ANTIFUNGAL EFFICACY OF CRUDE AQUEOUS WEED EXTRACTS AGAINST PATHOGEN OF COCOA BLACK POD ROT

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ABSTRACT

Black pod rot is the most economically important disease of cocoa in Malaysia which is mainly caused by a highly polyphagous *Phytophthora* species, called *Phytophthora palmivora*. The fungus could attack all parts of the cocoa plant organs and caused various diseases at any growth stage from seedling until the mature stages, especially during raining season. The application of synthetic fungicides has been widely recommended to manage the disease but their repeated use had led to other problems such as environmental, human health and development of fungicide resistance issues. This study isolated and identified *Phytopththora* isolate from a cocoa pod sample based on micro-morphological characters. Besides, the present investigation was undertaken to screen for the antifungal potency of different weed extracts against the *Phytophthora* pathogen using poisoned food technique. The fungal isolate was successfully recovered from pod tissues of clone PBC123 on 20% tomato juice agar culture (20T). Only one out of ten weed extracts tested showed a significant in vitro inhibitory effect towards mycelial growth of Ph*ytophthora* isolate, which was aqueous crude leaf extract of *Solanum torvum* (42.68%). This study indicated that the potential of weed extracts in the management of *Phytophthora* diseases, and may offer more natural, effective and economical control methods.

Keywords: Black pod rot, *Phytophthora palmivora*, poisoned food technique, tomato juice agar culture, *Solanum torvum*

1. Introduction

Black pod rot (BPR) is the most destructive and widespread disease of cocoa in all cocoa-producing regions of the world. The most recognizable symptoms on pods are firm, spreading, chocolate-brown lesions that eventually can cover a whole pod. The disease is caused by several *Phytophthora* species such as *Phytophthora palmivora*, *Phytophthora megakarya*, *Phytophthora citrophora*, *Phytophthora capsici*, *Phytophthora megasperma* and *Phytophthora katsurae* (Vanegtern *et al*. 2015; Guest, 2007). *P. palmivora* and *P. megakarya* have been identified as the most destructive species on cocoa thus limiting cocoa production worldwide. *P. palmivora* seems to be a cosmopolitan species but less virulent than *P. megakarya*. *P. megakarya* is restricted to only cocoa producing regions in Western and Central Africa. In Malaysia, *P. palmivora* has been reported as dominant species on cocoa (Drenth & Guest, 2004). Others are like *P. cinnamomi*, *P. capsici*, *P. nicotianae*, *P. botryose*, *P. citrophthtora*, *P. hevea* and *P. megasperma* (End *et al*., 2017; Drenth & Guest, 2004). Conventional methods were used in this study for detection and identification of *Phytophthora* isolate, which firstly involved using two commonly used media (potato dextrose agar and tomato juice agar) for supporting the growth and maintenance of the fungus; followed by examination upon

its micromorphology features such as sporangia, coenocytic hyphae and chlamydospores. Furthermore, Al-Hedaithy & Tsao (1979) suggested that *Phytophthora* spp. can be identified and grouped into three categories based on average pedicel lengths. They were (a) with short pedicels (< 5 µm): *P. cactum*, *P. infestans* (Mont.) deBary and *P. palmivora* (Butl.) Butl. (MF1); (b) with pedicels of intermediate lengths (5-20 µm): *P. botryose* Chee, *P. colocasiae* and *P. palmivora* (MF3) and (c) with long pedicels (> 20 µm): *P. hibernalis* Carne and *P. palmivora* (MF4). Recently, advanced detection methods included the use of electrophoretic banding patterns of soluble proteins, isoenzyme analyses, genome-based techniques such as restriction fragment length polymorphisms (RFLP) of mitochondrial DNA and sequencing of the internal transcribed spacer regions of ribosomal DNA (rDNA-ITS). Yet, morphological comparisons are still the most practical option in countries affected by severe cocoa *Phytophthora* diseases (Appiah *et al*., 2003).

