MOLECULAR DIAGNOSTIC KIT FOR DETECTION OF PATHOGENIC *Phytophthora*

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Phytophthora palmivora has been identified as the responsible pathogen of bud rot disease of oil palm in Colombia which also known as *Pudricion del cogollo* (PC) (Colombia) and *Amarelecimento fatal* (AF) (Brazil) (Turner and Gillbanks, 2003). The disease wiped out thousand hectares of oil palm plantations in South America in 5 years (Torres *et al*., 2016). To date, no outbreaks of the disease have been reported in oil palm in Malaysia or other Southeast Asian countries (SEA). The pathogen belong to the *Phytophthora* genus under family Pythiaceae (along with sister genus, *Pythium*) of order Peronosporales/Pythiales; class oomycetes, kingdom of Chromista and is not a fungi although their morphology resembles fungi. Most species of *Phytophthora*, which means 'plant destroyer' in Greek, are considered as the most damaging pathogens and responsible for some of the world's most destructive diseases of crops and native vegetation (Brasier, 1992). *Phytophthora* attack wide range of plants and can cause diseases in different part of plants such as shoots, buds, leaves, stems, collars, fruits and roots even for the same host. In SEA, *Phytophthora* infect several plant species including rubber, cocoa, durian, pepper, coconut, jackfruit and papaya. Among species of *Phytophthora* that important to SEA are *P. palmivora* (infecting rubber, cocoa, durian, jackfruit, coconut *etc*.), *P. nicotianae* (durian, citrus, tobacco, brinjal, strawberry, papaya, roselle), *P. capsici* (black pepper, bell pepper) and *P. infestans* (tomato, potato). Other species that had been isolated from several hosts in SEA since 1925 which includes *P. colocasiae* (betel vine, yam), *P. haveae* (rubber, cocoa), *P. meadi* (rubber), *P. cinnamomi* (quinine), *P. botryosa* (rubber) (Lee and Lum, 2004).

NOVELTY OF TECHNOLOGY

The detection method employ the used of isothermal amplification. The detection kit comprises of isothermal reaction reagents and special primer marker specially designed for detection of *Phytophthora* species. The marker comprised of a series of Phy-M primers. The isothermal amplification technique are fast, convenience and robust to be used in screening of the pathogens contamination in plant and soil samples. Contamination of plant materials with pathogens of genus *Phytophthora* can be diagnosed and eliminated at the early stage. Soil detection is also possible as the method is not highly affected by inhibitors in soil samples. Other available detection methods of the pathogens of *Phytophthora* usually employed conventional PCR method with combination with other techniques such as Random Amplified Polymorphic DNA (RAPD), which took at last two days before a diagnostic can be made. Most detections are designed to be species specific and cannot be used to eliminate a general *Phytophthora* genus contaminations which is usually needed by the quarantine agencies since almost all member of genus *Phytophthora* are known as plant pathogens with a wide-host range.

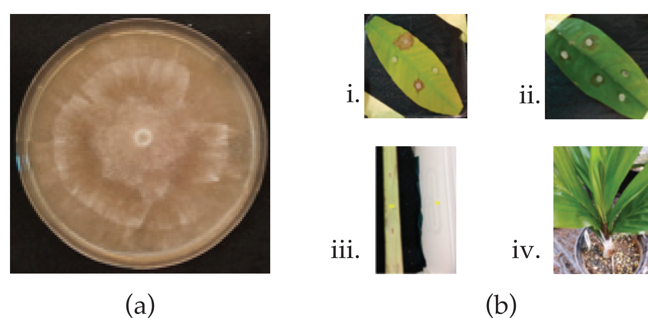

Figure 1. Samples used in the assay, (a) pure culture, (b) plant materials artificially inoculated with P. palmivora: *i. rubber, ii. durian, iii. oil palm unopen spear (in- vitro leaf detached) and iv. oil palm seedlings (glasshouse).*

DETECTION of *Phytophthora*

The method involve DNA extraction of the samples such as mycelial of pure culture (*Phytophthora* and others) scrapped from agar plate

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(*Figure 1a*), plants materials (*Figure 1b*) and soils using commercial plant and soil DNA extraction kit. The isothermal amplification mixture consists of reaction reagents mix, Phy-M primers mix and 1 μl DNA of samples. The isothermal amplification reaction was carried out in isothermal condition at 65°C for 30-60 min with two controls (positive and negative). Amplification products were detected using real time fluorescence detection system and were express as amplification time and curve by the fluorescent detector (*Figure 2*) but other detection systems such as by turbidity meter and colour dye are also possible. For plant samples, additional assays using plant COX primer marker were included to eliminate possible *pseudo* negative result which will give positive result to all plant materials, indicating the acceptable quality of DNA used. All *Phytophthora* isolates, samples artificially infected and soil contaminated with *Phytophthora* were detected based of the presence of amplification curve and time. Summary of the detection assays are as in *Tables 1* and *2*.

