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Antifungal Activity of *Cosmos caudatus* Extracts against Seven Economically Important Plant Pathogens

Nazihah Mohd Salehan[1], Sariah Meon[1, 2]* and Intan Safinar Ismail[3]
[1]*Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*
[2]*Department of Plant Protection, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*
[3]*Laboratory of Natural Product, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
*For correspondence: sariahm@putra.upm.edu.my

Abstract

Crude leaf extract of *Cosmos caudatus* Kunth was separated into hexane, ethyl acetate and aqueous fractions and screened for antifungal activity against selected plant pathogens using agar cup method. The ethyl acetate (EtOAc) fraction was the most active in inhibiting growth and spore germination of *Phytophthora palmivora* (Butl.) Butl., the causal pathogen of black pod of cocoa, with percentage inhibition in radial growth (PIRG) of 52%. Sporangial germination was recorded to be the lowest in the EtOAc fraction with value of 15.62%. Scanning electron microscopy of *P. palmivora* treated with the EtOAc fraction showed stunted growth and abnormalities in the mycelium with reduced spore production. The bioefficacy of the fractions was further tested on detached cocoa pods. The EtOAc fraction gave the highest inhibition of 57.46% on diameter of lesions. These findings indicate that the ethyl acetate fraction of *C. caudatus* contains antifungal agents effective against *P. palmivora*, which could be used in the development of biopesticide for the control of black pod disease.

Keywords: Antifungal activity; *Cosmos caudatus* Kunth; *Phytophthora palmivora*; Black pod disease; Biopesticide

Introduction

Use of synthetic pesticides has been the common practice to combat plant diseases due to its efficacy and convenience in agriculture. However, indiscriminate and excessive use of synthetic pesticides has led to deterimental consequences to humans and the environment. Even though synthetic pesticide use is a popular way of controlling pests because it is cheap, quick acting, and has direct results, public concerns have been raised over pesticide residues on fruits and food (Paster and Bullerman, 1988). Conventional pesticides have caused extensive side effects on ecological sustainability, health safety of farmers and consumers, and farmland biodiversity (Pimentel and Greiner, 1997). There has been ascending concerns over environmental and human health on persistence of chemicals in ground water (Hallberg, 1987), heavy metal toxicity (Huisingh, 1974), and carcinogenic threat (Tangley, 1987). Steen *et al.* (2001) have also reported that pesticide contamination occurs in coastal areas from the draining of hazardous effluents from farming activities located upstream. In addition, undiscerning application of chemical fungicides has led to pathogens becoming more resistant that require higher concentrations of fungicide application in the long run (Fu *et al.*, 2007). Increasing concern over the unfavorable environmental effects and decreasing efficacy of synthetic fungicides has brought about the need for the development of new and natural control alternatives (Soylu *et al.*, 2010).

Natural plant products can be alternatives to currently used synthetic pesticides, since they provide unlimited opportunities for the discovery of new pesticides because of their rich bioactive chemical constituents (Isman, 2000; Burt, 2004). Plants produce secondary metabolites such as flavonoids, saponins, alkaloids, tannins, and phenols that are important for survival. These metabolites allow plants to defend themselves from herbivory effects, pathogens (bacteria, fungi and viruses) and from other plants, and also provide protection from adverse physical effects, such as damaging UV-radiation, water loss, and low temperatures (Noor *et al.*, 1995; Auria and Gershenzon, 2005; Pelser *et al.*, 2005; Kong *et al.*, 2007). Thus, extensive explorations on exploitation of plants as natural commercial biopesticides have been actively undertaken for the past two decades, which have now become an intense and productive research field (Tegegne *et al.*, 2007; Haouala *et al.*, 2008).

