Phytophthora palmivora, the causal agent of bud rot disease of oil palm (*Elaeis guineensis* Jacq.): Biology, detection and control

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Abstract

Bud rot disease has been considered as a devastating disease of oil palm in Latin America. Severe outbreaks of this disease have been reported in Colombia, Brazil, Ecuador, Panama and Suriname. The causal agent of bud rot disease in Colombia has been identified as *Phytophthora palmivora*. This pathogen is known to be responsible for several tropical diseases such as black pod and stem canker disease of cocoa, especially during the rainy season. *Phytophthora palmivora* has also been reported to attack durian, rubber, pepper and jackfruit causing diseases in various parts of the plant such as fruit, leaves and stems. However, no outbreaks of the disease have been reported in oil palm in Malaysia or other Southeast Asian countries. Several aspects of research need to be conducted to understand why this pathogen causes problems in oil palm in South America but not in Southeast Asia.

This study aimed to analyze variation between the Colombian *P. palmivora* isolates that cause bud rot disease in comparison with Malaysian isolates and other isolates gathered from different hosts and regions. Our hypothesis was that *P. palmivora* isolates from the different regions and/or hosts have different molecular characteristics and have dissimilar levels of pathogenicity. Sequence alignments of several genetic markers, the internal transcribed spacer (ITS) of the ribosomal RNA (rRNA) gene cluster, beta-tubulin (*β-tubulin*), translation elongation factor 1 alpha (*EF-1α*), cytochrome c oxidase subunit I (*CoxI*) and subunit II (*CoxII*) genes failed to distinguish between Colombian oil palm isolates and *P. palmivora* from different hosts and regions. It was concluded that these markers are more suitable for inter-specific studies between species but not for intra-specific evaluation within species of *P. palmivora*. However, a new marker named as *P. palmivora* hypothetical avirulence

effector protein (PpHPAVR) along with analyses of amplified fragment length polymorphisms (AFLPs), separated the Malaysian and Colombian isolates into distinct clades. This indicates that there is genomic variation within *P. palmivora* isolates. The zoospores of *P. palmivora* from various hosts and demographic origin were shown to have the ability to cause infection to oil palm seedlings, durian and rubber. However, not enough evidence has been collected to confirm that pathogenicity correlates with the distinct clades observed with AFLPs and PpHPAVR. *Phytophthora palmivora* species-specific diagnostic using PCR and loop-mediated isothermal amplification (LAMP) have been developed based on the PpHPAVR region.

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List of Abbreviations and Symbols

a.i.	active ingredient
AFLP	Amplified Fragment Length Polymorphisms
ANOVA	Analysis of variance
AUMGC	Area Under Mycelial Growth Curve
Avr	avirulence
BLAST	Basic Local Alignment Search Tool
Bp	base pair
COX	Cytochrome Oxidase
DxD	Dura x Dura
DxP	Dura x Pisifera
EF-1α	Elongation factor 1-alpha
FAO	Food and Agriculture Organization of the United Nations
PpHPAVR	*Phytophthora palmivora* hypothetical avirulence effector protein
ITS	Internal transcribed spacer
Kb	kilobase pairs
LAMP	Loop-Mediated Isothermal Amplification
LSD	Least Significant Difference
MEGA	Molecular Evolutionary Genetics Analysis
n.d.	not done
NGS	Next Generation Sequencing
OxG	*E. oleifera* x *E. guineensis*
PCR	Polymerase Chain Reaction
PIRG	percentage inhibition of radial growth
PVPP	Polyvinyl-polypyrrolidone
Rpm	Rotation per minutes
SEA	South East Asia
TxT	Tenera x Tenera
β-tubulin	Beta-tubulin
v/v	Volume per volume
w/w	Weight per weight
≥	greater-than or equal to
≤	less than or equal to

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Chapter 1. General introduction

1.1 Oil palm and palm oil

Oil palm is widely used in the production of a type of edible vegetable oil known as palm oil. Edible vegetable oils are not just the most important cooking ingredients in the world but also play a role in the production of food based products such as margarine, spreads and confectionaries as well as in the production of non-food products such as cosmetics (soap, lotion, shower foam etc.), detergents, agrochemicals and production of biofuels.

Palm oil is derived from the fruit of the oil palm, either from the outer flesh pulp (mesocarp) or from the inner nut (kernel). Commercial palm oil extracted from the pulp is known as palm oil, whilst the oil derived from palm kernel (endosperm) is known as palm kernel oil. Both oils have unique nutritional and physical characteristic. The palm oil is orange-red in colour whilst the palm kernel oil is clear yellowish oil that is similar to coconut oil. Palm oil and its refined derivatives, palm olein and palm stearin, are the major commercial products of oil palm (Corley and Tinker, 2003). Palm oil is widely used as cooking and frying oil especially in Southeast Asia, Africa and some part of Latin America whilst palm kernel is used mainly in the production of food and non-food products. About 90% of palm oil is used for food consumption and the remaining 10% is used for non-food production. Palm oil has been use worldwide in commercial food industries because of its lower cost compared to other vegetable oils and fats and the high oxidative stability of the refined oil during frying (Matthäus, 2007).

The importance of palm oil has increased substantially during the last 20 years. The global consumption of palm oil has increased from 15.8 million metric tons in 1995/96 to 56.7 million metric tons in 2014 and is estimated to rise to approximately 60.7 million metric tons by the end of 2015 (USDA, 2015) which is approximately 36.97% of the overall vegetable oil consumption making it the highest among all oils (Figure 1-1). This trend is expected to continue as palm oil is a very competitive edible oil compared to other edible oils such as from soya beans, corn, rapeseeds (canola) and sunflower. This might largely be due to the fact that the oil palm has the highest yield per hectare when compared to other oil crops as reported in the database of the Food and Agriculture Organization of the United Nations (FAO) (Table 1-1)(FAO, 2015). One hectare of oil palm produces about 4-5 tonnes palm oil per year which is 10 times the yield of soybean (Mohd Hanif, 2000; Rajanaidu et al., 2000).

Palm oil is mostly extracted from the fresh fruit of African oil palm (*Elaeis guineensis*) and also from the American oil palm *Elaeis oleifera*; however, production of palm oil from American oil palm is much less than from African oil palm. Palm oil from *E. guineensis* has been used domestically in West African countries from as far back as 5000 years ago. People of Africa have been processing oil palm fruits for edible oil using simple but inefficient and tedious methods. The oil produced traditionally is highly coloured and flavoured. Aside from the African continent, palm oil is also believed to have been used by people in Ancient Egypt based on the finding of a jar of palm oil in the tomb excavated by archeologists in Abydos which dated from 3000 BC. It is thought that this was taken to Egypt by Arab traders as part of the spice trade (Obahiagbon, 2012).

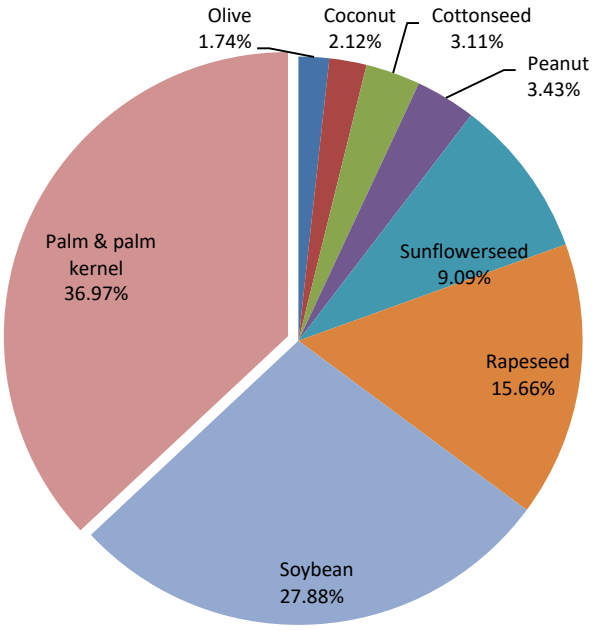
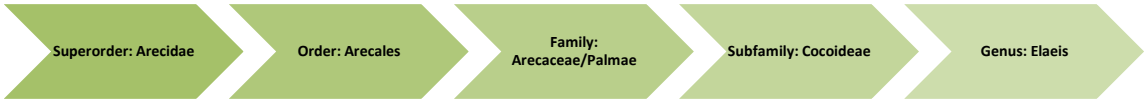


Figure 1-1 World consumption of vegetable oil 2013/2014
(adapted from USDA (2015))

Table 1-1 Average global yield of primary oil crops 2011-2013
(adapted from FAO (2015))

Oil crops	**Yield (metric tonnes/hectare)**		
	2011	**2012**	**2013**
Oil palm fruit	16.01	15.97	16.27
Sunflower seed	5.70	5.40	6.06
Olives	5.43	5.67	5.70
Rapeseed	2.78	2.53	2.73
Soybeans	2.04	2.08	2.20
Coconuts	2.23	1.80	2.18
Corn (Maize)	1.75	1.65	1.93

1.2 Classification of oil palms



The oil palm is grouped with *Cocos* (the coconut) and other genera in the subfamily of Arecoideae, tribe *Cocoeae*, and subtribe *Elaeidinae* (Price et al., 2007). The palm family, *Arecaceae* which was formally known as *Palmae,* is placed in the order *Arecales* (Cronquist, 1981) which forms a distinct group among the monocotyledons plants. The *Arecaceae* is considered as old as any other family of flowering plants with fossils discovered in Cretaceous rocks dating from around 120 million years ago, according to Purseglove (1972) as cited in Latiff (2000). The genus *Elaeis* is believed to have originated from Africa or America. There are three accepted species of genus *Elaeis*; *E. guineensis* (African oil palm), *E. oleifera* (American oil palm) and *E. odora.*

Elaeis guineensis, the African oil palm, is the first species in the genus, and was first described by Jacquin (1763) and also illustrated in the 3rd volume of the *Köhler's Medizinal-Pflanzen* (*Medicinal Plants*) in 1898 (Brandt et al., 1898) (Figure 1-2). The word '*Elaeis*' comes from a Greek word *elaion*, which means the oil, and the species name *guineensis* shows that Jacquin attributed its origin to the Guinea Coast and was based on palms introduced into Martinique. The second species, the South American oil palm, *E. oleifera* (H.B.K.) *Cortes*, was officially described by Cortes in 1897. This species had been described earlier by Gaertner in 1788 but with the name *E. melanococca* and later in 1816 as *Alfonsia oleifera* (Latiff, 2000) and also *Corozo oleifera.*

Figure 1-2* Elaeis guineensis*; schematic drawing by Jacquin (1763) (left) and Brandt et al. (1898)(right).

The third species, *E. odora* was previously known as *Barcella odora* but was renamed by Wessels-Boer (1965). However, some taxonomist place *E. odorata* in a separate genus, *Barcella*, rather than *Elaeis* and retain the name as *B. odorata*, which leaves the genus *Elaeis* with only two species. Both genera, however, are always recovered as monophyletic (97%) (Hahn, 2002). *Elaeis odora* is not cultivated and not much is known about the species. Some other species have also been placed in the genus *Elaeis* but not on a permanent basis except for *E. madagascariensis Becc.,* which was described by Beccari (1914) as cited in Corley and Tinker (2003). This species is distinguished from *E. guineensis* based on some flower and fruit characteristics but has an uncertain status. Some taxonomists

believe this species is a variant of *E. guineensis* which was introduced around the 10th century when African influence entered Madagascar (Purseglove, 1972).

1.3 African oil palm, *Elaeis guineensis*

Most commercial oil palm plantations worldwide are of *E. guineensis*. The other species, *E. oleifera* is not commercially exploited. *Elaeis guineensis* is believed to have originated from the wild and semi wild groves of tropical West Africa. Analysis of the species' natural genetic diversity suggests that populations of wild *E. guineensis* could be separated into three groups located at the extreme west of Africa, equatorial Africa and on the island of Madagascar, in the area of average annual rainfall of about 1780–2280 mm and temperature ranging from 24 to 30°C (Barcelos et al., 2015).

Elaeis guineensis is a large, unbranched plant with pinnate fronds (feather-leaf palm) and has a solitary columnar stem with short internodes (Figure 1-3)(Hartley, 1977). The palm is usually monoecious, producing alternately male or female inflorescences in a cycle around six months. Sometimes, a mixture of male and female (hermaphrodite) inflorescences can be observed from a single palm, typically in the young palm or between transitions of the cycle. Both female and male inflorescences are borne in the axils of the fronds. The oil palm is obligatory cross-pollinated since both female and male inflorescences do not occur together in the same palm, and fertilization of the female must occur by pollen transmission from another palm. The pollination can occur naturally by wind, or through assistance of pollinating insects such as the weevil, *Elaiedobius kamerunicus,* which is important in order to archive maximum yield (Turner and Gillbanks, 2003).

Figure 1-3 African oil palm tree in an oil palm plantation in Malaysia.
**Note the harvested fresh fruit bunches waiting for collection*

Each mature frond has a rachis, pinnae (leaflets) and spines on the leaf petiole. The pinnate frond can reach up to 8 metres in length and the palm tree can grow to a height of 15 to 20 metres in cultivation areas and up to 30 metres in dense forests. The life span of the oil palm is believed to be up to 200 years, although plantations usually start to replace palms at 25 years of age due to productivity decline. The cultivation time of oil palm from the seed to maturity is relatively long. The germination of the seeds takes around 100-120 days, followed by the seedling or nursery stage of 10 to 12 months. Usually, the seedlings are planted in the field after 12 months in the nursery. The oil palms will start producing fruits after 2 to 4 years depending on the variety and approach maturity around 10 years. The economic life of planting varies from 20-30 years.

The fruit is a sessile drupe, having an outer fibrous fleshy part (mesocarp/outer pulp), a thin skin (exocarp) and a central seed (kernel) with a hard shell (endocarp) (Figure 1-4). The fruit is borne on a large compact bunch. Each bunch can have up to 3,000 fruits on a mature palm but the average is around 1,500 fruits per bunch (Mayes et al., 2008). The ripe fruit bunch is commonly known as the fresh fruit bunch (FFB). The shape, size and colour of the fruits varies from spherical to ovoid, elongated and with a little bulge on the top around, 2 to 5 cm in length, with the individual weight of 3 g to 30 g. The most common type of fruit is deep violet to black at the apex with a pale greenish yellow base before ripening (Corley and Tinker, 2003) which turns to orange red when ripe.

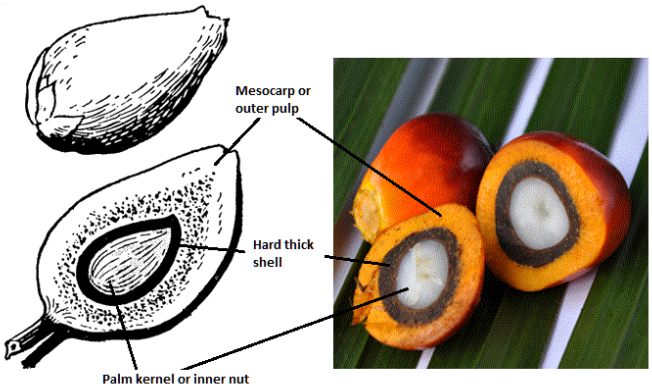


Figure 1-4 Fruits of oil palms
(Pictures obtained www.fao.org and http://etp.pemandu.gov.my)

There are no subspecies of *E. guineensis*, just types or varieties (the words have been used interchangeably). The most common (or probably the only) classification is based on the internal fruit structure; the variation in shell thickness (believed to be controlled by a single gene) of the fruits, which are known as *Dura*, *Pisifera* and *Tenera*. *Tenera* (heterozygote $Sh^{+}sh^{-}$) is the hybrid of the dominant homozygote *dura* ($Sh^{+}Sh^{+}$) and recessive homozygote *pisifera* ($sh^{-}sh^{-}$). *Dura* oil palm

bears a fruit with a thick shell and has relatively less oil bearing mesocarp (25%-65%), and *pisifera* has a shell-less fruit with a high proportion of mesocarp to fruit ratio (95%)(Figure 1-5). *Tenera* has a thin shell and high content of mesocarp oil (due to its high mesocarp to fruit ratio of 75%-85%) compared to the others. Based on the mesocarp ratio, *pisifera* may look like a better choice for commercial planting but the *pisifera* palms are usually female sterile or semi sterile where bunches do not develop well, thus reducing the yield. Therefore, *tenera* is the commercially important oil palm variety worldwide.

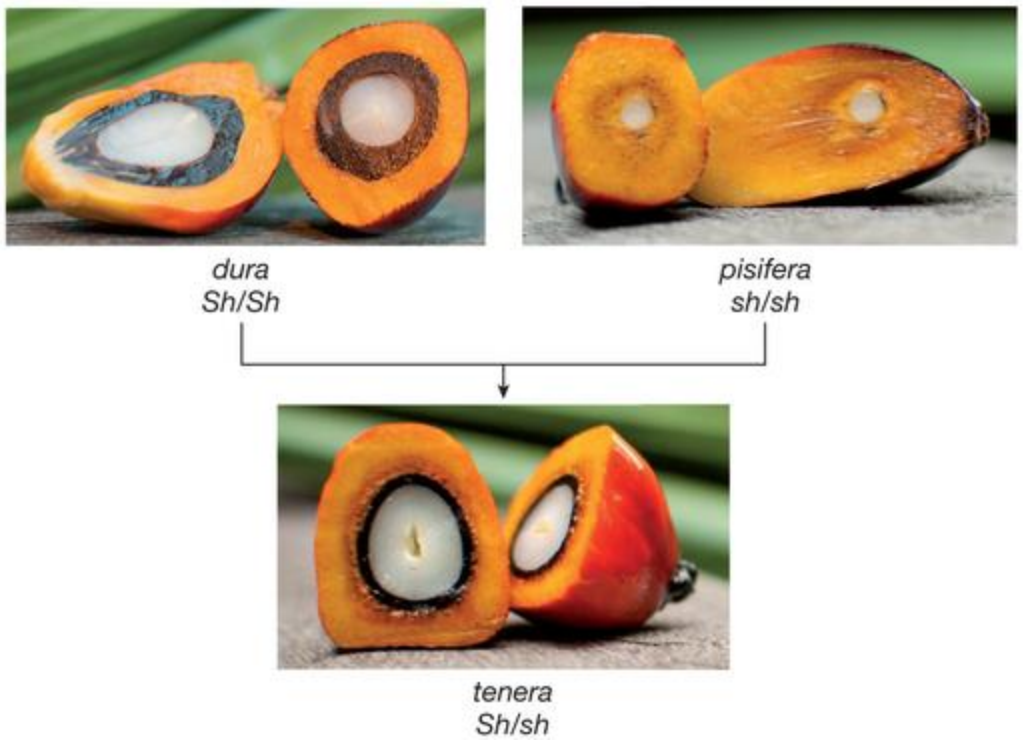


Figure 1-5 Dura (Sh^+/Sh^+) fruit with a thick shell around the kernel and shell-less pisifera (sh^-/sh^-)(adapted from (Singh et al., 2013))

1.3.1 The growth of *E. guineensis*

Oil palm propagates by means of seed. The seed of oil palm is the nut after the removal of the fleshy mesocarp, which consists of the hard shell (endocarp) and usually with one kernel inside, but two or three kernels are possible. The kernel consists of layers of hard oily greyish white endosperm surrounded by dark-brown testa with a network of fibres. Embedded in the endosperm and opposite one of the germ pores lies the embryo (Latiff, 2000). The plumule (seedling shoot) and the radicle emerge via a cylindrical ligule close to the embryo. The seedling depends on the nutrients stored in the endosperm during the first few weeks of growth. Two bladeless plumular sheaths are produced first before the emergence of the green leaf blade (lamina), which usually emerges a month after germination. One leaf is produced each month until the seedling is six months old. Early on, the leaves are lanceolate (with a midrib that divides half of the length of the leaf), but later, the leaf shape changes and becomes bifurcate where the leaves split, and this is then followed by more splitting which divides the laminea between the veins, resulting in a pinnae or feather like leaves. However, the young pinnate leaves are different from the mature.

The oil palm has one terminal growing point or apical meristem. The development of branched palms with two or more growing points is unusual but can happen occasionally as a result of damage to the apex. At the nursery stage, 3 to 4 months after germination, the base of the stem becomes a swollen bulb. At this stage, a true primary root emerges from the base. After the seedling stage, the early growth of the oil palm involves just the horizontal growth, which is the widening of the stem base. During this stage, no internodal elongation occurs. The wide base is important to firmly hold the stem column. The apical meristem is situated in a bowl-like cavity at the apex of the stem. The size of the bowl cavity is around 10-12 cm in

diameter and 2.5 to 4 cm deep in mature palms (Hartley, 1977; Corley and Tinker, 2003). The apex is conical in shape and is concealed in the crown of oil palms within the soft mass of young fronds and frond bases, known as the 'cabbage' palm heart.

The way in which the fronds and spikelets of male and female inflorescences are arranged with regard of the palm axis is call phyllotaxy. The meristem produces new leaf primordia every 2 weeks on mature palms and each remains enclosed in the crown for approximately 2 years. After that, the leaves rapidly develop into a central 'spear' and finally open (Broekmans (1957), cited in Corley and Tinker (2003)). The base of each leaf primordium completely encircles the next young leaf. The apical meristem is mainly involved in the production of the leaf. The horizontal growth or widening of the stem is carried out by the meristem situated just below the apical meristem. The young leaves are largely composed of leaf bases with lateral extensions but are not yet elongated. The rest of the leaf is only a small epical folding. There are approximately 30 to 50 leaves from the centre of the apical meristem in the middle of the cavity to the highest point of the ring of the crown of a mature palm. Once the formation of the wide base is completed (where the stem reaches its maximum diameter), the internodes begin to elongate and a column stem with adhering leaf bases is formed. Each stem segment can be considered as an internode with a leaf, but the node is the only external indication based on leaf scars on an old palm, without any boundary between adjacent internodes internally.

1.4 The American Oil Palm (*E. oleifera*) and OxG hybrids

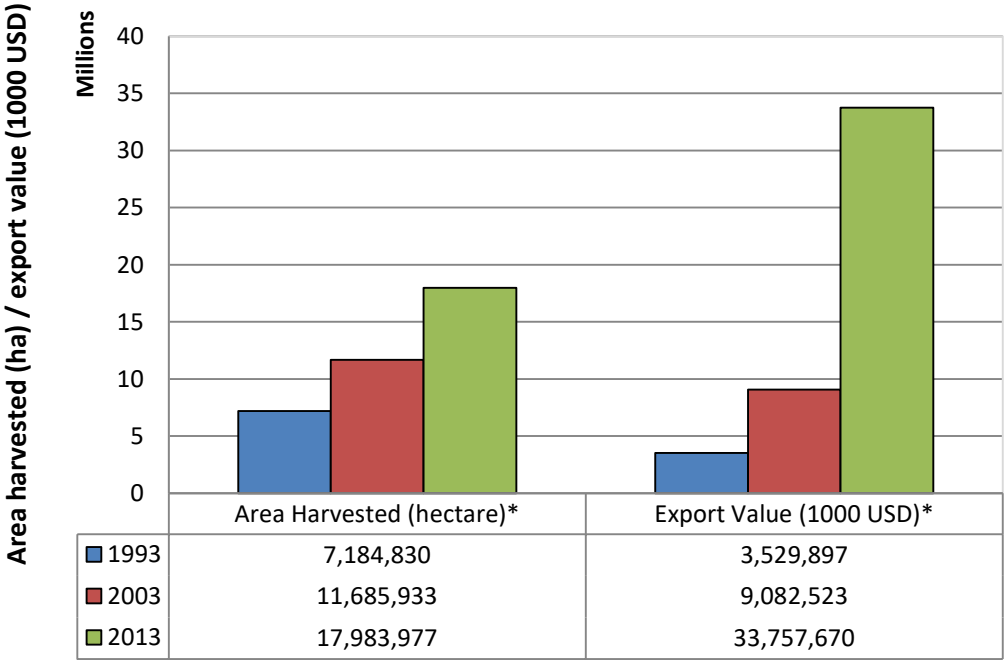
Elaeis oleifera is found in the tropical countries of Central and South America. This species originated from Brazil, Colombia, Costa Rica, Honduras, Panama, Venezuela,

Nicaragua, French Guiana and Surinam (Rajanaidu, 1986). *Elaeis oleifera* is shorter and grows slower (in term of height) than *E. guineensis* and usually has a procumbent trunk and is easily differentiated from *E. guineensis* as all its leaflets lie in one plane and do not have basal swellings with short and think spines of the petiole. Oil composition of *E. oleifera* is higher than *E. guineensis;* on the other hand the ratio of oil to bunch of *E. oleifera* is much lower.

The *E. oleifera* and *E. guineensis* are able to cross hybridise. The hybrids (OxG) usually have some intermediate characteristics/traits from both parents such as palm height and level of unsaturated fatty acid. Hormaza et al. (2012) undertook a comprehensive study on the phenology of the O×G hybrid. Appearance wise, the hybrids have comparatively larger fronds than both parents. FFB yield of the hybrid is similar to that of *E. guineensis* but with a lower oil to bunch ratio. In Latin America, specifically Colombia, the OxG hybrids have gained interest among planters/producers as a promising solution to problems caused by diseases such as bud rot because of the apparent partial resistance of this genotype to the disease, due to the inheritance of disease tolerance from *E. oleifera*. *Elaeis oleifera* has been observed to have some degree of resistance to diseases of Latin America such as bud rot disease, fatal yellowing disease and sudden wilt to which the *E. guineensis* is very susceptible. Turner and Gillbanks (2003) described *E. oleifera* as having complete resistance to sudden wilt disease and the hybrid OxG has proven to have tolerance to fatal yellow disease, which might be another name for bud rot disease. Apart from these major diseases, *E. oleifera* and its hybrids have also shown some substantial degree of resistance to leaf mottle disease and infection by *Ustulina* but are more susceptible to *Cercospora* leaf spot disease then *E. guineensis* and comparable in susceptibility to vascular wilt disease.

1.5 The oil palm industry

The increasingly high yield of oil palm per hectare has fueled the expansion of the oil palm industry worldwide. In the last two decades, the cultivation area of oil palm has increased greatly with a total harvested area increment of 10,799,147 ha from 1993 to 2013 and total export value increase of nearly US$30.23 billion (Figure 1-6) (FAO, 2015). The worldwide production of crude palm oil in 2013 was 56.27 million tonnes (MPOB, 2015) with approximately 87% was contributed by producers in Southeast Asia, mainly by Malaysia and Indonesia (adapted from FAO (2015)). At present, the top five producers of palm oil worldwide are Indonesia, Malaysia, Thailand, Colombia and Nigeria (Figure 1-7).



	Area Harvested (hectare)*	Export Value (1000 USD)*
1993	7,184,830	3,529,897
2003	11,685,933	9,082,523
2013	17,983,977	33,757,670

**Note: values are aggregate, may include official, semi-official, estimated or calculated data (adapted from FAO (2015))*

Figure 1-6 Total world oil palm harvested area and palm oil export value 1993-2013.

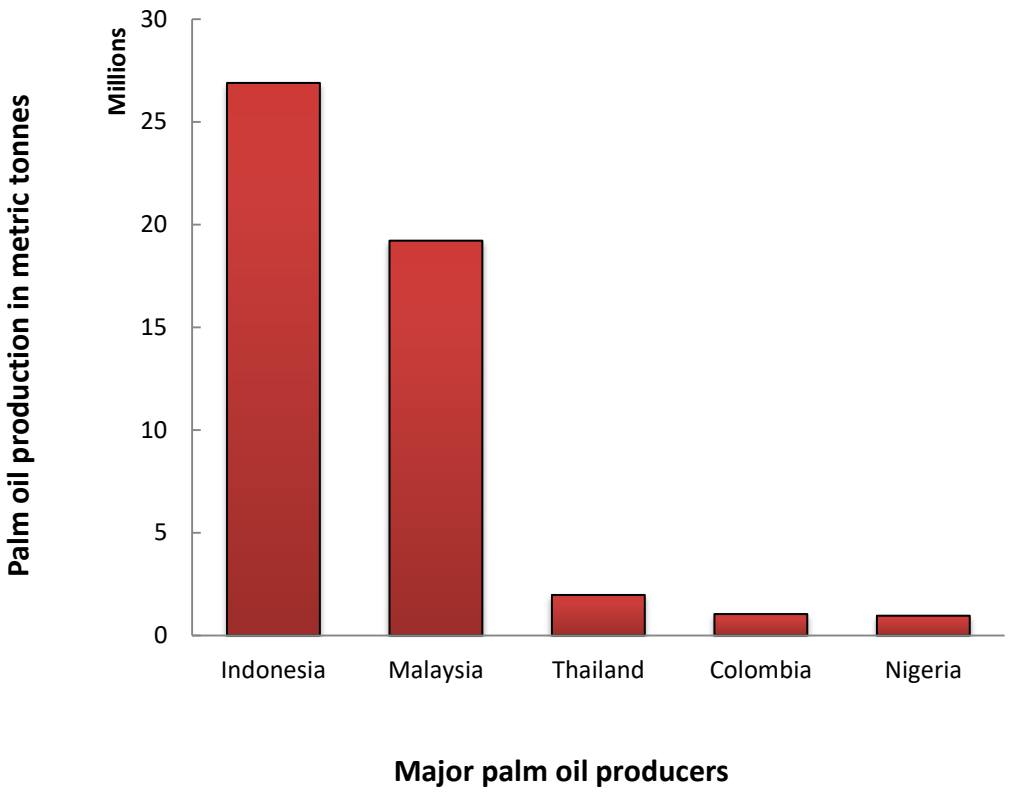


Figure 1-7 Major producers of palm oil worldwide
(adapted from (FAO, 2015))

1.5.1 Oil palm cultivation in Africa and South America

Even though oil palm originated from Africa, commercial cultivation of the crop in this region was not established until the 1920s with the opening of the first commercial plantation in Zaire, followed by other West African countries (Kushairi and Rajanaidu, 2000). In the Niger Delta, experimental plantations began as early as 1932. However, trading of the palm oil from West Africa had begun much earlier, and by the early 1870s exports of palm oil from the Niger Delta were 25,000 to 30,000 tonnes per annum and by 1911 the British West African territories exported

87,000 tonnes (Poku, 2002). In 1900, 89% of Nigeria's total exports were palm produce and the principal economic activity of the people was collecting palm nuts from the palm bush during January to June (Aghalino, 2000). In the first half of the 20th century, Nigeria and Zaire led as the leading producers of oil palm worldwide but were surpassed by Malaysia and Indonesia in 1966 (Poku, 2002). Today, Nigeria is the largest producer of palm oil within the African region and amongst other palm oil producers in Africa are the Democratic Republic of the Congo (Zaire), Ivory Coast, Cameroon, Ghana, Sierra Leonne and Guinea.

In South and Central America, the oil palm industry has been established in many countries; however, the export is quite small and the oil is largely used locally. Colombia is the largest producer of palm oil in the region, with commercial cultivation starting 50 years ago in four geographic regions referred to as central, northern, eastern and western regions, in 16 states (Henson et al., 2011). The limiting factors for growth of the oil palm industry in South America, including Colombia, have been the presence of pests and diseases not found in other oil palm growing regions. In 2010, oil palm plantations in Colombia covered 404,104 ha, and approximately 160,000 ha were used for biodiesel production (Castiblanco et al., 2013). As mentioned by Henson et al. (2011) the factors that hinder the oil palm production in Colombia are topographic, climatic (excessive rainfall, seasonal dry periods, high day temperature), edaphic (acidic, poorly structure, bad drainage system, shallow and infertile soils) and the presence of many pests and diseases. In addition to that, poor infrastructure, armed conflicts, and the existence of Collective Territories of Afro-Colombian communities is also limiting the expansion of the industry in the region (Seeboldt and Salinas (2010) cited in Castiblanco et al. (2013)).

1.5.2 Malaysian oil palm industry

Oil palm planted in Malaysia and Southeast Asia is believed to have originated from Africa. Four *dura* seedlings were planted at the Bogor Botanical Garden (formally known as Buitenzorg) in Java, Indonesia in 1848. Two of these seedlings were from the Amsterdam Botanical Garden and the other two were from Bourbon (Réunion) or Mauritius in the Indian Ocean; nevertheless, the origins are somewhat vague, but it is believed that they were originally produced in Amsterdam from seeds brought from Africa from the same parent palm, based on their uniformity (Hartley, 1977; Corley and Tinker, 2003).

The progeny from the four palms were distributed to Sumatra in the 1860-1870s. At first the oil palm was planted as an ornamental plant, but experimental plots were already established in 1860 and one of these was at Deli, Sumatra, Indonesia. The progenies, known as '*Deli palm'* from this plot became the base of the stock of the oil palm industry in Malaysia and Southeast Asia as the *Deli Dura* palms, which have been extensively exploited as the female parent (mother palm) in the seed production of commercial oil palm. This shows that the oil palm cultivated here has a narrow genetic background. The Deli Dura has a good crossing ability to AVROS (Algemene Vereniging van Rubberplanters ter Oostkust van Sumatra) Pisifera and other Pisifera parental palms which originated from 'Djongo' palm of Congo (Singh et al., 2013). The *Pisifera* palm is usually female-sterile. Most oil palm plantations in Malaysia are planted with Tenera which are the hybrids of *Dura* and *Pisifera* (DxP), due to the high oil extraction rate of *Tenera* fruits compared to *Dura*. The *Tenera* fruit forms were recognized and exploited in Africa long before the *Tenera* palms were being selected by pre-colonial cultures in West Africa (Singh et al., 2013). The genetics of the *tenera* hybrid as *dura* x *pisifera* (DxP) was discovered

in Zaire in a breeding program of *tenera* x *tenera* (TxT)(Kushairi and Rajanaidu, 2000).

The first large plantation was planted in Sumatra, Indonesia using *Deli* palms in 1911 where oil palm started to be commercially exploited as an oil crop. The establishment of commercial oil palm plantations in Malaysia did not start until 6 years later with the development of the Tennamaran Estate in the district of Selangor in 1917. In 1925, there were 3350 ha of oil palm planted in what is present day Malaysia and 31,600 ha in Sumatra, Indonesia (Corley and Tinker, 2003). At first, the growth of the industry in Malaysia was slow but it began to gain momentum after the end of World War II and has expanded rapidly since then. As of December 2015, there were about 5.64 million hectares of oil palm plantations in Malaysia (MPOB, 2016b) and more than 4000 oil palm plantations.

1.5.3 Economic importance of the oil palm industry in Malaysia

The Malaysian oil palm industry plays an important role in the growth of the agricultural sector of the country and has made a significant contribution to the Malaysian gross domestic product (GDP), foreign currency exchange and labour usage. The contribution of the agriculture sector to Malaysian GDP in 2012 was estimated to be RM 54 billion with 35% of this due to oil palm *(http://www.treasury.gov.my/).* Malaysia has been a primary producer and exporter of palm oil from 1970 until 2007. Malaysian production of palm oil in 2015 was recorded at 19.97 million tonnes (Table 1-2) with an export value from the palm oil and other oil palm products of approximately RM 60.17 billion (Table 1-3).

Table 1-2 Malaysia production of oil palms product 2015 (adapted from MPOB (2016a))

Palm products	**Production (Tonnes)**
Crude Palm Oil	19,961,581
Palm Kernel	4,915,661
Crude Palm Kernel Oil	2,276,466
Palm Kernel Cake	2,519,990

Table 1-3 Malaysian export value of oil palm products 2015 (adapted from (MPOB, 2016c))

Oil palm product	**Total export**	
	Tonnes	**MYR (million)**
Palm Oil	17,440,617	41,233.44
Palm Kernel Oil	1,066,662	4,079.99
Palm Kernel Cake	2,617,101	932.86
Oleochemicals	2,847,071	11,291.98
Finished Products	440,049	1,726.06
Biodiesel	178,942	483.57
Others	742,824	366.06
TOTAL	**25,333,265**	**60,106.30**

1.7 Diseases of oil palms

Oil palm, like other perennial crops, is not free from serious diseases. Serious outbreaks of devastating diseases have been reported in several part of the world since the Second World War (Hartley, 1977). Among important diseases of oil palm are basal stem rot, vascular wilt, bud rot and sudden wilt. The diseases are localized to regions, where those that are prevalent in some regions do not cause serious problems in others, such as basal stem rot which is devastating in Southeast Asia but not in Africa and South America and but rot disease, which is a significant problem in South America but not Southeast Asia and Africa.