REAL TIME QUANTIFICATION AND DETECTION LIMIT

The molecular diagnostic kit of *Phytophthora* can be used as real time quantification when using fluorescence detection system (*Figure 3*). The sensitivity of the detection kit is calculated at 8.3 x 10^{-8} ng μl^{-1} which is equivalent to 20 copies of the double stranded DNA template (*Figure 4*).

ECONOMIC ANALYSIS

The internal rate of return (IRR) for manufacturing of the diagnostic kit is estimated at 47.7%, net present value (NPV@10%), RM 2 006 350 and discounted benefit-cost ratio (BCR) at 1.21. The discounted payback period is estimated at 2 years. The cost of production is estimated at RM 2600 per box of 96 reactions. The values are subjected to change.

TABLE 1. RESULTS OF MOLECULAR DIAGNOSTIC KIT ASSAYS FOR DETECTION OF *Phytophthora* WITH PURE CULTURE

Isolate	No. of isolates*	*Phytophthora* diagnostic assay**
P. palmivora	27	+
P. colocasiae	1	+
P. citrophthora	1	+
P. infestans	2	+
P. parasitica	2	+
P. cryptogea	1	+
P. megakarya	3	+
Fusarium sp., *F. oxysporum & F. solani*	4	-
Gongronella butleri	1	-
Mortierella chlamydospora & M. echinosphaera	2	-
Purpureocillium lilacinum	1	-
Rhizomucor variabilis	1	-
Trichoderma asperellum & T. koningiopsis	3	-
Talaromyces aculeatus	1	-
Pythium cucurbitacearum, P. splendens & P. aphanidermatum	3	-
Lasiodiplodia theobromae	1	-
Colletotrichum gloeosporioides	1	-
Ganoderma boninense & G. miniatocintum	4	-
Phoma herbarum	1	-
Total	**60**	-

Notes: * from different host and demographic origins
**symbol ‘ + ’ is positive assay and ‘ – ‘ is negative assay based on presence of real-time fluorescence curve and amplification time.

TABLE 2. MOLECULAR DIAGNOSTIC KIT ASSAY FOR DETECTION OF *Phytophthora* WITH PLANT MATERIAL AND SOIL SAMPLES

Materials	*Phytophthora* diagnostic assay*	Plant DNA control assay **
Infected leaf tissue of oil palm (OP) seedling inoculated with *Phytophthora*	+	+
Healthy leaf tissue of OP seedlings inoculated with sterile distilled water (control)	-	+
Infected tissue of detached OP leaf inoculated with *Phytophthora*	+	+
Healthy tissue of detached OP leaf inoculated with sterile distilled water	-	+
Healthy tissue of non-inoculated OP seedling leaf	-	+
Infected tissue of detached rubber leaf inoculated with *Phytophthora* mycelial disc	+	+
Healthy tissue of detached rubber leaf inoculated with sterile carrot agar disc	-	+
Infected tissue of detached durian leaf inoculated with *Phytophthora* mycelial disc	+	+
Healthy tissue of detached durian leaf inoculated with sterile carrot agar disc	-	+
Infected tissue of detached durian cocoa inoculated with *Phytophthora* mycelial disc	+	+
Healthy tissue of detached cocoa leaf inoculated with sterile carrot agar disc	-	+
Pure culture *Phytophthora* (positive control)	+	-
Soil samples spiked with mycelial of *Phytophthora*	+	-
Soil samples	-	-
Water (negative control)	-	-

Notes: * Conducted using Phy-M primer.
** Conducted using plant cox primer (Tomlinson et al., 2010) and symbol of ' + ' is positive detection and ' – ' is negative detection based on presence of real-time fluorescence curve and amplification time.

