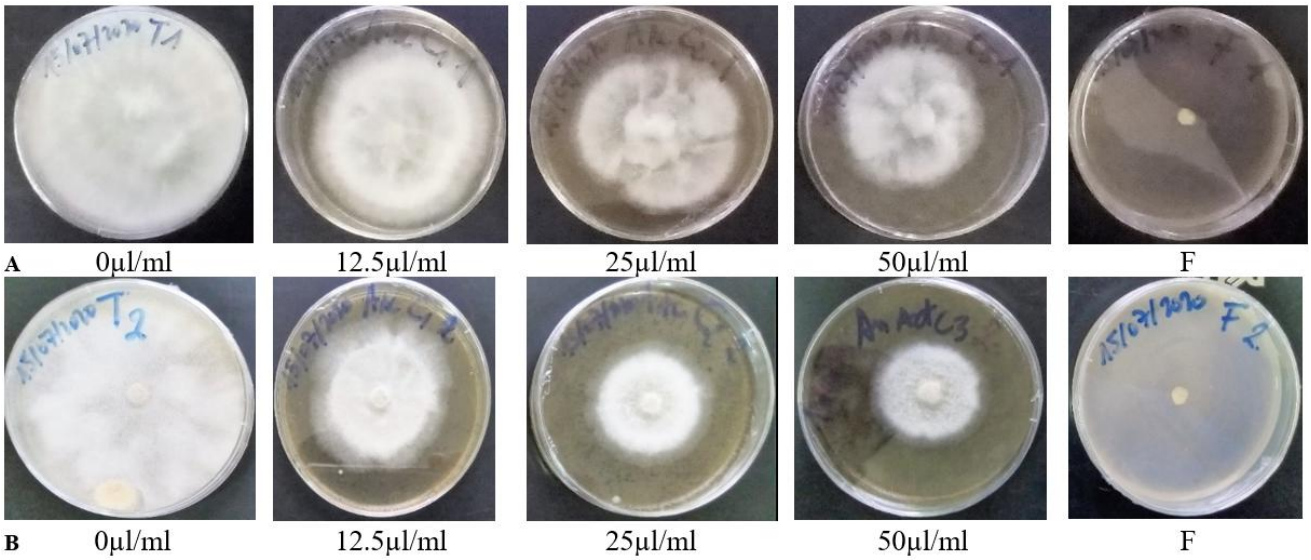

Fig. 1: Effect of acetone extract on radial growth of *C. lindemuthianum* strains, A: strain 1 B: strain 2.