To date, the most important disease in Malaysia and Indonesia has been basal stem rot (BSR) caused by the white rot basidiomycete fungus of genus *Ganoderma* (Ariffin et al., 2000) that attacks palm oil roots. The disease is also recorded in Thailand (Tummakate and Likhitakaraj, 1994), Colombia (Nieto, 1995), Honduras, Papua New Guinea and some African countries (Turner, 1981) but with lower incidence. The disease was first detected in old palms above 25 years old and was later identified in the younger palms as early as 12-24 months after planting (Turner, 1981). Increased incidence on 4-5 year old palms was reported particularly in replanted areas (Singh, 1991) and areas previously planted with coconut (Turner, 1965). Several species of *Ganoderma* have been found to be responsible for BSR with the most pathogenic being *Ganoderma boninense*. Flood et al. (2010) described the characterization of BSR as a decay of the bole, production of aerial symptoms such as multiple spears and production of brackets or fruit bodies on the base of the trunk and in severe cases, the palms fall over. Because of its economic importance, BSR is being extensively studied to further understanding of its biology, epidemiology, detection, control and management. Apart from basal BSR,

Ganoderma is also responsible for upper stem rot (USR) disease in Malaysia and Indonesia. In some estates in Indonesia, the ratio of USR to BSR is from 1:10 to 1:1 (Hasan et al., 2005). Akino and Kondo (2012) described a disease in Indonesia called common spear rot (CSR) also known as *'crown disease'* believed to be caused by *Ceratocystis paradoxa.* This disease is different from spear rot or bud rot disease in Latin America described by Chinchilla (2008). Among other insignificant diseases recorded in Malaysia are stem wet rot, charcoal wet rot, *Marasmius* bunch rot, algae leaf spot, *Anthracnose* and leaf spot of seedlings, brown germ and *Schizophyllum* seed disease and orange spotting.

In Africa, vascular wilt disease caused by *Fusarium oxysporum* f. sp. *elaeidis,* a soil borne fungus that produces macroconidia, microconidia, and chlamydospores, results in serious damage to oil palm cultivation, causing up to 70% mortality. The disease is also reported to attack *E. oleifera* but less aggressively (Aderungboye, 1977). Like BSR, vascular wilt is initially a disease of mature palm plantations developed on former forests or savannah. However, incidence on younger palms has been recorded in replanted areas of former oil palm areas affected with vascular wilt (Ntsefong et al., 2012). Amongst the external symptoms of disease are initial wilting followed by desiccation of the fronds, which finally break and hang around the trunk, and common internal symptoms are the browning of the vascular tissue of seedlings and mature palms (Flood, 2006). Aderungboye (1977) has reviewed some of the diseases of oil palm in Africa such as *Cercospora* leaf spot caused by an imperfect fungus, *Cercospora elaeidis* Stey, *Corticiurn* leaf rot (*Thanatephorus cucurneris*) and a blast believed to be caused by *Pythium splendens* and *Rhizoctonia lamellifera*.

The third major oil palm disease, known as bud rot, is prevalent only in South America. This disease will be explained in more detail in the next section due to its significance in this study. Apart from bud rot, other important diseases in this region

are sudden wilt associated with the flagellated protozoa *Phytomonas staheli*, red ring (a nematode disease caused by *Bursaphelenchus cocophilus*) and lethal wilt (*marchitez letal*) which is believed to be caused by a phytoplasma and has only been recorded in Colombia (Martinez, 2009a). In Colombia, lethal wilt disease has severely infected oil palms in the Upía River region and had eradicated about 690 ha with a total of 97,619 plants by 2010 (Alvarez et al., 2014).

1.8 Bud rot disease of oil palm

Bud rot disease of oil palm has been a serious problem in oil palm plantations in Latin America for more than 50 years with severe outbreaks in Brazil, Colombia, Ecuador, Panama and Surinam and some cases in Costa Rica, Nicaragua, Honduras, Peru and Venezuela (Martinez, 2009a; Martínez et al., 2010). It is also known by various other names. In Colombia, it is called the *pudricion del cogollo (PC),* which directly translates to English as '*bud rot*' by Google Translate (*https://translate.google.co.uk*). In some reviews such as in Darus (2000), bud rot disease is assumed to be similar to other oil palm diseases or disorders which have similar symptoms such as fatal yellowing, heart rot, lethal bud rot, lethal spear rot and sudden wilt (*Marchitez progresiva*), but the latter is now known to be a different disease. Kastelein et al. (1990) and Beuther et al. (1992) described fatal yellowing or '*Amarelecimento fatal (AF)*' in Brazil, also known as '*pudricion del cogollo'* in Spanish speaking countries and '*speerrot'* or *'lethal spear rot'* in Surinam. In Costa Rica, it is known as '*flecha seca'* (Henry et al., 2015). The description of spear rot disease in Surinam by Van de Lande and Zadoks (1999) matched the description in Martinez (2009a) when he mentioned about incidence of bud rot in Surinam.

However, Boari (2008) believed that in Brazil fatal yellowing (AF) is different to Colombian bud rot disease, although both have similar symptoms. Boari et al. (2012) have listed several studies conducted on AF to understand the epidemiology of the disease. Whether these diseases are actually the same as bud rot is still confusing. The various names are probably due to attempts to describe the disease in different locations with different levels of infections or just because of the linguistic problem. For example, Martínez et al. (2009) describe '*the palm heart'* as '*cogollo*' not '*the palm bud'* which probably translates to '*heart rot'* rather than bud rot for some authors. Since the etiologies of these diseases are unknown, it is rather difficult to differentiate them. The naming problem of the disease was also discussed by Chinchilla (2008). However, recently with improvements in the understanding of some diseases such as *Marchitez* disease, it is believed that some of the various names of bud rot (but not all) might not actually refer to the same disease.

1.8.1 Brief history and economic importance

Observations of bud rot symptoms were made in 1928 at the Almirante Estate in Panama (De Franqueville, 2003). Similar symptoms were observed in India by Vann Hall in 1920 (Benítez and García, 2014), in the Mbandaka region of the Democratic Republic of Congo (formally known as the Coquilhatville region of the Belgian Congo) in 1935 and in the Sibiti region, Congo Brazzaville in 1954 (De Franqueville, 2003). However, real damage from the disease was first reported in 1960 in the Turbo region (Uraba zone), in the northwestern region of Colombia (De Franqueville, 2003; Martinez, 2009a; Benítez and García, 2014) and destroyed all 2,800 hectares of African oil palm planted in that area and the attempt to replanting failed. In 1970, an estate in Colon, Panama was also destroyed.

In Tumaco (situated in the southwestern part of Colombia, near the border of Colombia and Panama), the disease was firstly reported in 1977. Between 2006 and 2009, a total of 30,000 hectares out of 35,000 hectares of oil palm in Tumaco and in the central zone (Puerto Wilches) were destroyed (Martinez, 2009a) and many plantations had 100% infected palms (Sarria et al., 2008a). In the affected plantations, over 90% of productive oil palms were affected by the disease often resulting in plant death (Moreno-Chacón et al., 2013). Corredor and Gómez (2009) reported that the disease had moved to the northwestern part of Ecuador and affected 20,000 hectares of oil palm. Apart from Turbo and Tumaco, the disease emerged in the eastern region, the Colombian Llanaos Orientales, in late 1960, and has also spread considerably in the northern and western zones of Colombia but is less severe and not as devastating as in Turbo (the southwest region) where high proportions of the infected oil palms in these areas recovered significantly (De Franqueville, 2003; Martinez, 2009a). In Surinam (Victoria), 95% of 5,000 hectares of oil palm planted in 1970 was destroyed in 1992 and 5,000 hectares in Denpasa, Brazil in 1980 with a similar disease, while in Peru, some cases of bud rot incidence were reported between 1992-1993 (Martinez, 2009a). De Franqueville (2003) has extensively reviewed the history of bud rot in other South American regions such as Surinam, Brazil, Ecuador, Peru and Venezuela.

In terms of economic losses due to this disease, in 2007 up to US$58.5 million was lost due to bud rot and lethal wilt and a report in 2008 stated that approximately 1,540 jobs had been lost directly (Fedepalma (2007), citied in Benítez and García (2014)). In Puerto Wilches (Santander) and Cantagallo (Bolívar), more than $5.5 billion and more than 8,000 jobs have been lost due to the disease (Fedepalma, 2016). According to Santacruz et al. (2004) bud rot is significantly reducing palm yields by 35% to 39%.

1.8.2 Disease symptoms

Even though there is some variation in the description of the disease especially at the advanced stages, most authors agree that the initial symptom is chlorosis of the young unopened frond or spear leaf (Kastelein et al., 1990; Darus, 2000; De Franqueville, 2003; Navia et al., 2014a). The infected tissue becomes desiccated and destroyed, leaving necrotic patches (Figure 1-8 & Figure 1-9). Browning of internal tissue can be observed when the unopened spear is unfolded (Figure 1-10). If the infection stops, the small desiccated, necrotic patches damage the frond formation creating various external symptoms such as the '*shark bite*' look on the frond, desiccated and necrotic leaflets and loss of some part of the frond (Figure 1-11) depending on the level of infection during the initial stages. If the infection continues, the whole spear leaf might become infected, necrotic and turn brown, and then the infection continues to the other fronds in the centre of the palm crown (Figure 1-12). In the advanced stage, the fronds snap, followed by collapse of the upper crown (Figure 1-13), but the mature leaves (lower crown) remain green for several months, because they are not affected by the pathogen, although the palms cease production. Usually at this stage, the basal tissue rots, as indicated by the presence of dark brown tissue internally (Figure 1-14).

If the infections have not yet gone too deep into the apical meristem and the rotting stops, the palm can recover. Leaf production resumes, but the first new frond is usually smaller, shorter, more erect and slightly more chlorotic than normal fronds and the growth is slower causing the stunted appearance of the new crown (Figure 1-15). The palms can suffer for six months to three years before recovering to normal (Rocha et al., 2005). The next leaves become longer and less malformed and eventually the palm produces nearly normal fronds which indicate full recovery of the palm. It has been suggested that bud rot disease should be classified into two forms,

and the form when the palm can recover is a non-lethal form. The bud rot found in the eastern region of Colombia (Llanos) is believed to be the non-lethal form, whilst in the southwestern region it is the lethal form. The lethal form is aggressive and can cause total destruction and palm death. Turner (1981) suggested that the non-lethal form of bud rot should be called *'bud rot little leaf'*, due to the formation of the malformed fronds during recovery, and the lethal form as *'lethal bud rot'*. In the lethal form, the infection and rotting does not stop and advances to the heart of the palm (*cogollo*) and eventually affects the leaf primordia and apical meristem. If the apical meristem, which is the growing point of the palm, is destroyed, the palm will not produce leaves and fruits, and eventually die. It is not known why there are lethal and non-lethal forms of bud rot; either it is because of pathogenicity factors, physiology of the palm or other biotic and abiotic reasons.

Figure 1-8 Initial symptoms of bud rot disease
(adapted from Martinez (2009b))

Figure 1-9 Early external and internal symptoms on spear leaves
(adapted from Martinez (2009c) and Drenth et al. (2012))

Figure 1-10 Internal tissue damage of the unopened spear

Figure 1-11 External symptoms of bud rot due to tissue necrosis in the immature spear (unopen spear)(adapted from Martinez (2009b))

Figure 1-12 Infected spears (Martinez, 2009b)

(a)  (b) 

(c)  (d) 

Figure 1-13 Advance stage of the bud rot a) inoculated seedlings b, c) collapse of upper crown fronds and d) palms without upper crown fronds (adapted from Martinez (2009b))

Figure 1-14 Internal tissue damage of the advance stage of bud rot disease (adapted from Martinez (2009c))

Figure 1-15 Newly deformed fronds during palm recovery (adapted from Drenth et al. (2012)).

1.8.3 Biology and epidemiology

The causal agent of bud rot disease has been debated for over a decade. There has also been a consideration as to whether the disorder is caused by one single agent or a combination of several. Since the discovery of the disease, extensive research into its etiology has been conducted by many researchers, looking at different angles such as the role of microbial pathogens, insects and other pests, plant physiological or environment conditions such as soil, water management, climate, agronomic management and climatic conditions that effect plant mechanisms, particularly plant defences.

Work on the role of microbes started as early as 1928 by Reinking during the outbreak in Almirante, Panama where he discovered the presence of *Fusarium* and possibly of *Phytophthora* (De Franqueville, 2003; Benítez and García, 2014). Since

then, various microorganisms have been associated with the disease such as oomycetes (genus *Pythium*, *Phytophthora* spp. in particular), fungi (*Fusarium oxysporum, F. solani, F. roseum, Thielaviopsis paradoxa*), and bacteria (*Bacillus coli*, *Erwinia* spp.). Even though many studies have focussed on the involvement of microbial pathogens in the disease etiology, no concrete findings were established for nearly 40 years, leading to an alternative hypothesis of abiotic factors either directly or as predisposing factors. The disease has been associated with poor soil aeration causing asphyxia of root systems due to poor drainage, heavy compacted soils, accumulation of nitrites due to poor fertilization systems and unbalanced nutrients. In addition, soil acidity has also been linked to the disease (Turner, 1981; De Franqueville, 2003; Albertazzi-Leandro et al., 2005). Bud rot has also been considered as a growth disorder associated with one or more types of stress that trigger a susceptibility response, and pathogens associated with symptoms are considered opportunistic (Albertazzi-Leandro et al., 2005). However, in Denpasa, Brazil, the disease spread was significantly reduced after systematic eradication of diseased palms indicating the role of a biotic factor (De Franqueville, 2003).

Research aimed at finding the causal agent of the disease has been carried out by various groups since the 1980s in Colombia, Surinam, Brazil and Ecuador but initially failed to identify any concrete causal agent. For example, work by Beuther et al. (1992) ruled out viroids as the causal agent of *fatal yellowing* in Brazil. However, in 2008 Cenipalma was able to establish Koch's postulates for *Phytophthora palmivora* as the causal agent of bud rot disease studied from four growing areas of Colombia and two from Ecuador (Corredor and Gómez, 2009). *Phytophthora palmivora* and other species of *Phytophthora* were already known as the causal agents of various diseases of palms (Garofalo and McMillan, 1999) such as bud rot and heart rot of coconut (Uchida et al., 1992; Dollet et al., 2012). *Phytophthora*

palmivora is now believed to be the primary pathogen that causes the initial damage, which then attracts opportunistic microorganisms such as bacteria and fungi that exacerbate the damage to the tissue (Martínez et al., 2009). It was found from the artificial inoculation (Koch's postulate assay), that lesions were visible on the sides of the spear leaf (youngest, unexpanded leaf) at 3-4 days after inoculation on 85 % of the treated oil palm seedlings and the sizes of the lesions remained static as tissues emerged, expanded, and matured (Martínez et al., 2009; Torres et al., 2010). When the conditions were favourable (temperature around 26°C and relative humidity above 80%), new infections took place and the severity of the disease increased as more and more lesions appeared and ended up destroying all of the spear leaves, that are infected by contact between infected and healthy tissue in the heart of the palm as observed in 15% of the inoculated seedlings (Martinez, 2009a; Martínez et al., 2010; Torres et al., 2010).

It is believed that the disease spreads in two stages. The initial stage is random, but these random incidences then become the foci for further spread in stage two, which correlates to the rate of spread that is initially linear but later becomes exponential as described by Corredor and Gómez (2009) and Van de Lande and Zadoks (1999). According to Santacruz et al. (2004), in the eastern zone of Colombia, disease progress has been observed to be up to 12% annually especially in the regions of Cumaral and Upía in 1997 and in 2001 and 2002 in the region of San Carlos de Guaroa. There is evidence that insects from the *Tettigoniidae* family also play a role in spread of this disease (Figure 1-16), but once there is sufficient inoculum there are also many other ways for dissemination of the pathogen.

***Figure 1-16 Insect from the family Tettigoniidae associated with the dissemination of* P. palmivora.**

Note: The long ovipositor is used to lay eggs deep in the young immature spear leaf (adapted from Martinez (2009a)

Genetic backgrounds of the palms have also been shown to contribute to disease severity. The DxP materials from Asia have shown the highest incidence rates (65%-85%) compared to African materials in studies in the eastern region of Colombia (Santacruz et al., 2004). Navia et al. (2014a) carried out assessment of two interspecific OxG hybrids and two cultivars of *E. guineensis* by planting them in a disease hot spot zone in Tumaco. Their findings show significantly slower disease progression rates for the hybrids compared to *E. guineensis* but not between the two hybrids. A similar study was also conducted using six planting materials from Malaysian seed producers and 16.15% of the palms were infected with the disease within three years. Nevertheless, some materials showed lower susceptibility and various recovery rates (Navia et al., 2014b). Disease progression and development are also closely related to climatic conditions especially , precipitation, since the disease is more severe in areas with high humidity and a short dry season and less severe in areas with a long dry season (Santacruz et al., 2004; Martinez, 2009a). Acosta and Munévar (2003) showed that soil characteristics also affect the disease. Higher disease levels were recorded in soils with higher proportions of clay and soil

compaction. In addition, soils with high hydraulic conductivity, porosity and good drainage systems had lower bud rot incidence and severity.

1.8.4 Management

There are many approaches taken by growers to manage the disease. Early detection is crucial, followed by removal of infected tissue and treatment with chemicals consisting of insecticides, fungicides and bacteriocides to protect from subsequent infection. Henry et al. (2015) showed that palms receiving these recovery treatments had a remission of symptoms in approximately 18 months, whilst the control palms were still showing major symptoms after two years. To assist the growers with diagnosis, Cenipalma has established a grading system for disease severity (Figure 1-17). In addition, a spray program is conducted to protect the surrounding palms and total eradication of severely infected palms is implemented to reduce inoculum pressure. Improvement of drainage and nutrient balancing also reduces the impact of the disease (Martinez, 2009a). In Ecuador, plantations have shifted from using *E. guineensis* particularly DxP to the OxG hybrids in 1960s, and this strategy is now adopted in Colombia.

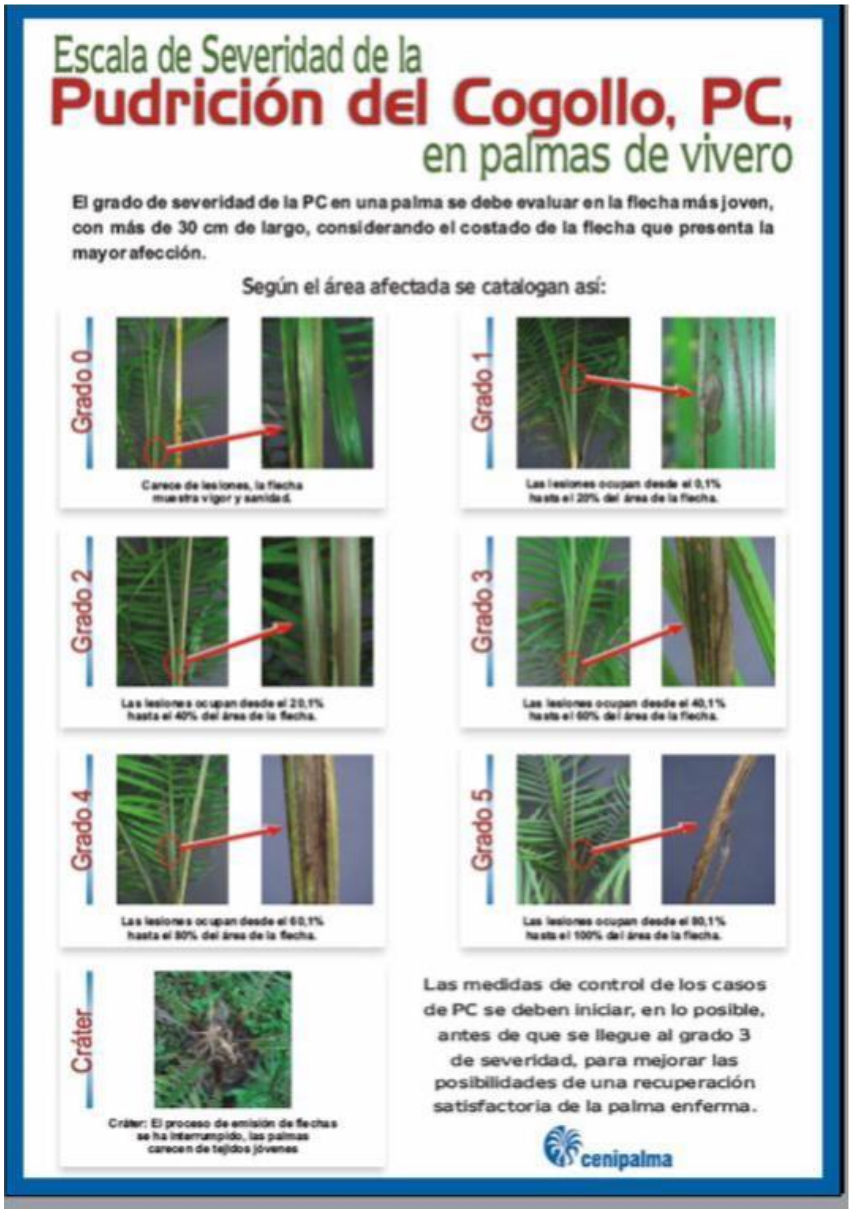


Figure 1-17 Grading system for physical diagnosis of bud rot disease introduced by Cenipalma (source: Martinez (2009b))

1.9 Bud rot disease of coconut and other palms

The coconut (*Cocos nucifera* L.) is a monoecious plant and another member of the Arecaceae (palm) family, and is an important crop in some tropical countries such as Indonesia, the Philippines, Sri Lanka, India, the Ivory Coast and Papua New Guinea. As with oil palm, coconut is also affected by bud rot disease. Bud rot of coconut is not a new disease. According to Tucker (1926) coconut with bud rot symptoms was first reported on Grand Cayman Island of the British West Indies in 1834 and since then the symptoms have been seen in many regions of the world. In the 1920s, bud rot symptoms in coconut were identified in Jamaica, Puerto Rico, Africa, Peninsular Malaysia and the Philippines but later in other part of the world such as India, Sri Lanka, Central America, the West Indies, Fiji and Vanuatu (Dollet et al., 2012). In Malaysia, the bud rot was first observed to cause death of coconut in local planting by Reinking (1919). However, the disease did not become epidemic and was known to be prevalent only in the highlands where the climatic conditions favour disease development. The outbreak of the disease in the Philippines was reported in 1989, where over 3000 Malayan Yellow Dwarf and West African Tall (MAWA) hybrid trees in large coconut plantations were affected; meanwhile in the Ivory Coast, the disease was reported to be widespread in 50,000 ha plantations of the same coconut hybrids (Concibido-Manohar, 2004).

In North Sulawesi, Indonesia, coconut with symptoms of bud rot and premature nutfall were observed in the early 1980's and the spread of the disease was rapid and the losses due to the diseases were high (Figure 1-18) (Smith and Flood, 2001). In India, bud rot was reported by Butler as early as in 1906. According to Sharadraj and Mohanan (2013b), the disease incidence has increased each year and attained high levels in certain disease endemic areas of the coconut growing

states in India due to the inoculum build up leading to heavy economic loss to the coconut growers with both rainfall and temperature playing vital roles in the intensity of the disease (Sharadraj and Mohanan, 2013b).

Figure 1-18 Coconut plantation with budrot diseased palm (centre)(adapted from (Smith and Flood, 2001)

The disease is considered as a lethal and serious fatal disease (Sharadraj and Mohanan, 2013b). Palms of all ages are susceptible to the bud rot disease, but it is more frequent on young palms (Srinivasulu et al., 2008). Dense under planting practices (planting new plants under existing old coconut trees) are vulnerable to the disease (Liyanage, 1999). Research has shown that the bud rot premature nutfall diseases of coconut in Indonesia (Smith and Flood, 2001), India (Sharadraj and Mohanan, 2014b) and the Philippines (Concibido-Manohar, 2004) are caused by *P.*

palmivora which was found to be the causal agent of bud rot disease in Colombia. Apart from *P. palmivora* other *Phytophthora* spp. have been associated with the disease(s) of similar etiology in some countries such as *P. katsurae, P. nicotianae* and *P. arecae* (*P. arecea* is now identified as *P. palmivora*) (Quillec et al. (1984) cited in Concibido-Manohar (2004)). *Phytophthora katsurae* is the main causal agent in the Ivory Coast (Hall and Warokka, 1994; Pohe et al., 2011).

The first symptom is discoloration and wilting of the spear leaf and often the first spear leaf and later the spear turns brown, dries and bends down (tilting) (Radha and Joseph, 1980; Drenth and Sendall, 2001; Srinivasulu et al., 2008; Dollet et al., 2012). Tilting of the spear leaves while all other leaves stay green and healthy is the most typical symptom. At this stage, the tissues surrounding the terminal bud (meristematic zone) usually have a foul-smell and the rot is surrounded by a yellow/brown border and central rot of the stem may be found in palms (Srinivasulu et al., 2008; Dollet et al., 2012). The spear can be pulled out easily due to the rotten base (Garofalo and McMillan, 1999; Drenth and Sendall, 2001). In the severally infected mature palms, the crown droops or snaps followed by the collapse of the spear leaves (or) crown (Figure 1-19)(Liyanage, 1999). Similar symptoms are also observed in other palms such as an ornamental palm *Washingonia robusta* (Figure 1-20)(Garofalo and McMillan, 1999; Elliott, 2006). The roots are not usually affected and look like healthy roots. In the Ivory Coast, affected palms died slowly in 6 to 8 months and sometimes much later (Quillec et al., 1984).

Figure 1-19 Coconut bud rot symptoms: cross section of meristem (left) and spear droop (right)

Figure 1-20 Spear leaf and next youngest leaf exhibiting typical symptoms of Phytopthora bud rot (adapted from (Elliott, 2006)

1.10 Oomycetes and the *Phytophthora* genus

The *Phytophthora* genus was first described by Heinrich Anton de Bary in 1876 as cited by Erwin and Ribeiro (1996). The first key, developed by Rosembaum in 1917, included eleven species and was entirely based on morphological characteristics (Gerrettson-Cornell et al., 1994).

1.10.1 Taxonomy and characteristic

Species in the *Phytophthora* genus have sometimes been considered and called fungi even though they do not belong to the fungal kingdom. Blackwell (2009) remarked that *Phytophthora* is clearly not a true fungus but is rather a "*pseudo-fungus*" and not related to fungi but more closely related to types of *protozoa* such as *ciliates, dinoflagellates* and *apicomplexans*. Fungi are more closely related to animals than to *oomycetes*, and *oomycetes* are more closely related to algae and to green plants (Figure 1-21) (Fry and Grünwald, 2010). *Phytophthora* is one the member of *oomycetes*, also known as the water moulds, along with a sister genus, *Pythium*.

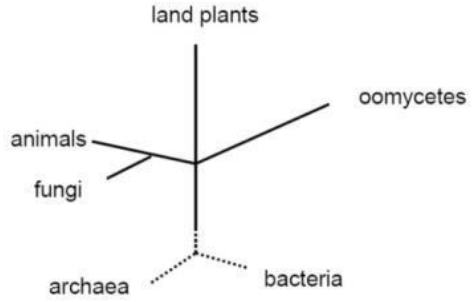


Figure 1-21 Schematic relationship among oomycetes, land plants, animals and fungi
(adapted from Fry and Grünwald (2010))

Phytophthora share many common physiological, ecological and life cycle characteristics with true fungi. This includes the presence of fine filaments called hyphae, the presence of spores and nutrition by absorption. However, they are clearly distinguished from true fungi (*Basidiomycetes*, *Ascomycetes*, and others) in many other characteristics including their genetics and reproductive mechanisms (Erwin and Ribeiro, 1996) and metabolic pathways (Elliott, 1983), notably the highly conserved lysine synthesis path. The taxonomical classification and nomenclature of the *Phytophthora* genus or even its class, *oomycetes,* is still quite dynamic amongst taxonomists. At present most taxonomists agree that the *oomycetes* are placed in the *Chromista* kingdom (which is sometimes also known as the *Stramenopile* kingdom). This kingdom also includes diatoms and brown algae. The name *Chromista* was initially introduced by Cavalier-Smith (1981), who proposed that oomycetes and two other classes (*Chytridiomycetes* and *Hyphochytridiomycetes*) be placed in the kingdom *Chromopytha,* one of his nine kingdoms of *Eukaryota*, under subphylum, *Phycomycotina* or the alga-like fungi, phylum Heterokonta (Table 1-4 and Table 1-5). This proposal was based on the presence of two anterior cilia; one clothed in tubular mastigonemes and the other smooth, which resembled the *chromophyta* algae.

Table 1-4 Cavalier-Smith 1981's: The Nine kingdoms of the superkingdom of eukaryote.

	Kingdom	Characteristic
1.	Eufungi	The non-ciliated fungi
2.	Ciliofungi	The posteriorly uniciliate fungi
3.	Animalia	Animals, sponges, mesozoa, and choanoflagellates
4.	Biliphyta	Phycobilisome-contalning algae
5.	Viridiplantae	Green plants
6.	Eugienozoa	Euglenophyta and kinetoplastida.
7.	Protozoa	Protozoa, excluding choanoflagellates but including Dinophyta.
8	Cryptophyta	Cryptomonads.
9	Chromophyta	Algae with chlorophyll c, plastid endoplasmic reticulum and no phycobilins; plus the anteriorly ciliated fungi

Table 1-5 Cavalier-Smith 1981's: Kingdom Chromophyta Distinguishing characters and constituent Phyla

Phylum	Subphylum	Characteristic
1. Heterokonta	i. Chrysophytina	Chrysophyceae (including Bicoecida and Silicoflagellida), Xanthophyceae, Phaeophyceae, Chloromonadophyceae (= Raphidiophyceae).
	ii. Phycomycotina	Oomycetes, Hyphochytridiomycetes, Thraustochytrids, Labyrinthulids.
2. Bacillariophyta		Diatoms.
3. Eustigmatophyta		-
4. Haptophyta		Biciliates with haptonema

Since then, there have been many changes to the classification and placement of *oomycetes* within the *chromista* kingdom by Cavalier-Smith and his colleague. Cavalier-Smith et al. (2015) and Ruggiero et al. (2015) proposed a new classification that put the genus *Phytophthora* into *Kingdom:Chromista> Subkingdom:Harosa> Infrakingdom: Halvaria> Superphylum:Heterokonta (=supergroup Stramenopiles)> Phylum: Pseudofungi (=oomycota)> Class:Oomycetes> Subclass:Peronosporidae> Order:Pythiales*. However, some other reviews place it in the *Peronosporales*, family *Pythiaceae* (Hawksworth et al., 1995). The family of *Pythiaceae* contains a number of genera, the best-known of which are *Phytophthora* and its sister group, *Pythium* (Drenth and Sendall, 2001). The *oomycete* genus *Phytophthora* encompasses over 100 morphological species, (and growing) making this genus the second largest in the *Peronosporales* family (Thines, 2013).

There are many characteristics of the *Phytophthora* and other *oomycetes* that make them different from fungi. The cell wall of *Phytophthora* consists of cellulose (β-glucans), similar to algae and plants, rather than chitin (the polymer of N-acetyl glucose amine) as in the walls of true fungi (Deacon, 2005). Like true fungi, the life cycle of *Phytophthora* can consist of asexual and sexual phases (Figure 1-22). However, in the asexual phase, the nuclei of vegetative structures are diploid. The

diploid phase is predominant in *Phytophthora*'s life cycle rather than haploid as in fungi (Erwin and Ribeiro, 1996). *Phytophthora* have fine filament structure called hyphae or mycelia similar to fungi which allows it to infect and grow within the plant cells. The septa (cell walls) in the hyphae of *Phytophthora* are multinucleate (coenocytic). Sporangia are formed from mycelia or from sporangiophores, which develop from the germination of chlamydospores or oospores.

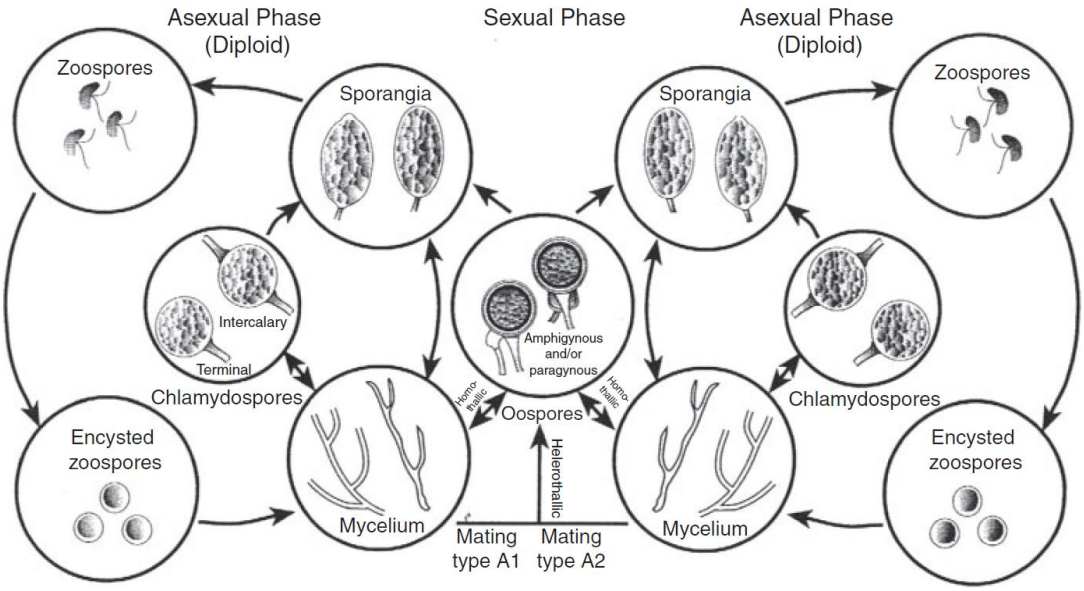


Figure 1-22 Representation of the* Phytophthora *life cycle
(adapted from Ribeiro (2013))

Shape and characteristic of the sporangia are various among species and are used to aid in the species identification (Figure 1-23). They may be terminal or intercalary (within a hyphal filament), bulbous or not. Terminal sporangia in some species can be readily detached (caducous) by water, wind, changes in humidity or by contact with vectors. Sporangia produce wall-less, bi-flagellated motile or swimming spores, called zoospores (Desjardins et al., 1969). Sporangia germinate in

the presence of water either in soil, ponds or films of water on plant surfaces and a sporangium can release around 30 zoospores (Drenth and Guest, 2004a). The zoospores, although wall-less, retain a consistent but flexible shape which probably helps to enhance their ability to swim in water films on plant surfaces (leaf, stem, fruit) and in soil water, hydroponic media and natural water bodies (Fry and Grünwald, 2010) towards the suitable penetration sites (such as stomata or anticlinal wall junctions) of the susceptible host plants by chemotaxis or electro-tactically. However, zoospores are short-lived structure. After a period of time (of several hours) or upon finding a suitable infection site, zoospores will encyst, stop swimming, drop their flagella and develop a cell wall. Cysts then germinate by producing germ tubes that are tactically attracted to suitable plant entry sites. Once in the host, the tubes will grow as hyphae, and the life cycle continues inside the host. In some species, sporangia can also germinate directly to form a germ tube. When the host tissue is available, the germ tube forms an appressorium that attaches to the host surface, penetrates and infects (Drenth and Guest, 2004a). The development of appressoria is induced by surface topology and/or hydrophobicity (Latijnhouwers et al., 2003). In general, the function of oomycete appressoria is the penetration of the outermost epidermal cell layers (Fawke et al., 2015). After successful penetration, the ability for invasive hyphal growth by elongation and ramification through the host tissue determines disease incidence (Larousse and Galiana, 2017).

Phytophthora also produces long-term survival, thick-walled, usually spherical and pigmented spores called chlamydospores, which play an important role in the pathogen survival under harsh conditions. Another thick-walled spore is the oospore. Oospores function as resting spores and are produced in infected plant tissue and released into soil as the plant tissue degrades. Oospores and chlamydospores

germinate directly by a germ tube with or without a sporangium on the end. The oospore is a sexual spore produced from the sexual reproduction between two dissimilar gametangia (sexual structures, singular=gametangium), a large round oogonium (plural=oogonia) containing one to several eggs, and a smaller antheridium (plural=anteridia)(Heffer-Link et al., 2002). However, the formation of sexual structures is relatively rare (Nicholls, 2004). During mating, the antheridium fertilizes or introduces gametes into the oogonium. The antheridial attachment is called paragynous when the antheridium is attached to the side, usually on the lower half of the oogonium. In an amphigynous arrangement, the oogonium grows through the antheridium, which remains as a collar at its base or the antheridium surrounds the base of the oogonium (Figure 1-24). *Phytophthora* are able to produce both gametangia, but only half of the genus is able to produce both gametangia (female and male) and subsequently produce oospores in a single culture, which is known as homothallic (Drenth and Sendall, 2001). Others are heterothallic species that produce gametangia with the response of chemical stimulation from an isolate of the opposite mating type designated as A1 and A2 (Drenth and Sendall, 2001). Oospores are rare in tropical species, although both gametangia are present (Drenth and Guest, 2004a).