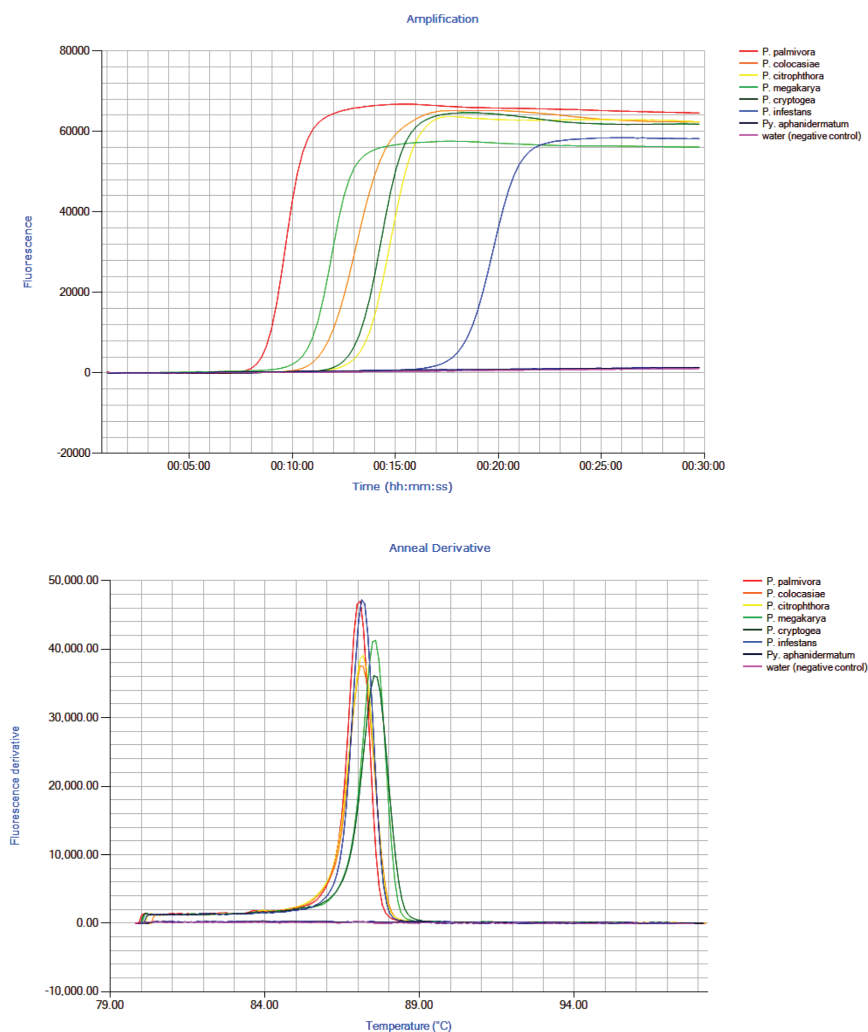

Figure 2. Positive amplification of Phytophthora *is represented by the presence of sigmoidal curve, annealing/melt and amplification time (in seconds) produced by fluorescent detector.*

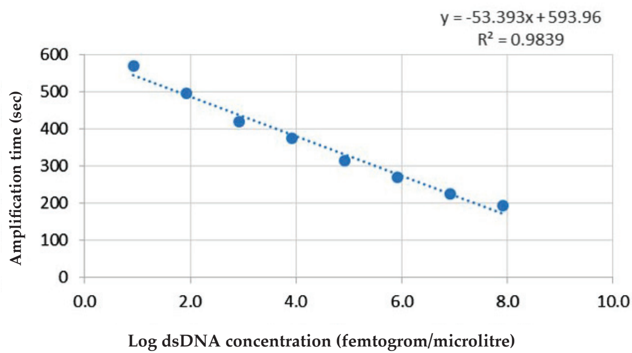

Figure 3. Real-time (quantification) curve of Phytophthora *diagnostic kit using fluorescence detection system.*

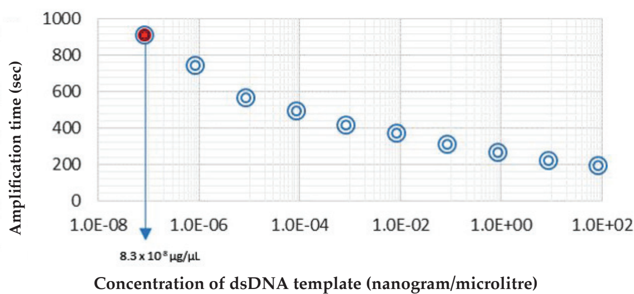

Figure 4. Sensitivity of the Phytophthora *diagnostic kit is at 8.3 x 10^{-8} ng μl^{-1} (red dot), calculated based on the concentration of DNA extracted from pure culture of* Phytophthora palmivora.

BENEFITS

The technology offer a simple and rapid detection tool of *Phytophthora* that can be used to screen for the pathogenic *Phytophthoras* in plant and soil materials. This is essential to reduce the risk of entry of exotic disease cause by *Phytophthora* such as bud rot disease of oil palm caused as well as monitoring the incidence of these pathogens locally as part of mitigation measures in the biosecurity plan for Malaysian oil palm industry.

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