Several methods have been adopted by farmers to control the disease such as the use of chemical compounds, genetically resistant trees, biocontrol and phytosanitary methods but none of them have completely controlled the disease so far (Guest, 2007; Ploetz, 2007). In contrast, Barreto *et al*. (2015) reported that the most efficient way to control black pod disease was to use resistant crop varieties by suggesting the strategies to use them in a cocoa breeding program that could accelerate the process of selecting cocoa genotypes resistant to black pod disease caused by multiple *Phytophthora* spp.

Plant extracts have great potential as alternatives to synthetic fungicides because they are easily available at low cost, lack of toxicity, local availability and biodegradability (Maswada & Abdallah, 2013). Such study showed that plant extracts can be used to control cocoa black pod disease caused by *Phytophthora* spp. and it was done by Mohsan *et al*. (2017) which reported that the crude extracts of *Parthenium hysterophorus* (Parthenium), *Nerium oleander* (Kaner) and *Oscimum basilicum* (Niazbo) showed inhibitory efficacy against *P. capsici in vitro*. Besides, Ngoh Dooh *et al*. (2015) *in vivo* study revealed that aqueous extract of *Thevetia peruviana* could produce cheap and effective formulation to control the black pod disease caused by *P. megakarya.* In addition, rosemary and lavender leaf extracts were also found to be effective in reducing germination of *P. capsici*, *P. megakarya* and *P. palmivora* zoospores (Widmer & Laurent, 2006). In this study, ten weed species have been tested for their antifungal activity against pathogen of black pod disease on PDA medium incorporated with different weed extracts using food poisoned technique, which was commonly used to evaluate the antifungal effect of certain antifungal agents or extracts against molds (Kumar *et al*., 2014; Ali-Shtayeh & Abu Ghdeib, 1999; Mukherjee & Raghu, 1997).

Thus, the main objectives of this study were to isolate and identify the pathogen that caused BPR on a cocoa pod sample obtained from CRDC, Jengka, Pahang and to recognize weed species with anti-*phytophthora* activity. Consequently, the study may be able to reveal weed extracts as a good alternative in developing potent plant based fungicides or as an integrated approach to combat fungal plant pathogens.

2. Materials and Methods

2.1. *Collection and identification of weed species*

Field-collected weed samples were obtained from various locations in UiTM Jasin Campus and Kampung Seri Mendapat, Merlimau, Melaka. Weeds were identified on the basis of pectoral characteristics guided by Chung *et al.* (2014) and Zakaria & Mohd (2010). Table 1 shows a list of weeds selected and part of the weed used in the study. The selection of the weeds were based on their potential medicinal values and their availability.

Table 1: Weeds used in the study.

Name of the Weeds	Scientific Name	Family	Part Used
Common Asystasia, Chinese violet, Creeping foxglove, Ganges primrose	*Asystasia gangetica*	Acanthaceae	Leaf
Broad sword fern, Paku larat, Giant sword fern, Sword fern	*Nephrolepis biserrata*	Oleandraceae	Leaf
Fireweed, Tetracera, Hedge Row Tetracera, Puson Dumarun, Akar Pulas Duyio, Akar Mempelas, Empelas	*Tetracera indica*	Dilleniaceae	Leaf
Siam Weed, Bitter bush, Devil Weed, Hagonoy, Jack in the bush, Triffid weed	*Chromolaena odorata*	Asteraceae	Leaf
Lantana, Common Lantana, Shrub verbena, Spanish flag, Tick berry, Bunga tahi ayam, Bunga pagar	*Lantana camara*	Verbenaceae	Leaf
Ivy gourd, Scarlet-fruited gourd	*Coccinia indica*	Cucurbitaceae	Leaf
Castor oil bean, Castor oil plant, Palma christi, Castor bean plant, Jarak	*Ricinus communis*	Euphorbiaceae	Leaf
Turkey berry, Devil's fig, Terung pipit	*Solanum torvum*	Solanaceae	Leaf
Peacock flower, Barbados flower fence, Flower fence, Jambol merak, Cana, Barbados pride, Red bird of paradise, Paradise flower, Flamboyant tree, Gold mohur, Pride of barbados, Jambul merak	*Caesalpinia pulcherrima*	Fabaceae	Leaf
Spreading dayflower, Climbing dayflower, Scurvy weed	*Commelina diffusa*	Commelinaceae	Leaf