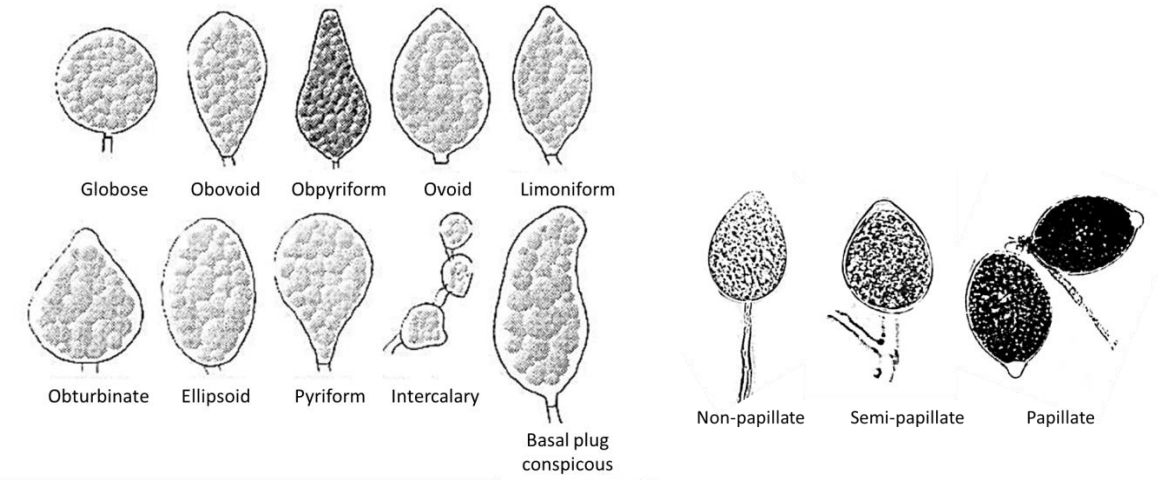


Figure 1-23 (a) Sporangium shapes (b) papillation and (c) Sporangiophore morphology (adapted from Drenth and Sendall (2001))

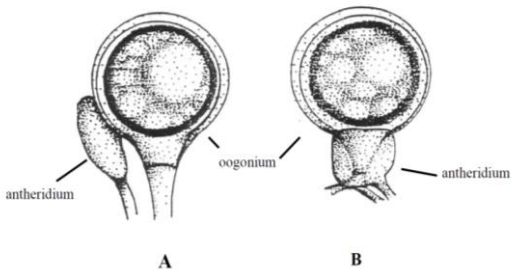


Figure 1-24 Attachment of antheridium A) paragynous, B) amphigynous (adapted from Erwin and Ribeiro (1996))

1.10.2 Oomycete interactions with plant hosts

Oomycete pathogens display biotrophic, necrotrophic, or hemibiotrophic lifestyles. Biotrophs grow and reproduce in living plant tissue and obtain nutrients (energy) through intimate interaction with living host plant cells. Some biotrophic oomycetes such as *Albugo laibachii* are completely reliant on host tissues (obligate biotrophs) and therefore need to maintain a close interaction with the hosts while keeping the plant alive for their own benefit (highly specific infection mechanism) which somehow significantly restrict their host range. Obligate biotrophs usually form haustoria to get nutrients from the plant host (Latijnhouwers et al., 2003) and the presence of haustoria is one of the key characteristics of the biotrophs (Kemen et al., 2015). However, among biotrophic oomycetes there is considerable variation in the duration of the biotrophic relationship and their capacity for saprotrophic growth *in vitro* or necrotrophic growth on dead plant tissues. For example, hemibiotrophs initially feed biotrophically for varying periods before switching to necrotrophy (O'Connell and Panstruga, 2006).

Hemibiotrophic pathogens have a combination of both biotrophic and necrotrophic lifestyles. Initially, hemibiotrophs oomycetes establish a biotrophic relationship with the host, but later switch to a necrotrophic phase when the host cells die as the infection proceeds. This lifestyle is observed in some species of genus *Phytophthora* and *Pythium*. *Phytophthora palmivora*, is an aggressive biotrophic pathogen of *Medicago truncatula*, a model legume plant that is widely used in symbiosis research (Rey et al., 2015). In contrast with biotrophs, the hemibiotrophs can infect hundreds of different plant species. Hemibiotrophs commonly have the ability to survive in axenic culture (facultative) similar to the necrotrophs. Necrotrophic pathogens kill their host cells and feed on dead cells. Oomycete necrotrophs are found among the genera *Pythium* and *Aphanomyces*.

Oomycetes may enter the tissue of the host through epidermal cells, between cells or via stomata (Figure 1-25) and trichromes as observed for the *P. palmivora* interaction with oil palm leaves by Sarria et al. (2016). Entry into host cells requires penetration of the plant cell wall including the protective coating of waxes and cutin of the epidermal cells. Some pathogens are thought to crack these barriers using physical forces based on appressorial turgor pressure while others appear to use a combination of lytic enzymes and turgor pressure (O'Connell and Panstruga, 2006). Upon penetration of the cell wall by appresoria, the vegetative hyphae colonize plant tissue by several different routes. The hyphae may spread over the plant cutical, under the cuticle, between host cells or inside host cells. Haustoria (the specialized structures that penetrate the host cell wall) develop as side branches from intercellular, intracellular and epicuticular hyphae and terminate inside the penetrated host cell (Fawke et al., 2015). However, some hemibiotrophs and necrotrophs such as *Pythium ultimum* do not form haustoria.

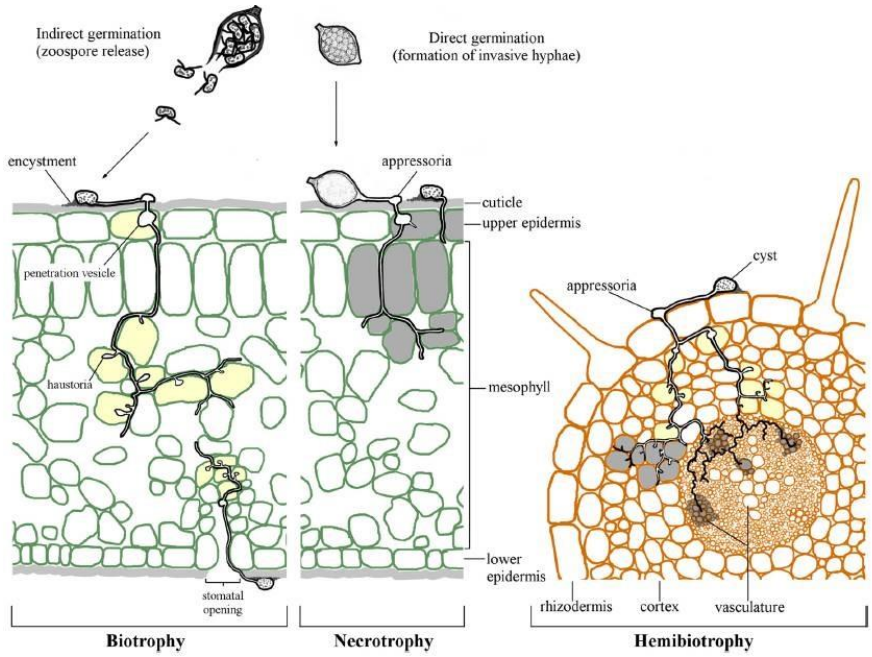


Figure 1-25 Leaf (left) and root (right) colonization by oomycetes (adapted from (Fawke et al., 2015)

Plants have several defence mechanisms to overcome the invasion of the pathogen. For example, the penetration attempt of the pathogen may lead to cellular rearrangement of the plant host tissue including reorganization of the actin cytoskeleton and organelle movements. This results in a polarization of the host cell at the site of attack which will usually result in the reinforcements of the local cell wall which is believed to function as physical and chemical barriers to the pathogen invasion (O'Connell and Panstruga, 2006). Pathogen invasions is sensed by plants in some ways such as by associated mechanical wounding or the detection of released plant wall fragments (Vorwerk et al., 2004) and elicitors. Elicitors are molecules which stimulate a defence response in a host plant and include pathogen-associated molecular patterns (PAMPs). PAMPs are structurally conserved and thought to be indispensable components or products of a pathogen's infection process. For example, elicitors such as elicitin, a family of small extracellular protein produced by many *Phytophthora* species induces a vigorous defense response, the hypersensitive response, locally and distally in some species of Solanaceae (Kamoun et al., 1993).

Oomycetes release protein effectors to suppress immune responses triggered by their own elicitors. Effector proteins act in several different cellular compartments and alter the physiological state of plants to favour and facilitate colonization. Effectors can be released to the apoplast (the space outside plant cell membranes) (apoplastic effectors/extracellular effectors) or translocated into the host cell (cytoplasmic effectors/intracellular)(Bozkurt et al., 2012). Several apoplastic effectors contribute to counter-defence by inhibiting host enzymes, such as proteases and glucanases, that accumulate in response to pathogen infection (Morgan and Kamoun, 2007). Apoplastic effectors act in the apoplast surrounding plant and microbial cells. There are two major categories of apoplastic effectors, the effectors mediating protection against host defences and effectors mediating invasion

(Wawra et al., 2012). Cytoplasmic effectors such as RxLR effectors enter the plant cell and have to cross the plant cell wall and the plant plasma membrane. Haustoria release these effector proteins to the host cytoplasm via the extrahaustorial matrix and the extrahaustorial membrane (Figure 1-26)(Ellis et al., 2006).

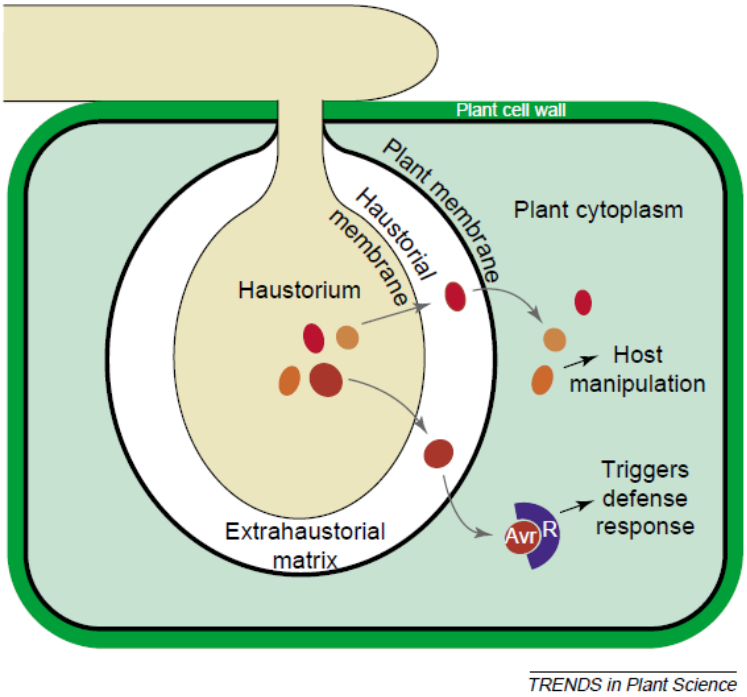


Figure 1-26 The oomycete pathogen penetrates the plant cell wall but not the host plasma membrane. Effector proteins (orange and red ovals) are secreted by the pathogen and are postulated to enter the host cytoplasm to alter host metabolism and defence pathways. When recognized by a corresponding resistance protein (R) the effector proteins are referred to as avirulence (Avr) proteins (adapted from (Ellis et al., 2006)

Cytoplasmic effectors of oomcetes have been discovered based on their avirulence (Avr) function which is their ability to trigger hypersensitive cell death on host genotypes with corresponding disease resistance (*R*) genes (Morgan and Kamoun, 2007). Genes encoding pathogen effectors that induce *R* gene resistance response

are defined as avirulence (*Avr*) genes. Pathogen *Avr* genes can contribute to virulence and cause disease of the host plant when the plant lacks an appropriate *R* gene. Recognition of an Avr protein by a plant R protein has been called effector triggered immunity (ETI) (Qutob et al., 2006).

1.10.3 Phytophthora diseases

Most of the species in this genus are plant pathogens responsible for some of the world's most destructive diseases of crops and native vegetation (Brasier, 1992). The name, *Phytophthora* is a Greek word for "*plant destroyer*". *Phytophthora* species are among the most destructive pathogens of agricultural crops and forests in the world. There are many factors that make almost all species of this genus successful as plant pathogens such as the rapid production of various reproductive structures, rapid sporulation on host tissue, the ability of zoospores to target hosts, resilient spores (oospores and chlamydospore), rapid evolution of new races and/or strains and ability to produce hybrid species. As water moulds, they thrive in wet conditions; therefore, the infection by the initial inoculum usually takes place during the wet season in tropical monsoon climates, but stops during the dry season. But if the conditions are relatively wet all year round, the disease cycle may continue unbroken. The hyphae and chlamydospores on infected plants (fruits, bark and roots) can then serve as inoculum for secondary infections where disease will become worse. *Phytophthora* are soil-borne microorganisms, but they can cause infection in the upper plant canopy suggesting that inoculum may move from soil assisted by vectors such as insects, rain, soil and water splash and human activities.

Phytophthora species attack a wide range of plants and can cause diseases in different parts of the plant such as roots, leaves, buds, stems, collars and fruits even

of the same host causing multiple diseases such as stem canker, leaf blight, root rot, fruit rot, collar rot, tuber rot and fruit rots. Some *Phytophthora*, such as *P. infestans* and *P. fragariae* are relatively host specific infecting potato and strawberry respectively, but others have very wide host ranges. One of the most famous pathogens of the *Phytophthora* genus is *P. infestans*, which causes potato blight disease that was responsible for the European potato famine of the middle 19th century (Cooke and Anderson, 2013). *Phytophthora cinnamomi*, *P. cryptogea*, *P. citricola*, *P. cactorum* and *P. cambivora* are among commonly found species that affect ornamentals plants, meanwhile *P. ramorum* causes sudden oak death of various oak species as well as Ramorum blight and shoot dieback on ornamental plants (Fry and Grünwald, 2010). In South East Asia, *Phytophthora* infect several plant species including rubber, cocoa, durian, pepper, coconut, jackfruit and papaya.

1.10.4 Phytophthora palmivora

Phytophthora palmivora can cause numerous diseases on many different crops in the tropics such as black pod and stem canker of cocoa, black stripe in rubber, and trunk canker, root rot, fruit rot in durian, bud rot, fruit rot in coconut and canker, fruit rot, and root rot in citrus. Recently it has been identified as the causal agent for bud rot disease of oil palm in Colombia. It is the most commonly found *Phytophthora* species in the tropics and was first described by Butler in 1919 (Drenth and Guest, 2004a). Earlier, this species was divided into four groups M1, M2, M3 and M4. Later, M1 and M2 were put together and M3 and M4 were renamed to *P. megakarya* and *P. capsici,* which was confirmed by Cooke et al. (2000). *Phytophthora palmivora* was placed in morphological group II by Stamps (1990) and in clade 4 together with *P. megakarya* and *P. quercetorum* (Cooke et al., 2000) based on molecular characterization. *Phytophthora palmivora* is heterothallic with amphigynous antheridia and spherical

oogonia. The sporangia are caducous, conspicuous papillate, varying in shape and size mostly ovoidal, elliptical and obpyriform with average length of 40-60 µm and width of 25-35 µm and short pedicel (<5 µm) (Erwin and Ribeiro, 1996). Chlamydospores are terminal and intercalary averaging 33 µm in diameter. Colony morphology on V8 is a stellate pattern with aerial mycelium; hyphae are coralloid meanwhile growth on CMA is sparse with no aerial mycelium (Widmer, 2014).

1.11 Research aims and objectives

In Malaysia, the current status of bud rot disease incidence in oil palm plantations is very vague. To date, disease outbreaks have not been reported in Malaysia or other Southeast Asian countries despite the fact that *P. palmivora* is a common pathogen to this region on other plant species. However, Albertazzi-Leandro et al. (2005) cited Turner (1981) who wrote that symptoms similar to '*pudrición del cogollo'* were not unknown in Asia, but incidence has never reached the levels observed in South America. It is not known why there are lethal and non-lethal forms of bud rot, and whether this is because of pathogenicity factors, physiology of the palm or other biotic and abiotic reasons. Thus, several avenues of research need to be pursued to fill knowledge gaps in understanding why this pathogen causes problems in oil palm in South America but not in Southeast Asia, and to develop suitable diagnostic tools to monitor the pathogen. The knowledge obtained from this study will aid efforts towards developing preventive and control measures for bud rot disease of oil palm in Southeast Asia, especially in Malaysia, and reduce potential losses due to *Phytophthora* infections.

The key aim of this thesis is to know if there is variation between the *P. palmivora* isolates that were isolated from diseased oil palm in Colombia in comparison with Malaysian isolates and other isolates gathered from different hosts and regions. This study has focused on the molecular characterization, particularly examining genomic DNA sequences from *P. palmivora,* pathogenicity and also to develop a suitable detection and diagnostic method to detect the causal agent for disease monitoring either as part of border controls or locally. The main hypothesis of the study is that *P. palmivora* isolates from the same host and region have the same molecular characteristics particularly in genomic DNA sequences, and have similar levels of pathogenicity. Therefore, the aims of the research were:

1. To isolate and identify *Phytophthora* isolates obtained from various hosts and regions especially from Colombia and Malaysia
2. To characterize the collected *P. palmivora* isolates from Colombia, Malaysia and other regions using molecular approaches
3. To study their pathogenicity against commercial oil palm seedlings
4. To develop species specific diagnostic methods for detection of *P. palmivora*
5. To examine potential fungicides/chemicals to suppress the growth of *P. palmivora in vitro*.

Chapter 2. Isolation, characterization and identification of *P. palmivora* isolates

2.1 Introduction

In order to understand why *P. palmivora* causes devastating bud rot disease in oil palm in Colombia and other Latin America regions but not in other regions such as Malaysia and South-east Asia, several studies have to be carried out, such as on the genetic variation between isolates of *P. palmivora* collected from different regions in comparison with the oil palm pathogenic isolates. Isolation of *Phytophthora* often requires special techniques and media, and a lack of knowledge of isolation techniques of this genus has led to negative findings in the past. In many cases such as in root rot disease, other pathogens have been wrongly cited as being the causal agent of the disease.

Recovery of *Phytophthora* spp. from soil using conventional direct plating methods is difficult compared to other fungi. Tsao (1960) tried to establish conventional direct plating of serial dilutions for isolation of *Phytophthora* of citrus from soil but failed to do so. It is known now that these species are weak competitors in non-sterile soil as well as in cocktail cultures, mainly due to their slow growth (Manning and Crossan, 1966). According to Hendrix and Kuhlman (1965), *Phytophthora* normally give rise to fewer infective propagules per gram of soil than most other soil fungi. There is evidence that some microorganism have direct inhibitory effect on the growth of *Phytophthora* species (Brodrick et al., 1975). The use of baits has been found to be particularly successful in the isolation of some soil species. Various plant species and parts susceptible to *Phytophthora* have been

tested and used as baits for isolation and detection of *Phytophthora* species, not only from soil but also from infected plants. For example, Chee and Newhook (1965) and Darvas (1979) successfully used blue lupin germinated seedlings (*Lupinus angustifolius* L.) as bait to isolate and detect *P. cinnamomi* from forest soil in New Zealand and reported that this technique also works to recover *P. syringae*, *P. megasperma*, *P. nicotianae* var. *parasitica* and *P. boehmeriae* from soil. Furthermore, Pratt and Heather (1972) used lupin seedlings to differentiate between some *Phytophthora* species (*P. cinnamomi, P. dreschleri* and *Pythium* spp.) based on morphological characterization of the infection on lupin seedlings bait. Among other baits used are pear (Van Der Scheer, 1971), apple (Newhook, 1959) and cocoa pod (Dakwa, 1974; Newhook and Jackson, 1977).

Isolation of *Phytophthora* from diseased tissue such as bark, leaves and fruits is less troublesome compared to the isolation from soil and has been done by direct plating of the infected tissue from the marginal or advancing area onto selective media. Low nutrient media such as corn meal, carrot, vegetable juice (for example V8) and lima bean agar media are usually used for isolation to inhibit other contaminants such as fungi. Pentachloronitrobenzene (PCNB) is also usually added to help inhibit the growth of fungi, whilst antibiotics such as pimaricin, vancomycin, nystatin, mycostatin, polymyxin, streptomycin and rose Bengal are generally added to facilitate elimination of bacteria (Hendrix and Kuhlman, 1965). Combinations of media with these antibiotics and fungicides usually provide selective growth of *Phytophthora* and *Pythium* (Brodrick et al., 1975).

Like fungi, identification and classification of *Phytophthora* can be made based on morphological characterization. Among criteria used to distinguish between species are morphology of colonies on media, sporangia, gametangia; the presence or absence of hyphal swellings, chlamydospores and zoospore formation; patterns of

sexuality in the genus; host range and optimal growth in specific media (Gallegly and Hong, 2008). Identification keys developed by Waterhouse (1963) and later revised by Stamps (1990) have been widely used. However, identification based on morphological characteristics of isolates is time-consuming, requires trained experts with a good eye and attention to detail (Kroon et al., 2012), and is unreliable (Truong et al., 2010). Advances in DNA-based molecular diagnostics and DNA sequencing has increased our ability to accurately detect and characterize *Phytophthora* spp. (Cooke et al., 2007). The most common region used in the identification of fungi and oomycetes to species level is the internal transcribed spacer (ITS) of ribosomal RNA (rRNA) gene cluster of genomic DNA and the sequences encoding for rRNA. The rDNA region is highly stable and exhibits a mosaic of conserved and diverse regions within a genome (Hibbett (1992), as citied in Liew et al. (1998)).

Ribosomal RNA genes cluster are found as parts of repeat units that are arranged in tandem arrays. Each repeat unit consists of a transcribed region consisting of genes encoding for small subunit (SSU) and large subunit (18S, 5.8S and 28S genes) and the external transcribed spacers (ETS1 and ETS2) and a non-transcribed spacer (NTS) region. The ITS region is situated within ribosomal gene clusters between the conserved flanking regions of the small and large subunit of ribosomal RNA on either side of the 5.8S rRNA gene and are described as ITS1 and ITS2 (Figure 2-1) (Liew et al., 1998).

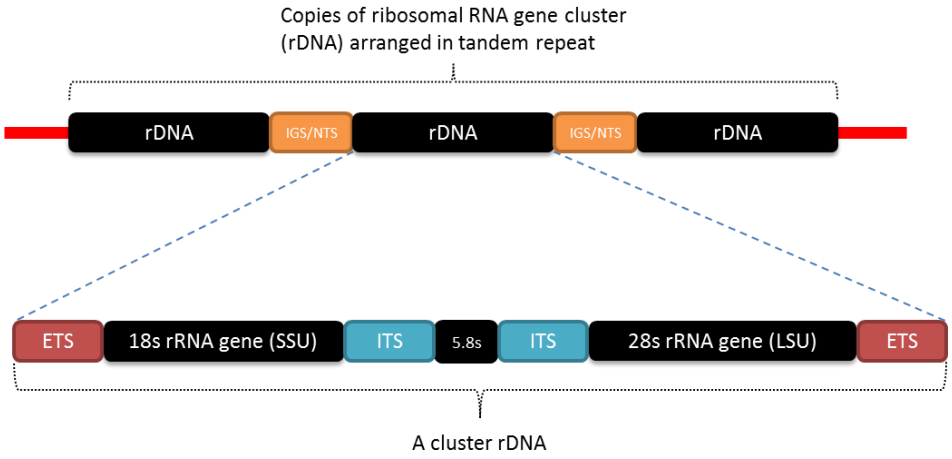


***Figure 2-1 Ribosomal RNA (rRNA) gene cluster of* Phytophthora**

Usually, molecular identification of plant pathogenic fungi is accomplished by polymerase chain reaction (PCR) amplification of the ITS region followed either by direct sequencing and Basic Local Alignment Search Tool (BLAST) searching against databases such as GenBank® as described by White et al. (1990). The ITS regions can be amplified for DNA sequencing in most species with the use of universal eukaryotic PCR primers (Robideau et al., 2011). GenBank® is part of the International Nucleotide Sequence Database Collaboration, which comprises the DNA DataBank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank® at the National Center for Biotechnology Information (NCBI)(https://www.ncbi.nlm.nih.gov/). Alternatively, the sequence can be subjected to restriction enzyme digestion that will cut the PCR product to small pieces at specific cutting sites to get a DNA fingerprinting, which is known as the internal transcribed spacer (ITS) region of the ribosomal DNA-restriction fragment length polymorphism (ITS-RFLP) as carried out by Cacciola et al. (2001).

This chapter aimed to collect and identify *Phytophthora* isolates from bud rot diseased oil palm in Colombia and from other hosts and locations around the world.

2.2 Materials and methods

2.2.1 Isolation of *Phytophthora* from diseased tissue

Infected tissue samples (cocoa pods and leaves, durian canker bark)(Figure 2-2) were collected, washed with distilled water and surface sterilized using 70% ethanol, followed by 2x washes with sterile distilled water. Small pieces of the freshly cut tissue from the margins of infection were placed directly onto *Phytophthora* selective media ($P_{10}VP$) containing corn meal agar (CMA), with pentachloronitrobenzene (PCNB) and two antibiotics, pimaricin and vancomycin, to inhibit the growth of unwanted fungi and bacteria as described by Tsao and Ocana (1969). Plates were incubated at 25°C +/- 2°C. Cultures grown on the media were sub-cultured onto fresh selective media after 4-5 days depending on their growth.

Isolation from Colombian diseased oil palm samples was done in Cenipalma Phytopathology Laboratory in Colombia with the help of the Colombian Oil Palm Research Center (Cenipalma) during a sampling trip to Colombia in June 2014. The isolates originated from the serious outbreak area in Tumaco, Colombia and were reported by Cenipalma as highly virulent to the oil palm. Infected young unopened spear leaves were collected from the oil palm plantation near Cenipalma's Experimental field station, Palmar de la Vizcaina, Colombia. Inoculation of *P. palmivora* from the young leaf samples was done using pear as bait. A triangular cut was aseptically made in two opposite sides of a single pear (Figure 2-3). Diseased leaf tissue taken from the marginal infection area was put in the triangular cut and the other side served as control (Figure 2-4). The pear bait containing diseased leaf samples was then incubated at room temperature in humidity chambers for up to 8 days (Figure 2-5). A small part of the pear tissue was taken from the advancing

stage near the lesion and was then inoculated onto selective media $P_{10}VP$. All cultures are maintained on carrot agar, CMA or V8 agar with or without antibiotic supplements (see Appendix 1 for media preparation) at temperatures of 25°C +/- 2°C and the stock cultures were kept in 20°C +/- 2°C in sterile universal bottles (Appendix 2).

Figure 2-2 Infected cocoa parts (a) young leaves of cocoa seedling (b) cocoa fruit pod.

Figure 2-3 Triangular cuts were made on surface sterilized pears using a sterile spatula.

(a)  (b) 

Figure 2-4 (a) Samples of infected young oil palm spear fronds (internal folds) (b) Small samples of tissue were taken from marginal infection areas.

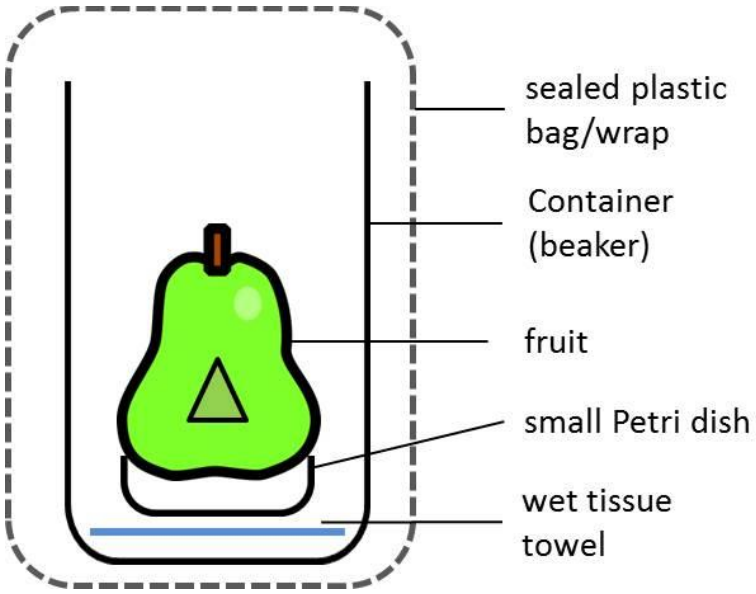


Figure 2-5 Schematic diagram of a humidity chamber. *Two layers of paper towels were wetted with sterile distilled water to create humid conditions. The re-sealable plastic bag was used to keep the chamber moist.*

2.2.2 Isolation of *Phytophthora* from soil

Soils samples were collected from oil palm plantations in Perak (Seberang Perak), Johor (Kluang) and Selangor (Sepang), Malaysia with peat, inland and coastal soil types, respectively. Soil samples were also collected from cocoa experimental plots in Hilir Perak, Malaysia. The samples were taken from the surface of the soil (≤ 30 mm in depth), around the base of the palm/cocoa (approximately within a metre radius), between the planting rows, and also in shallow drainage ditches (Figure 2-6). Isolations were done by direct plating of soil washes onto *Phytophthora* selective media and using a baiting technique with apple and cocoa pod using the same method as in section 2.2.1 (Figure 2-7). In the initial sampling, the soil washes were plated in Malaysia, and then sent to the United Kingdom for further selection and colony purification, but the isolations from the second sampling were carried out in Malaysia. Apart from *Phytophthora*, other soil fungi were also collected from the soil samples by direct plating of the soil washes onto Potato Dextrose Agar (PDA) supplemented with antibiotics (ampicillin or penicillin).

Figure 2-6 Sampling of the soil from an oil palm plantation in Sepang, Selangor, Malaysia

(a)

(b)

(c)

Figure 2-7 Isolation of* Phytophthora *by fruit baiting method using (a) apple (b) cocoa pod which was then assembled in a humidity chamber using a clean sterile container and a re-sealable plastic bag as shown in (c).

Other isolates including a *Pythium* were obtained and purchased from The World Oomycetes Genetic Resource Collection (WOGRC), formerly World Phytophthora Collection (WPC), University of California-Riverside, USA; culture collection center, the Netherlands (CBS-KNAW); Oil Palm Research Institute of Ghana (OPRI); Malaysian Cocoa Board (MCB); Malaysian Agriculture and Research Development Institute (MARDI); Malaysian Palm Oil Board; FERA, UK and University of Nottingham, UK.

2.2.3 Morphological characterization of *P. palmivora*

Microscopic morphological structures of the isolates were examined under light microscopy based on the characteristics described by Waterhouse (1963) and Gallegly and Hong (2008) from the randomly picked 14-40 days old plate cultures grown on carrot agar supplemented with antibiotics and PCNB and incubated at 25°C, under light illumination of 12-14 h light, 10-12 h dark cycle. The cultures were flooded with water and suspensions mounted on microscope slides, stained with lacto phenol cotton blue if needed.

2.2.4 DNA Extraction

Between 50-100 mg of *Phytophthora* mycelium was scraped from the 7 to 10 day old agar plate cultures using a sterile surgical blade and placed into a sterile screw-capped tube. The tubes were then kept in liquid nitrogen to prevent degradation and facilitate the disruption of tissue. Tissue disruption was carried out using a mixture of sterile 2 mm and 4 mm diameter glass beads and a homogenizer (FastPrep®, QBiogene) at a speed of 6.5 rpm for 45 s repeated thrice. DNA extractions of the cultures were then carried out using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol (Appendix 3).

2.2.5 Identification of the isolates using molecular methods

2.2.5.1 Amplification of internal transcribed spacer regions and cytochrome oxidase I gene

PCR amplifications of the ITS regions of nuclear rDNA of the isolates were carried out using primers ITS1 5'-TCC GTA GGT GAA CCT GCG G-'3 and ITS4 5'-TCC TCC GCT TAT TGA TAT GC-'3 as described by White et al. (1990) and (Crawford et al., 1996). Amplification of the cytochrome oxidase I (*CoxI*) mitochondrial gene was carried out using the primer pair of OomCoxILevup (5'-TCAWCWMGATGGCTTTTTTCAAC-3') and Fm85mod (5'-RRHWACKTGACTDATRATACCAAA-3') as described by Robideau et al. (2011) and Ginetti et al. (2014). All oligonucleotides were synthesized by Sigma-Aldrich.

The amplifications were performed in 30 µl volumes consisting of 15 µl of master mix (2x MangoTaq™ DNA Polymerase), 1 µl (10 pmol/ul) each of forward and reverse primers, 12 µl sterile distilled water and 1 µl of template DNA. The reactions were performed in a BIO-RAD S1000 Thermal Cycler with the amplification conditions of 95°C for 2 min for initial denaturation, followed by 35 cycles of denaturation at 95°C for 2 min. The annealing was carried out at 55°C for ITS and 41°C for *CoxI* followed by extension/elongation at 72°C for 1 min 30 sec. The final extension was set at 72°C for 10 min. The amplicons were then run in 1.2% agarose gels stained with ethidium bromide in Tris-borate-EDTA (TBE) buffer at 100 volts for 25-40 min alongside with 1kb DNA marker ladder to determine and estimate amplicon size and concentrations. The presence of single clear bands was checked for successful amplification using a gel imager. The amplified products were then purified with the QIAquick® PCR Purification Kit (QIAGEN), following manufacturer's instructions (Appendix 4) and were then sent for DNA sequencing to Eurofins MWG

using the same primer pairs used in PCR amplifications. Cloning of PCR amplicons was performed when the PCR amplicon was more than 500 bp to obtain good quality sequences.

2.2.5.2 Cloning of PCR products

Cloning of PCR amplicons was conducted using the cloning kit, pGem®-T Easy Vector System I (Promega). Ligation was carried out in 1.5 ml Eppendorf tubes by gently mixing 0.5 µl pGem® vector, 0.5 µl T4 DNA ligase and 2.5 µl 2x rapid ligation buffer (all three regents were supplied in the kit) with 1.5 µl purified PCR amplicon. The ligation solution was then incubated at 4°C overnight. Transformation was conducted using chemically treated competent cells of *E. coli* (DH5α) using heat shock treatment. The ligation mixture was gently mixed with 40 µl of competent cells and incubated on ice for 5 min. The mixture was then put in a heated block or water bath set at 42°C for 50 sec and then quickly placed in ice for 15 to 20 min. Luria - Bertani/Lysogeny broth (LB) medium (0.7 ml) was then added to the transformation mixture and incubated in a shaking incubator at 37°C for a minimum of 90 min. LB agar plates for selection of transformed colonies were prepared by adding 50 µl of 0.1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG), 120 µl of 0.05 M 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and 100 µl of 100 mM ampicillin into 100 ml molten LB agar cooled down to approximately 60°C to avoid denaturation of temperature sensitive reagents. The mixture was then poured into a 90 mm Petri dish and allowed to cool and solidify in sterile chamber. Fifty ml-100 ml of the bacteria culture was then platted on the LB plate using an L-spreader and incubated at 37°C overnight. PCR amplification of transformed regions from the white colonies was conducted using primer M13 forward (5'-GTAAAACGACGGCCAGT-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3'). The amplification was checked using gel

electrophoresis. The amplicons were purified using QIAQuick® PCR Purification Kit (Qiagen) according to the manufacturer's protocol (Appendix 4) prior to sending for sequencing at Eurofin MWG Operon, UK.

2.2.5.3 Sequence analysis

GAP4 software package (Staden-package, USA) was used to check and clean the sequence data obtained from the sequencing company by removing the vector sequences, correcting the base errors and generating contig sequences from forward and reverse sequences of each individual isolate. The ITS sequence of each isolate was then subjected to nucleotide-nucleotide searches with the Basic Local Alignment Search Tool - BLASTn algorithm at the NCBI website (*http://www.ncbi.nlm.nih.gov/BLAST/*). The outputs from the BLAST searches were sorted based on the maximum identity. Identification of each isolate was based on the maximum scoring of identity value and query coverage.

2.3 Results

2.3.1 Isolation and collection of *P. palmivora*

Isolations from the diseased tissues of cocoa pods, cocoa leaves and durian bark by direct plating onto selective media $P_{10}VP$ were successfully carried out with no serious cross contamination problems from bacteria or other fungi. The hyphal growth can be observed within 2-4 days after plating and transferred to fresh $P_{10}VP$ (Figure 2-8). Subsequent re-culturing was carried out using either carrot agar or CMA with/without antibiotic supplements. For isolation from tissues of diseased oil palm using pear bait, lesions at the inoculation site of the pear were observed within 3-4 days (Figure 2-9). Hyphal growth of *P. palmivora* on the selective media from infected tissue of the fruit bait was also observed 2-4 days after plating. The sub-culturing of the infected bait tissue was observed to be similar to direct isolation using tissue of diseased cocoa pods. A total of 42 isolates collected from oil palm, cocoa, coconut, durian, rubber, betel palm, kentia palm and cymbidium from various locations such as Malaysia, Colombia, Indonesia, Sri Lanka, India, Ghana and South Korea were further identified using molecular techniques (Table 2-1). There were 28 isolates retrieved from the soil samples taken from two oil palm plantations in Malaysia using *Phytophthora* selective media $P_{10}VP$ (Table 2-3). However, based on the colony morphology of the isolates, it was believed that they were non-*Phytophthora* and molecular techniques were used to confirm this as described in the next section.