Essential oils and extracts of numerous plants known for their antimicrobial and antifungal activity are widely used in medicine and in the food industry (Kalemba and Kunicka, 2003). Essential oils of a number of medicinal plants have been reported to be effective against postharvest fungal diseases (Shahi *et al.*, 2003; Haikal, 2007; Kumar *et al.*, 2007; Tzortzakis and Economakis, 2007; Haouala *et al.*, 2008). Some medicinal plants have also been shown to be effective *in-vivo*, confirming potential in the search for

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plant-derived fungicides to be commercialized. Promising results by Amadioha (2000) showed that extracts of *Azadirachta indica* had the potential as a preventive control measure that reduce disease incidence of rice blast, both *in-vitro* and *in-vivo*. Dikbas *et al*. (2008) reported that essential oils of the medicinal plant *Satureja hortensis* L. showed strong antifungal activity, which was able to suppress the growth of *Aspergillus flavus* on lemon fruits under storage conditions.

Cosmos caudatus Kunth, a medicinal plant belonging to the Asteraceae family, is a traditional herb eaten as salad to cure and improve human ailments such as infectious diseases, body heat, blood circulation and aging (Guanghou *et al.*, 2005). It is locally known as Ulam Raja in Malaysia and is an annual herb that grows to about 1-8 feet tall in tropical climates and has fine dissected leaves of 10-20 cm long. This plant is widely grown and can be readily established at low cost. *C. caudatus* has antioxidative and radical-scavenging activities and possesses highly active phenolic compounds that have the potential to reduce the onset of cancer (Abas *et al.*, 2003; 2006). Rasdi *et al.* (2010) also reported that *C. caudatus* could become a novel antibiotic agent due to the significant antimicrobial properties of its polar and non-polar leaf extracts. The extracts showed a significant degree of inhibition against five human pathogenic strains; *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli,* and *Candida albicans* at minimum inhibitory concentrations (MIC) of 25 mg/mL for the hexane extract, 6.25 mg/mL for the ethanol extract and 6.25 mg/mL for the diethyl ether extract, suggesting high concentrations of antimicrobial constituents in *C. caudatus*.

C. caudatus has been shown to have antimicrobial activity against human pathogens. However, its activity on plant pathogens has not been scientifically investigated to date. Hence, this study was carried out to obtain crude leaf extract of *C. caudatus* and to separate the crude into hexane, ethyl acetate (EtOAc), and aqueous fractions. The crude and the fractions were screened for antifungal activity against seven economically important plant pathogens. The activity was determined in terms of mycelial growth inhibition, sporulation and spore germination. Subsequently, *in-vivo* control of black pod disease caused by *Phytophthora palmivora* was observed by testing *C. caudatus* fractions on detached cocoa pods.

Materials and Methods

Plant Materials

Seeds of *C. caudatus* supplied by the Herbal Unit, Universiti Putra Malaysia (UPM), were grown at the UPM Agricultural Park on a Serdang series soil amended with organic fertilizer. The plants were exposed to direct day sunlight and grown in a tropical climate with a mean monthly temperature of 27.8°C with an annual rainfall of 2443 mm at an elevation of 39.7 m in Serdang (Derso *et al.*, 2006). The soil was fertilized and covered with a black plastic, on which equidistant holes were made in two rows and 20 plants were planted in every row. The plants were irrigated automatically with a hydraulic system as well as manually. Five grams of commercial organic fertilizer (Harvester PCM®) was applied to each plant on a monthly basis, and plants were harvested three months after sowing (Mediani *et al.*, 2012). Fresh leaves were collected, washed thoroughly under running water, dried with blotting paper and cut into small pieces. The plant was identified and authenticated (voucher specimen number: SK1926/11) and deposited at the Biodiversity Unit of Institute of Bioscience, Universiti Putra Malaysia.

Extraction

Extraction of the antimicrobial compounds was performed according to the method described in Shaari *et al.* (2011) with some modifications. Fresh leaves of *C. caudatus* (3.06 kg) were soaked in ethanol (HmbG Chemicals) and ground to fineness in a mechanical blender. The mixture was sonicated for 10 min five times and then filtered through Whatman filter paper. The filtrate was evaporated to 1/10 of initial volume using a rotary evaporator at 40°C (Buchi Rotavapor), yielding 128.2 g of crude extract. The crude extract was then successively partitioned with *n*-hexane (Systerm ChemAR) and ethyl acetate (EtOAc) (Systerm ChemAR) to give hexane, EtOAc and aqueous fractions, which were evaporated under reduced pressure and dried, to yield 9.1, 12.2, and 83.2 g, respectively. The fractions were stored at -20°C until required for analysis.