2.2. *Preparation of weed leaf aqueous extracts*

A method according to Prasad & Anamika (2015) was followed but with some modifications. Fresh weed leaves were washed thoroughly 2-3 times with running tap water and once with distilled water before placing them inside the hot air oven at 40$_o$C (Memmert Universal Oven UF260, Germany) till it dried and crumbled by hand within 4 to 7 days. The dried samples were pulverized with blender machine (MX-GM1011 H [Grey], Panasonic, Malaysia) and finely blended with coffee bean grinder (D&K M1000 coffee bean grinder [silver], China) before packed in clean and dark plastic containers; and stored in refrigerator at 4$_o$C for a long-term storage. Next, the extraction of antifungal compounds was done by freshly macerating 24 g of each oven-dried selected-weed species into 400 ml sterile distilled water in a 500-ml flat bottom and narrow neck flask (Simax glass, Czech Republic) before keeping them on a rotary shaker (Stuart Orbital Shakers SSL1, UK) for 24 h at 120 rpm; and incubated at room temperature. After maceration, the aqueous extract was filtered through a commercial cloth coffee filter to remove large weed leaf tissue residues prior to antifungal activity assay by poisoned food technique.

2.3. Survey, sample collection and *Phytophthora* isolation

A disease survey in the cocoa fields at Malaysian Cocoa Board, CRDC, Jengka, Pahang showed that many cocoa pods from different clones (PBC 123, PBC 140, KKM 1, KKM 5 and KKM 22) were severely infected by black pod disease. In this study, an isolate of *Phytophthora* was obtained from a naturally infected cocoa pod tissues of clone PBC 123.

Isolation of pathogen from infected pod tissues was done between the margin of the infected and healthy areas. The infected tissues were cut by sterilized scalpels about 5 mm length x 5 mm width x 5 mm depth. The samples were washed and surface sterilized by soaking in sodium hypochlorite-containing commercial bleach solution (5-10% [w/v] Clorox, USA) for 10 minutes followed by triple rinsing in sterilized deionized distilled water (ddH_2O) for 5 minutes each before blotted dry with tissue paper. After that, three pieces of the infected tissues were placed on 20% tomato juice agar (20T) (20% Campbell's Tomato Juice [Malaysia], 0.04% $CaCO_3$ and 2% agar) and PDA media, respectively. A previous study has shown that 20T could induce the growth and both asexual and sexual reproductions of some *Phytophthora* species (Guo & Ko, 1993). The incubation was made in the incubator at 30_oC for five to seven days. Fungal growths from tissue segments were then transferred onto another 20T medium to obtain pure culture of the isolated fungus. Identification of the *Phytophthora* isolate was based on the microscopic characters. According to Stamps *et al*. (1990), Newhook (1978) and Waterhouse (1963) studies, production of oogonia, antheridia and oospores (sexual spores) and the morphological of sexual spores (zoosporangium and chlamydospores) produced by *Phytophthora* spp. can be used as the basis for species identification and taxonomy of *Phytophthora* species. Pure cultures obtained from infected tissues were maintained on 20T slants at 4^oC for future use.