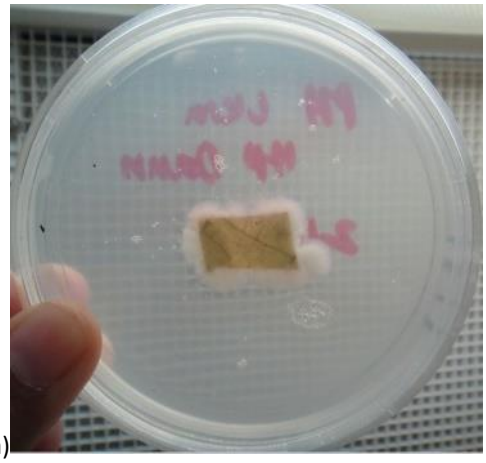
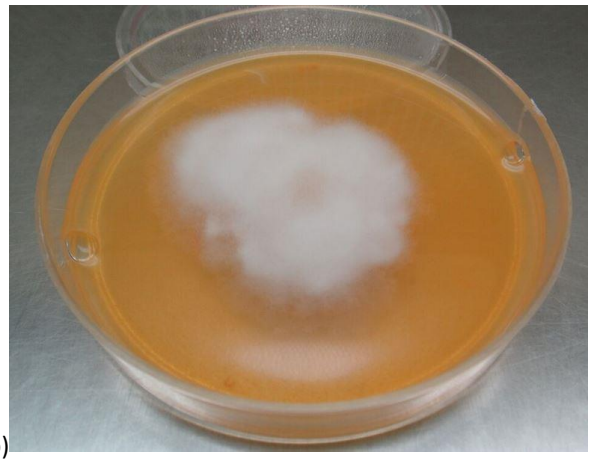
(a)  (b) 

Figure 2-8 (a) Hyphal growth of* Phytophthora *from the infected tissue of young leaves of cocoa seedlings after 4 days of inoculation on $P_{10}VP$ and (b)* Phytophthora *culture on carrot agar supplemented with PCNB and antibiotics at 4 days after subculture

Figure 2-9 Lesion on the inoculated site of the pear bait 3 days after inoculation with oil palm diseased leaf tissue

Table 2-1 Details of isolates collected in this study

No.	Isolate	Origin	Ex-host	Source	Species based on the source database
1	IMI382528	Indonesia	*Cocos nucifera* (coconut)	CABI Bioscience	*P. palmivora*
2	IMI382544	Indonesia	*Cocos nucifera* (coconut)	CABI Bioscience	*P. palmivora*
3	CBS111346	South Korea	*Cymbidium spp.* (orchid)	CBS-KNAW	*P. palmivora*
4	CBS179.26	Sri Lanka	*Theobroma cacao* (cocoa)	CBS-KNAW	*P. palmivora*
5	CBS236.30	India	*Cocos nucifera* (coconut)	CBS-KNAW	*P. palmivora*
6	CBS298.290	Trinidad & Tobago	*Theobroma cacao* (cocoa)	CBS-KNAW	*P. palmivora*
7	CCO2083	unknown	unknown	FERA	*P. palmivora*
8	P0497	Colombia	*Theobroma cacao* (cocoa)	WOGRC	*P. palmivora*
9	P11007	Guam	*Areca catechu* (betel Palm)	WOGRC	*P. palmivora*
10	P16385	California, USA	*Howea forsteriana* (kentia palm)	WOGRC	*P. palmivora*
11	P16828	Colombia, Central Zone	*Elaeis guineensis* (African oil palm)	WOGRC	*P. palmivora*
12	P16831	Colombia Tumaco	*Elaeis guineensis* (African oil palm)	WOGRC	*P. palmivora*
13	P3767	Indonesia	*Cocos nucifera* (coconut)	WOGRC	*P. palmivora*
14	P6948	Malaysia	*Hevea brasiliensis* (rubber)	WOGRC	*P. palmivora*
15	P8513	Colombia	*Theobroma cacao* (cocoa)	WOGRC	*P. palmivora*
16	PPC2614P	Colombia, Tumaco	*Elaeis guineensis* (African oil palm)	CENIPALMA	*P. palmivora*
17	PPC280574	Colombia, Tumaco	*Elaeis guineensis* (African oil palm)	CENIPALMA	*P. palmivora*
18	PPC3614L	Colombia, Tumaco	*Elaeis guineensis* (African oil palm)	CENIPALMA	*P. palmivora*
19	PPG1	Ghana	*Theobroma cacao* (cocoa)	OPRI	unknown
20	PPG11	Ghana	*Theobroma cacao* (cocoa)	OPRI	unknown
21	PPG13	Ghana	*Theobroma cacao* (cocoa)	OPRI	unknown
22	PPG8	Ghana	*Theobroma cacao* (cocoa)	OPRI	unknown
23	PPM1	Malaysia	*Theobroma cacao* (cocoa)	MCB	*P. palmivora*
24	PPM2	Malaysia	*Theobroma cacao* (cocoa)	This study	unknown
25	PPM3	Malaysia	*Theobroma cacao* (cocoa)	This study	unknown
26	PPM4	Malaysia	*Durio zibethinus* (durian)	This study	unknown
27	PPM5	Malaysia	*Durio zibethinus* (durian)	MARDI	*P. palmivora*
28	PPM6	Malaysia	*Theobroma cacao* (cocoa)	This study	unknown
29	PPM7	Malaysia	*Theobroma cacao* (cocoa)	This study	unknown
30	PPM8	Malaysia	*Durio zibethinus* (durian)	MPOB	unknown
31	CBS358.30	Sri Lanka	*Hevea brasiliensis* (rubber)	CBS-KNAW	*P. palmivora*
32	P19537	Colombia	*Elaeis guineensis* (African oil palm)	WOGRC	*P. palmivora*
33	P19538	Colombia	*Elaeis guineensis* (African oil palm)	WOGRC	*P. palmivora*
34	PPG3	Ghana	*Theobroma cacao* (cocoa)	OPRI	unknown
35	PPG4	Ghana	*Theobroma cacao* (cocoa)	OPRI	unknown
36	PPG12	Ghana	*Theobroma cacao* (cocoa)	OPRI	unknown
37	PC01	unknown	unknown	UoN	*P. cryptogea*
38	13-A2	United Kingdom	*Solanum tuberosum* (potato)	UoN	*P. infestans*
39	2009-7654A	United Kingdom	*Solanum tuberosum* (potato)	UoN	*P. infestans*
40	CBS581.69	Malaysia	*Hevea brasiliensis* (rubber)	CBS-KNAW	*P. botryosa*
41	CBS148.88	USA (Florida)	*Chamaedorea sefritzii* (bamboo palm)	CBS-KNAW	*P. arecae*
42	PYT01	Unknown	unknown	UoN	*Pythium aphanidermatum*

2.3.2 Isolation of fungi from soil of oil palm plantations

Colony purification from the consortium cultures of the soil wash plates sent from Malaysia were carried out by transferring each single colony onto fresh agar plates of PDA and CMA selective media. Initially, a total of 120 isolates were isolated using both media, but some of them were lost due to contamination, stunted growth and failed to be re-cultured. A total of 70 isolates were successfully obtained from the soil washes from oil palm plantations in Malaysia and Ghana (Table 2-2). Isolates were grouped together based on colony morphology grown of PDA (Figure 2-10) with a total of 20 groups (Table 2-3). Twenty-eight isolates retrieved from the *Phytophthora* selective media were grouped into 12 groups. Identification of all isolates was carried out using molecular methods as described in Section 2.2.5 but only with representatives from each group.

Table 2-2 Number of isolates obtained from three sampling sites using soil wash isolation

Sampling site	**No. of isolate grow on each media**		**Total**
	Phytophthora* selective media	**Potato Dextrose Agar (PDA)**	
Seberang Perak, Perak, Malaysia	8	25	33
Kluang, Johor, Malaysia	18	15	33
Ghana	2	2	4
Total	**28**	**42**	**70**

**CMA+PCNB+nyastatin+penicillin*

Table 2-3 Fungal isolates retrieved from soil wash inoculation of samples

No.	Isolate code	Origin	Media	Group
1.	MN1A	Ghana	CMA	1
2.	MN1B	Ghana	PDA	1
3.	MN2	Ghana	CMA	2
4.	MN3	Ghana	PDA	3
5.	C1	Seberang Perak	PDA	4
6.	K4	Seberang Perak	CMA	4
7.	B1	Seberang Perak	CMA	4
8.	C2	Seberang Perak	CMA	4
9.	C3	Seberang Perak	CMA	4
10.	D1	Seberang Perak	CMA	4
11.	E3	Seberang Perak	CMA	4
12.	F1	Seberang Perak	CMA	4
13.	F2	Seberang Perak	CMA	4
14.	H1	Seberang Perak	CMA	4
15.	K6	Seberang Perak	CMA	4
16.	H1	Seberang Perak	PDA	4
17.	B1	Seberang Perak	PDA	4
18.	C1	Seberang Perak	PDA	4
19.	C2	Seberang Perak	PDA	4
20.	C3	Seberang Perak	PDA	4
21.	D1	Seberang Perak	PDA	4
22.	E3	Seberang Perak	PDA	4
23.	F1	Seberang Perak	PDA	4
24.	F2	Seberang Perak	PDA	4
25.	H2	Seberang Perak	PDA	4
26.	C1	Kluang	CMA	4
27.	D1	Kluang	CMA	4
28.	C1	Kluang	PDA	4
29.	D1	Kluang	PDA	4
30.	IC8-11	Kluang	PDA	4
31.	IF4	Kluang	PDA	4
32.	IA1-4	Kluang	PDA	4
33.	D2	Seberang Perak	CMA	5

34.	D2	Seberang Perak	PDA	5
35.	H4	Seberang Perak	PDA	7
36.	H3	Seberang Perak	PDA	8
37.	H3	Seberang Perak	PDA	8
38.	K1	Seberang Perak	CMA	12
39.	K2	Seberang Perak	CMA	13
40.	K3	Seberang Perak	CMA	13
41.	K2	Seberang Perak	CMA	13
42.	D3	Seberang Perak	CMA	14
43.	D7	Seberang Perak	CMA	14
44.	E2	Kluang	PDA	24
45.	D3	Kluang	PDA	24
46.	D4	Kluang	PDA	24
47.	D4	Kluang	CMA	24
48.	IA9	Kluang	PDA	28
49.	IA10	Kluang	PDA	28
50.	IA8	Kluang	PDA	29
51.	UG1A	Kluang	CMA	30
52.	UG2D	Kluang	CMA	30
53.	UG2C	Kluang	CMA	30
54.	UG2A	Kluang	CMA	30
55.	IB8	Kluang	PDA	31
56.	IB5	Kluang	PDA	31
57.	IB7	Kluang	PDA	31
58.	IB1	Kluang	PDA	32
59.	IE1	Kluang	PDA	32
60.	IC1	Kluang	CMA	33
61.	IC1	Kluang	PDA	33
62.	IF1	Kluang	PDA	33
63.	IB2	Kluang	PDA	33
64.	IB6	Kluang	PDA	33
65.	IC2	Kluang	PDA	33
66.	ID1	Kluang	PDA	36
67.	IF5	Kluang	PDA	36
68.	IF3	Kluang	PDA	36
69.	IA11	Kluang	PDA	36
70.	H4	Seberang Perak	CMA	38

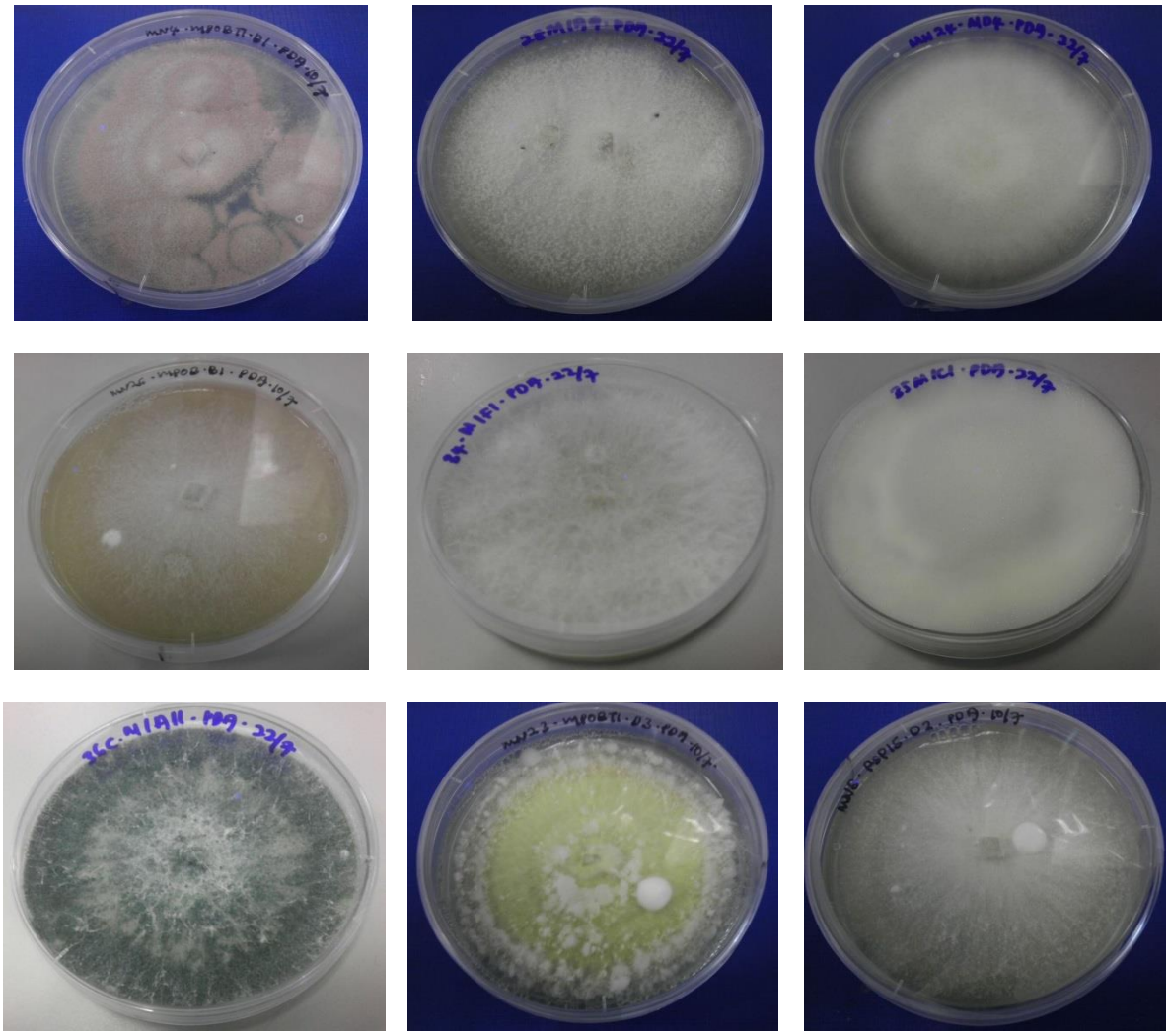


Figure 2-10 Some of the isolates of fungi collected from the soil samples taken from oil palm plantations in Malaysia

2.3.3 Morphological characterization of *P. palmivora*

Phytophthora palmivora cultures produce sporangia readily on the surface of agar media and do not need to be cultured in water in order to observe them. Sporangia were not observed on the V8 or carrot agar plates incubated without illumination for all isolates of *P. palmivora*, but were found abundantly when the plates were transferred and incubated under light for 2-3 days. The sporangia of *P. palmivora* isolate PPC280574, pathogenic to oil palm collected from Colombia, were observed to be distinctly papillate and caduceus with short pedicels. The shape of the sporangia varied from ellipcoidal, ovoid, limoniform and orturbinate (Figure 2-11a). The size of the sporangia varied from 40-54 µm in length and 17–30 µm in breadth. The production of intercalary thick walled chlamydospores was observed in all isolates (Figure 2-11b). The hyphae grew smooth and very thin on corn meal agar (CMA) for all isolates but looked denser on carrot agar and V8 agar with stellate to rosaceous growth patterns. However, the patterns were not consistently shown. Some plates of the same isolates were faintly more stellate than the others. Several attempts to grow *P. palmivora* on PDA and MEA failed.

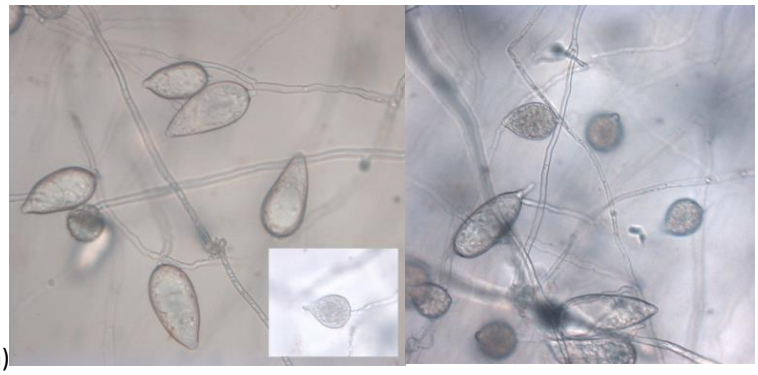
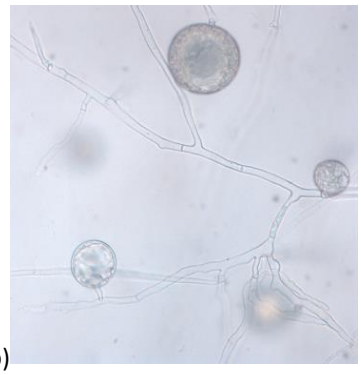
(a)  (b) 

Figure 2-11(a) Sporangia and (b) chlamydospores of* P. palmivora *isolate PPC280574 observed under light microscopy under magnification of 400x

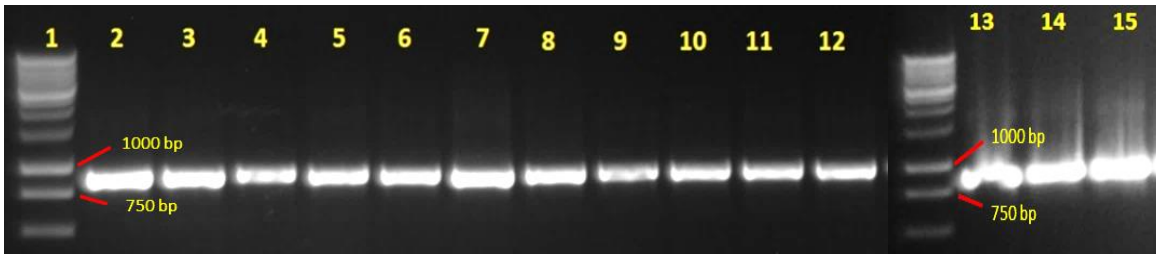
2.3.4 Molecular identification of isolates

2.3.4.1 PCR Amplification

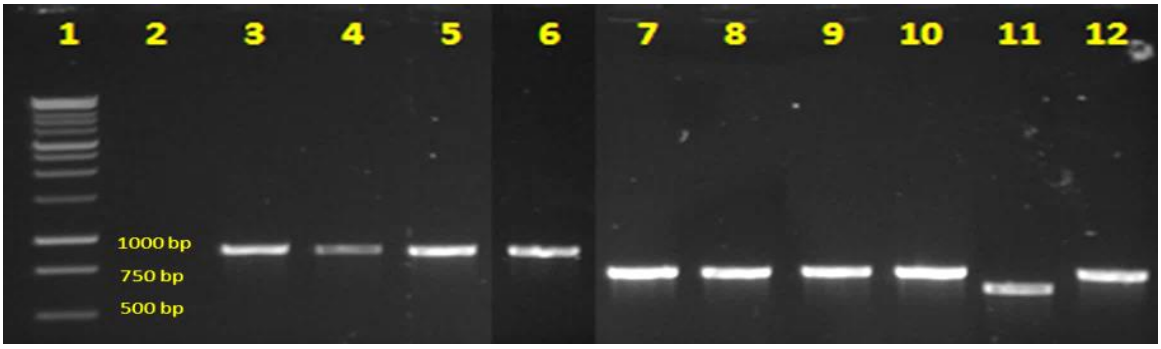
Amplification with ITS1 and ITS4 primers produced amplicons of approximately 900 bp based on the 1kb marker for all *Phytophthora* isolates and *Pythium aphanidermatum* (Figure 2-12). PCR amplicons of fungi isolated from soil were smaller than those of *Phytophthora* isolates and *Pythium* which were 700 bp and 750 bp. According to White et al. (1990), the PCR amplification using ITS1 and ITS4 primer pairs will produce a DNA fragment containing sequences encoded for a small portion of small ribosomal subunit (18S rRNA), ITS1, 5.8S rRNA, ITS2 and a small portion of large ribosomal subunit (28S rRNA) of nuclear DNA. Meanwhile, amplification of the *CoxI* gene using OomCoxILevup and Fm85mod produced amplicons of a size smaller than ITS primers (approximately 800 bp) for both *Phytophthora* and *Pythium* isolates (Figure 2-13). Amplification of fungal DNA was not carried out using the *CoxI* primer pair.

2.3.4.2 Cloning of PCR products

After overnight incubation, single colonies of white and blue were observed on the LB plate containing IPTG, X-gal and ampicillin spread with *E. coli* competent cells that had been subjected to the transformation procedure (Figure 2-14). The amplification products of the while colonies using M13 reverse and M13 forward primers were bigger (Figure 2-15) than amplification from genomic DNA because the M13 primers amplified several nucleotides upstream and downstream from the cloning vector's insertion site (Figure 2-16). The sequences length of the amplicons after removal of vector sequences was between 870-900 bp for ITS and 750-800 bp for *CoxI*.

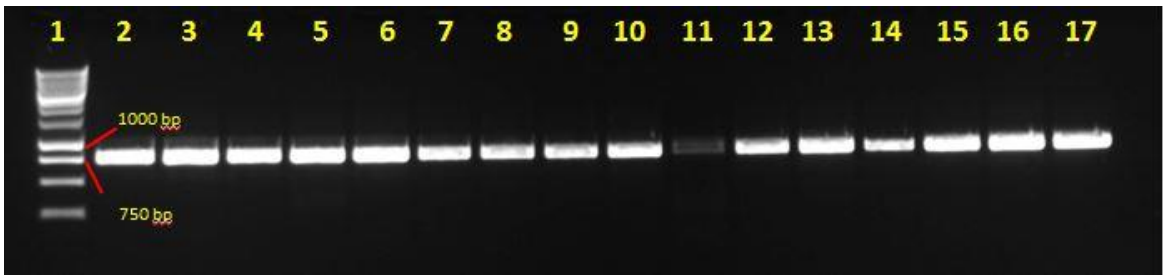


Lane 1; 1kb Marker, Lane 2-15: *Phytophthora* isolates P16828, P16831, P19537, P8513, P6948, P3767, P11007, P16385, PPG3, PPG4, PPG12, IMI382528, IMI382544, PPC280574, respectively



Lane 1; 1kb Marker, Lane 2: negative control (distilled water), Lane 3-4 & 6: *Phytophthora* isolates PC01, CBS 358.59, PPM1, respectively, Lane 5: *Pythium* PYT01, Lane 7-12: fungal isolates 33MIC1, 38FH4, 12FH1, 12MIUG1, 36MIF5, and 30MIUGe, respectively.

Figure 2-12 PCR amplification of nuclear ITS region on 1.2% agarose gel



Lane 1; 1kb Marker, Lane 2-8: *Phytophthora* isolates PPM2, PPM3, PPM5, PC01, CBS358.30, CBS1113346, CBS179.26, respectively, Lane 9: *Pythium* PYT01, Lane 10-17: *Phytophthora* isolates CCO2083, CBS236.30, PPM4, 13-A2, 2009-7654A, PPM1, CBS298.290 and PPG1, respectively

Figure 2-13 PCR amplification of mitochondrial cytochrome oxidase I genes on 1.2% agarose gel

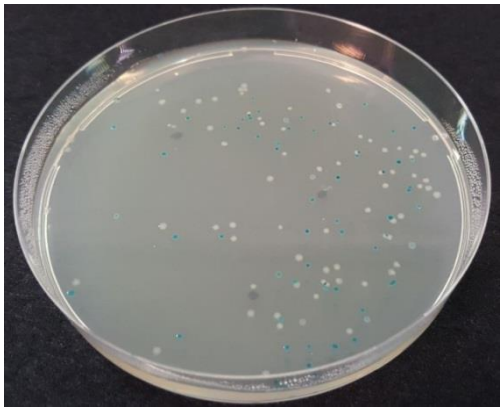
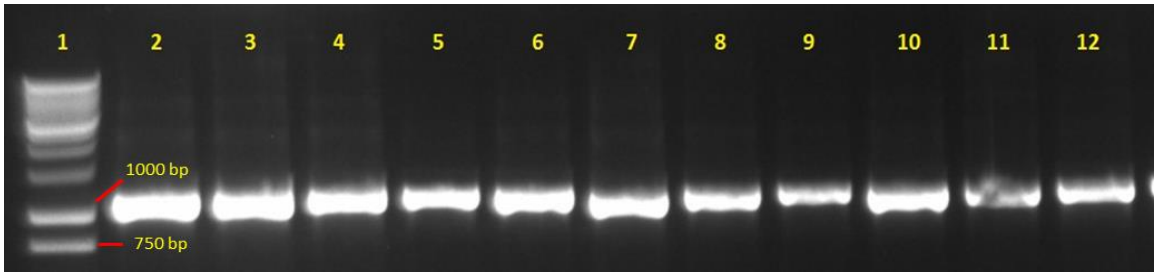


Figure 2-14 Blue and white colonies of* E. coli *after transformation using heat shock technique



Lane 1; 1kb Marker, Lane 2-12 were *Phytophthora* isolates (Lane 2: P16828, Lane 3: P1683, Lane 4: P19537, Lane 5: P8513, Lane 6: P. P6948, Lane 7: P3767, Lane 8: P11007, Lane 9: P16385, Lane 10: PPG3, Lane 11: PPG4, Lane 12: PPG12)

Figure 2-15 PCR amplification of clone plasmid inserted with partial ITS genes amplicon using M13 reverse and forward primers on 1.2% agarose gel.

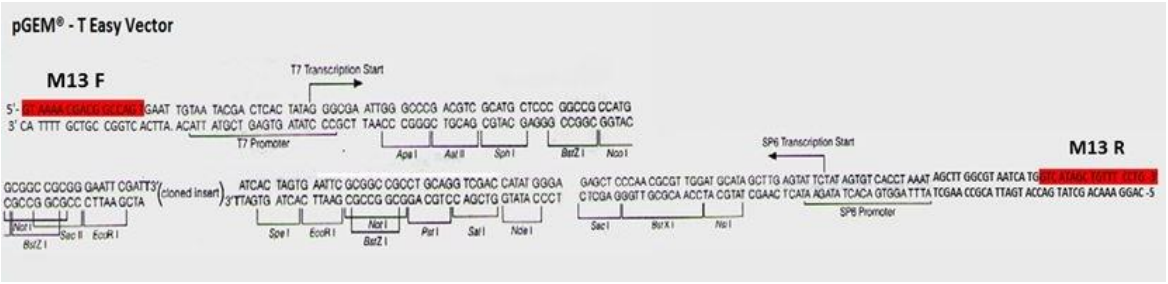


Figure 2-16 Position of M13 forward and reverse primer (highlighted red) in the vector (adapted from PROMEGA (2014))

2.3.4.3 Sequence analysis

Sequence alignments (BLAST) of the rDNA in the ITS region with nucleotide sequences in the GenBank® database showed a similarity to *P. palmivora* for 31 isolates, whilst the other 11 isolates were similar to *P. colocasiae*, *P. megakarya*, *P. cryptogea*, *P. infestans, P. citrophthora* and *Pythium aphanidermatum* with the maximum percentage of identity and the maximum query cover value of all being ≥ 99% except for isolate P3767 (ident; 97%, query cover; 100%), PPG4 and PPG12 (ident; 99%, query cover; 95%) (Table 2-4). Isolates P19537 and P19538 were initially identified as *P. palmivora* by WOGRC but were identified as *P. parasitica*; meanwhile isolate CBS148.88 is named as *P. arecae* and isolate CBS581.69 as *P. botryosa* in the CBS-KNAW database, but were identified as *P. citrophthora* in this study based on the BLAST results of ITS sequences. *Phytophthora arecea* was previous considered as a distinct species with close evolutionary relationship to *P. palmivora*, but recently has been considered to be synonymous with *P. palmivora* (*http://www.phytophthoradb.org).*

BLAST results using *CoxI* sequences also showed that these isolates are *P. palmivora* (maximum scores of % identity ≥ 99%, % query cover ≥ 87%), *P. infestans* (ident; 99%, query cover; 98%), and *Pythium aphanidermatum* (ident; 99%, query cover; 99%). Five isolates showed different identity when using *CoxI* sequences compared to ITS. Maximum scores of the BLAST result for P19537 and P19538 came back as *P. nicotianae,* while CBS581.69, CBS358.30 and PC01 were *P. insolita, P. citricola* and *P. parsiana*, respectively with the % identity value ≥ 97% and query cover ≥ 87%) (Table 2-4). Identity of the representative isolates of the fungi retrieved from soil samples taken from the oil palm plantations based on BLAST results of alignment of ITS sequences of each isolates are presented in Table 2-5. Maximum scoring of the BLAST in terms of identity and query cover percentage of all

sequences are ≥ 97%. Based on this identification, it was shown that the twenty-eight isolates retrieved from *Phytophthora* selective media were confirmed as non *Phytophthora* and identified as species of *Purpureocillium, Fusarium*, *Mortierella* and *Gongronella.* Apart from that, 20 isolates of *Mortierella* species; *Mortierella chlamydospora* (11) and *Mortierella echinosphaera* (9), 9 isolates of *Fusarium* species, 4 isolates of *Trichoderma* were among others that were successfully retrieved from the soil wash isolates.

Table 2-4 Identification of* Phytophthora *isolates collected in this study based on BLAST alignment results (maximum scoring) of their sequences from the ITS region and CoxI genes

No.	Isolate	Origin	Summary of BLAST results (maximum score) based on ITS							Summary of BLAST results (maximum score) based on *CoxI*						
			Identity	Score	Total score	Query cover	*	Ident	Accession	Identity	Score	Total score	Query cover	*	Ident	Accession No.
1.	IMI382528	Indonesia	*P. palmivora*	1611	1611	100%	0	99%	KF263691.1	*P. palmivora*	1256	1256	87%	0	100%	HQ261380.1
2.	IMI382544	Indonesia	*P. palmivora*	1611	1611	100%	0	99%	KF263691.1	*P. palmivora*	1240	1240	87%	0	99%	HQ261380.1
3.	CBS111346	South Korea	*P. palmivora*	1611	1611	100%	0	99%	KF263691.1	*P. palmivora*	1251	1251	87%	0	99%	HQ261380.1
4.	CBS179.26	Sri Lanka	*P. palmivora*	1611	1611	100%	0	99%	KF263691.1	*P. palmivora*	1245	1245	87%	0	99%	HQ261380.1
5.	CBS236.30	India	*P. palmivora*	1611	1611	100%	0	99%	KF263691.1	*P. palmivora*	1256	1256	87%	0	100%	HQ261380.1
6.	CBS298.29	Trinidad & Tobago	*P. palmivora*	1605	1605	100%	0	99%	KF263691.1	*P. palmivora*	1256	1256	87%	0	100%	HQ261380.1
7.	CCO2083	unknown	*P. palmivora*	1605	1605	100%	0	99%	KF263691.1	----------Not available----------						
8.	P0497	Colombia	*P. palmivora*	1616	1616	100%	0	100%	KF263691.1	*P. palmivora*	1243	1243	87%	0	99%	HQ261380.1
9.	P11007	Guam	*P. palmivora*	1596	1596	100%	0	99%	KP183963.1	*P. palmivora*	1245	1245	87%	0	99%	HQ261380.1
10.	P16385	California	*P. palmivora*	1616	1616	100%	0	100%	KF263691.1	*P. palmivora*	1256	1256	87%	0	100%	HQ261380.1
11.	P16828	Colombia	*P. palmivora*	1611	1611	100%	0	99%	KF263691.1	*P. palmivora*	1247	1247	87%	0	99%	HQ261380.1
12.	P16831	Colombia	*P. palmivora*	1611	1611	100%	0	99%	KF263691.1	*P. palmivora*	1256	1256	87%	0	100%	HQ261380.1
13.	P3767	Indonesia	*P. palmivora*	1458	1458	100%	0	97%	KF263691.1	*P. palmivora*	1245	1245	87%	0	99%	HQ261380.1
14.	P6948	Malaysia	*P. palmivora*	1616	1616	100%	0	100%	KF263691.1	*P. palmivora*	1251	1251	87%	0	99%	HQ261380.1
15.	P8513	Colombia	*P. palmivora*	1616	1616	100%	0	100%	KF263691.1	*P. palmivora*	1240	1240	87%	0	99%	HQ261380.1
16.	PPC2614P	Colombia	*P. palmivora*	1611	1611	100%	0	99%	KF263691.1	*P. palmivora*	1251	1251	87%	0	99%	HQ261380.1
17.	PPC280574	Colombia	*P. palmivora*	1616	1616	100%	0	100%	KF263691.1	*P. palmivora*	1256	1256	87%	0	100%	HQ261380.1
18.	PPC3614L	Colombia	*P. palmivora*	1616	1616	100%	0	100%	KF263691.1	*P. palmivora*	1256	1256	87%	0	100%	HQ261380.1
19.	PPG1	Ghana	*P. palmivora*	1611	1611	100%	0	99%	KF263691.1	*P. palmivora*	1256	1256	87%	0	100%	HQ261380.1
20.	PPG11	Ghana	*P. palmivora*	1616	1616	100%	0	100%	KF263691.1	*P. palmivora*	1245	1245	87%	0	99%	HQ261380.1
21.	PPG13	Ghana	*P. palmivora*	1616	1616	100%	0	100%	KF263691.1	*P. palmivora*	1256	1256	87%	0	100%	HQ261380.1
22.	PPG8	Ghana	*P. palmivora*	1613	1613	100%	0	99%	KP183963.1	*P. palmivora*	1251	1251	87%	0	99%	HQ261380.1
23.	PPM1	Malaysia	*P. palmivora*	611	1611	100%	0	99%	KF263691.1	*P. palmivora*	1245	1245	87%	0	99%	HQ261380.1

24.	PPM2	Malaysia	*P. palmivora*	1600	1600	100%	0	99%	KF263691.1	*P. palmivora*	1240	1240	87%	0	99%	HQ261380.1
25.	PPM3	Malaysia	*P. palmivora*	1600	1600	100%	0	99%	KF263691.1	*P. palmivora*	1245	1245	87%	0	99%	HQ261380.1
26.	PPM4	Malaysia	*P. palmivora*	1613	1613	100%	0	99%	KP183963.1	*P. palmivora*	1243	1243	87%	0	99%	HQ261380.1
27.	PPM5	Malaysia	*P. palmivora*	1613	1613	100%	0	99%	KP183963.1	*P. palmivora*	1234	1234	87%	0	99%	HQ261380.1
28.	PPM6	Malaysia	*P. palmivora*	1583	1583	100%	0	99%	KF263691.1	*P. palmivora*	1245	1245	87%	0	99%	HQ261380.1
29.	PPM7	Malaysia	*P. palmivora*	1583	1583	100%	0	99%	KF263691.1	*P. palmivora*	1245	1245	87%	0	99%	HQ261380.1
30.	PPM8	Malaysia	*P. palmivora*	1613	1613	100%	0	99%	KP183963.1	*P. palmivora*	1243	1243	87%	0	99%	HQ261380.1
31.	PPG3	Ghana	*P. megakarya*	1528	1528	95%	0	99%	AF467100.1	*P. megakarya*	1245	1245	87%	0	99%	HQ261357.1
32.	PPG4	Ghana	*P. megakarya*	1528	1528	95%	0	99%	AF467100.1	*P. megakarya*	1256	1256	87%	0	100%	HQ261357.1
33.	PPG12	Ghana	*P. megakarya*	1522	1522	95%	0	99%	AF467100.1	*P. megakarya*	1256	1256	87%	0	100%	HQ261357.1
34.	13-A2	UK	*P. infestans*	1628	1628	100%	0	99%	EF126351.1	*P. infestans*	1387	1387	98%	0	99%	AY129165.1
35.	2009-7654A	UK	*P. infestans*	1622	1622	100%	0	99%	EF126351.1	*P. infestans*	1406	1406	98%	0	99%	AY129165.1
36.	CBS148.88	USA	*P. palmivora*	1609	1609	100%	0	99%	KP183963.1	*P. palmivora*	1256	1256	87%	0	100%	HQ261380.1
37.	PYT01	Unknown	*Pythium apha-nidermatum*	1580	1580	99%	0	99%	KJ162355.1	*Pythium apha-nidermatum*	1402	1402	99%	0	99%	AY129164.1
38.	CBS358.30	Sri Lanka	*P. colocasiae*	1587	1587	100%	0	99%	GU111605.1	*P. citricola*	1273	1273	98%	0	97%	FJ237512.1
			P. colocasiae	1583	1583	99%	0	99%	JN661139.1	*P. capsici*	1267	1267	98%	0	97%	AY129166.1
			P. colocasiae	1581	1581	100%	0	99%	GU111604.1	P. colocasiae	1264	1264	99%	0	96%	AY129173.1
39.	P19537	Colombia	*P. parasitica*	1648	1648	100%	0	100%	KC768775.1	*P. nicotianae*	1380	1380	96%	0	100%	EU660844.1
			P. parasitica	1644	1644	100%	0	99%	GU111667.1	*P. nicotianae*	1380	1380	96%	0	100%	EU660844.1
			P. nicotianae	1642	1642	100%	0	99%	KJ494902.1	*P. nicotianae*	1375	1375	96%	0	99%	EU660846.1
40.	P19538	Colombia	*P. parasitica*	1637	1637	100%	0	99%	KC768775.1	*P. nicotianae*	1378	1378	98%	0	99%	AY129169.1
			P. parasitica	1633	1633	100%	0	99%	GU111667.1	*P. nicotianae*	1369	1369	96%	0	99%	EU660844.1
			P. nicotianae	1631	1631	100%	0	99%	KJ494902.1	*P. nicotianae*	1363	1363	96%	0	99%	EU660846.1
41.	PC01	unknown	*P. cryptogea*	1600	1600	100%	0	99%	AF087475.1	*P. parsiana*	1260	1260	93%	0	98%	HM749282.1
			P. cryptogea	1594	1594	98%	0	99%	GU111626.1	*P. cryptogea*	1256	1256	87%	0	100%	HQ261290.1
			P. cryptogea	1589	1589	100%	0	99%	GU111631.1	*P. cryptogea*	1251	1251	87%	0	99%	HQ261294.1
42.	CBS581.69	Malaysia	*P. citrophthora*	1580	1580	100%	0	99%	GU133066.1	*P. insolita*	1321	1321	93%	0	99%	GU594821.1
			P. colocasiae	1572	1572	100%	0	99%	GU111605.1	*P. colocasiae*	1310	1310	97%	0	98%	AY129173.1
			P. citrophthora	1572	1572	100%	0	99%	GU111603.1	*P. citrophthora*	1277	1277	95%	0	98%	GU133458.1