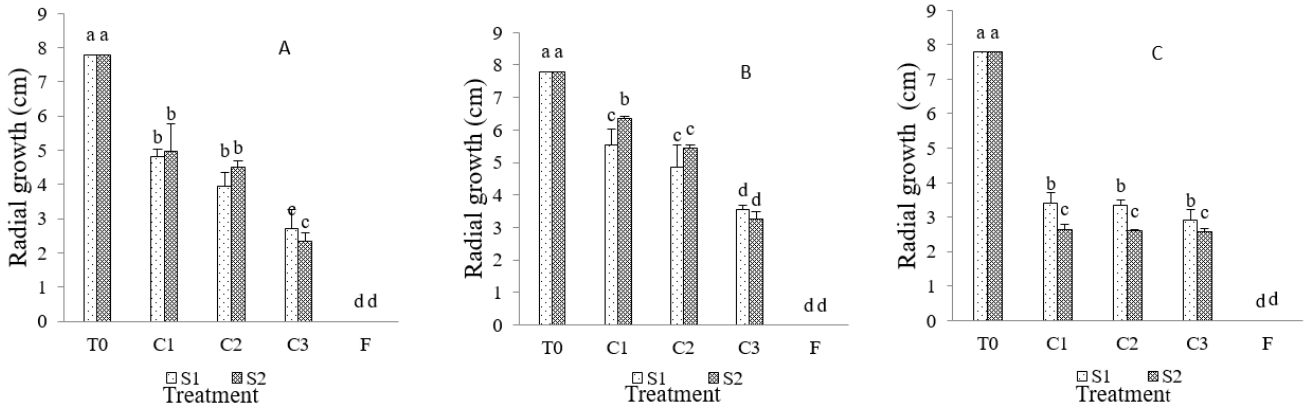

Fig. 2: Effect of seed extracts on mycelial growth of *C. lindemuthianum* at different concentrations. Histograms with the same letter do not have a significant difference at the threshold of 5%. S1: strain 1; S2: strain 2; C0: 0µl/ml; C1: 12.5 µl/ml; C2: 25 µl/ml; C3: 50 µl/ml and 3.33 g/l for F; AcE: Acetone extract; ME: Methanol extract; AqE: Aqueous extract; F: Chemical fungicide; T0: Negative control.

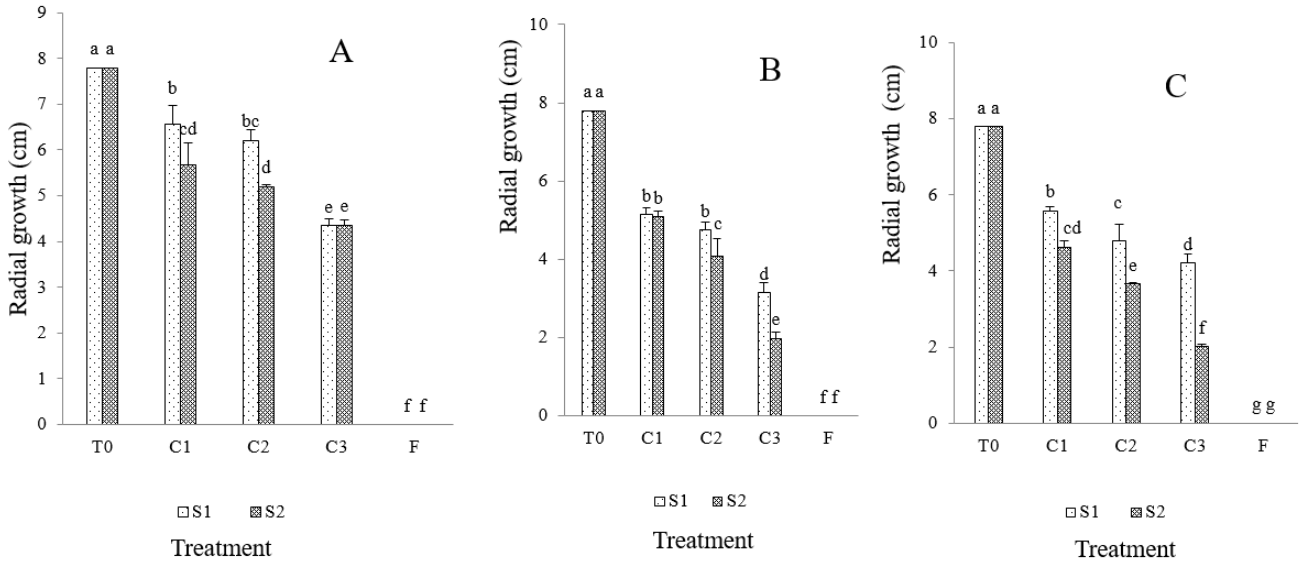

Fig. 3: Effect of seed extracts on the mycelial growth of *F. solani* as a function of different concentrations. Columns with the same letter do not differ significantly at a threshold of 5%. S1: strain 1; S2: strain 2; C0: 0 µl/ml: C1: 12.5 µl/ml; C2: 25 µl/ml; C3: 50 µl/ml and 3.33g/l for F; AcE: Acetone extract; ME: Methanol extract; AqE: Aqueous extract; F: Chemical fungicide; T0: Negative control.