Source of Plant Pathogens

Pure cultures of seven economically important pathogens; *Phytophthora palmivora* (*Theobroma cacao*), *Pythium spp.* (*Axonopus compressus*), *Colletotrichum gloeosporioides* (*Mangifera indica*), *C. gloeosporioides* (*Carica papaya*), *C. truncatum* (*Glycine max*), *C. capsici* (*Capsicum annuum*), and *Pestalotiopsis spp.* (*Garcinia mangostana*) were obtained from the Department of Plant Pathology, Universiti Putra Malaysia. Cultures were maintained on corn meal agar (CMA) (Oxoid Thermo Scientific) or potato dextrose agar (PDA) (Oxoid Thermo Scientific) for the following studies. Stock cultures were maintained at -4^{o}C on agar slants.

In-vitro Antifungal Screening of *C. caudatus* Crude and Fractions against Plant Pathogens

The antifungal activity of the different *C. caudatus* fractions (crude, hexane, ethyl acetate and aqueous fractions) against the selected fungal pathogens was evaluated using the agar cup method (Igbinosa *et al.*, 2009). The dried crude, hexane, EtOAc and aqueous fractions were dissolved in 90% ethanol at 200 mg/mL. 20 mL of CMA or PDA cooled at 45°C was poured into 90 mm diameter petri dishes and allowed to solidify. Six mm diameter wells were cut in the agar plates and 20 µL of fraction or pure extraction solvent

as negative control were loaded individually in the wells, taking care to avoid spillage on the surface of the agar medium. Inoculum discs of 6 mm diameter, from the edge of 7-day old actively growing cultures of the fungal isolates, were transferred aseptically 2-cm away from the wells containing the fractions. The plates were pre-incubated at room temperature to allow for uniform diffusion before sealing with parafilm. Plates were incubated in an incubation chamber at room temperature of 25 ± 2°C and radial growth of mycelia was observed and measured after six days of co-incubation and the percentage inhibition was calculated as follows:

$$\text{Percentage of inhibition of mycelial growth (\% PIRG)} = \frac{\text{mycelial growth in control} - \text{mycelial growth in fraction}}{\text{mycelial growth in control}} \times 100$$

The antifungal effect was determined using a completely randomized design with five replications for each fraction. Analysis of variance on transformed data was performed using the SAS software to determine the differences in radial growth between treatments (Meneses *et al.*, 2009).

Effect of *C. caudatus* Crude and Fractions on Sporangia Production and Sporangia Germination of *P. palmivora*

Mycelial growth in the control was permitted to extend to the edge of the plate. After seven days of incubation, plates were flooded with sterile-distilled water and spores were gently dislodged from the mycelium using a hockey stick and the resulting spore suspension was filtered through two layers of cheesecloth and the spore density of the suspension was determined using a haemocytometer (Neubauer, Marienfeld, Germany). Percent sporulation was determined as: (treatment/control) × 100% (Mills *et al.*, 2004). Data were recorded with three replicates in each treatment.

Spore germination test was performed by transferring 30 µL of spore suspension of each treatment to a depression slide and adding 10 µL of 200 mg/mL extract fraction and sterile distilled water to make up to 70 µL per slide. All experimental units were placed on moist paper towel in a plastic incubation box and then placed in an incubation chamber for 24 h at room temperature (25±2°C). Sporangia were considered germinated by either forming germ tubes or producing zoospores (Clerk, 1971). Percent spore germination was determined as: [treatment (germinated/total sporangia accessed)]/[control (germinated/total sporangia accessed)] x 100% (Mills *et al.*, 2004). Data were recorded with three replicates per treatment and analysis of variance on transformed data was performed using the SAS software to determine the differences in sporulation and spore germination between treatments.