**E-value. The rows highlighted in blue and grey show up BLAST scores up to 3rd rank of the maximum*

Table 2-5 Identification of representatives of fungal isolate groups retrieved from soil samples

No.	Isolate	Group	Summary of result of BLAST (maximum score) of ITS sequences with GenBank®				
			Identity	Query	E-value (%)	Ident	Accession No.
1.	MN1A	1	*Fusarium oxysporum*	99%	0	100%	KC215112.1
2.	MN1B	1	*Fusarium oxysporum*	99%	0	100%	KT719193.1
3.	MN2	2	*Purpureocillium lilacinum*	99%	0	99%	KC790527.1
4.	MN3	3	*Purpureocillium lilacinum*	99%	0	99%	KC790527.1
5.	D1	4	*Purpureocillium lilacinum*	99%	0	99%	KC790527.1
6.	D4	24	*Gongronella butleri*	99%	0	98%	KP067277.1
7.	B1	26	*Fusarium* sp.	99%	0	99%	JQ364975.1
8.	IA8	28	*Fusarium* sp.	99%	0	99%	JQ364975.1
9.	IA9	29	*Fusarium* sp.	100%	0	99%	JQ364975.1
10.	UG2A	30	*Mortierella chlamydospora*	99%	0	98%	AB476422.1
11.	UG2C	30	*Mortierella chlamydospora*	99%	0	97%	AB476422.1
12.	UG1A	30	*Mortierella chlamydospora*	99%	0	97%	AB476422.1
13.	IB8	31	*Mortierella echinosphaera*	97%	0	99%	JX976015.1
14.	IB1	32	*Rhizomucor variabilis*	100%	5.00E-141	99%	KJ862066.1
15.	IC1	33	*Mortierella echinosphaera*	98%	0	99%	JX976015.1
16.	IC1	33	*Mortierella echinosphaera*	97%	0	99%	JX976015.1
17.	IF1	34	*Trichoderma koningiopsis*	99%	0	99%	KP340235.1
18.	IA11	36	*Trichoderma asperellum*	99%	0	100%	KU215913.1
19.	IF5	36	*Trichoderma asperellum*	99%	0	100%	KU215913.1
20.	IF3	36	*Trichoderma asperellum*	99%	0	100%	KU215913.1
21.	D2	5	*Fusarium solani*	99%	0	99%	KT211526.1
22.	H4	7	*Talaromyces aculeatus*	97%	0	99%	KF741981.1
23.	H3	8	*Mortierella chlamydospora*	99%	0	98%	AB476422.1
24.	K1	12	*Mortierella chlamydospora*	99%	0	98%	AB476422.1
25.	K3	13	*Mortierella chlamydospora*	99%	0	98%	AB476422.1
26.	K2	13	*Mortierella chlamydospora*	99%	0	98%	AB476422.1
27.	D3	14	*Fusarium* sp.	100%	0	99%	JQ364975.1
28.	H4	38	*Mortierella chlamydospora*	99%	0	98%	AB476422.1

2.4 Discussion

2.4.1 Isolation and collection of *P. palmivora*

Selective media $P_{10}VP$ containing CMA worked well for isolating *Phytopthora* from the diseased tissue of cocoa and durian without any serious contamination from unwanted fungi and bacteria. Pimaricin, a polyene antibiotic is known to suppress almost all fungi but not the *Pythiaceae* (*Phytophthora* and *Pythium*)(Jeffers and Martin, 1986). Further suppression of fungi was provided by pentachloronitrobenzene (PCNB) which is a narrow spectrum fungicide. The antibiotic, vancomycin is a broad spectrum antibiotic that suppresses both Gram positive and negative bacteria. The use of $P_{10}VP$ is known to work successfully for isolates of *Phytophthora* and eliminate most fungi except *Pythium* and *Mortierella* (Brodrick et al., 1975). However, during the colony purification from the soil wash plates, the selective media was prepared using CMA supplemented with PCNB, nyastatin and penicillin instead of pimaricin and vancomycin as used in $P_{10}VP$. Nyastatin also works as an antifungal agent and can be used to replace Pimaricin, while penicillin can replace vancomycin as a broad-spectrum antibiotic as described by Drenth and Sendall (2001). However, nyastatin might not eliminate all fungal species when compared with pimaricin, which might explain why it was possible to retrieve 28 cultures on the selective media that were believed to be non *Phytophthora* species based on morphological characteristic of their colonies. The identification using ITS sequencing confirmed that they were *Purpureocillium, Fusarium, Gongronella* and *Mortierella*.

The attempts to retrieve any *Phytophthora* species from soil samples of several sampling sites in Malaysia were therefore not successful, neither by using direct

plating of soil dilutions nor baiting techniques. The methods to retrieve *Phytophthora* from soil samples using both techniques are not newly developed and have already been established by many researchers. For example, Tsao and Ocana (1969) successfully carried out direct isolation of some *Phytophthora* spp. from soil by plating soil dilutions. However, the use of baiting is preferred by many researchers in isolating *Phytophthora* from soil samples (Duncan, 1976; Hargreaves and Duncan, 1978; Anandaraj and Sarma, 1990; Eden et al., 2000) and has proved to be successful to isolate many *Phytophthora* species. Isolating *Phytophthora* from soil samples is difficult due to high risk of contamination from other microorganism. The use of baits such as pear and apple can help eliminate many unwanted contaminations. *Phytophthora* species attack living tissue, so the use of baiting is useful when isolating from dormant propagules such as chlamydospores and oospores as these spores are slow to germinate in artificial media, and the use of fresh plant bait can facilitate the germination (Drenth and Sendall, 2001). Using baiting to isolate *Phytophthora* from water samples is semi-selective as the sporangia of *Phytophthora* release swimming zoospore that are negatively geotactic and exhibit chemotaxis, therefore being attracted to the bait, whilst other soil microbes lack swimming spores and therefore are not baited (Mohammadi, 2012; Huai et al., 2013).

Failure in the first attempt (sampling from oil palm plantations in Kluang and Sebarang Perak) was probably due to the fact that the soil wash plates were sent from Malaysia to the UK and the transportation period was approximately five days. Within this period, any *Phytophthora* in the soil washes were probably out-competed by other microbes since most species of *Phytophthora* grow slower *in vitro* compared with saprophytic fungi and bacteria. However, isolation from the samples of the second samplings were carried out entirely in the MPOB's pathology lab in Malaysia but also

failed to collect any *Phytophthora* species using both direct plating and fruit baiting with apple and cocoa pod including samples taken from the cocoa experimental plots that have some incidence of cocoa pod disease cause by *P. palmivora*. A discussion with a researcher from the Malaysian Cocoa Board, who has experience with isolation of *Phytophthora* from the soil of the cocoa plantations, revealed that he was able to isolate them only after many attempts and the chance of successfully getting the isolates was better during the wet season where the incidence of the diseases caused by *Phytophthora* is higher. Our samplings were not done during the wet season and only a couple of black pod infections were spotted in the plot. Therefore, the low concentration of the *Phytophthora* in the soil during the sampling time might be another factor for not being able to isolate any of them. In addition, the concentration of pimaricin used also played an important role as high concentrations of this reagent will suppress the germination of resistant chlamydospores, sporangia and zoospore of many *Phytophthora* species but not the mycelia (Tsao and Ocana, 1969); however, most *Phytophthora* do not exist in soil as mycelia but mainly as resistant spores (Tsao, 1970). According to Jeffers and Martin (1986), direct isolation of *Phytophthora* species from the soil was not achieved until the concentration of pimaricin was suitable to allow germination of resistant spores. Although, P_{10}VP media used in this study is known to allow the isolation of many *Phytophthora* species, more assays should be carried out in the future to improve the isolation technique so that field surveys on the occurrence of the *Phytophthora* in the soil of the oil palm plantations can be carried out to understand more about the bud rot disease of oil palm.

2.4.2 Isolation of fungi from soil of oil palm plantations

Isolation of fungi from the soil dilutions was initially performed to obtain microbes for screening of potential biological agents against *Phytophthora.* The isolations from soil dilutions plated on PDA media supplemented with antibiotics were successful even though the plates of the soil washes were sent from Malaysia to the UK and took about 5 days to arrive. However, some species might have been lost during this period and also during colony purification. Use of the grouping method based on colony morphology for identification helped to expedite and reduce the costs of identification of all isolates but some isolates with similar colony characteristics might have been mistakenly identified during groupings. Hence, the finding from isolation of fungi from these soils can only give a rough idea of the microbial populations there and should not be treated as the total population or diversity of fungi at the sampling sites.

2.4.3 Morphological characterization of *P. palmivora*

The isolate pathogenic to oil palm (PPC280574) and the other isolates used in this study were observed to share common characteristics with other isolates as described by Waterhouse (1963) and other observations such as Blaha et al. (1994), Harris et al. (1984) and Turner (1960) such as in terms of shape and size of sporangia and chlamydospores. The size of sporangia and chlamydospores of this isolates are within the range for *P. palmivora* described earlier in Chapter 1. The shape and size of sporangia are said to depend on the isolation from different hosts (*http://www.phytophthoradb.org*); however, based on the table obtained from the *http://www.phytophthoradb.org* (Table 2-6), the size of sporangia of different *P. palmivora* isolates is mostly in range and is not clearly distinct among different

isolates. In this study, these variations were observed either in the same or separate culture plates. Colony morphology is also found to vary from one plate to another for the same isolate. According to Duncan and Cooke (2002) some morphological characteristics of *Phytophthora* are not constantly expressed in cultures and may vary even within isolates.

Table 2-6 A table of data on the sporangia, chlamydospores, oogonia and oospore of P. palmivora extracted from http://www.phytophthoradb.org

Host	Sporangia (µm)	Chlamydospore diameter (µm)	Oogonia diameter (µm)	Oospores diameter (µm)	Min:opt:max temperatures (°C)	Length-breadth ratio	Reference
Palm	38–72 × 33–42 (av. 50 × 35)	25–45 (av. 40)	NO[a]	NO	NO	1.43:1	Butler (1907)
Cacao	34–63 × 19–49 (av. 48.5 × 32.3)	23–55 (av. 38.9)	NO	NO	NO	1.47:1	Rosenbaum (1917)
Coconut	19–83 × 13–45 (av. 52 × 31)	19–61 (av. 41.6)	NO	NO	NO	1.68:1	Reinking (1923)
Rubber	31–62 × 22–39 (av. 39.6 × 24)	22–48 (av. 35.5)	NO	17–29	NO	1.65:1	Gadd (1924)
Citrus	23–65 × 15–39 (av. 44.7 × 29.5)	19–49 (av. 31)	NO	NO	NO	1.45:1	Ocfemia and Roldan (1927)
Cacao	36–62 × 24–36 (av. 49 × 30.6)	21–49 (av. 38)	NO	19–29 (av. 24.1)	NO	1.6:1	Ashby (1929)
Coconut	36–66 × 24–27 (av. 53.5 × 31)	19–49 (av. 36.1)	NO	27–41 (av. 31.5)	NO	1.73:1	Ashby (1929)
Cacao	30–78 × 18–42 (av. 49.7 × 30.3)	21–45 (av. 35.9)	NO	NO (av. 23)	NO	1.66:1	Thompson (1929)
Rubber	36–75 × 21–36 (av. 50.9 × 26.9)	27–34	NO	NO (av. 24.1)	NO	1.87:1	Thompson (1929)
Coconut	26–88 × 18–41 (av. 52.7 × 31)	19–49 (av. 34.8)	NO	NO	NO	1.7:1	Tucker (1931)
Bougainvillea	28–56 × 19–45 (av. 40 × 31)	25–40 (av. 34)	24–31 (av. 26)	16–25 (av. 22)	NO	NO	Ramakrishnan & Seethalakshmi (1956)
Hibiscus	22–102 × 19–53 (av. 62 × 37)	31–71 (av. 50)	23–31 (av. 28)	16–23 (av. NO)	NO	NO	Ramakrishnan & Seethalakshmi (1956)
Black pepper[b]	28–76 × 20–40 (av. 43 × 30)	12–40 (av. 25)	21–31 (av. 25.5)	17–30 (av. 22.7)	10:25–29:35	1.5:1	Holliday and owat (1963)
Petunia	27–62 × 19–37 (av. 45.1 × 23.9)	14–43 (av. 28.9)	23–37 (av. 28.6)	18–31 (av. 23.4)	12:25–27:35	1.88:1	Ershad (1971)
Cacao 'S' type (MF1)	27–65 × 17–34 (av. 43.8 × 19.6)	NO	25–34 (av. 29)	19.6–34 (av. 24.7)	10:28–30:34	1.82:1	Brasier and Griffin (1979)
Wide range of hosts	31.0–56.4 × 20.7–36.7	36.2 ± 9.6	NO	NO	5:24–30:35	1.2:1–1.8:1	Mchau and Coffey (1994)

[a] Not observed by reference cited. [b] Probably MF4 = *P. capsici.*

2.4.4 Molecular identification of isolates based on internal transcribed spacer (ITS) DNA marker

Amplification of the ITS region using primer pair ITS1 and ITS4 was easily being carried out for all *Phytophthora* and non *Phytophthora* isolates used in this study. This might be due to the short length of the region amplified and high copy number of the rDNA repeat, which can be up to 200 copies per haploid genome (Bruns et al., 1991; Capote et al., 2012). According to White et al. (1990), PCR amplification using the ITS1 and ITS4 primer pair will produce a DNA fragment containing sequences encoding a small portion of the small ribosomal subunit (18S rRNA), ITS1, 5.8S rRNA, ITS2 and a small portion of the large ribosomal subunit (28S rRNA) of nuclear DNA. These regions are the most common DNA regions being sequenced for identification of oomycetes up to species level. The use of the ITS region as a genetic marker is extensive, not only in identification of strains at and even below the species level but also to address research questions relating to systematics and phylogeny (Diaz et al., 2012). In the ribosomal RNA gene cluster, the ITS region (and the intergenic spacer) of the nuclear rDNA evolve fastest compared to the other regions in the rRNA gene cluster such as the small subunit and larger subunit sequences. Therefore, the sequences in this region may vary among species within a genus or among populations making the possibility of identification among species more likely (Lee and Taylor, 1992). The small subunit sequences are more useful for studying distantly related organisms (White et al., 1990). Cooke et al. (2000) have sequenced the ITS region of 50 taxa of *Phytophthora* and related oomycetes, which covered all the known and available species of the oomycetes genus. The ITS has been accepted as the *de facto* DNA barcode for identification of *Phytophthora* and also *Pythium* (Robideau et

al., 2011). Furthermore, sequence databases of the ITS region especially for *Phytophthora* are larger than for other molecular markers.

Apart from the use of the ITS region for identification of oomycetes, other molecular markers are also being explored by many researchers, such as the cytochrome oxidase I and II genes, including in the development of barcoding for oomycetes and true fungi. In this study, 30 isolates of *Phytophthora* were identified as *P. palmivora* based on analysis of sequences from the ITS region. Similar results were observed from the analysis using mitochondrial *cytochrome oxidase I (CoxI). CoxI* is the default DNA barcode approved by GenBank® and the Consortium for the Barcode of Life (CBOL) and has proven useful in phylogenetic studies of the oomycete genus *Phytophthora* (Robideau et al., 2011). However, there were five isolates that were identified differently using both molecular markers. These isolates were P19537 and P19538, which were named as *P. parasitica* using ITS sequences, but *P. nicotianae* by *CoxI* gene. However, the identification was based on the identity of sequences that have maximum scoring from the BLAST when compared to the sequences of the query. When, the next scores were evaluated, it also showed high homology to ITS sequences of *P. nicotianae* (identity 99%, query cover 100%). These two species are probably evolutionarily closely related which explains the high homology. The same situation was also observed for isolate CBS581.69. The sequences of ITS and *CoxI* of *P. citrophthora*, *P. colocasiae* and *P. insolita* are highly homologous with only 1% differences, making identification to species level using these markers difficult. For isolate PC01C, even though the analysis of *CoxI* sequences suggested the isolate as *P. parsiana* based on the identity of the maximum score of the BLAST, the 2nd and 3rd scores suggested it was *P. cryptogea,* as suggested by identity of the 3rd highest scoring of ITS sequences. On the other hand, for isolate CBS358.30, even though the

analysis of *CoxI* sequences suggested the isolate as *P. citricola, P. capsici* or *P. colocasiae* based on the 3rd highest scoring of the BLAST, the scores using ITS are quite conclusive with all 3rd highest scoring as *P. colocasiae.* However, the ITS region sequences of this isolate were also shown to be highly homologous to *P. citrophthora* (99%). In order to identify these isolates correctly, it is suggested that identification based on the morphological characteristics is also required. However, since the objective of this chapter was to collect *P. palmivora* species, the other species are just for use as references, therefore further identification of these isolates to species level was not important and the isolates have been named in this thesis based on identification using the maximum BLAST score for ITS sequences.

Identification of 28 isolates retrieved from the *Phytophthora* selective media using ITS sequences and alignment with the GenBank® database confirmed the identity of all as non *Phytophthora* as mentioned earlier. The details of their identity and other fungal isolates collected from the soil samples is presented in Table 2-7 below. Overall, a total of 30 out of 70 isolates obtained from all sites were *Purpureocillium lilacinum*, 20 isolates were *Mortierella chlamydospora*, several species were *Fusarium* and some isolates were *Trichoderma asperellum* and other common soil microbes. It will be interesting to see the interaction of these fungi with *P. palmivora,* even by *in vitro* studies, to see if any of them have antagonistic potential against *P. palmivora*, which was proposed as a line of investigation at the start of this study, but unfortunately was not carried out due to time constraints.

Table 2-7 Identity of fungi isolated from soil from the oil palm plantations

Fungi	*Phytophthora* Selective media	PDA	Total
Fusarium oxysporum	1	1	2
Fusarium solani	1	1	2
Fusarium sp.	2	3	5
Gongronella butleri	1	3	4
Mortierella chlamydospora	10	10	20
Purpureocillium lilacinum	13	17	30
Rhizomucor variabilis	0	2	2
Talaromyces aculeatus	0	1	1
Trichoderma asperellum	0	4	4
Total	**28**	**42**	**70**

Chapter 3. Analysis of genetic variation of *P. palmivora* using sequence analysis and amplified fragment length polymorphism (AFLP)

3.1 Introduction

Phytophthora palmivora was identified as the causal agent for bud rot disease of oil palm in Colombia (Torres et al., 2016). The same species is common in Malaysia and other oil palm producing countries but no outbreaks of bud rot disease of oil palm have been documented to date outside Latin America. It is unknown why this species causes devastating damage to oil palm in Colombia and other Latin American countries. There is a question whether the *P. palmivora* pathogenic to oil palm in Colombia is a different strain from *P. palmivora* in Malaysia. One of the steps for addressing this question is to identify the phylogenetic relationship and genetic variation of the *P. palmivora* species from both places and also other regions around the world.

The advances in molecular techniques, particularly PCR and DNA sequencing, have fueled bioinformatics studies of DNA data of organisms. DNA nucleotide sequence analysis has contributed to the understanding of the phylogenetic and molecular diversity of organisms including in the *Phytophthora* genus (Scibetta et al., 2012). Sequencing of specific target regions (single and multiple) has been widely used to study the diversity of *Phytophthora* (Hu et al., 2013; Rahman et al., 2015), *Pythium* (Arcate et al., 2006) and other microbes such as fungi (Korabecna, 2007), phytoplasmas (Jović et al., 2011) and plants (Ritland et al., 1993). Molecular analysis

of DNA sequences by Crawford et al. (1996), Cooke and Duncan (1997), Cooke et al. (2000) and Förster et al. (2000) have increased the understanding of the phylogenetic relationships between *Phytophthora* species. Their work has been based mainly on the nucleotide sequence data of a single DNA region, the rDNA internal transcribed spacer (ITS). Earlier work on analysis of sequences to investigate genetic diversity, phylogenetics and genetic variation of *Phytophthora* and fungi were also based on this rDNA and ITS region (Bruns et al., 1992); however, other regions and genes of nuclear or mitochondrial DNA have more recently been explored extensively, such as beta-tubulin (*β-tubulin*), translation elongation factor 1 alpha (*EF-1α*), NADH dehydrogenase subunit I, cytochrome c oxidase subunit I (*CoxI*) and subunit II (*CoxII*) either being analyzed individually or as multi-locus/multi-gene combinations (Martin and Tooley, 2003b; Kroon et al., 2004; Villa et al., 2006; Blair et al., 2008). Phylogenetic analysis based on multiple genes has also been reported for many fungal species such as *Fusarium* (Nalim et al., 2009) and *Corynespora* (Shimomoto et al., 2011).

Apart from the analysis of DNA sequences using selected regions as molecular markers, DNA fingerprinting methods such as amplified fragment length polymorphism (AFLP) have also been widely used to study genetic variation, phylogenetic relationships, population evolution, and diversity without knowing the DNA sequences of the studied organism, such as for plants (Zhang et al., 2000; Huang et al., 2002), fungi (Mueller et al., 1996; Baayen et al., 2000; Abdel-Satar et al., 2003), microalgae (Muller et al., 2007), and oomycetes (Samen et al., 2003; Ivors et al., 2004). AFLP is a PCR-based fingerprinting technique that is similar to the random amplified polymorphic DNA (RAPD) but offers higher stringency while retaining time efficiency (Mueller et al., 1996) and has proven useful for investigating genetic variation among

individuals (Mueller and Wolfenbarger, 1999). This method involves two main steps; the cleavage of the genomic DNA into restriction fragments using restriction enzymes and the PCR amplification. Other examples of DNA fingerprinting methods and random markers that are available include RAPDs (Samen et al., 2003; Sudheesh and Sreekumar, 2006), microsatellites/simple sequence repeats (SSR) (Lees et al., 2006; del Castillo-Munera et al., 2013), inter-simple sequence repeats (ISSR)(Muthusamy et al., 2008; Mohammadi et al., 2014; Yugander et al., 2015) and single nucleotide polymorphisms (SNP)(Quesada-Ocampo et al., 2011), each with their own advantages and disadvantages.

In this chapter, DNA sequence analysis and the AFLP fingerprinting technique were adopted to molecularly characterize *P. palmivora* isolates from Colombia and Malaysia, particularly focusing on the study of the genetic variations between these isolates. The null hypothesis is that there is no genetic difference between Colombian and Malaysian isolates.

3.2 Material and Methods

3.2.1 Sequence analysis of selected genetic markers

3.2.1.1 PCR amplification of the markers

DNA extraction of all isolates was carried out as described in Chapter 2 using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. The PCR amplification was performed in 25 µl volumes consisting of 12.5 µl of master mix (MangoTaq™ DNA Polymerase), 1 µl each of forward and reverse primers (10 pmol/µl), 10.5 µl sterile distilled water and 1 µl of template DNA. Fragments of six molecular markers; the internal transcribed spacer (ITS) regions, translation elongation factor 1 alpha gene (*EF-1α*), beta-tubulin gene (*β-tubulin*), *cytochrome oxidase II* (*CoxII*), *cytochrome oxidase I* (*CoxI*) genes of the mitochondrial DNA and the *ras*-related protein gene (*Ypt1*) were amplified for all isolates except for *Ypt1* which was only used for selected isolates as shown in Table 3-1. All oligonucleotides were synthesized by Sigma-Aldrich, UK. Amplifications were performed in a BIO-RAD S1000 Thermal Cycler.

The thermo cycle for the amplification of all regions was set at 95°C for 2 min for initial denaturation, followed by 30 cycles of denaturation at 95°C for 1 min. Annealing was set for 1 min at 41°C for *CoxI*, 48°C (spacer between *CoxI* and *II*), 55°C (ITS, *CoxII*), 64°C (*β-tubulin*, *EF-1α*) and 50°C for *Ypt1*, followed by the extension/elongation at 72°C for 1 min 30 sec. The final extension was set at 72°C for 10 min.

Table 3-1 Primers used for PCR amplification

Marker	Oligo-nucleotide	Sequence 5′ to 3′	Reference
ITS regions	ITS1 ITS4	TCC GTA GGTGAA CCTGCG G TCCTCCGCTTAT TGATATGC	White et al. (1990)
EF-1α	EF1AF EF1AR 5′	TCACGATCGACATTGCCCTG ACGGCTCGAGGATGACCATG	Kroon et al. (2004)
CoxII	FM82 FM78	TTGGCAATTAGGTTTTCAAGATCC ACAAATTTCACTACATTGTCC	Martin and Tooley (2003b)
CoxI	OomCoxILevup Fm85mod	TCAWCWMGATGGCTTTTTTCAAC RRHWACKTGACTDATRATACCAAA	Ginetti et al. (2014).
β-tubulin	BT5 BT6	GTATCATGTGCACGTACTCGG CAAGAAAGCCTTACGACGGA	Villa et al. (2006).
Ypt1	Ypt1	CGACCATYGGYGTKGACTTT	Chen and Roxby (1996)
	Ypt4	TTSACGTTCTCRCAGGCGTA	Moorman et al. (2002)

3.2.1.2 Development of primers for PCR amplification of new markers

In addition to using the ITS and some housekeeping genes, it was felt useful to study additional genomic markers. Since there has been no whole genome sequence of *P. palmivora* published to date, it was hypothesized that one region that might have genetic variation within species would be the gene clusters that encode effector/avirulence proteins that are involved in the infection process and colonization of plant tissue.

Since there were no published *Avr* gene sequences for *P. palmivora* available during the study, we attempted to amplify DNA regions equivalent to the *Avr4* gene of

P. infestans using primers PiAvr4F (5'-ATGCGTTCGCTTCACATTTTGCTGG-3') and PiAvr4R (5'-CTAAGATATGGGCCGTCTAGCTTGGAG-3') as described by van Poppel (2009). The PCR amplification was conducted using genomic DNA of *P. infestans* (13-A2) and *P. palmivora* (PPC280574). The amplification was also conducted using a new primer set *ARP1F and ARP1R* designed based on the sequences of the *P. infestans* avirulence (PiAvr4) gene obtained from GenBank® (accession no. EF672355.1), using the Primer BLAST tool from NCBI (*http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi*). The optimization of the amplification for both sets of primers was carried out with genomic DNA of *P. infestans* (13-A2) and *P. palmivora* (PPC280574) using gradient PCR to determine the optimum annealing temperature for the primers using a BIO-RAD S1000 Thermal Cycler with the amplification conditions of 95°C for 2 min for initial denaturation, followed by 30 cycles of denaturation at 95°C for 2 min. The range of the annealing gradient was set up at 53°C to 61 °C, followed by extension/elongation at 72°C for 1 min 30 sec. The final extension was set at 72°C for 10 min. Amplification of some other isolates were conducted at an annealing temperature of 58°C, for 35 cycles.

Successful amplification was confirmed by gel electrophoresis using 1.2% (w/v) agarose gels. The bands from the amplification of genomic DNA of *P. palmivora* of approximately the same size as amplification bands from *P. infestans* genomic DNA were cut from the gel and purified using a QIAquick® Gel Extraction Kit (Qiagen, USA) following the manufacturer's instructions (Appendix 5). Data for sequences and phylogenetic analysis for all *P. palmivora* isolates in the study based on the new marker was obtained by PCR amplification using another primer AVR1F and AVR1R that amplifies a nested region of ARP1F and ARP1R.

3.2.1.3 Phylogenetic analysis

All purified PCR amplicons were cloned prior to sequencing to obtain good quality sequences. Cloning of PCR amplicons was conducted using the pGem®-T Easy Vector System I (Promega) and the transformation was conducted using chemically treated competent cells of *E. coli* DH5α using heat shock treatment as described in Section 2.2.5. GAP4 software package (Staden-package, USA) was used to clean the sequence data obtained from the sequencing company by removing the vector sequences, correcting the base errors and generating contig sequences from forward and reverse sequences of each individual clone. Sequence alignments and phylogenetic analyses were conducted using *MEGA 6.06* (Tamura et al., 2013) using the data from the sequences obtained in this study, combined with additional sequences obtained from GenBank®, usually indicated by the presence of accession number in the bracket.

Sequence alignments were performed using ClustalW (Thompson et al., 1994) using default settings. The phylogenetic trees were constructed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The evolutionary distances were compared nucleotide-by-nucleotide using the nucleotide substitution model of maximum composite likelihood with rate uniformity and homogeneity pattern as implemented in *MEGA version 6.06* with bootstrap tests of 1000 replicates to estimate error. All alignment gaps and missing data were completely deleted before the calculation using the complete-deletion option. The Maximum Likelihood trees were constructed using individual datasets of each marker. For the concatenated analysis, the dataset was limited with nucleotide data only available for all five markers of the internal transcribed spacer (ITS) regions, translation elongation factor 1 alpha gene (*EF-1α*), beta-tubulin gene (*β-tubulin*) and the cytochrome oxidase II (*CoxII*) and the cytochrome oxidase I (*CoxI*) genes of the

mitochondrial DNA. Nucleotide sequences of all markers were concatenated using *SequenceMatrix* (Vaidya et al., 2011). All external gaps were manually inspected and deleted before alignment. Alignment and phylogenetic analyses of concatenated datasets were carried out with the same method as individual datasets.

3.2.2 Amplified fragment length polymorphism (AFLP)

3.2.2.1 Adapter preparation

*Eco*RI and *Mse*I adapters were prepared by mixing 20 µl of forward adapters (100 pMol), 20 µl of reverse adapter (100 pMol) and 160 µl distilled sterile water. The mixture was then incubated in a water bath set at 65°C for 10 min and then left to cool down slowly to room temperature. The sequences of each adapter are as in Table 3-2.

Table 3-2 Adapter and primer sequences used in AFLP analysis

Adapter/primer	Sequence
*Eco*RI adapter (forward)	5'-CTCGTAGACTGCGTACC-3'
*Eco*RI adapter (reverse)	5'-AATTGGTACGCAGTCTAC-3'
*Mse*I adapter (forward)	5'-GACGATGAGTCCTGAG-3'
*Mse*I adapter (reverse)	5'-TACTCAGGACTCAT-3'
*Eco*RI universal primer	5'-CGTAGACTGCGTACCAATTC-3'
*Mse*I universal primer	5'-GACGATGAGTCCTGAGTAA-3'
*Eco*RI selective primer	5'-GACTGCGTACCAATTC-3' plus selective nucleotides labeled with fluorescence dye D3 or D4
*Mse*I selective primer	5'-GACGATGAGTCCTGAGTAA-3' plus selective nucleotides

3.2.2.2 Digestion and ligation

Approximately 400-500 ng genomic DNA was double digested with two restriction enzymes, *Eco*RI and *Mse*I in restriction enzyme buffer in a total volume of 25 µl (Table 3-3) at 37°C for 3 hours. The reaction was then heated at 65°C to deactivate the enzymes. A small aliquot (5 µl) of digested and undigested DNA was checked on a 1.2% (w/v) agarose gel with 80-90 volts for 40-60 min before proceeding with adapter ligation steps. Only genomic DNA of a few representative isolates was used in this AFLP analysis. Ligation was carried out by adding 1 µL *Eco*RI adapter (10 pMol), 1 µl of *Mse*I adapter (10 pMol), 1 µl of T4 DNA ligase enzyme (1U/ul), 8 µl 10x T4 DNA ligase buffer and 11 µl of sterile distilled water to the ligation mixture tube and incubated at 4°C overnight. The digestion-ligation solution was then diluted with TBE at 1:10 ratio and kept at -20°C until needed for further steps.

Table 3-3 Digestion mixture component

Item	Final concentration	Volume (µl)
*Eco*RI (10 U/µl)	10 U	0.5
*Mse*I (5 U/µl)	5 U	0.5
*Eco*RI restriction enzyme buffer Tango™ (10x)	2x	5.0
Genomic DNA	400-500 ng	15.0
Sterile distilled water	-	4.0
Total	**-**	**25.0**

3.2.2.3 PCR amplifications

For pre-amplification, 5 µl of diluted ligation mixture, 1 µl of 10 pmol/µl *Eco*RI-universal primer and *Mse*I-universal primer each, and 18 µl sterile distilled water were added into Illustra™ puReTaq Ready-To-Go™ PCR Beads (GE Healthcare, UK). Amplification conditions were 94°C for 1 min followed by 10 cycles at 94°C for 40 sec, 65°C for 1 min and 72°C for 1 min and then 25 additional cycles at 94°C for 40 sec, 56°C for 1 min and 72°C for 1 min. The amplified products were checked in 1.2% w/v agarose gels run at 80-90 volts for 40 to 60 min. Selective amplification was done using a mixture of 5 µl of diluted pre-amplification product (1:20 dilution), 1 µl of each selective primer and 18 µl of sterile distilled water and Illustra™ puReTaq Ready-To-Go™ PCR Beads (GE Healthcare, UK). Primers tested were combinations of *Eco*RI selective primers labelled with WellRED® fluorescence, D3 or D4 dyes and *Mse*I selective primers with different randomly selected additional nucleotide tails. The primer combinations used in the study are listed in Table 3-4.

Amplification products were separated using 2% w/v agarose gels run at 120 volts for 60 min. The assays were carried out in two replicates to validate the results. Selective amplification products from the AFLP were then sent for automated capillary electrophoresis using the CEQ™ 8000 System. Only AFLPs with good banding patterns/polymorphisms were selected to be separated with CEQ. Volumes of samples loaded onto the CEQ were 1 µl with 600 bp standard ladder.

Table 3-4 Selective EcoRI and MseI primers used

No.	D3 labeled	No.	D4 labeled
1.	*Eco*RI - AA / *Mse*I-AA	37.	*Eco*RI - AC / *Mse*I-AA
2.	*Eco*RI - AA / *Mse*I-AC	38.	*Eco*RI - AC / *Mse*I-AC
3.	*Eco*RI - AA / *Mse*I-AG	39.	*Eco*RI - AC / *Mse*I-AG
4.	*Eco*RI - AA / *Mse*I-AT	40.	*Eco*RI - AC / *Mse*I-AT
5.	*Eco*RI - AA / *Mse*I-CA	41.	*Eco*RI - AC / *Mse*I-CA
6.	*Eco*RI - AA / *Mse*I-CC	42.	*Eco*RI - AC / *Mse*I-CC
7.	*Eco*RI - AA / *Mse*I-CG	43.	*Eco*RI - AC / *Mse*I-CG
8.	*Eco*RI - AA / *Mse*I-CT	44.	*Eco*RI - AC / *Mse*I-CT
9.	*Eco*RI - AA / *Mse*I-GA	45.	*Eco*RI - AC / *Mse*I-GA
10.	*Eco*RI - AA / *Mse*I-GC	46.	*Eco*RI - AC / *Mse*I-GC
11.	*Eco*RI - AA / *Mse*I-GG	47.	*Eco*RI - AC / *Mse*I-GG
12.	*Eco*RI - AA / *Mse*I-GT	48.	*Eco*RI - AC / *Mse*I-GT
13.	*Eco*RI - AA / *Mse*I-TA	49.	*Eco*RI - AC / *Mse*I-TA
14.	*Eco*RI - AA / *Mse*I-TC	50.	*Eco*RI - AC / *Mse*I-TC
15.	*Eco*RI - AA / *Mse*I-TG	51.	*Eco*RI - AC / *Mse*I-TG
16.	*Eco*RI - AA / *Mse*I-TT	52.	*Eco*RI - AC / *Mse*I-TT
17.	*Eco*RI - AA / *Mse*I-CAA	53.	*Eco*RI - AC / *Mse*I-CAA
18.	*Eco*RI - AA / *Mse*I-CAT	54.	*Eco*RI - AC / *Mse*I-CAT
19.	*Eco*RI - AA / *Mse*I-CTC	55.	*Eco*RI - AC / *Mse*I-CTC
20.	*Eco*RI - AA / *Mse*I-CTG	56.	*Eco*RI - AC / *Mse*I-CTG
21.	*Eco*RI - AA / *Mse*I-A	57.	*Eco*RI - AC / *Mse*I-A
22.	*Eco*RI - A / *Mse*I-AA	58.	*Eco*RI - TA / *Mse*I-AA
23.	*Eco*RI - A / *Mse*I-AC	59.	*Eco*RI - TA / *Mse*I-AC
24.	*Eco*RI - A / *Mse*I-AG	60.	*Eco*RI - TA / *Mse*I-AG
25.	*Eco*RI - A / *Mse*I-AT	61.	*Eco*RI - TA / *Mse*I-AT
26.	*Eco*RI - A / *Mse*I-CA	62.	*Eco*RI - TA / *Mse*I-CA
27.	*Eco*RI - A / *Mse*I-GA	63.	*Eco*RI - TA / *Mse*I-GA
28.	*Eco*RI - A / *Mse*I-GC	64.	*Eco*RI - TA / *Mse*I-TA
29.	*Eco*RI - A / *Mse*I-CAA		
30.	*Eco*RI - A / *Mse*I-CAT		
31.	*Eco*RI - A / *Mse*I-CTC		
32.	*Eco*RI - A / *Mse*I-CTG		
33.	*Eco*RI - C / *Mse*I-AG		
34.	*Eco*RI - C / *Mse*I-CA		
35.	*Eco*RI - C / *Mse*I-GA		
36.	*Eco*RI - C / *Mse*I-TC		

3.2.2.4 Fragment analysis

The data from the CEQ Genetic Analysis System was exported to *MS Excel* and manually examined, cleaned up and edited before transformation to binary coding. The absence of a peak/band is indicated by '0' and the presence of a band is indicated by '1'. Monomorphic fragment peaks were not scored. The phylogenetic analysis of the AFLP data was done using *FreeTree* software using UPGMA (Pavlicek et al., 1999; Hampl et al., 2001). The distance matrix was calculated using Nei and Li distance (Nei and Li, 1979). Resampling was done by bootstrapping with 1000 replicates. The phylogenetic tree derived from *FreeTree* was viewed using *Treeview* and *MEGA 6.0*.6.