Furthermore, fungal plugs (about 5 mm x 5 mm x 5 mm) from a 7-day-old culture were used as inoculum before placing them on lesions made on healthy, half maturity and detached pods collected from trees of the same clone to examine its pathogenicity in stab method with some modifications (Iwaro *et al*., 1997). Next, the inoculated pods were placed in clean plastic containers in which the humidity maintained by a plug of sterile cotton wool soaked with ddH_2O; and tightly covered with a few sheets of old and clean newspaper to create dark condition (Mpika *et al*., 2011; Omokolo *et al*., 2003). The incubation was done at room temperature (RT) in laboratory and daily observation made for seven days on black pod symptom development. Then, the fungus was re-isolated to fulfil Koch's postulates.

2.4. *Poisoned food technique*

Assessment of fungal toxicity was carried out by poisoned food technique followed the procedure of Schmitz (1930) with some minor modifications, intentionally to evaluate or screen the antifungal efficacy of aqueous crude weed extracts against black pod rot pathogen of cocoa. PDA dissolved in aqueous weed extracts, respectively before autoclaved (121_oC, 20 min, 15 psi) and poured into petri dishes. With the help of a sterile scalpel, a cubic shape of fungal culture plug (about 5 mm x 5 mm x 5 mm) was cut from the periphery of 7- day-old culture of *Phytophthora* isolate and transferred aseptically on PDA plates impregnated with crude weed extracts before incubated at 30°C for seven days. A plate without crude weed extract served as a control for the calculation of percentage inhibition of mycelial growth of test fungus. The experiments were carried out in triplicates and percent reduction of mycelial growth over control calculated using the following formula proposed by Harlapur *et al*. (2007).

Percentage Inhibition (%I) = *(dc-dt)/dc x 100* (1)
Where, dc – Average increase in mycelial growth in control.
dt – Average increase in mycelial growth in treatment.

3. Data Analysis

All statistical analyses were performed using SPSS Version 24 (IBM SPSS, Chicago, IL, USA). One way ANOVA test (Analysis of Variance) at alpha ≤ 0.05 was used to determine the significant differences between the treatments.

4. Results and Discussions

4.1. *Observation on symptoms of cocoa pod rot disease*

Survey results on cocoa pod rot disease in CRDC, Jengka, Pahang showed that the disease could infect all stages of pod development, starting from cherelles, immature to mature pods. Symptoms of infected pods were browning, blackening and rotting cocoa pods with clear boundary between healthy part and infected part. This discoloration generally started from pod stem (Figure 1) or pod tip.

Figure 1. Pod of *Theobroma cacao* in CRDC Jengka, Pahang displays enlargement of black pod lesion started from the stalk. This pod was infected during the last stage of ripening

4.2. *Cultural and morphological characteristics*

An isolation trial on 20T medium resulted in mycelial growth and sporulation of *Phytophthora* isolate. Colony of the fungal isolate visible in five to seven days after introduction to the medium, which surface filled with creeping whitish fluffy mycelium (Figure 2a). The growth medium also could induce production of asexual structures such as abundant of chlamydospores (Figure 2c), ellipsoid to ovoid sporangium with an apical papillate, short pedicel and coenocytic hyphae (Figure 2d) after the fungus stained with lactophenol cotton blue for microscopic observation (Leck, 1999). The findings were in accordance with the detection and identification of *Phytophthora* guided by Drenth and Sendall (2011). Guo & Ko (1993) reported that tomato

juice agar (TJA) was found to be comparable to or in some cases better than the V8 vegetable juice agar in supporting the growth of *Phytophthora cactorum*, *Phytophthora capsici* and *Phytophthora parasitica*; sporangium production of *P. capsici* and *P. palmivora*; and oospore formation of *P. cactorum*, *P. parasitica*. Besides, tomato agar was stated to be conducive to formation of amphigynous antheridia in sexual reproduction of *Phytophthora boehmeriae* (Gao *et al.*, 1998). In addition, TJA was found to be able to support better growth of *P. palmivora* and *P. megakarya* compared to cocoa beans agar (CBA) and cocoa pod and beans agar (CPBA) (Oluyemi *et al*., 2014). In this study, no sporulation was observed on PDA. In future, further study will be carried out to identify the fungal isolate using polymerase chain reaction (PCR) as suggested by Alsultan *et al*. (2017).