Scanning Electron Microscopy

Agar discs from the inhibition zone in the *in vitro* plate assay was carefully cut (1 cm^3) and fixed in 4% glutaraldehyde (Sigma, USA) for 6 h at 4°C. After fixation, the samples were washed using 0.1 M sodium cacodylate buffer three times for 10 min, then fixed in 1% osmium tetroxide (Sigma, USA) for 2 h at 4°C and washed again with 0.1 M sodium cacodylate buffer three times for 10 min. Dehydration was carried out at room temperature for 10 min treatment each in graded concentrations of acetone (35, 50, 75, and 95%, v/v). After final dehydration in 95% acetone, samples were left overnight at 4°C, and then dehydrated in 100% acetone for 15 min for three times. The samples were then transferred to a critical point dryer (Bal-Tec CPD 030) for half an hour, mounted onto stubs and subjected to gold coating in a sputter coater (Bal-Tec Scd 005, USA). The specimens were viewed under LEO 1455 VPSEM attached with EDX. The energy of the electron source was 10KV (Soylu *et al.*, 2006).

***In-vivo* Screening of *C. caudatus* Crude and Fractions on Detached Cocoa Pods**

Mature green cocoa pods (Hybrid PBC 104: Prang Besar Clone 104) were collected from the field, washed under running water, surface sterilized, and air-dried. A 50 µL aliquot of each fraction was applied on the lateral side of the cocoa pods, spread to a size of approximately 1 cm^2 and allowed to air dry. The treated cocoa pods were pricked with sterilized needle and agar disc of *P. palmivora* (1 cm^2) from a 9-day old culture was placed on the spot (Koranteng and Awuah, 2010). Wet cotton was placed on top and secured with a cellophane tape and pods were incubated in a moist chamber at room temperature (25 ± 2°C) for seven days and were observed for lesion development at intervals throughout the week. Untreated spots inoculated with *P. palmivora* served as control. The diameter of lesion was recorded and the percentage of inhibition in lesion diameter relative to the control was calculated. Each fraction treatment was replicated five times with one point of inoculation per pod. Analysis of variance using SAS software was performed on transformed data to determine the differences in lesion development between fractions.

Results and Discussion

***In vitro* Antifungal Screening of Extracts against Plant Pathogens**

The effectiveness of the antifungal activity of the crude, hexane, ethyl acetate and aqueous fractions of *C. caudatus* against the selected phytopathogenic fungi was assessed based on PIRG values (Table 1). Among the four fractions, the EtOAc fraction showed antifungal activity on most tested isolates with PIRG values of 4.7 to 52%. Highest inhibition was observed on *P. palmivora* (*Theobroma cacao*) (52%), followed by *C. gloeosporioides* (*Carica papaya*) (23.5%) and *C. gloeosporioides* (*Mangifera indica*) (18%). The rest of the fractions (crude, hexane and aqueous) gave low inhibition on all tested pathogens.

Table 1: Effect of *Cosmos caudatus* crude and fractions on percentage inhibition of radial growth (PIRG) of fungal pathogens using agar cup method, 6 days after co-incubation