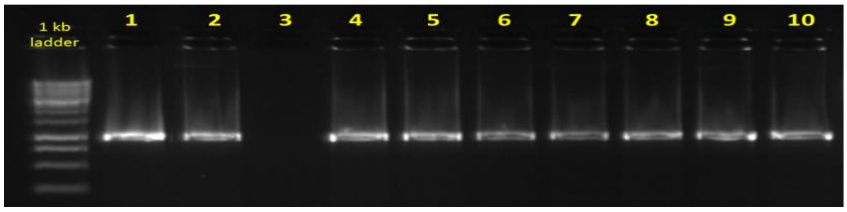
3.3 Results

3.3.1 PCR amplification of the markers

The fragment sizes obtained from the PCR amplifications varied among all markers depending on the primers used for amplification (Table 3-5, Figure 3-1). Amplifications with all 37 species of *Phytophthora* and a species of *Pythium* were observed to have fragments of the same size for each primer pair/marker. All primers can be used to amplify the markers studied for species of the genus *Phytophthora* and *Pythium* except for primers EF1AF and EF1AR, which were unable to amplify from the translation elongation factor 1 alpha gene (*EF-1α*) of *Pythium*.

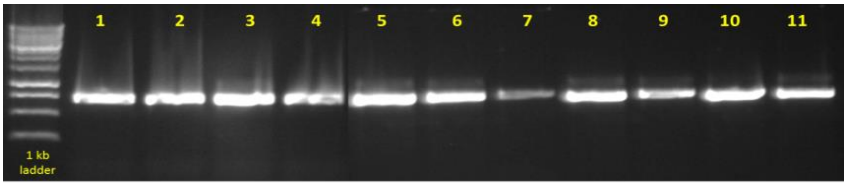
Table 3-5 The size of amplified fragments of each marker

Marker	Primer pair	Fragment size (approximately)	
		***Phytophthora* spp.**	***Pythium* spp.**
ITS regions	ITS1/ITS4	900 bp	900 bp
EF-1α	EF1AF/EF1AR	1000 bp	not amplified
CoxII	FM82/FM78	600 bp	600 bp
CoxI	OomCoxILevup/Fm85mod	800 bp	800 bp
β-tubulin	BT5/BT6	750 bp	750 bp
Ypt1	Ypt1/Ypt4	450 bp	not carried out



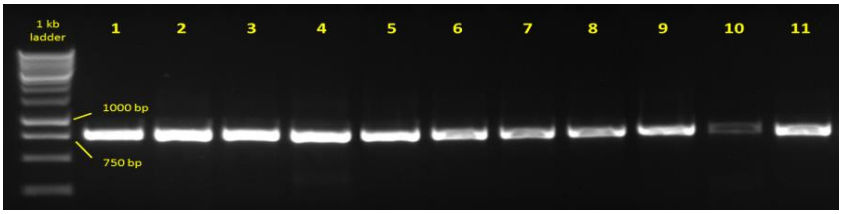
(a)

Lane 0: 1 kb ladder/marker
Lane 1: *P. palmivora* Malaysia-Cocoa PPM3
Lane 2: *P. cryptogea* PC01
Lane 3: *Pythium aphanidermatum* PYT01
Lane 4: *P. palmivora* CCO2083
Lane 5: *P. palmivora* India-Coconut CBS236.30
Lane 6: *P. palmivora* Malaysia-Durian PPM4
Lane 7: *P. infestans* United Kingdom-Potato 13-A2
Lane 8: *P. infestans* United Kingdom-Potato 2009-7654A
Lane 9: *P. palmivora* Trinidad & Tobago-Cocoa CBS298.29
Lane 10: *P. palmivora* Ghana-Cocoa PPG1



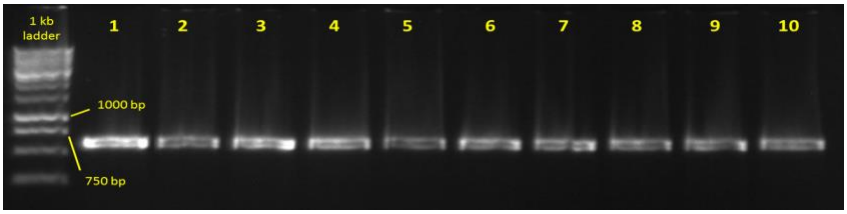
(b)

Lane 0: 1 kb ladder/marker
Lane 1: *P. palmivora* Malaysia-Cocoa PPM3
Lane 2: *P. palmivora* CCO2083
Lane 3: *P. palmivora* India-Coconut CBS236.30
Lane 4: *P. palmivora* Malaysia-Durian PPM4
Lane 5: *P. palmivora* Colombia-African Oil Palm P16828
Lane 6: *P. palmivora* Colombia-African Oil Palm P16831
Lane 7: *P. parasitica* Colombia-African Oil Palm P19537
Lane 8: *P. palmivora* Colombia-Cocoa P8513
Lane 9: *P. palmivora* Malaysia-Rubber P6948
Lane 10: *P. palmivora* Indonesia-Coconut P3767
Lane 11: *P. palmivora* Guam-Betel Palm P11007



(c)

Lane 0: 1 kb ladder/marker
Lane 1: *P. palmivora* Malaysia-Cocoa PPM2
Lane 2: *P. palmivora* Malaysia-Cocoa PPM3
Lane 3: *P. palmivora* Malaysia-Durian PPM5
Lane 4: *P. cryptogea* PC01
Lane 5: *P. colocasiae* Sri Lanka-Rubber CBS358.30
Lane 6: *P. palmivora* South Korea-Cymbidium CBS1113.46
Lane 7: *P. palmivora* Sri Lanka-Cocoa CBS179.26
Lane 8: *Pythium aphanidermatum* PYT01
Lane 9: *P. palmivora* CCO2083
Lane 10: *P. palmivora* India-Coconut CBS236.30
Lane 11: *P. palmivora* Malaysia-Durian PPM5



(d)

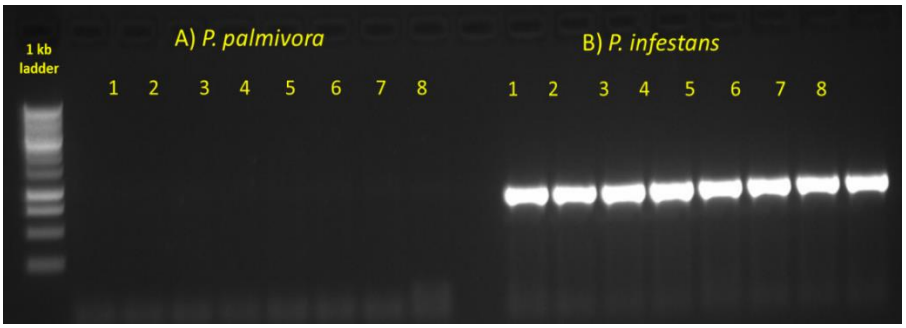
Lane 0: 1 kb ladder/marker
Lane 1: *P. palmivora* Malaysia-Cocoa PPM3
Lane 2: *Pythium aphanidermatum* PYT01
Lane 3: *P. palmivora* CCO2083
Lane 4: *P. palmivora* India-Coconut CBS236.30
Lane 5: *P. palmivora* Malaysia-Durian PPM4
Lane 6: *P. infestans* United Kingdom-Potato 13-A2
Lane 7: *P. infestans* United Kingdom-Potato 2009-7654A
Lane 8: *P. palmivora* Malaysia-Cocoa PPM1
Lane 9: *P. palmivora* Trinidad & Tobago-Cocoa CBS298.29
Lane 10: *P. palmivora* Ghana-Cocoa PPG1

Figure 3-1 Amplification bands of some Phytophthora isolates consisting of partial fragments of (a) elongation factor 1 alpha (b) β-tubulin (c) cytochrome oxidase I (CoxI), (d) Cytochrome oxidase II (CoxII)

3.3.2 Development of a new marker

PCR amplification using primer pair PiAvr4F and PiAvr4R (van Poppel, 2009) was shown to be specific to *P. infestans* with no amplification for *P. palmivora* (Figure 3-2). Amplification using primer ARP1F and ARP1R (Table 3-6) designed from the sequence of the *P. infestans* avirulence protein gene obtained from GenBank® gave a very clear band of approximately 1,000 bp using the genomic DNA of *P. infestans* at annealing temperatures between 56.2°C to 60.5°C but not for *P. palmivora* (Figure 3-3). However, multiple weak and unspecific bands were produced from *P. palmivora* at lower annealing temperatures below 56.2°C. The sequences of the bands (lane 6, 7, 8 highlighted with green box) produced from the amplification of genomic DNA of *P. palmivora* that were of similar size to the bands produced from *P. infestans* were successfully obtained by cloning the fragment into the pGem®-T Easy Vector System I (Promega) followed by PCR amplification using M13F and M13R primers and sequencing. A sequence of 1040 bp was retrieved after removing vector sequences and clean-up (Appendix 6). The sequence was compared to the nucleotide and protein database in GenBank® using BLAST programs (blastn and blastx). Alignments using high similarity blast (megablast) was not able to find any similarity, but when '*discontiguous megablast'* was used some similarities were acquired, with the highest scored to *Phytophthora parasitica INRA-310 hypothetical protein partial Mrna*; Accession no. XM_008893943.1, with query cover and identity of 35% and 74% respectively. The alignment with the non-redundant protein sequence database showed the highest score with hypothetical protein F441_00386 [*Phytophthora parasitica* CJ01A1]; Sequence ID: ETP27065.1, with query cover and identity of 96% and 46% (Appendix 7).

The primer pair AVR1F and AVR1R amplified all *P. palmivora* isolates and there was no amplification from genomic DNA of *P. megakarya, P. infestans, P. colocasiae, P. parasitica, P. cryptogea, Pythium aphanidermatum* and some other soil fungi (refer to Chapter 4, section 4.3.1). The amplification fragment was approximately 1000 bp. The sequences of the PCR fragments from primer AVR1F and AVR1R of all *P. palmivora* isolates were subjected to phylogenetic analyses. The region of the new marker is named as the *P. palmivora* hypothetical avirulence effector protein (PpHPAVR) region.



Annealing temperature gradients:

Lane 1: 64.0°C
Lane 2: 63.3°C
Lane 3: 62.1°C
Lane 4: 60.3°C
Lane 5: 58.0°C
Lane 6: 56.3°C
Lane 7: 55.0°C
Lane 8: 54.0°C

Figure 3-2 Gradient amplification of with primer pair PiAVR4F and PiAVR4R using genomic DNA of (A)* P. infestans *isolate 13-A2 and (B)* P. palmivora *isolate PPC280574.

Table 3-6 Details of primer pair ARP1F and ARP1R

Primer	Sequence (5'->3')	Template strand	Length	Start	Stop	GC%
ARP1F	TTCGACGGAATAGCCCATCC	Plus	20	1394	1413	55
ARP1R	GCAGTAAAAGCGGAATGACGG	Minus	21	2384	2364	52.38

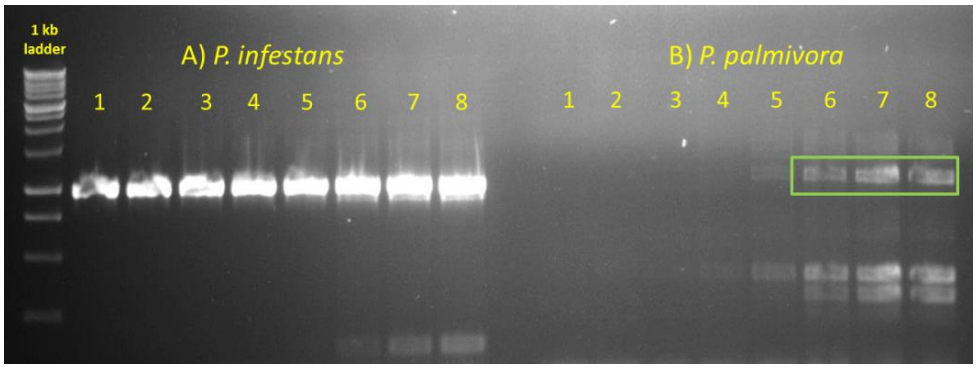


Figure 3-3 Gradient amplification of with primer pair ARP1F/ARP1R using genomic DNA of (A)* P. infestans *isolate 13-A2 and (B)* P. palmivora *isolate PPC280574.

3.3.3 Sequence and phylogenetic analyses

3.3.3.1 Internal transcribed spacer region of rDNA

Low intraspecific variation was observed in the ITS sequence data for all 24 isolates of *P. palmivora*. Isolates of *P. palmivora* originating from oil palm in Colombia (PPC280574), which were believed to be pathogenic to oil palm, showed a high similarity (97% to 100% identity, based on BLAST report) with other isolates obtained from various hosts and regions, including all six Malaysian isolates. Further assessments using phylogenetic analysis, showed similar results. The evolutionary history inferred using the Maximum Likelihood method based on the Tamura-Nei model grouped all *P. palmivora* isolates into one clade (Clade 1) with a strong bootstrap value regardless of the host and demographic origin of the isolates (Figure 3-4). The tree was constructed using 37 nucleotide sequences and involved 813 nucleotides in the final dataset. Heuristic searches of the initial tree(s) were automatically calculated based on the Maximum Parsimony method. A similar finding was also observed from the tree reconstructed using the same method but with

additional sequences gathered from GenBank®, which incorporated more *P. palmivora* isolates (a total of 58 isolates) from a wider range of hosts and origins (Figure 3-5). The accession numbers of all sequences taken from NCBI are written in parentheses in the phylogenetic tree. The tree was reconstructed using 75 sequences with a total of 663 positions in the final dataset.

In the initial analysis consisting of 37 taxa, with 26 isolates of *P. palmivora* (Figure 3-4), sub-branching of Clade 1 was observed (bootstrap value 84%), which consisted of two isolates originating from Malaysian durian (PPM4 and PPM5) and isolates from betel palm, Guam (P11007), cocoa, Ghana (PPG8) and *P. arecea* (=*P. palmivora*), bamboo palm, USA (CBS148.88). There was no consistent pattern for the origin/host of these isolates except that two isolates were from Malaysia. Sub-branching was also observed in the second ITS tree condensed to a 50% bootstrap value cut off. The sub–clades observed also showed no definite patterns with the exception of isolates from durian PPM4, PPM5, and Pp43 from Indonesia, which grouped together in sub-branch 4. Apart from these isolates (PPM4 and PPM5), the other Malaysian and Colombian isolates were randomly distributed in the large *P. palmivora* clade (Clade 1) in both trees. Interestingly, one isolate of *P. palmivora* (PPM3) isolated from cocoa in Malaysia was separated from the large *P. palmivora* clade with a high bootstrap value. However, this was not observed from the phylogenetic analyses with a larger number of taxon (Figure 3-5).

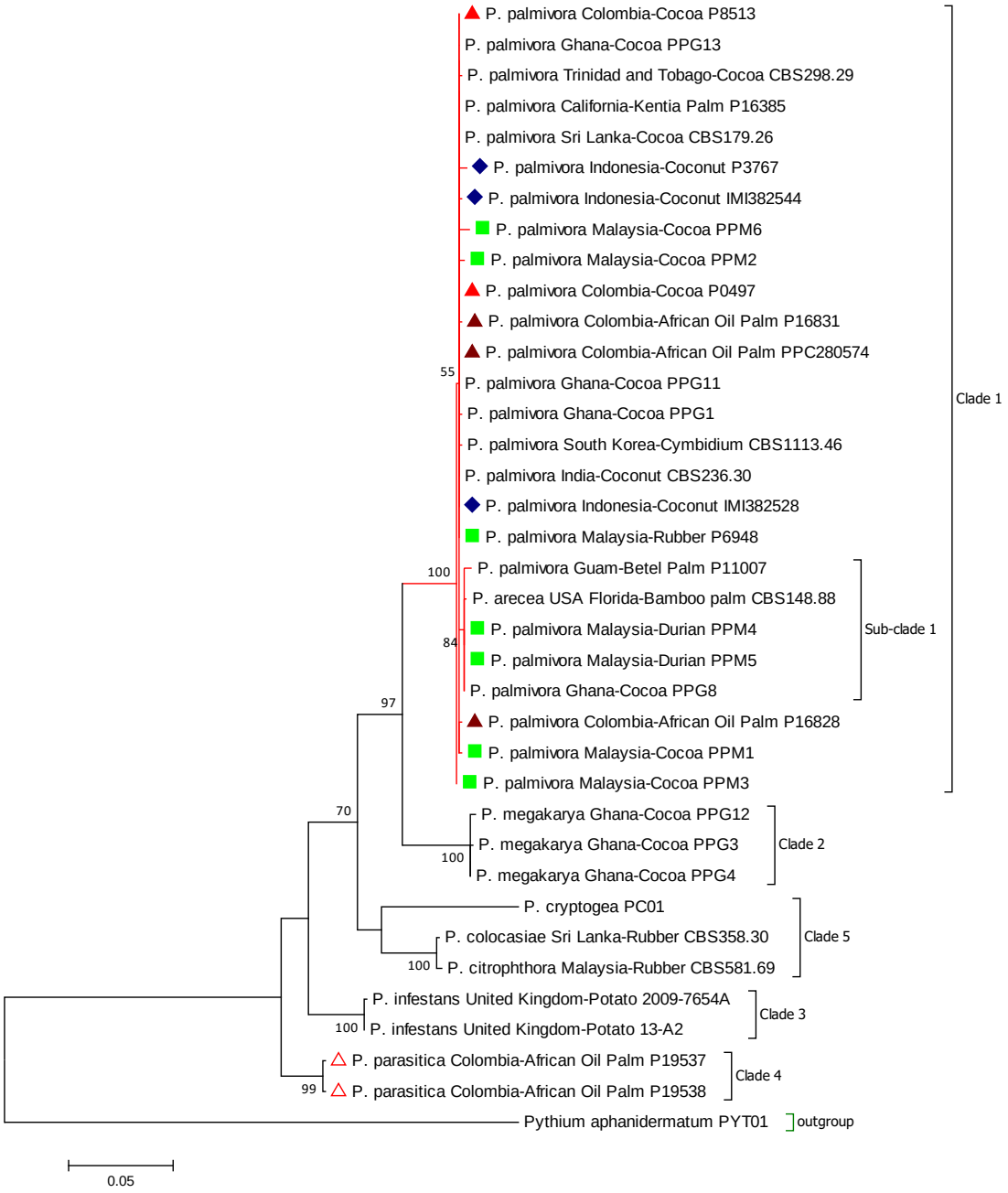


Figure 3-4 Molecular phylogenetic tree showing the relationship of 26 isolates of* P. palmivora *and other* Phytophthora *spp. from different hosts and demographic origin constructed from ITS rDNA data using maximum likelihood method based on the Tamura-Nei model.

Note: The tree with the highest log likelihood (-3196.6823) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches when ≥ 50. The labeled sub-clade is with ≥50% bootstrap value.

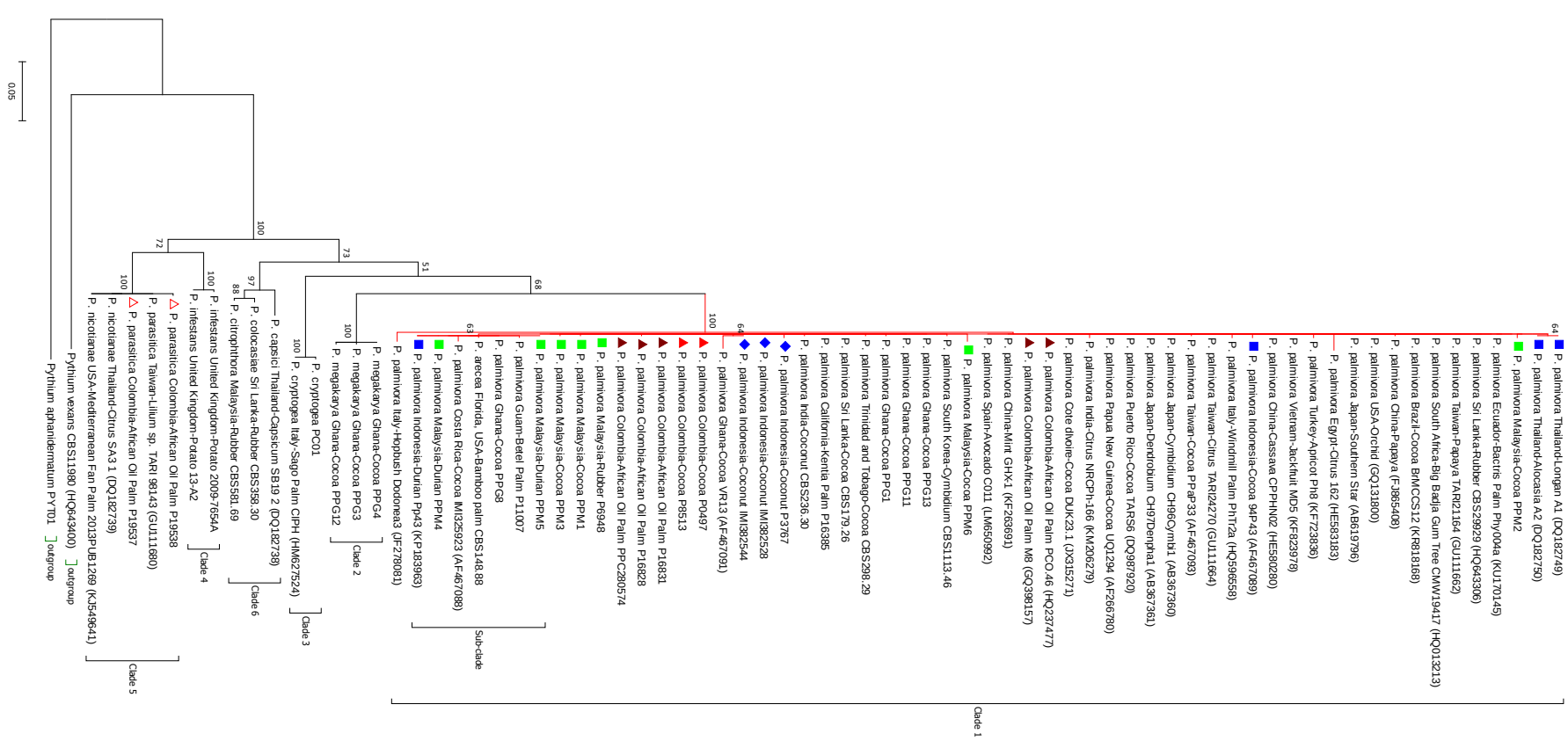


Figure 3-5 Molecular phylogenetic tree showing the relationship of 58 isolates* of P. palmivora *and other* Phytophthora *spp. from different hosts and demographic origin constructed from ITS rDNA data using maximum likelihood method based on the Tamura-Nei model.

Note: The tree with the highest log likelihood (-3645.0969) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches when ≥ 50. The labeled sub-clade is with ≥50% bootstrap value.

3.3.3.2 Translation elongation factor 1 alpha (EF-1α), β-tubulin, CoxI and CoxII

Initial tree(s) for the heuristic search using partial nucleotide sequences of *EF-1α*, *β-tubulin*, *CoxI* and *CoxII* were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analyses involved 44 sequences with a total of 868 positions in the final dataset for *EF-1α* and 41 sequences with a total of 648 positions in the final dataset for *β-tubulin*. For both *CoxI* and *CoxII*, the analysis involved a total of 38 sequences which incorporated 773 and 619 nucleotides, respectively. All the *P. palmivora* isolates (including *P. arecea*) were clustered in one clade (Clade 1) with bootstrap values of more than 97% for all trees. Sub-branching was observed for condensed trees with 50% bootstrap value cut off for all datasets except *β-tubulin*. In the *EF-1α* tree, isolates PPM4 and PPM5 were grouped together in a sub-clade, branched out from Clade 1, similar to the ITS tree (Figure 3-6). The other Malaysian isolates were distributed randomly in Clade 1. Some Colombian isolates were grouped in sub-clade 1 and sub-clade 2 with other isolates from Ghana and Sri Lanka also randomly distributed in Clade 1. Some sub-clades were also observed with the *β-tubulin* tree but with low bootstrap values (<50%) (Figure 3-7). In the *CoxI* tree, two isolates from Malaysia, PPM1 and PPM2, were grouped in a sub-clade. There were other sub-clades but with lower than 50% bootstrap values (Figure 3-8). The *CoxII* tree also grouped all 27 isolates of *P. palmivora* in one clade, but the clade was sub-branching into another sub-clade of 26 isolates with one isolate separated (isolate P11007)(Figure 3-9). The *ras*-related protein tree was constructed from just some taxa but also gave similar results to the other datasets (Figure 3-10).

Interspecific variation among other species of *Phytophthora* was clearly observed using all nucleotide datasets. *Phytophthora palmivora* is clearly distinguished from other species included in this study. Some species with more than one isolate were grouped together into the same clade such as of *P. megakarya*. Isolate CBS358.30, originally identified as *P. colocasiae* in this study, was always grouped with isolate CBS581.69 from Malaysia in all trees. Both original hosts of these isolates are rubber.

Figure 3-6 Molecular phylogenetic tree showing the relationship of* P. palmivora *and other* Phytophthora *from different hosts and demographic origin constructed from partial gene sequences of translation elongation factor 1 alpha (EF-1α) using maximum likelihood method based on the Tamura-Nei model.

Note: The tree with the highest log likelihood (-2859.6731) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches when ≥ 50. The labeled sub-clade is with ≥50% bootstrap value.

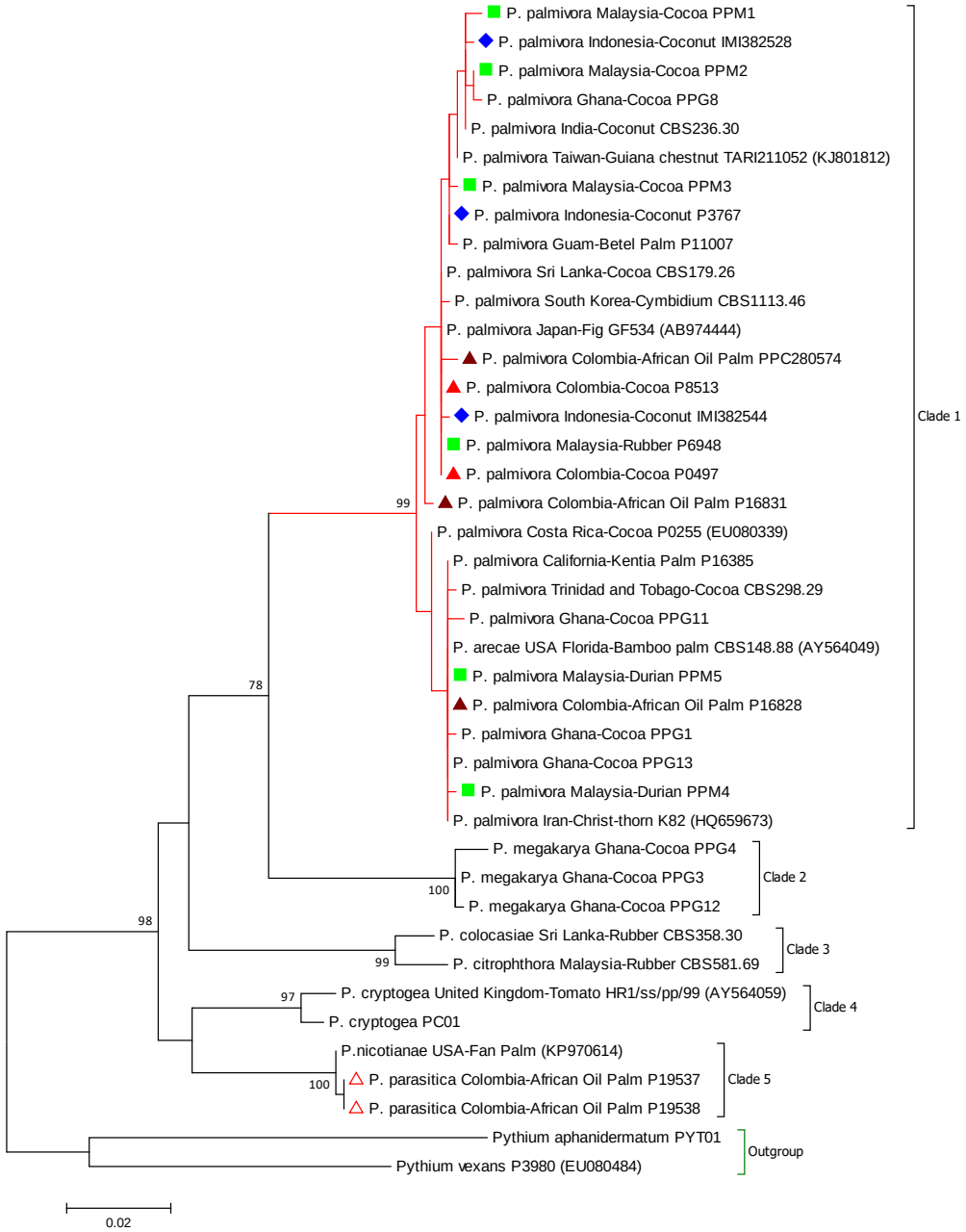


Figure 3-7 Molecular phylogenetic tree showing the relationship of* P. palmivora *and other* Phytophthora *from different hosts and demographic origin constructed from partial gene sequences of β-tubulin using maximum likelihood method based on the Tamura-Nei model.

Note: The tree with the highest log likelihood likelihood (-2351.2154) shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches when ≥ 50. The labeled sub-clade is with ≥50% bootstrap value.

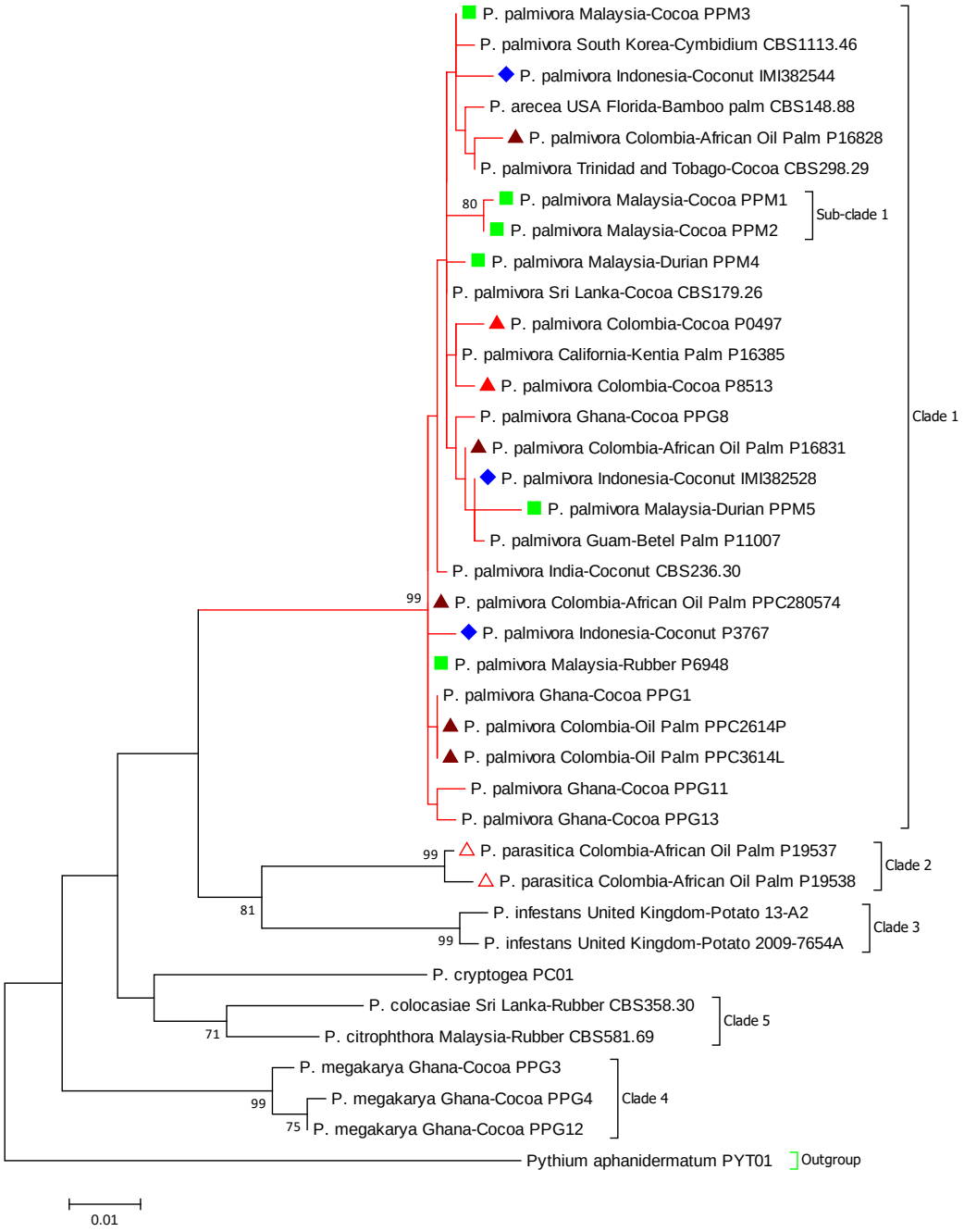


Figure 3-8 Molecular phylogenetic tree showing the relationship of* P. palmivora *and other* Phytophthora *from different hosts and demographic origin constructed from partial gene sequences of cytochrome c oxidase subunit I (CoxI) using maximum likelihood method based on the Tamura-Nei model.

Note: The tree with the highest log likelihood likelihood (-2770.5889) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches when ≥ 50. The labeled sub-clade is with ≥50% bootstrap value.