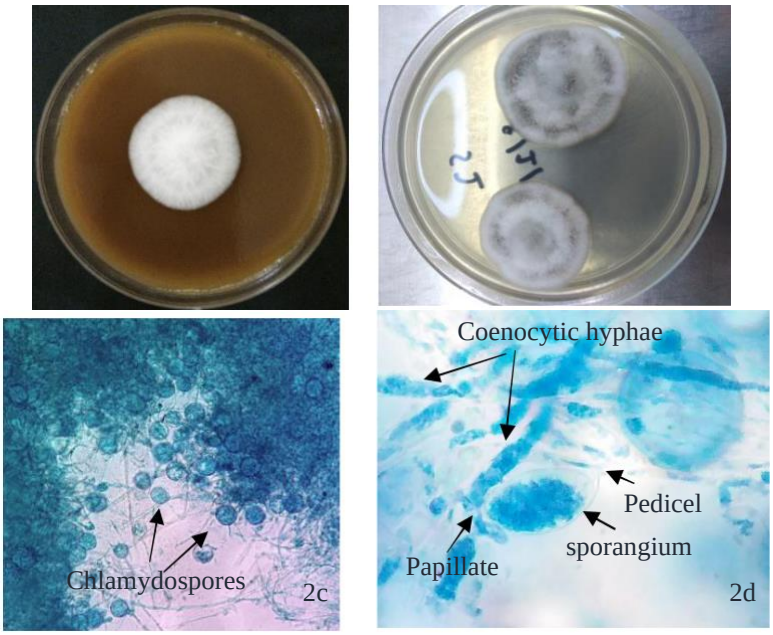
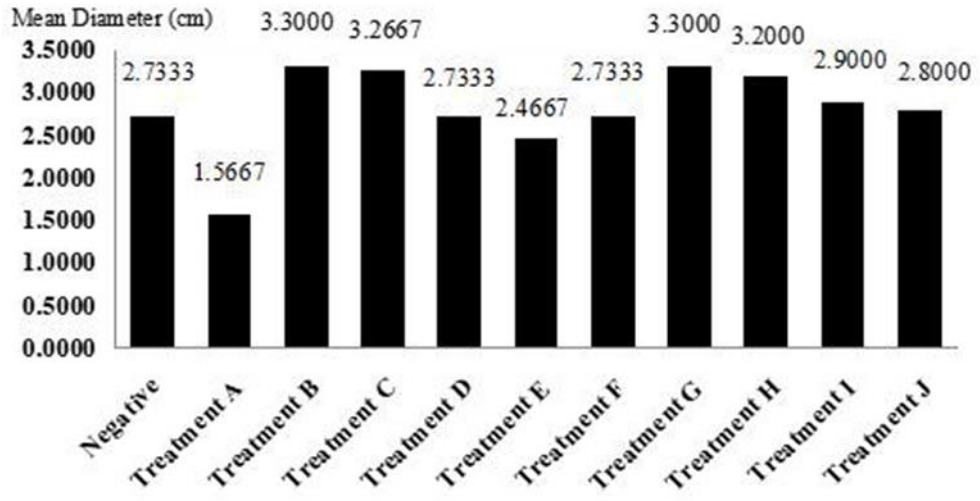


Figure 2. *Phytophthora* isolate. 2a: Colony observed on 20T culture plate; 2b: Colonies observed on PDA culture plate; 2c: Chlamydospores at 100x magnification; 2d: Sporangium and coenocytic hyphae at 400x magnification.