Pathogen	Host	Inhibition of mycelial growth (%)* (± SE) *Cosmos caudatus* leaves							
		Crude extract		Hexane fraction		Ethyl acetate fraction		Aqueous fraction	
Phytophthora palmivora	*Theobroma cacao*	21.2	(± 0.5) b*	13.0	(± 0.4) c	52.0	(± 0.3) a	20.6	(± 0.3) b
Phytium spp.	*Axonopus compressus*	0.0	(± 0.3) a	0.0	(± 0.3) a	0.0	(± 0.4) a	0.0	(± 0.5) a
Colletotrichum gloeosporioides	*Mangifera indica*	19.0	(± 0.3) a	19.0	(± 0.3) a	18.0	(± 0.3) a	10.6	(± 0.3) b
Colletotrichum gloeosporioides	*Carica papaya*	17.1	(± 0.5) b	15.0	(± 0.4) b	23.5	(± 0.3) a	11.7	(± 0.3) c
Colletotrichum truncatum	*Glycine max*	5.4	(± 0.3) b	7.6	(± 0.4) b	12.1	(± 0.3) a	7.1	(± 0.2) b
Colletotrichum capsici	*Capsicum annuum*	0.0	(± 0.5) b	0.0	(± 0.5) b	4.7	(± 0.4) a	3.4	(± 0.4) a
Pestalotiopsis spp.	*Garcinia mangostana*	0.0	(± 0.6) a	0.0	(± 0.7) a	0.0	(± 0.5) a	0.0	(± 0.6) a

*Means within a row followed by the same letters are not significantly ($P < 0.05$) different according to LSD's multiple comparison test

No inhibition was observed on the growth of *Phytium spp. (Axonopus compressus)* and *Pestalotiopsis spp.* (*Garcinia mangostana*). Previous antifungal studies utilized crude plant extracts and their essential oils in the search for sustainable biopesticides (Amadioha, 2000; Bajpai *et al.*, 2007; Tzortzakis and Economakis, 2007; Haouala *et al.*, 2008). In the present study, the crude extract of *C. caudatus* was separated into different organic solvents to test the effects of compound polarity on the mycelial growth of pathogens. Compounds in the solvent with intermediate polarity (ethyl acetate, EtOAc) had the highest inhibition, which may be attributed to the solvent's ability to extract and isolate secondary metabolites (alkaloids, phenolic, flavonoids and terpenoids compounds), which were characteristic of the plant's antifungal activity (Eloff, 1998; Mohamed and El-Hadidy, 2008). Sporulation of *P. palmivora* was not significantly different among treatments, but germination of *P. palmivora* was recorded to be the lowest in the EtOAc extracts with a value of 15.62% (Table 2). There were no significant differences in either sporulation or spore germination in the rest of the fungal pathogens tested.

SEM micrographs of *P. palmivora* from the inhibition zones of dual culture plates showed morphological abnormalities in hyphal structure with sparse production of sporangium (Fig. 1A, B). Sporangia produced were elongated in length (average: 38.41 µm) as compared to those produced from cultures free of EtOAc fraction (average: 30.49 µm) (Fig. 1C, D). Essential oils and extracts of various plants have been shown to have remarkable antifungal effects exhibited by retardation in mycelial growth and sporulation (Tzortzakis and Economakis, 2007; Soylu *et al.*, 2010; Tian *et al.*, 2011). Suppression of mycelial growth and inhibition in spore germination of *P. palmivora* shown by the EtOAc fraction might be essential for the control of black pod disease. The suppression would contribute in limiting the spread and occurrence of *P. palmivora* infections by lowering the spore load in the field atmosphere and on surfaces of cocoa pods (Soylu *et al.*, 2010).

In vivo Screening of Extract Fractions on Detached Cocoa Pods

Phytophthora palmivora was selected for further bioefficacy tests and the effects of *C. caudatus* crude and fractions on *P. palmivora* infection on detached cocoa pods are shown in Fig. 2. Lesion development was observed on all cocoa pods, with the EtOAc fraction exhibiting the highest inhibition (57.46%) with the smallest lesion development (Fig. 3D), followed by the aqueous fraction (29.07%), the crude fraction (7.89%) and the hexane fraction (2.21%).