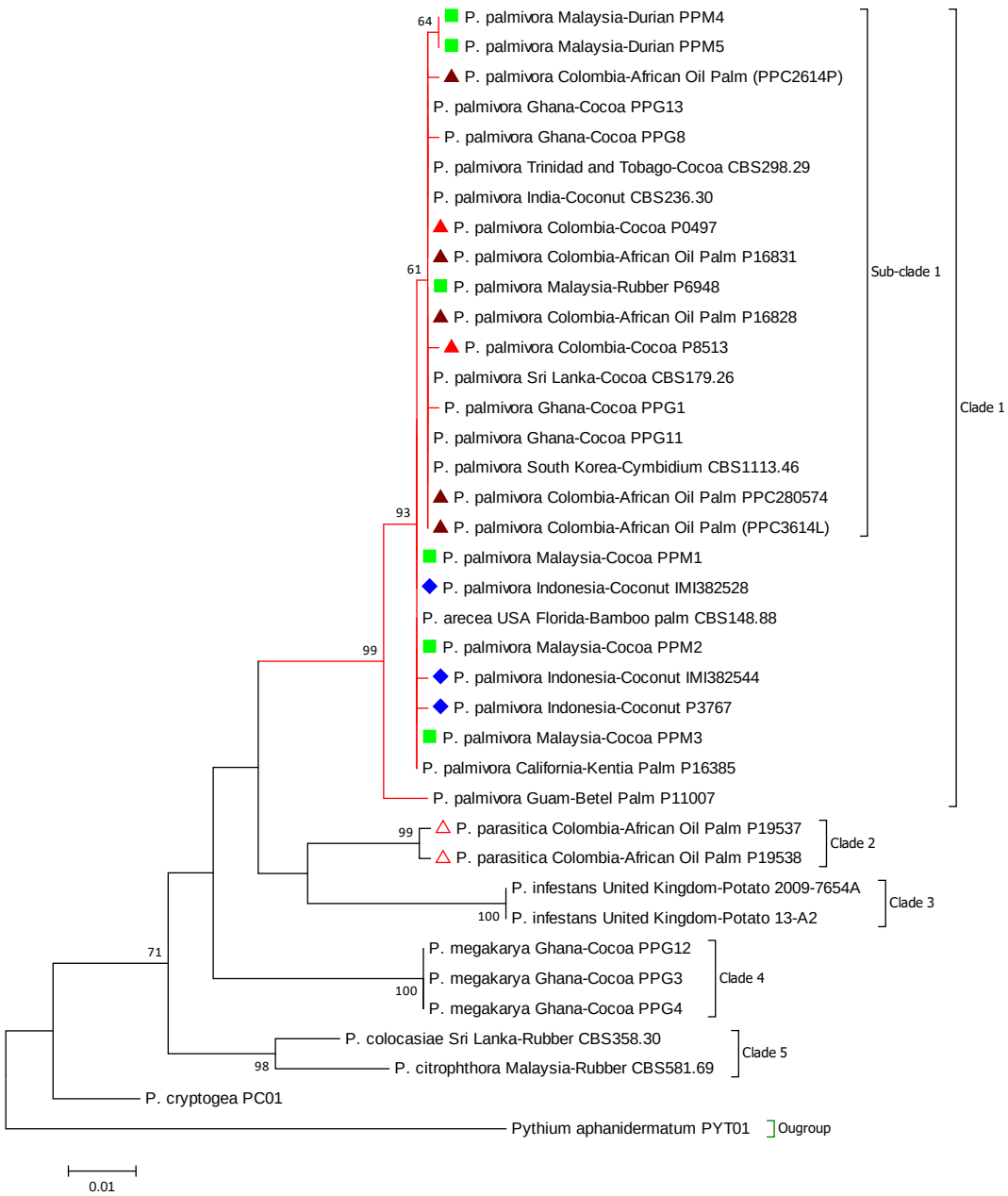


Figure 3-9 Molecular phylogenetic tree showing the relationship of* P. palmivora *and other* Phytophthora *from different hosts and demographic origin constructed from partial gene sequences of cytochrome c oxidase subunit II (CoxII) using maximum likelihood method based on the Tamura-Nei model.

Note: The tree with the highest log likelihood likelihood (-1815.3126) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches when ≥ 50. The labeled sub-clade is with ≥50% bootstrap value.

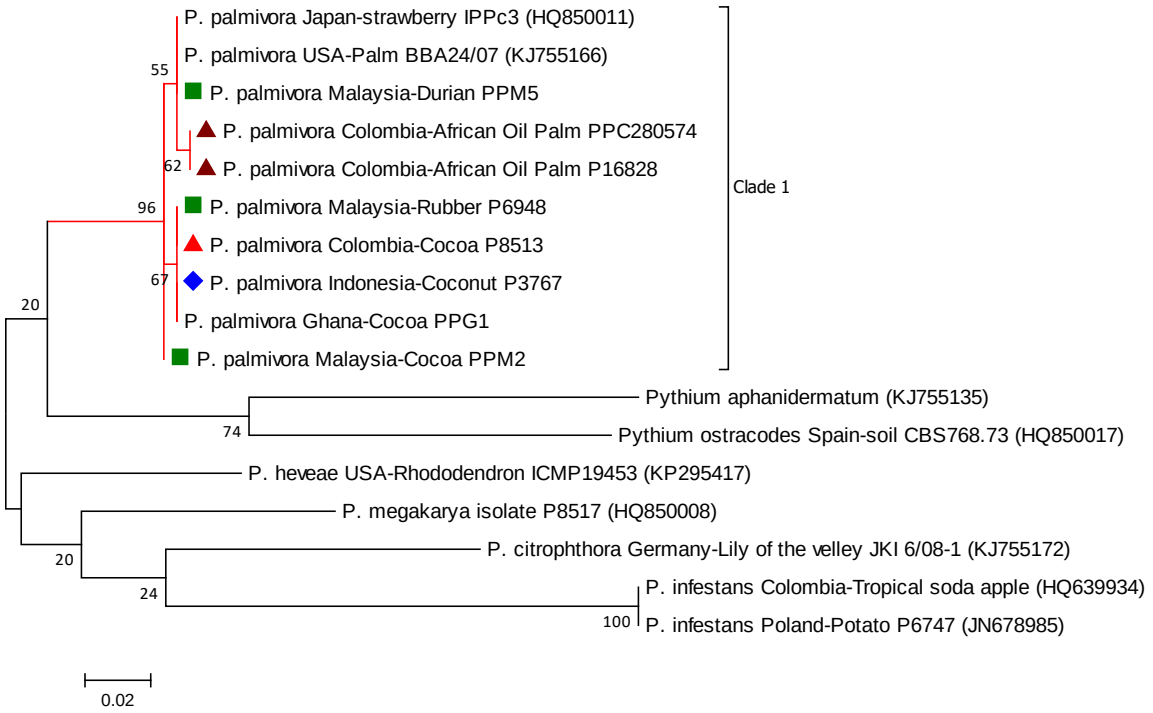


Figure 3-10 Molecular phylogenetic tree showing the relationship of* P. palmivora *and other* Phytophthora *from different hosts and demographic origin constructed from partial gene sequences of ras-related protein gene (Ypt1) using maximum likelihood method based on the Tamura-Nei model.

Note: The tree with the highest log likelihood likelihood (-1108.7618) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches when ≥ 50. The labeled sub-clade is with ≥50% bootstrap value.

3.3.3.3 Concatenated tree of ITS and other housekeeping genes

The concatenated tree was constructed from sequences of five different markers (Figure 3-11). The reconstruction of the tree was done using the same methods as previous trees. The tree involved 35 sequences from this study and from GenBank® marked with an asterisk (*). There was a total of 3773 nucleotide positions in the final concatenated dataset. As in other trees, all *P. palmivora* isolates were grouped in one clade, Clade 1. There are three sub-clades branching out from Clade 1 with more than 50% bootstrap value. Observation of the members of each sub-clade show no relationship in terms of host and demographic origin of the isolates involved.

3.3.3.4 Phylogenetic analysis of the new PpHPAVR marker

The initial tree(s) for the heuristic search from datasets of PpHPAVR sequences were obtained by using the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analysis involved 31 sequences with a total of 958 positions in the final dataset. The final tree consists of several major clades. *Phytophthora palmivora* Colombian isolates and Malaysian isolates were clearly separated in different clades. All the Colombian isolates were strongly grouped (bootstrap value of 98%) in Clade 1 together with isolates from the USA, Ghana, Trinidad & Tobago, Guam, Sri Lanka and India including *P. arecae* from the USA which later separated as an outgroup from the rest of Clade 1 members which were further grouped in sub-clade 1 (Figure 3-12). Isolates from Malaysia and Indonesia from different hosts of cocoa, durian, coconut and rubber clustered in several clades. Clade 2 consists of three Malaysian isolates obtained from cocoa (PPM1, PPM2 and PPM3). Clade 3 consist of a mixture of Malaysian and Indonesian

isolates form coconut, cocoa and durian. Clade 4 also contains a mixture of Malaysian and Indonesian isolates from various hosts. One isolate from South Korea (CBS1111.46) was also included in this clade.

Figure 3-11 Molecular phylogenetic tree showing the relationship of* P. palmivora *and other* Phytophthora *from different hosts and demographic origin constructed from concatenated sequences of ITS, EF-1α, β-tubulin, CoxI and CoxII using maximum likelihood method based on the Tamura-Nei model.

*Note: The tree with the highest log likelihood likelihood (-13551.6711) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches when ≥ 50. The labeled sub-clade (s) is/are with ≥ 50% bootstrap value. Three sequences of taxa with * were obtained from* GenBank® *and may not from the same isolate (EU080485 (EF-1α), AY564049 (EF-1α), AY564037 (β-Tubulin))*

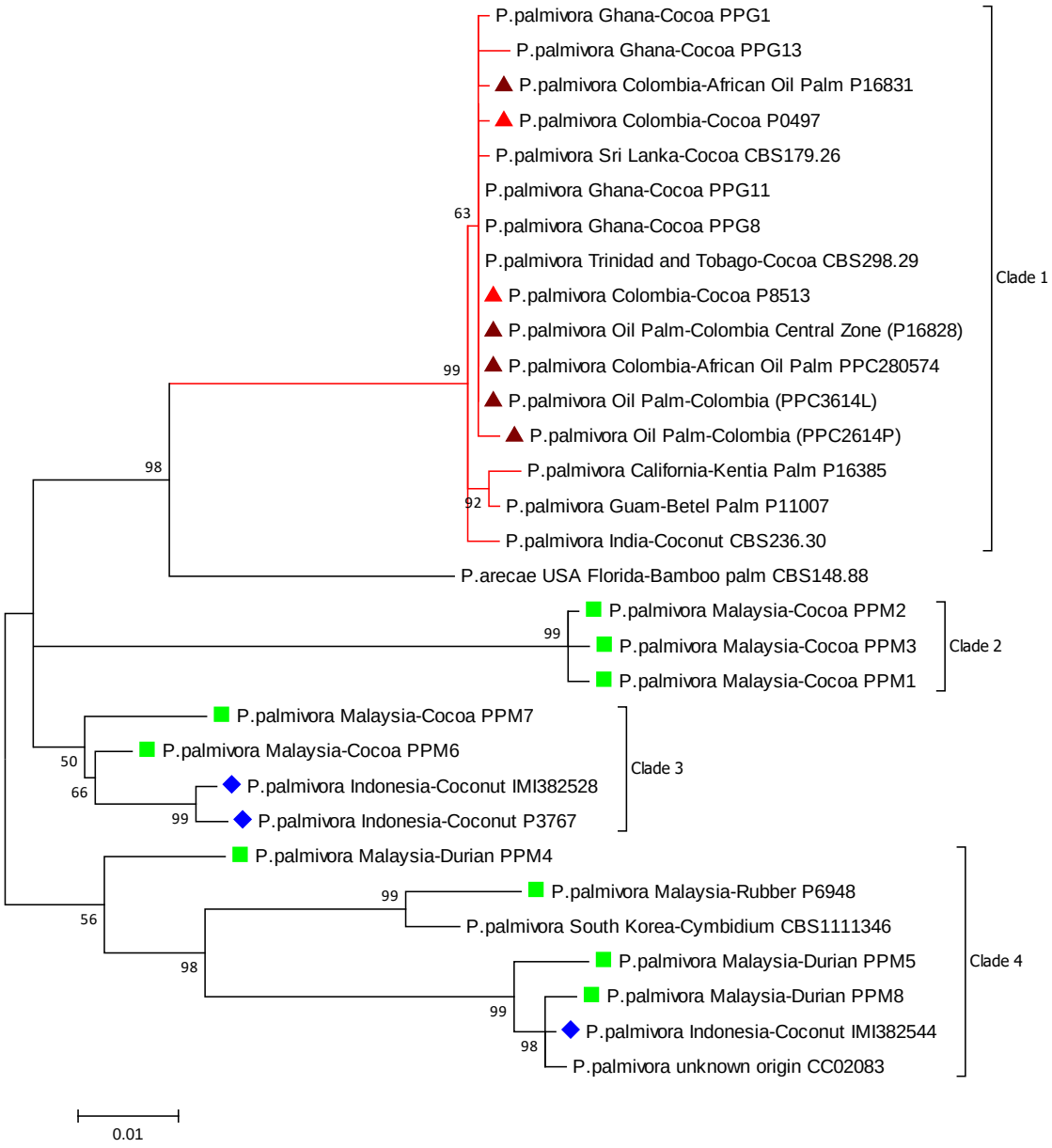


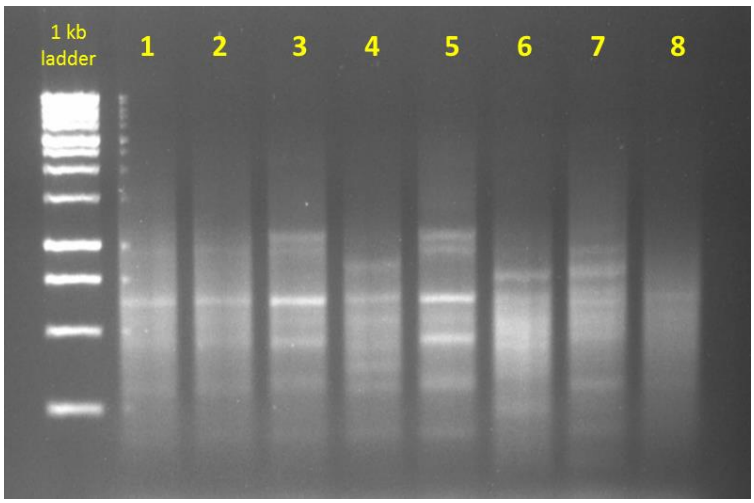
Figure 3-12 Molecular phylogenetic tree showing the relationship of* P. palmivora *pathogenic to oil palm obtained from bud rot disease hotspot zone in Colombia with other isolates of* P. palmivora *from different hosts and demographic origin constructed from our new PpHPAVR marker using maximum likelihood method based on the Tamura-Nei model.

Note: The tree with the highest log likelihood likelihood (-3080.8112) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site represented by the bar on bottom left. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches when ≥ 50. The labeled sub-clade (s) is/are with ≥50% bootstrap value.

3.3.4 Amplified fragment length polymorphism (AFLP)

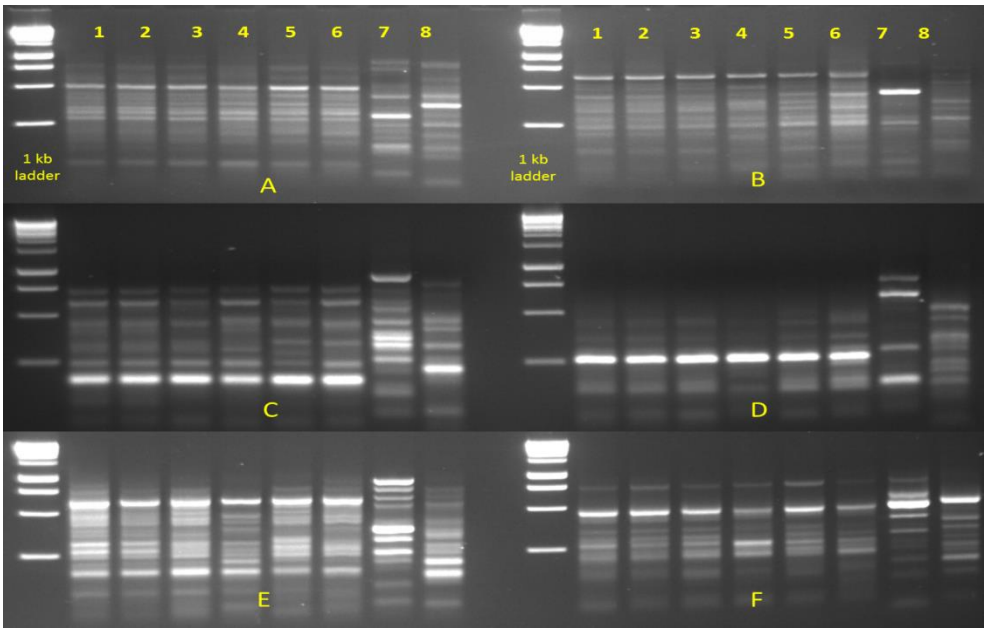
3.3.4.1 PCR amplification and capillary electrophoresis

Pre-amplification with *Eco*RI-universal primer and *Mse*I-universal primer produced smear-like unspecific bands for all the samples as expected (Figure 3-13). Most of the specific primer pair combinations showed good banding patterns on gel electrophoresis using 2% w/v agarose gels with clear polymorphic patterns observed between *P. palmivora* and other *P. infestans and P. parasitica* isolates (Figure 3-14), but only a few showed polymorphisms within the *P. palmivora* isolates tested, such as primer pairs *Eco*RI-A/*Mse*I-AG and the patterns obtained were observed to be consistent with the replicate set (Figure 3-15).



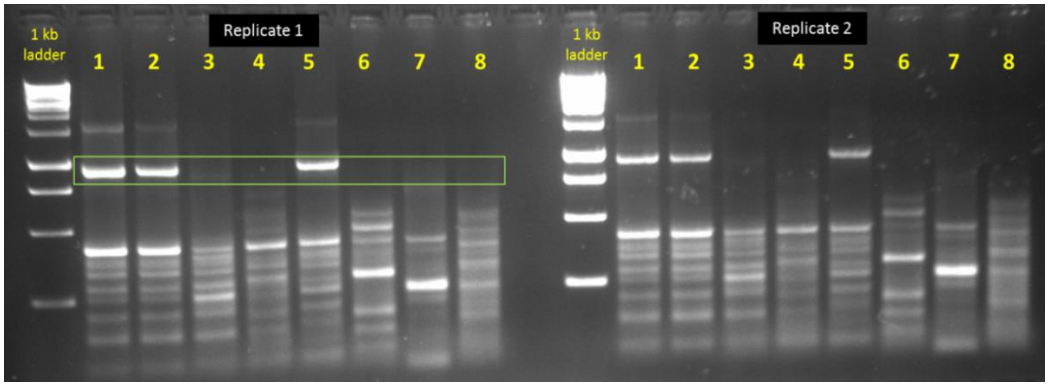
Lane 0: 1 kb ladder/marker
Lane 1: *P. palmivora* PPC280574
Lane 2: *P. palmivora* P16831
Lane 3: *P. palmivora* PPM2
Lane 4: *P. colocasiae* CBS358.30
Lane 5: *P. palmivora* PPM4
Lane 6: *P. parasitica* P19537
Lane 7: *P. infestans* 13-A2
Lane 8: *Fusarium oxysporum* MN1B

Figure 3-13 Electrophoresis of pre-amplification using EcoRI and MseI universal primers.



Lane 0: 1 kb ladder/marker
Lane 1: *P. palmivora* PPC280574
Lane 2: *P. palmivora* P16828
Lane 3: *P. palmivora* P16831
Lane 4: *P. palmivora* PPM2
Lane 5: *P. palmivora* P6948
Lane 6: *P. palmivora* PPM4
Lane 7: *P. parasitica* P19537
Lane 8: *P. infestans* 13-A2

Figure 3-14 Example of banding patterns obtained from gel electrophoresis on 2% w/v gel agarose with 7 µl of selective amplification using primer combinations (A) EcoRI-AC/MseI-TG, (B) EcoRI-AA/MseI-TG, (C) EcoRI-AC/MseI-CTC, (D) EcoRI-AA/MseI-CTC, (E) EcoRI-AC/MseI-TT, (F) EcoRI-AA/MseI-TT



Lane 0: 1 kb ladder/marker
Lane 1: *P. palmivora* PPC280574
Lane 2: *P. palmivora* P16831
Lane 3: *P. palmivora* PPM2
Lane 4: *P. colocasiae* CBS358.30
Lane 5: *P. palmivora* PPM4
Lane 6: *P. parasitica* P19537
Lane 7: *P. infestans* 13-A2
Lane 8: *Fusarium oxysporum* MN1B

Figure 3-15 Some polymorphic bands (highlighted green) observed between some Colombian and Malaysian isolates using primer combination EcoRI-A/MSeI-AG.

3.3.4.2 Capillary electrophoresis and phylogenetic analysis

A total of 31 sets of AFLP products were observed to have polymorphism within *P. palmivora* isolates based on the gel electrophoresis. AFLP products were separated using capillary electrophoresis (CE) which produces AFLP peak data such as in Figure 3-16. Based on the phylogenetic analysis of the CE data of all 31 sets, three selective primer pairs; *Eco*RI-A/*Mse*I-AG, *Eco*RI-AC/*Mse*I-AG and *Eco*RI-TA/*Mse*I-AG were found to have potential as suitable AFLP markers with good polymorphisms and were able to separate Colombian and Malaysian isolates into two distinct clades (Figure 3-17). The individual phylogenetic analysis of each combination involved 75, 121 and 149 random markers of polymorphic bands, using primers *Eco*RI-A/*Mse*I-AG, *Eco*RI-AC/*Mse*I-AG and *Eco*RI-TA/*Mse*I-AG, respectively. The concatenated tree of the three primer datasets involved 345 random markers (Figure 3-18). All trees had similar patterns in which the three Colombian isolates were grouped together in Clade 1, whilst the two Malaysian isolates were clustered in Clade 2. Other species were clearly distinguished as outgroups.

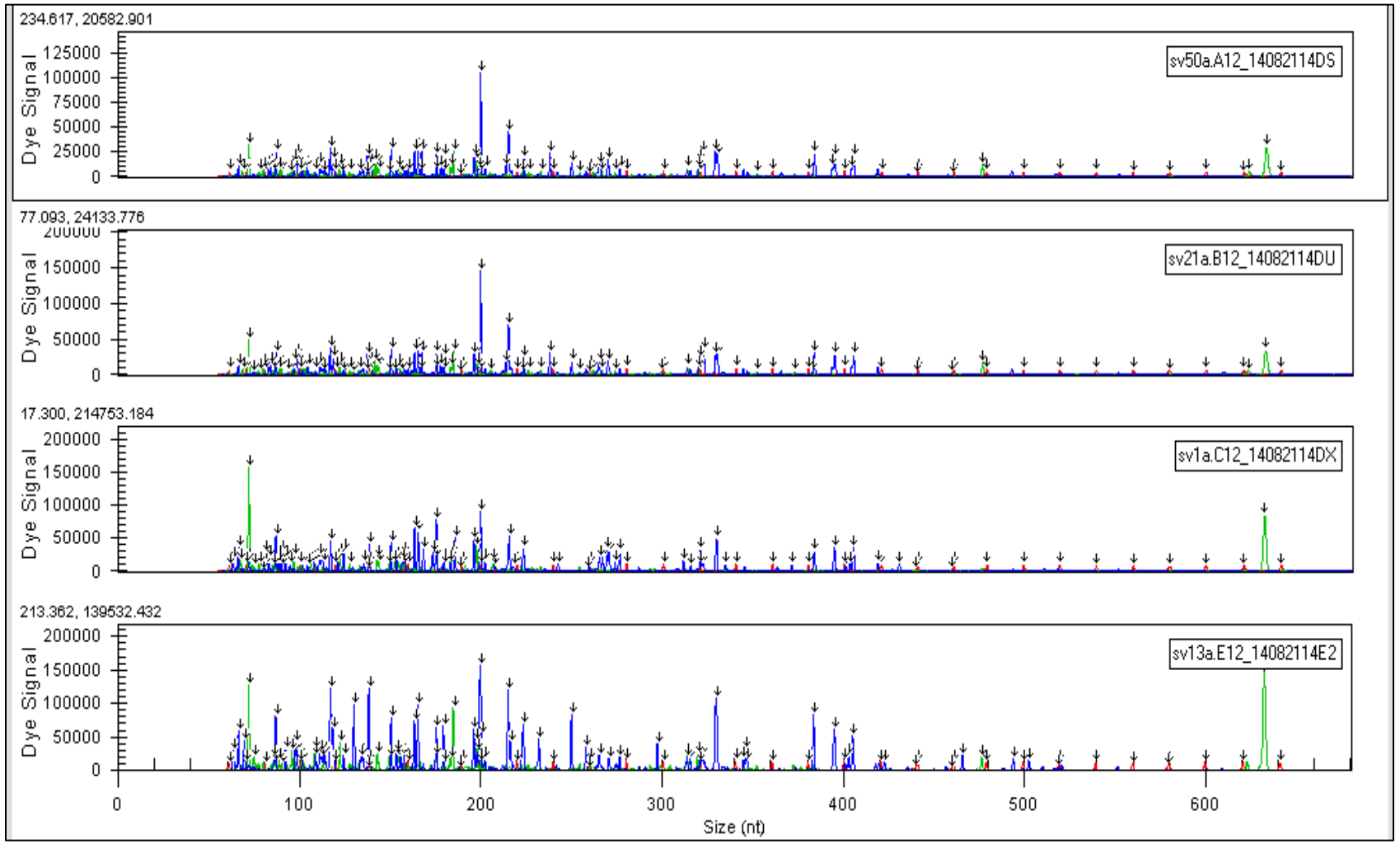
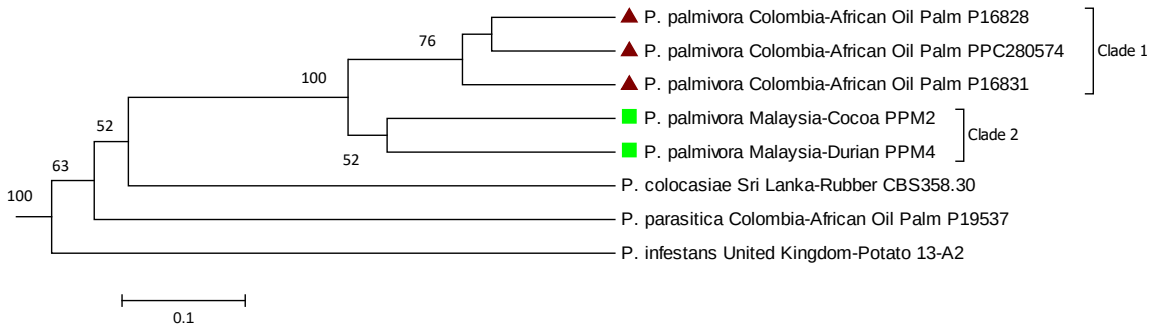
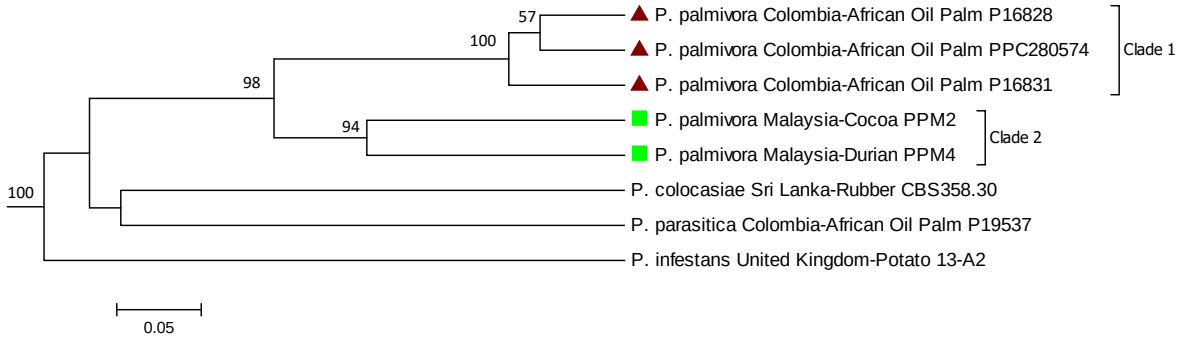


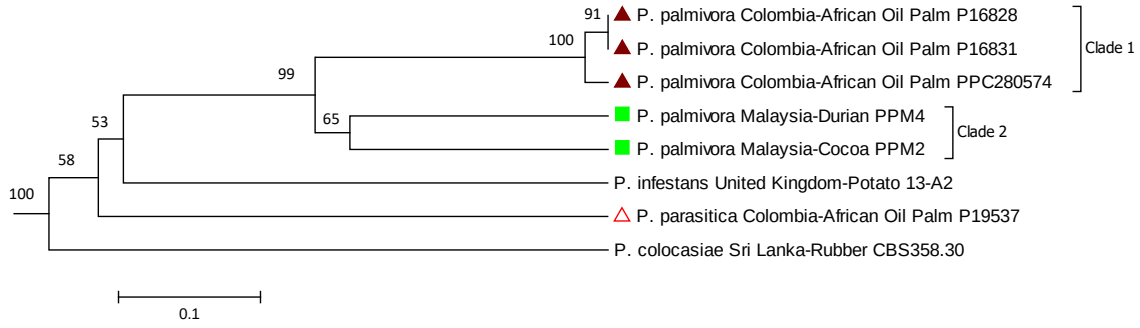
Figure 3-16 Example of AFLP DNA fingerprint data from four different* P. palmivora *isolates originating from Colombia and Malaysia; PPC280574 (Colombia-Oil palm), P16828 (Colombia-oil palm), PPM2 (Malaysia-cocoa) and PPM4 (Malaysia-durian) using primer pair EcoRI-TA/MseI-AG (tagged with D4 dye, indicated as blue peaks) and EcoRI-C/MseI-AG (tagged with D3, indicated as green peaks).



(a)



(b)



(c)

Figure 3-17 Phylogenetic tree constructed from AFLP data using primer a). EcoRI-A/MSeI-AG, b). EcoRI-AC/MseI-AG c). EcoRI-TA/MseI-AG

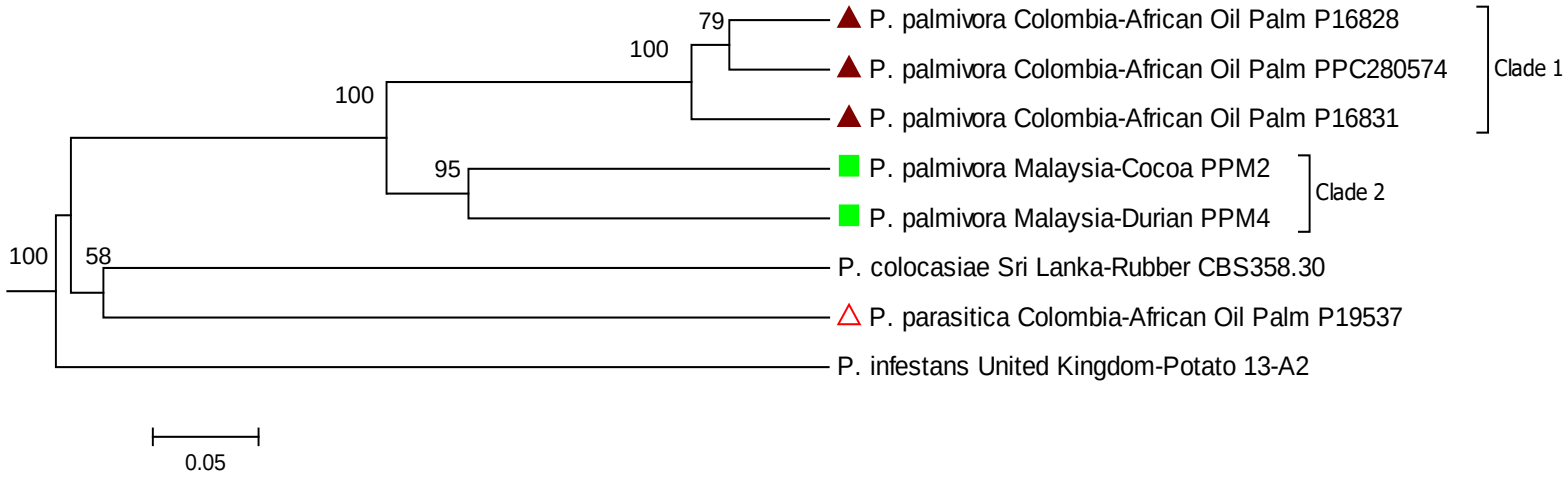


Figure 3-18 Phylogenetic tree constructed from concatenated AFLP data using three primer pairs of EcoRI-A/MSeI-AG, EcoRI-AC/MseI-AG and EcoRI-TA/MseI-AG.

3.4 Discussion

Studies on diversity, phylogenetic and polymorphisms among oomycetes, particularly *Phytophthora,* are not a new topic and have been carried out using various molecular tools including analysis of DNA sequences of target regions or genes. DNA sequences of rDNA gene clusters have been extensively used; however, work by Briard et al. (1995) found only minor variations in sequences of 28S rDNA for several *Phytophthora* species studied. The ITS, which is the non-coding spacer region between the 28S and 18S rDNA, has been shown to be more useful to study variation and phylogenetic relationships among species of *Phytophthora,* such as in the work of Lee and Taylor (1992) and Cooke and Duncan (1997) where high resolution of interspecific levels were achieved. However, intraspecific variations using this region are rather limited and rarely encountered (Sorensen et al., 1998), although not impossible for some species of *Phytophthora* and fungi. For example, Cohen et al. (2003) demonstrated some intraspecies variations and phylogenic separation of *P. citrophthora,* whilst Vinuesa et al. (2001) showed up to 16% variation for *Mycocalicium substantial* but only 1% for *M. albonigrum*.

In our study, variation at the intraspecific level within 26 isolates of *P. palmivora* from various host and demographic origins was not clearly observed in the DNA sequences of the ITS region. Analyses with 32 additional ITS sequences of *P. palmivora* obtained from GenBank® also showed similar findings. In addition, the small percentage of DNA nucleotide variations (0-3%) between some isolates might not be true ITS sequence variations but due to errors during PCR and sequencing even

though effort was taken to minimise such errors, for example by replicating the processes involved in obtaining the sequences. Apart from ITS, phylogenetic analyses of individual sequences from *CoxI, CoxII, β-tubulin* and *EF-1α* were carried out in order to avoid bias, since the evolution of one gene may not represent the entire genome (Villa et al., 2006). *Cox* genes of subunit I and II code for enzymes that catalyze the terminal step in the electron transport chain and are encoded in the mitochondria, which is considered generally to be more variable than nuclear DNA and has proven to be good for studying the relationship at the sub-generic level for various taxa (Villa et al., 2006). Phylogenetic relationships of the *Phytophthora* genus based on the *CoxI* and *CoxII* genes has been established by Martin and Tooley (2003a), whilst Villa et al. (2006) used *β-tubulin* data along with *ITS* and *CoxI*. Blair et al. (2008) used seven multi-locus markers (28S rDNA*, 60S ribosomal protein L10, β-tubulin, EF-α1, Enolase, heat shock protein 90* and *TigA gene fusion protein*) and found that *β-tubulin* provided the highest level of phylogenetic variation across the *Phytophthora* genus.

However, in this study, all the individual phylogenetic trees reconstructed using sequence data for *CoxI, CoxII*, *β-tubulin* and *EF-1α*, along with one additional marker, the *ras*-related protein gene, demonstrated similar findings to the ITS, with low intraspecific variations in DNA sequences. The trees did not exhibit consistent similarities in grouping based on demographic and host origin. Attempts were made to enhance the phylogenetic inference by combining or concatenating all five datasets, as combining sequences from multiple loci is theoretically expected to improve the phylogenetic analysis (Bininda-Emonds et al., 2001; Sanderson et al., 2003) and has been demonstrated in many studies such as Bapteste et al. (2002), Kroon et al. (2004), Martin and Tooley (2003a) and Blair et al. (2008). The multi-locus tree

constructed from the five loci also showed no clear separation of *P. palmivora* isolates based on demographic origin and host. Malaysian and Colombian isolates were not separated into different clusters and the sub-clusters did not show any meaningful characteristics based on host and origin of the isolates. From this study, it can be concluded that these five molecular markers are more suitable for inter-specific studies between species but not for intra-specific evaluation within species of *P. palmivora.*

The findings from the analyses of six loci showed that there were no differences between sequences of Colombian isolates pathogenic to oil palm with other isolates, particularly Malaysian and Indonesian isolates. Nevertheless, as mentioned before, the loci studied may not represent the whole genome. Whole genome sequencing using next generation sequencing (NGS) would give us more insight into the genetic variation within species of *P. palmivora* but this method is expensive; therefore, in this study the AFLP fingerprinting technique was used since the key feature of this method is the ability to simultaneously screen many DNA regions distributed randomly throughout the genome. However, AFLP bands are dominant markers, so they cannot differentiate homologous alleles, making it less useful for studies that involve allelic states such as heterozygosity analyses (Mueller and Wolfenbarger, 1999).

In this study, AFLP analyses were only carried out using some representative isolates of *P. palmivora* from Colombia and Malaysia, since the main objective was to study the variation between isolates obtained from these two regions. From a total of 64 primer pairs screened, three primer pairs showed a good separation of Malaysian and Colombian isolates. This indicates that there is genomic variation within *P. palmivora* isolates. It would have been useful to extend the AFLP analyses using these primers to other *P. palmivora* isolates to see the phylogenetic relationship of all

isolates; however, we were unable to conduct the assay due to time constraints. Based on the AFLP results it was decided to try exploring other loci that might be potentially useful as molecular markers to study variations among Colombian isolates and Malaysian isolates, and one region of interest was the gene clusters or regions encoding effector/avirulence proteins that are involved in the infection process and colonization of plant tissue. The genome sequencing of *Phytophthora* species such as *P. infestans* has revealed a diverse and large class of effectors (Bozkurt et al., 2012) such as AVR3a (Armstrong et al., 2005; Bos et al., 2009), AVR1b (Shan et al., 2004) and PiAVR4 (van Poppel et al., 2008; van Poppel, 2009). The effector proteins can be targeted to the apoplast (the space outside plant cell membranes)(apoplastic effectors) or translocated into the host cells (cytoplasmic effectors)(Bozkurt et al., 2012; Wawra et al., 2012)..