Percentage inhibition of extracts

The percentage inhibition of radial growth of strains 1 and 2 of *C. lindemuthianum* and *F. solani* increased with the concentration of the aqueous and organic extracts. At 6 days of incubation, there was complete inhibition with the chemical fungicide and the aqueous and organic extracts at the concentration of 50µl/ml for *C. lindemuthianum* but no inhibition with the negative control. At the same concentration, the percentage inhibition was highest for the aqueous and methanol extracts (Table 3).

Table 3. Percentage inhibition of seed extracts and synthetic fungicide on mycelial growth of *C. lindemuthianum* and *F. solani.*

Pathogens	Concentration (µl/ml)	Percentage inhibition (%)							F
		T0	EAc		EM		EAq (mg/ml)		
			S1	S2	S1	S2	S1	S2	
C. lindemuthianum	12.5	0	37.43	32.54	32.07	24.02	55.33	65.97	100
	25	0	50.89	49.53	49.06	43.90	58.67	66.49	100
	50	0	64.36	66.52	66.05	63.77	62.00	67.00	100
F. solani	12.5	0	12.61	26.27	31.36	32.22	28.86	39.25	100
	25	0	26.53	34.62	44.18	52.22	37.60	55.98	100
	50	0	40.45	42.96	57.01	72.22	46.34	72.71	100

EAc: Acetone extract; EM: Methanol extract; EAq: Aqueous extract; T0: Negative control; S1: Strain 1; S2: Strain 2.

Minimum inhibition concentrations of the different extracts

The values of the minimum inhibitory concentrations (MIC_{50} and MIC_{90}) of the extracts are presented in Table 4. For *C. lindemuthianum* strains 1 and 2, the MIC_{50} minimum inhibition concentrations are higher with the methanol extract (45.86 µl/ml and 45.42 µl/ml) than with the acetone extract (32.24 µl/ml and 33.79 µl/ml), respectively, while the values with the aqueous extract are not determined. With regard to MIC_{90}, the aqueous extract has the highest values (91.9 mg/ml 84.17 mg/ml), followed by the methanol extract (78.4 µl/ml and 58.97 µl/ml) and the acetone extract the lowest values (61.77 µl/ml and 53.03 µl/ml). For *F. solani* strains, MIC_{50} for strain S2 were lower for the aqueous (27.38 µl/ml) and methanol (31.48 µl/ml) extracts compared to the acetone extract (44.09 µl/ml).

For *F. solani* strain S1, MIC_{50} values are lower for the organic extracts (41.50 µl/ml for the acetone extract, 40.91 µl/ml for the methanol extract) compared to the

aqueous extract (56.98 µl/ml). MIC_{90} values are lowest with the methanol extract (89.32 µl/ml and 64.82 µl/ml) and highest with the acetone extract, 103.26 µl/ml and 74.02 µl/ml for the two strains respectively.

Table 4. MIC_{50} and MIC_{90} (µl/ml) values on mycelial growth of strains of pathogens as a function of seed extracts.

Pathogens	Solvent	MIC_{50}		MIC_{90}	
		S1	S2	S1	S2
C. lindemuthianum	Acetone	32.24	33.79	61.77	53.03
	Methanol	45.86	45.42	78.40	58.97
	Water	nd	nd	91.90	84.17
F. solani	Acetone	41.50	44.09	103.26	74.02
	Methanol	40.91	31.48	89.32	64.82
	Water	56.98	27.38	92.91	67.22

nd: not determined, AcE: Acetone extract; ME: Methanol extract; AqE: Aqueous extract; T0: Negative control; S1: Strain 1; S2: Strain 2.

Discussion

Plant extracts used against crop pathogens have shown many properties which make them an alternative to be used as synthetic pesticides (Zirihi et al., 2008; Zakaria et al., 2020). From the result, the effectiveness of *A. mannii* seed extracts on the *in vitro* growth of two strains of *C. lindemuthianum* and *F. solani* was studied.