Table 2: Effect of *C. caudatus* crude and fractions on sporulation and sporangial germination of *P. palmivora in-vitro* relative to the control

Fraction	Sporulation (%) (± SE)[a]		Spore germination (%) (± SE)[b]	
Crude	46.77	(±0.5) a	91.11	(± 0.3) a
Hexane	79.57	(±0.4) a	38.27	(± 0.3) b
Ethyl acetate	47.31	(±0.8) a	15.62	(± 0.2) b
Aqueous	37.10	(±0.3) a	23.43	(± 0.3) b

[a]Values were determined by: (treatment value)/(control value) x 100%
[b]Values were determined by: treatment [(germinated/total sporangia accessed)]/ control [germinated/total sporangia accessed)] x 100%
Means within a column followed by the same letters are not significantly ($P < 0.05$) different according to LSD's multiple comparison test

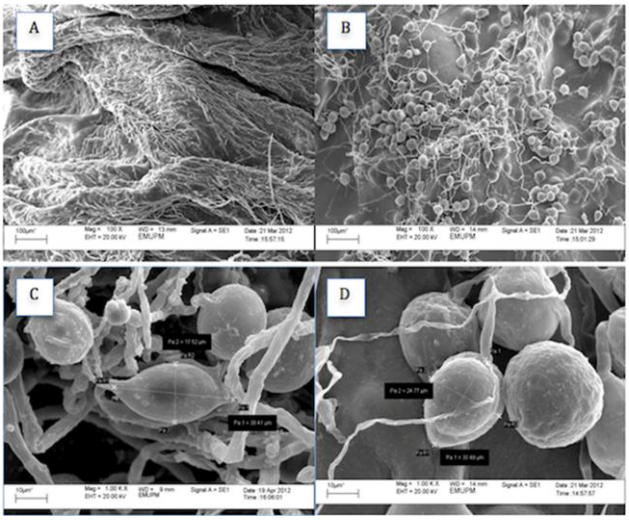

Fig. 1: Scanning electron micrographs of *P. palmivora* exposed to EtOAc fraction. [(A) EtOAc fraction, suppression in sporulation and abnormal mycelia development as compared to (B) control, (C) elongated spores of EtOAc fraction while normal spore morphology from (D) control]

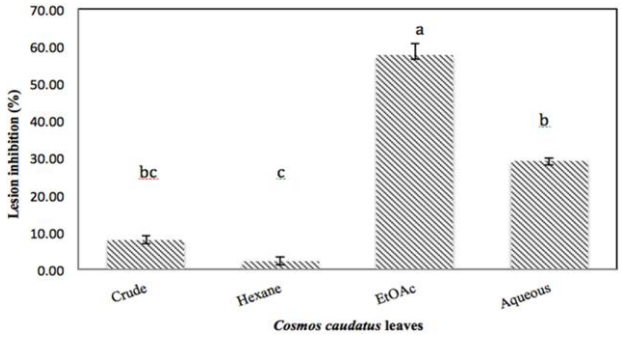

Fig. 2: Percentage inhibition in lesion development of black pod infection on detached cocoa pods by *C. caudatus* crude and fractions at concentration of 200 mg/mL
[Bars designated by the same letters are not statistically different ($P< 0.05$) at LSD's multiple comparison test]

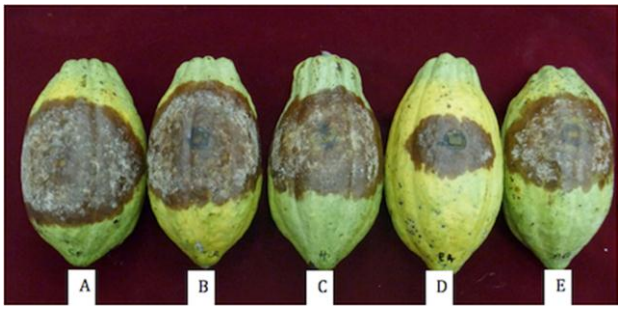

Fig. 3: Effect of *C. caudatus* crude and fractions on lesion development in pre-treated detached cocoa pods [Challenge inoculated with *P. palmivora* after 7 days of inoculation; (A) control; (B) crude extract; (C) hexane fraction; (D) ethyl acetate fraction; (E) aqueous fraction]