4.3. *Evaluation of weed extracts for Phytophthora mycelial growth inhibition*

The antifungal activity of crude aqueous extracts for ten weed species belonging to ten different families was evaluated against mycelial growth of *Phytophthora* isolate using poisoned food technique. Its effects on fungal colony diameters were measured and reported in Graph 1. The efficacy of the weed extracts was expressed as percent inhibition of mycelial growth over control which is show in Table 2. Among all weed extracts that have been evaluated for their efficacy against the black pod disease pathogen, *S. torvum* was found to be the most effective (42.68% inhibition, Table 2, Figure 3) followed by *R. communis* with lesser percentage (9.75%). No antifungal activities were observed for crude aqueous extracts of *T. indica* and *C. odorata*. While the rest six weed extracts showed stimulatory effect on the growth of *Phytophthora* isolate. The antifungal activity of the above weeds against *Phytophthora* isolate

was suspected due to the presence of few secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, steroids and etc. (Gurjar *et al*., 2012).



Graph 1: Effects of different crude weed extracts against mycelial growth of *Phytophthora* isolate. Abbreviations - Negative: Control; Treatment A: Treated with crude aqueous extract of *S. torvum*; Treatment B: *N. biserrata*; Treatment C: *C. diffusa*; Treatment D: *T. indica*; Treatment E: *R. communis*; Treatment F: *C. odorata*; Treatment G: *C. grandis*; Treatment H: *A. gangetica*; Treatment I: *C. pulcherrima*; Treatment J: *L. camara*.

Table 2: Inhibition percentage.

Botanical Name	% Inhibition
S.torvum	42.68
N. biserrata	-20.73
C. diffusa	-19.51
T. indica	0.00
R. communis	9.75
C. odorata	0.00
C. grandis	-20.73
A. gangetica	-17.07
C. pulcherrima	-6.10
L. camara	-2.44

Abbreviations: (+) – Inhibitory growth effect; (-) – Stimulatory growth effect

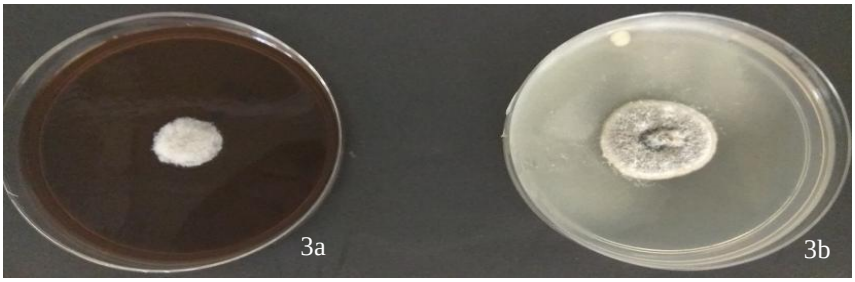


Figure 3. *In vitro* inhibition of mycelial growth of *Phytophthora* isolate by crude aqueous extract of *S. torvum*; 3a: Crude aqueous extract of *S. torvum* leaves showing inhibition in the fungal growth; 3b: Control (PDA + *Phytophthora* isolate) after a week incubation period at 30°C.

4.4. *Statistical analysis*

Comparison of means for the mycelial growth of *Phytophthora* isolate on PDA amended with different crude aqueous weed extracts, is respectively shown in Table 3. With a probability value of 0.000 and a level of significance fixed at 5%, it becomes clear after the analysis of means for variances that the crude aqueous weed extracts did cause the inhibitory effect on the mycelial growth of *Phytophthora* isolate which induced black pod disease on infected cocoa pod samples.

Table 3. Analysis of variance.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.589	10	0.759	6.843	0.000
Within Groups	2.440	22	0.111		
Total	10.029	32			

5. Conclusion

The results of this study showed that 20% tomato juice agar (20T) was a better medium compared with PDA for growth and reproduction of *Phytophthora* isolate from an infected cocoa pod sample. Crude aqueous weed extract of *S. torvum* leaves had the highest or the most effective antifungal activity against the test fungus as shown. 42.68% inhibition in mycelial growth inhibitory assay using poisoned food technique. Hence, it can be used as a new source for antifungal substance for management of *Phytophthora* pod rot infection in the field and further phytochemical studies are recommended to purify and characterize the active ingredients of this weed species.

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