Although studies on traditional medicine have shown the bactericidal and antimicrobial activity of *A. mannii* leaf, root and stem extracts (Donfack et al., 2014), the antifungal activity of *A. mannii* seed extracts is poorly documented. The results of the extraction of 500 g of *A. mannii* powder show a variation in yield according to the solvents used, with a yield of 32.89% with methanol higher than 25.40% obtained with water in this study. This variation in yield could be due to the state of the plant material at the time of collection, the plant cycle and the environmental conditions. Using leaves of *Moringa oleifera* and using seeds of *Thevetia peruviana*, Okumu et al. (2016) and Essomé et al. (2020), respectively, reported that extraction yields were better with methanol than water. Phytochemical analysis of the extracts showed the presence of numerous secondary compounds such as alkaloids, phenols, saponins, sterols and triterpenes. According to Boulogne (2011), Boulenouar (2011), Okumu et al. (2016), Zouaoui et al. (2018), Kossonou et al. (2019) and Essomé et al. (2020), the molecules mostly responsible for antifungal activity are phenolic compounds, terpenoids and alkaloids respectively.

Antifungal activity of *A. mannii* seed extracts on strains 1 and 2 of *C. lindemuthianum* and *F. solani* pathogens was recorded. These extracts significantly reduced the development of colonies of *C. lindemuthianum* compared to the control. A concentration of 50µl/ml was the most effective in reducing the radial growth of the fungi. Studies have shown the fungistatic effect of methanolic and aqueous extracts of *Cola gigantea* on *F. oxysporum* and *Colletotrichum* sp. (Kossonou et al., 2019), *Acacia raddiana* and *Asteriscus graveolens* (Boulenouar, 2011), *Euphorbia* sp. (Hajji et al., 2016). The inhibition percentages of plant extracts increased according to the concentrations used and the nature of the solvent. At the highest concentration (50 µl/ml), the extracts did not kill the pathogen strains as was seen with the fungicide, but showed a slow radial growth. On the other hand, a complete inhibition of radial growth can be obtained with an increase in concentration of extracts. Kone et al. (2018) obtained inhibited growth of *Cercospora malayensis* with different solvents at the concentration of 120 µl/ml for the extracts of *Jatropha curcas*.

Kossonou et al. (2019) showed the fungistatic activity of plant extracts on *F. oxysporum* and *Colletotrichum* sp. In this study, the aqueous extract showed an inhibition percentage of 72.71% on *F. solani*. Similarly, Doga et al. (2017) showed that aqueous extract of *Crotalaria retusa* had an inhibition rate of 92.04% on *F. solani* while Obi and Barriuso (2013) showed that aqueous extract of *Xylopiaa ethiopica* (Annonaceae) inhibits the growth of colonies of *Colletotrichum destructivum*. Furthermore, Znaïdi (2020) showed that extracts from compost inhibit the growth of *F. solani* in Tunisia. With regard to the minimum inhibition concentrations MIC_{50} and MIC_{90}, the organic extracts were more effective than the aqueous extract showing their high fungistatic properties. Low MICs of the acetone extract on *C. lindemuthianum* and the methanol extract on *F. solani* show their effectiveness. Doumbouya et al. (2012) showed that the low MIC

values of *Ocimum gratissimum* extracts would induce an inhibition of growth and development of phytopathogenic fungi.

Conclusions

Many research works have shown the therapeutic and medicinal uses of *A. mannii* in tropical Africa. This work has shown the remarkable and significant antifungal power of this plant against two most dreaded cryptogams of common bean crop. *A. mannii* seed extracts contain active substances that significantly inhibit the radial growth of *C. lindemuthianum* and *F. solani* strains in six days of incubation. It would be interesting to test *A. mannii* seed extracts on other phytopathogenic agents in order to determine the action spectrum for the development of an alternative to synthetic fungicides without harmful effects on human health and environment.

Conflict of interest statement

Authors declare that they have no conflict of interest.

Acknowledgement

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