Cross section of infections differed significantly between treatments at seven days after inoculation (Fig. 4). Infection began as a discolored spot on the inoculation spot and progressed as a brown lesion with whitish mycelium on the surface of the lesion. The most severe infection was seen in the control where only *P. palmivora* was inoculated on untreated pods (Fig. 4A). The exocarp and mesocarp were severely blackened and the infection penetrated deeper into the endocarp, evidenced by rotten segments and beans. Hexane and aqueous fractions showed low inhibition against *P. palmivora* infection (Fig. 4C, E), while crude extract and EtOAc fraction gave a high degree of protection, as only the external part of the pods was blackened by *P. palmivora* infection (Fig. 4B, D). Similar internal protection may be due to some common metabolites in the crude extract and EtOAc fraction. The EtOAc fraction in this study was fungistatic rather than fungicidal as the EtOAc fraction inhibited mycelia growth and reproduction of *P. palmivora*. Banihashemi and Abivardi (2011) reported that essential oils, particularly citral, a key compound of herbal plants, showed fungicidal effects against *Phytophthora* species, while other essential oils tested showed fungistatic effects.

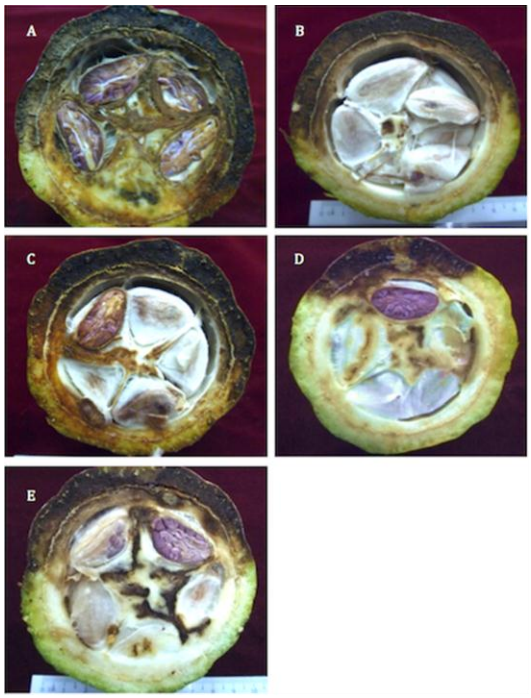

Fig. 4: Internal symptoms of black pod in cross-section of pre-treated cocoa pods [Challenge inoculated with *P. palmivora* after 7 days of inoculation; (A) control; (B) crude extract; (C) hexane fraction; (D) ethyl acetate fraction; (E) aqueous fraction]

According to Drenth and Guest (2004), an effective way to control *Phytophthora* diseases is to use a number of different approaches including cultural, biological and chemical measures in an integrated manner; i.e. integrating basic hygiene, disease-free planting materials, site preparation, drainage, soil health, and disease-resistant germplasm, together with biological and chemical controls. The EtOAc fraction can be an additional method of biological control that can help in reducing the disease to economically viable levels with a concomitant decrease in the use of chemicals, as advocated by Integrated Pest Management strategies (Krauss and Soberanis, 2001; Bajwa and Kogan, 2004). Most reported biological control methods of *P. palmivora* diseases involve microorganisms such as fungi, bacteria and arbuscular mycorrhizal fungi (AMF) (Odigie and Ikotun, 1982; Hanada *et al.*, 2009; Tchameni *et al.*, 2011), and only a few plant extracts had been screened to suppress black pod disease (Awauh, 1994), implying that attempts to control *P. palmivora* diseases using natural biopesticides is still ongoing. Results with the EtOAc fraction of more than 50% inhibition against *P. palmivora*, both *in vitro* and *in vivo*, suggest that the EtOAc fraction has good potential for further development. Additional studies on the isolation and identification of the active compound(s) in the EtOAc fraction would enable a better understanding of the mechanism of the antagonistic effects.

Conclusion

The results of the present study highlight the importance of the EtOAc fraction of *C. caudatus* as a potential component for the biological control of *P. palmivora*, the causal agent of black pod disease of cocoa.

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