The effector proteins are secreted by the oomycetes to suppress the immune responses of the host plant (such as pathogen associated molecular patterns (PAMPs) trigger immunity (PTI) triggered by their own elicitors. For example *P. infestans* effector AVR3a suppresses perception of the PAMP INFI through stabilization of the U-box protein CMPG1 (Fawke et al., 2015). The AVR3a protein is encoded by avirulence gene, *Avr3a* and belongs to a large, oomycete-specific family of highly divergent effectors that share a conserved domain named RXLR-dEER (Tyler et al., 2006) which triggers disease resistance and the hypersensitive response (HR) (Armstrong et al., 2005). The corresponding resistance *R* gene of the host plant to *Avr3a* is the *R3a*, and R proteins generally activate resistance responses effector-triggered immunity of the plant host (ETI). The *P. infestans Avr4* (PiAvr4) sequences used as the reference to design the primers for the new marker PpHAVR in this study encodes a typical oomycete RXLR effector molecule (van Poppel et al., 2008).

Although the primers did not amplify *P. palmivora* under optimal PCR conditions, it was possible to get faint bands at low annealing temperatures which were then sequenced, and this has been termed the PpHPAVR sequence. New primers were then designed from the sequences that could amplify all *P. palmivora* isolates in this study. The PpHPAVR sequences did not match closely to DNA or protein sequences in the GenBank® database, probably because whole genome sequencing and studies on effector proteins and avirulence genes of *P. palmivora* have not yet being published and are still on going.

Although the nature of the PpHPAVR sequences is vague, the locus was shown to have some intraspecific variation within *P. palmivora* species, at least between Colombian and Malaysian isolates. Phylogenetic analyses using PpHPAVR sequences separated all the Colombian isolates into one clade along with other isolates except isolates from Malaysia, Indonesia and South Korea, which were clustered in separated clades. However, these three clades did not show any other characteristics based on host and origin, but the phylogenetic observation suggested that they share common ancestry. It will be interesting to explore further isolates from South East Asia such as Thailand, the Philippines and Myanmar to confirm the distinct nature of SE Asian isolates.

Chapter 4. Development of molecular detection and diagnostic techniques for *P. palmivora*

4.1 Introduction

Plant diseases are inevitable problems to all crops. Nevertheless, a good biosecurity and contingency plan can usually help mitigate the problems. Efficient diagnosis and detection of plant diseases and the pathogens is crucial in the management of plant diseases. Plant diseases can be managed most effectively if the control measures (such as eradication and containment) are introduced at an early stage of the disease development to prevent and limit the spread before it causes significant economic losses (Miller and Martin, 1988; Tomlinson et al., 2010). Conventionally, plant diseases can be diagnosed and monitored by physically examining the symptoms of disease or pathogen in the plant, microscopic examination of diseased material and isolation of the pathogens from infected plant tissues, followed by pure-culturing of the pathogen and identification based on morphological characteristics. Baiting techniques are also widely explored for detection of the genus *Phytophthora* especially from soils. These biological techniques are usually accurate but are time-consuming and need to be carried out by highly trained and experienced personnel with specialization in taxonomical identifications.

Advances in developments of molecular biology offer rapid, robust, specific and sensitive tools for detection of plant pathogens (Ward et al., 2004) including of the genus *Phytophthora*. Some antibody-based methods have been developed over the past three decades, such as dipstick immunoassays to detect *P. cinnamomi* (Cahill and

Hardham, 1994) and a commercial *Phytophthora F* kit (Ellis and Miller, 1993), along with nucleic acid based assays such as dot blot hybridization assays using specific DNA probes (Goodwin et al., 1990), lab-on-a-chip DNA hybridization arrays (König et al., 2015), conventional polymerase chain reactions (PCR) and other PCR-based methods. PCR-based detection methods have been used extensively due to their sensitivity, specificity, speed and high sample throughput (Martin et al., 2000; Mumford et al., 2006). PCR (conventional and PCR-based techniques) have the potential to detect single copies of the target gene contained in single propagules (Lee and Taylor, 1990) and are widely reported as effective detection methods for plant pathogens including *Phytophthora*, which can potentially detect to the genus, species or strain level depending on the specificity of the primers designed.

Recently, a novel nucleic acid amplification technique called loop-mediated isothermal amplification (LAMP) has been developed which has gained momentum in the field of diagnostics. The robustness of LAMP has been proven in various studies with outstanding results when compared to other pre-existing molecular techniques (Abdullahi et al., 2015). LAMP can amplify a few copies of DNA to 10^9 in less than an hour under isothermal conditions. The LAMP reaction involves a special DNA polymerase (commonly *Bst* DNA polymerase) with displacement activity which displaces and releases a single stranded DNA and a minimum of four to six oligonucleotide primers consisting of two inner primers (namely, forward inner primer (FIP) and backward inner primer (BIP), two outer primers (F3 and B3) (Notomi et al., 2000) and additional loop primers (loop B and Loop F) that bind to those loops for the internal primers to bind and accelerate the amplification (Nagamine et al., 2002; Tomlinson et al., 2010).

The reaction starts with the annealing of the inner primer FIP to the target DNA template which initiates the synthesis of a new strand complementary to template DNA. The outer primer F3 then hybridizes to the target DNA template and initiates a strand displacement DNA synthesis, releasing the first complementary strand with the FIP tail. This FIP-tailed stand served as template for a new DNA stand synthesis initiated by BIP, followed by stand displacement DNA synthesis initiated by B3. The BIP-initiated strand will have two tails FIP and BIP which then form stem-loop structures that look like dumbbells which act as templates for subsequent LAMP cycles where one inner primer hybridizes and initiates DNA synthesis with strand displacement that produces new stem-loop DNA and a new stem-loop DNA with a stem twice as long. The final products are the stem-looped DNAs with several inverted repeats of the target DNA and with multiple loops (Nagamine et al., 2002; Njiru et al., 2008).

Since the reaction is carried out at an isothermal temperature ranging from 60°C to 65°C, the need for expensive thermo-cyclers can be omitted. The reaction can simply be done using a heat block or water bath or simple heating device allowing the possibility of real-time diagnostic in field. LAMP reactions can be observed by gel electrophoresis or visualization/measurement of turbidity caused by the formation of magnesium pyrophosphate precipitate as a by-product of the positive amplification process (Mori et al., 2001). The turbidity of the solution in highly correlated with the amount of DNA synthesized and can be quantified by a turbidimeter (Mori et al., 2004) or calorimetrically with the help of metal indicators such as hydroxyl naphthol blue (Goto et al., 2009), calcein (Tomita et al., 2008) or DNA intercalating dyes such as SYBRGreen or PicoGreen such as used in the OptiGene real-time LAMP system. OptiGene's Isothermal Mastermix, together with the OptiGene LAMP instrument, Genie

II or Genie III provides a real-time LAMP assay based on a fluorescence detection system, which allows the LAMP amplification to be monitored in real-time, based on the detection of the fluorescent dye incorporated into double stranded DNA generated during the amplification allowing direct detection of dsDNA (Bekele et al., 2011) not detection of magnesium pyrophosphate. This detection system works in the same way as real-time PCR and produces similar amplification plots as real-time PCR (Tomlinson, 2013) which potentially allows us to do quantification based on prepared standard curves (Kawicha, 2014). This system eliminates the need for gel electrophoresis or turbidity detection and allows a closed-tube system reducing cross contamination of samples.

LAMP assays are considered inexpensive, simple yet rapid diagnostic tools for detection (Almoammar et al., 2013) of microorganism such as pathogens/diseases of animals (Luo et al., 2013), humans (Parida et al., 2008; Moslemi et al., 2009; Surabattula et al., 2013), plants (Zhao et al., 2012; Tomlinson et al., 2013; Luo et al., 2014; Duan et al., 2015), as well as pathogenic and allergenic microbes in the environment (Cao et al., 2010; Sun et al., 2010) ranging from bacteria, protozoa, virus, fungi, phytoplasma, as well as oomycetes. Both conventional PCR and LAMP are also being used as diagnostic tools to detect plant pathogens of the genus *Phytophthora.* Many studies on the development of techniques for identification, detection and diagnosis of genus *Phytophthora* up to species level have been successfully undertaken using conventional PCR and other PCR-based methods and LAMP assays, but not many studies have been done on *P. palmivora* particularly on oil palm. This chapter aims to develop species specific detection tools for *P. palmivora*, and produce a diagnostic technique to differentiate between Colombian and Malaysian isolates using both conventional PCR and/or LAMP assays.

4.2 Materials and methods

4.2.1 DNA extraction

All DNA extractions of *Phytophthora*, *Pythium*, and fungi were carried out using DNeasy Plant Mini Kit (Qiagen) as described in Section 2.2.4. Concentration of DNA obtained was determined using a Thermo Scientific NanoDrop™ 2000 spectrophotometer. All DNA was subjected to dilution of 1:10 using TE buffer prior to all assays.

4.2.2 Polymerase Chain Reaction (PCR)

4.2.2.1 Primer design

Three primers AVR1F/R, AVR2F/R and AVR3F/R were designed from the initial sequence of PpHPAVR of the oil palm Colombian isolate PPC280574, which originated from the bud rot outbreak zone of Tumaco, Colombia (see Chapter 3), using the Primer BLAST tool from NCBI (*http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi*). Sequences of several *P. palmivora* isolates, particularly Colombian and Malaysian, amplified by primer pair AVR1F/AVR1R were aligned to design additional primers that might help to distinguish Malaysian and Colombian isolates using either the Primers BLAST tools, manually handpicked or a combination of both methods. Sequence alignments were performed using *MEGA 6.0.6* software, by ClustalW. Details of all the primers are as in Table 4-1. All primers were synthesis by Sigma Aldrich, UK.

Table 4-1 Details of the designed primers

Primer	Sequence (5'-> 3')	Template	Template strand	Length	Start	Stop	GC%
ARP1F	TTCGACGGAATAGCCCATCC	EF672355.1	Plus	20	1394	1413	55
ARP1R	GCAGTAAAAGCGGAATGACGG		Minus	21	2384	2364	52.38
AVR1F	AATGACGGCTTCTGCGTTTG	PpHPAVR-PPC280578	Plus	20	14	33	50
AVR1R	GGCGTGACTACAGAGTGTCC		Minus	20	992	973	60
AVR2F	GCACCTCAGTCAGGTAAGCC		Plus	20	105	124	60
AVR2R	TTCGACGGAATAGCCCATCC		Minus	20	1044	1025	55
AVR3F	CATGACAGTCGCACCTCAGT		Plus	21	183	203	42.86
AVR3R	TGCAGCCATAGCACCTTTCA		Minus	21	952	932	57.14
AVM1F	GATAYAGCATAAGCTATYCAG	PpHPAVR-AV-PPM1	Plus	21	183	203	42.86
AVM1R	RATCGGCTTYACGCGTTCAAG		Minus	21	952	932	57.14
AVM2F	GTKGCCGWAGAYGRTGTCAATG		Plus	22	390	411	54.55
AVM2R	CCGGYCAATTTCRGTTCATC		Minus	20	684	665	50
AVM3F	CAAGCCAGAAGCGGTAAGTCTGG		Plus	23	597	619	56.52
AVM4F	GATGAACYGAAATTGRCCGG		Plus	20	665	684	50
AVM5F	CTAGACGCACAAGCCAAGGA		Plus	20	869	888	55
AVM5R	CACGCGTTCAAGTTGGGAAG		Minus	20	943	924	55
AVM6F	GCACATTCGAGCAAGACGAC		Plus	20	541	560	55
AVM6R	CTTYACGCGTTCAAGTTGRG		Minus	20	946	927	55
AVM7F	GGGACGCGTTGGATGTATCT		Plus	20	460	479	55
AVM7R	GTCGTCTTGCTCGAATGTGC		Minus	20	560	541	55
AVM8F	CCAGAAGCGGTAAGTCTGGG		Plus	20	601	620	60
AVM8R	ACCGAGGCGGTACTTATTGC		Minus	20	829	810	55

4.2.2.2 Amplification and validation of primer specificity

Amplifications with all the designed primers were done using the same protocol as described in section 2.2.5.1. The annealing temperature and the amplification cycles of each primer pair are summarized in Table 4-2. The amplifications were carried out with the genomic DNA of *Phytophthora* isolates and other organisms. In addition, the amplifications were also carried out with genomic DNA of soil fungi obtained from Malaysian oil palm plantations (see Chapter 2) to check for primer specificity and cross-reactions. Amplification using ITS1 and ITS4 universal primers (White et al., 1990) were carried out as controls and references in some of the assays.

Table 4-2 Annealing temperature and number of PCR cycles used for amplification with each primer pair

Primer		Annealing Temperature (°C)	Amplification cycles
Forward	**Reverse**		
ARP1F	ARP1R	60.0	35
AVR1F	AVR1R	60.0	35
AVR2F	AVR2R	60.0	35
AVR3F	AVR3R	60.0	35
AVM1F	AVM1R	60.0	30
AVM3F	AVM1R	68.9	35
AVM2F	AVM1R	68.9	35
AVM4F	AVM1R	64.4	35
AVM1F	AVM2R	68.9	35
AVM2F	AVM2R	68.9	35
AVM3F	AVM2R	68.9	35
AVM4F	AVM2R	68.9	35
AVM5F	AVM5R	60.9	30
AVM6F	AVM6R	60.9	30
AVM7F	AVM7R	67.1	30
AVM8F	AVM8R	67.1	30

4.2.3 Loop-mediated isothermal amplification (LAMP)

4.2.3.1 Primer design

Several sets of LAMP primers, each set containing internal (F3, B3), external (FIP, BIP) and loop (loop-F, loop-B) primers, were designed using the LAMP Designer software provided by OptiGene Limited (Table 4-3). All primers were synthesised by Sigma Aldrich, UK.

***Table 4-3 LAMP primers designed from several regions of* P. palmivora**

Primer set	Primer	Sequence	Target region
PPALML	F3	GACATCTTCCTGTGTCACTAC	Cellulose synthase (JN561775.1)
	B3	CACGTCTTCAAGCTCTGG	
	FIP	GAAGTAGCTCGGGAGGGTACTGGAACCTGTTACGGATTCAA	
	BIP	TACACCAAGTCCGTTTGGGATGTCTCCAGCAATCTCAATAACC	
	LOOP-F	GCATGGCGAGACAGTTCT	
	LOOP-B	CAAGGTGACGGTGAACACTA	
AVRL1	F3	GTGGGTGTCTTTGACTCG	PpHPAVR (PPC280574)
	B3	AACCAAGACTTACCACTTATGG	
	FIP	CCGTCGTCAGCATCTTCTTCATGGACCAGTGACCGTAGAT	
	BIP	GAGTGACGTTGACTCCGATGGGTCGTCTTGCTCGAATGT	
	LOOP-F	CGTCACTTATCATCTCCTCACC	
	LOOP-B	TTCGAAGACGACAGCGAC	
AVRL2	F3	TGGCTGCATATTGCTGAC	
	B3	CCACAGTGCTTCTCCAAG	
	FIP	TCGAAGGGTCCTTGGTTTGTGTACCAGTTGATGCCGAGA	
	BIP	AGAGTTCAGCCTCTCGCAGATAACATGCAGAACTTCGTGAG	
	LOOP-F	TCTAGCTGCTTCATGCGAAT	
	LOOP-B	CCAACTTGAACGTGCGAAG	
AVRL3	F3	AAAGTCTCCGTTAGGAATGTTT	
	B3	CCACAGTGCTTCTCCAAG	
	FIP	TCGAAGGGTCCTTGGTTTGTGCCTCGGTCGTATAACCAGT	
	BIP	AGAGTTCAGCCTCTCGCAGATAACATTGCAGAACTTCGTGAG	
	LOOP-F	TCTAGCTGCTTCATGCGAAT	
	LOOP-B	CCAACTTGAACGTGCGAAG	
AVML1	F3	GATGTCAATGTGGGTGAGG	PpHPAVR (PPM1)
	B3	ACCGATTGTAGAAATCAGTAGC	
	FIP	AATGTGCCGCTGTCGTCTTTGCAGCATCTCTGGAGTA	
	BIP	CAAGACGACGATGCCACGACTGTATAACCCAGACTTACCG	
	LOOP-F	GAAGTTGCCATCGGAGTCA	
	LOOP-B	ATCTTCGACCAAGCCAGAAG	
AVML2	F3	CAGGGAACAGTTGTTTGGA	
	B3	CTGGCTTGGTCGAAGATG	
	FIP	CGCCGTCGTCAGCATCTTACGATGTCAATGTGGGTG	
	BIP	TCTCTGGAGTACGAGAGTGACGTCGTCTTGCTCGAATGTG	
	LOOP-F	CATCGTCGCTTATCATCTCCT	
	LOOP-B	TGACTCCGATGGCAACTTC	
AVCL1	F3	GTGGGTGTCTTTGACTCG	PpHPAVR (PPC280574)
	B3	AACCAAGACTTACCACTTATGG	
	FIP	CCGTCAGTCAGCATCTTCTTCATGGACCAGTGACCGTAGAT	
	BIP	CGAGAGTGACGTTGACTCCGCGTCTTGCTCGAATGTGT	
	LOOP-F	CGTCACTTATCATCTCCTCACC	
	LOOP-B	GCGACTTCGAAGACGACA	

4.2.3.2 Reaction mixtures and optimal conditions

All primers were diluted to make 100 µM stocks. Primer cocktails were freshly prepared by mixing 152 µl sterile distilled water, 4 µl primer B3, 4 µl F3 and 20 µl each of primer BIP, FIP, LoopF and LoopB. Reaction mastermixes were then prepared by mixing 115 µl of GspSSD Isothermal Mastermix (ISO-001) (OptiGene Limited, UK), 46 µl sterile distilled water, and 23 µl of primers cocktail. Twenty microlitres of the reaction mastermix was then aliquot into each of 8 tubes. The amplification was performed in 20 µl volume consisting of 1x concentration of Isothermal Mastermix (OptiGene Limited, UK) with 1 µl of DNA sample. The final concentration of primers in the reaction was 0.2 µmol/µl for primer B3, 0.2 µmol/µl F3 and 1 µmol/µl of each FIP, BIP, LoopB and LoopF. The loop mediated isothermal amplifications were carried out using either a Genie II or Genie III (OptiGene Limited, UK) at 65°C for 30 min and results were analysed based on T_p values (the time taken to generate a positive result) and annealing temperature of amplicons as described by Kawicha (2014) and Bekele et al. (2011). The LAMP assays were initially tested with genomic DNA of some *P. palmivora* isolates (mainly Malaysian and Colombian), including some other species of *Phytopthora* and non *Phytophthora* (soil fungi) to check for cross reactivity. Primer sets with potential were then tested with all isolates available in this study.

4.2.4 Detection and diagnosis of diseased samples

One hundred mg of diseased tissue of oil palm leaves artificially inoculated with *P. palmivora* (Table 4-4) were weighted and keep in a 2 ml screwed cap tube containing approximately 15-20 glass beads with the diameter of 2 mm and 4 mm. The tubes containing sample were submerged in liquid nitrogen for 10-20 min prior to grinding

using FastPrep® (QBiogene) homogenizer at a speed of 6.5 rpm for 45 s repeated at least thrice. Extraction of genomic DNA was carried out using DNeasy Plant Mini Kit (Qiagen). Amplifications of the samples were carried out using both PCR and LAMP methods using primers AVR1F/AVR1R and ITS1/ITS4 for PCR and primer sets AVR2 and AVM2 for LAMP amplification, including a control primer targeting plant *Cox* (Tomlinson et al., 2010) as reference and control. A preliminary study to see if both assays can detect *P. palmivora* in soil samples was carried out by spiking the soil with mycelia of PPC280574 scraped from the surface of a carrot agar culture and extracted using E.Z.N.A.® soil extraction kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and DNeasy Plant Mini Kit (Qiagen) following the manufacturers protocol; some samples were further cleaned with polyvinylpolypyrrolidone (PVPP) spin columns as suggested by Berthelet et al. (1996) (Table 4-5). The conditions for amplifications by PCR and LAMP were as described earlier.

Table 4-4 Leaf samples tested using PCR and LAMP assays

Sample	Inoculation technique	Sample type and location of tissue taken	Pre-extraction treatment
A1	Seedling + *P. palmivora* isolate P16835	diseased tissue, marginal, upper section	none
A2		diseased tissue, lesion zone, upper section	
B1		diseased tissue, marginal, lower section	
B2		diseased tissue, lesion zone, lower section	
C1		diseased tissue, marginal, upper section	Surface sterilization with 70% ethanol followed by 2x sterile distilled water
C2		diseased tissue, lesion zone, upper section	
D1		diseased tissue, marginal, lower section	
D2		diseased tissue, lesion zone, lower section	
E	Seedling+sterile distilled water	Healthy tissue (no lesion), inoculation site	none
74-s1	leaf detached + *P. palmivora* isolate PPC280574	Diseased tissue (random)	
74-s2		Diseased tissue (random)	
Ctrl-s1	leaf detached+ sterile distilled water	Healthy tissue (no lesion), inoculation site	
PPM1	Pure culture	DNA extracted from pure culture of PPM1	

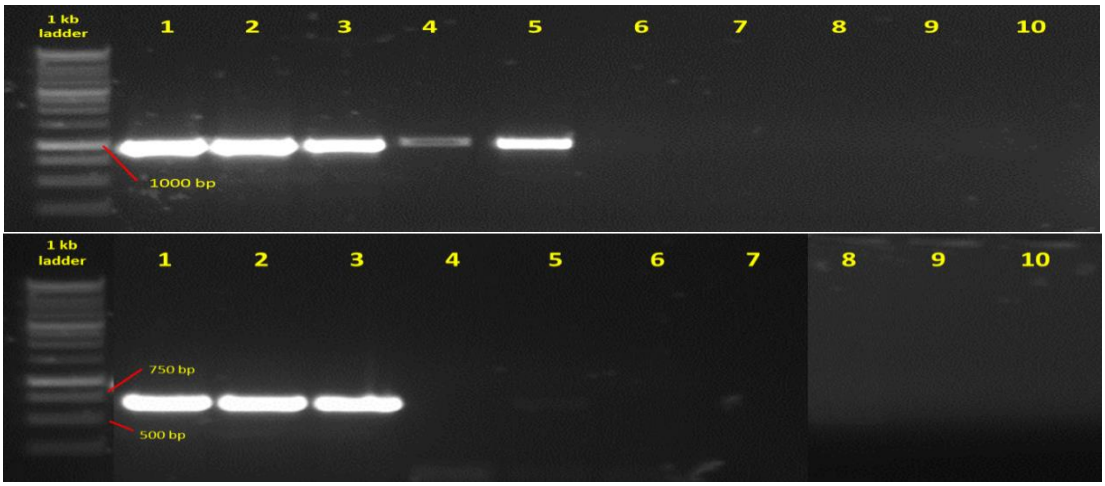
Table 4-5 DNA extraction methods of soil samples tested with PCR and LAMP assays

Samples	Description
S1	Soil+mycelial of PPC280574, extracted with soil DNA kit
S2	Same as S1 with further clean up with PVPP column
S3	Soil+mycelial of PPC280574, extracted with plant DNA kit
S4	Same as S3 with further clean up with PVPP column

4.3 Results

4.3.1 Polymerase Chain Reaction (PCR)

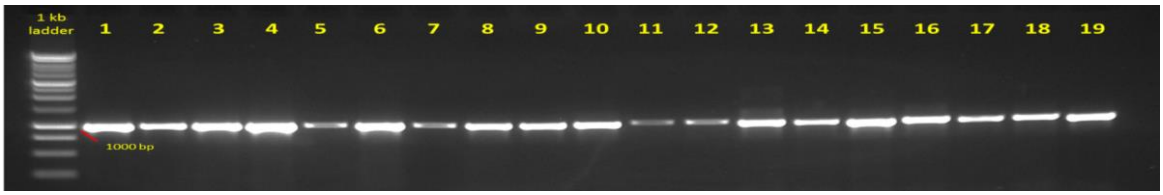
First amplifications with the primers AVR1 and AVR3 using genomic DNA of some Malaysian and Colombian isolates of *P. palmivora* produced PCR fragments of approximately 1000 bp and 700 bp, respectively (Figure 4-1). Primer pair AVR1 amplified all six *P. palmivora* isolates, but amplified none of the other *Phytophthora* species tested; *P. parasitica*, *P. infestans*, *P. cryptogea* and *P. megakarya*. Neither set of primer pairs amplified *Pythium aphanidermatum*, a species belonging to a genus that is closely related to *Phytophthora*. However, primer pair AVR3 did not amplify the three Malaysian isolates of *P. palmivora* (PPM2, PPM5 and P6948) and just amplified the three Colombian isolates. No amplifications were observed with primer pair AVR2.



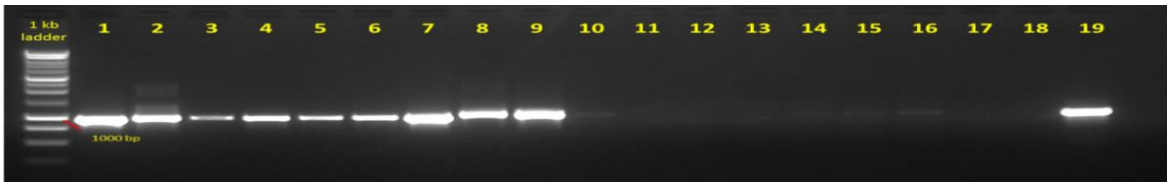
Lane 0:	1 kb ladder/marker	Lane 5:	*P. palmivora* P6948 (Malaysia)
Lane 1:	*P. palmivora* PPC280574 (Colombia)	Lane 6:	*P. parasitica* P19538 (Colombia)
Lane 2:	*P. palmivora* P16828 (Colombia)	Lane 7:	*P. infestans* 13-A2
Lane 3:	*P. palmivora* P16831 (Colombia)	Lane 8:	*Pythium aphanidermatum* PHY01
Lane 4:	*P. palmivora* PPM2 (Malaysia)	Lane 9:	*P. cryptogea* PC01
Lane 5:	*P. palmivora* P6948 (Malaysia)		

Figure 4-1 First round amplifications using primers AVR1 (top) and AVR3 (bottom)

After several amplifications, it was observed that primer pair AVR1 amplified all 31 *P. palmivora* isolates (Figure 4-2). Meanwhile, primer pair AVR3 was observed to have selective amplifications on *P. palmivora* isolates. Both primer pairs were shown to amplify none of the other species of *Phytophthora* in this study, namely *P. parasitica* isolate P19537 and P19538, *P. colocasiae* CBS358.30, *P. megakarya* isolates PPG4 & PPG12, *P. cryptogea* PC01, *P. infestans* 2009-7654A and *P. citrophthora* CBS581.48, showing a potential as species specific primers. No cross-amplification was observed with a *Pythium* species (*Pythium aphanidermatum* PYT01) and several soil fungi; *Mortierella echinosphaera, M. chlamydospora, Gongronella butleri, Trichoderma asperellum, Fusarium* sp*., Fusarium oxysporum, Purpureocillium lilacinum,* and *Talaromyces aculeatus* (Figure 4-3).



Lane 0: 1 kb ladder/marker
Lane 1: *P. palmivora* PPC280574 (Colombia)
Lane 2: *P. palmivora* P16828 (Colombia)
Lane 3: *P. palmivora* P16831 (Colombia)
Lane 4: *P. palmivora* P8513 (Colombia)
Lane 5: *P. palmivora* P0497 (Colombia)
Lane 6: *P. palmivora* CBS298.290 (Trinidad & Tobago)
Lane 7: *P. palmivora* P16385 (California, USA)
Lane 8: *P. palmivora* PPC2614P (Colombia)
Lane 9: *P. palmivora* PPC3614L (Colombia)
Lane 10: *P. palmivora* PPM1 (Malaysia)
Lane 11: *P. palmivora* PPM2 (Malaysia)
Lane 12: *P. palmivora* PPM3 (Malaysia)
Lane 13: *P. palmivora* PPM4 (Malaysia)
Lane 14: *P. palmivora* PPM5 (Malaysia)
Lane 15: *P. palmivora* P6948 (Malaysia)
Lane 16: *P. palmivora* IMI382528 (Indonesia)
Lane 17: *P. palmivora* IMI382544 (Indonesia)
Lane 18: *P. palmivora* P3767 (Indonesia)
Lane 19: *P. palmivora* CBS179.26 (Sri Lanka)



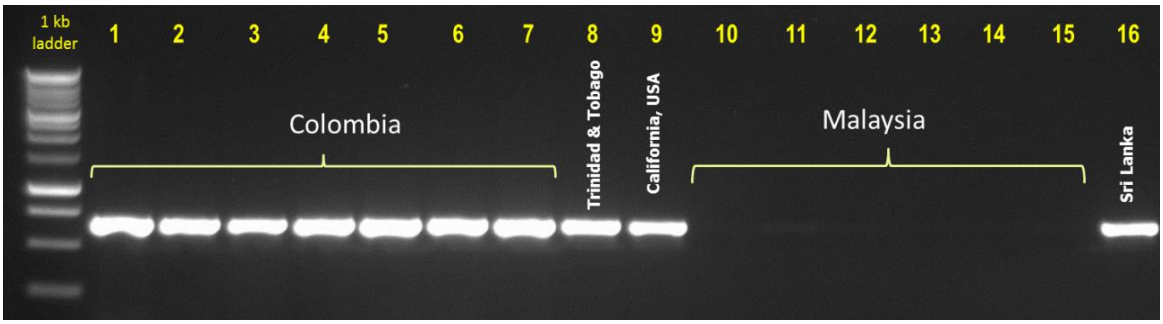
Lane 0: 1 kb ladder/marker
Lane 1: *P. palmivora* CBS236.30 (India)
Lane 2: *P. palmivora* CBS111346 (South Korea)
Lane 3: *P. palmivora* P11007 (Guam)
Lane 4: *P. palmivora* PPG1 (Ghana)
Lane 5: *P. palmivora* PPG8 (Ghana)
Lane 6: *P. palmivora* PPG11 (cocoa-Ghana)
Lane 7: *P. palmivora* PPG13 (cocoa-Ghana)
Lane 8: *P. palmivora* PPM6 (Malaysia)
Lane 9: *P. palmivora* CBS148.88 (Florida, USA)
Lane 10: *P. parasitica* P19537 (Colombia)
Lane 11: *P. parasitica* P19538 (Colombia)
Lane 12: *P. colocasiae* CBS358.30 (Sri Lanka)
Lane 13: *P. megakarya* PPG4 (Ghana)
Lane 14: *P. megakarya* PPG12 (Ghana)
Lane 15: *P. cryptogea* PC01
Lane 16: *P. infestans* 2009-7654A
Lane 17: *Pythium aphanidermatum* PHY01
Lane 18: *P. citrophthora* CBS581.48 (Malaysia)
Lane 19: *P. palmivora* PPM7 (Malaysia)

Figure 4-2 PCR amplifications of Phytophthora isolates using primers AVR1F and AVR1R

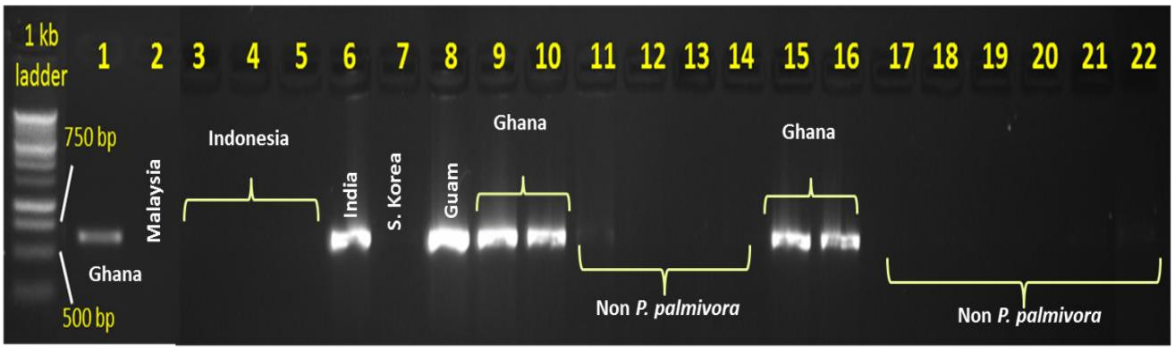
Lane 0: 1 kb ladder/marker
Lane 1: *Fusarium oxysporum*
Lane 2: *Mortierella echinosphaera*
Lane 3: *Mortierella chlamydospora*
Lane 4: *Mortierella chlamydospora*
Lane 5: *Gongronella butleri*
Lane 6: *Mortierella echinosphaera*
Lane 7: *Trichoderma asperellum*
Lane 8: *Fusarium* sp.
Lane 9: *Purpureocillium lilacinum*
Lane 10: *Talaromyces aculeatus*
Lane 11: *Fusarium* sp.
Lane 12: *P. palmivora* PPM1 (+ve)
Lane 13: *P. palmivora* PPM3 (+ve)
Lane 14: *P. palmivora* PPM2 (+ve)
Lane 15: *Purpureocillium lilacinum*
Lane 16: *Mortierella chlamydospora*
Lane 17: *Mortierella echinosphaera*
Lane 18: negative control (distilled water)

Figure 4-3 PCR Amplification of soil fungi using primer pair AVR1F/AVR1R

Meanwhile, amplification using primer pair AVR3F and AVR3R showed a selective amplification within *P. palmivora* isolates as observed in the initial assays. This primer pair amplified all isolates of *P. palmivora* from Colombia, Trinidad & Tobago, the USA, Sri Lanka, India and Ghana but did not amplify any from Malaysia, Indonesia and South Korea. No cross-reaction with any non *P. palmivora* isolates was observed. Amplifications with universal rRNA primers ITS1 and ITS4 to check the integrity of the DNA samples showed all samples to be positive (refer Chapter 2, Figure 2-15). The amplification outcomes were consistent in several repeats of amplifications either using the same or different batches of DNA or thermo-cyclers.



Lane 0: 1 kb ladder/marker
Lane 1: *P. palmivora* PPC280574 (Colombia)
Lane 2: *P. palmivora* P16828 (Colombia)
Lane 3: *P. palmivora* P16831 (Colombia)
Lane 4: *P. palmivora* PPC2614P (Colombia)
Lane 5: *P. palmivora* PPC3614L (Colombia)
Lane 6: *P. palmivora* P8513 (Colombia)
Lane 7: *P. palmivora* P0497 (Colombia)
Lane 8: *P. palmivora* CBS298.290 (Trinidad & Tobago)
Lane 9: *P. palmivora* P16385 (California, USA)
Lane 10: *P. palmivora* P6948 (Malaysia)
Lane 11: *P. palmivora* PPM4 (Malaysia)
Lane 12: *P. palmivora* PPM5 (Malaysia)
Lane 13: *P. palmivora* PPM1 (Malaysia)
Lane 14: *P. palmivora* PPM2 (Malaysia)
Lane 15: *P. palmivora* PPM3 (Malaysia)
Lane 16: *P. palmivora* CBS179.26 (Sri Lanka)



Lane 0: 1 kb ladder/marker
Lane 1: *P. palmivora* PPG11 (Ghana)
Lane 2: *P. palmivora* PPM7 (Malaysia)
Lane 3: *P. palmivora* IMI382528 (Indonesia)
Lane 4: *P. palmivora* IMI382544 (Indonesia)
Lane 5: *P. palmivora* P3767 (Indonesia)
Lane 6: *P. palmivora* CBS236.30 (India)
Lane 7: *P. palmivora* CBS111346 (South Korea)
Lane 8: *P. palmivora* P11007 (Guam)
Lane 9: *P. palmivora* PPG1 (Ghana)
Lane 10: *P. palmivora* PPG8 (Ghana)
Lane 11: *P. parasitica* P19537 (Colombia)
Lane 12: *P. parasitica* P19538 (Colombia)
Lane 13: *P. colocasiae* CBS358.30 (Sri Lanka)
Lane 14: *P. megakarya* PPG3 (Ghana)
Lane 15: *P. palmivora* PPG11 (cocoa-Ghana)
Lane 16: *P. palmivora* PPG13 (Ghana)
Lane 17: *Pythium aphanidermatum* PHY01
Lane 18: *P. cryptogea* PC01
Lane 19: *Fusarium oxysporum* MN1B
Lane 20: *P. megakarya* PPG4 (Ghana)
Lane 21: *P. megakarya* PPG12 (Ghana)
Lane 22: *P. infestans* 2009-7654A

Figure 4-4 PCR amplification using primers AVR3F and AVR3R

Apart for primers that can amplify Colombian isolates but not Malaysian, several primers were designed to amplify just Malaysian isolates. A total of 12 primer pairs were tested with genomic DNA of both Malaysian and Colombian *P. palmivora* isolates and the results are as summarised in Table 4-6. All primers tested were unable to differentiate Malaysian and Colombian isolates. Eight primers amplified both sets of isolates and the others did not work on either.

Table 4-6 Screening for* P. palmivora*-Malaysian specific primers based on hypothetical avirulence protein (PpHPAVR) sequences

	Primer		Amplification status		Size of amplicon (approx.)
	Forward	**Reverse**	**Malaysian isolate**	**Colombian isolate**	
1.	AVM1F	AVM1R	+	+	750
2.	AVM1F	AVM2R	-	-	-
3.	AVM2F	AVM2R	-	-	-
4.	AVM2F	AVM1R	+	+	500
5.	AVM3F	AVM2R	-	-	-
6.	AVM3F	AVM1R	+	+	370 - 400
7.	AVM4F	AVM1R	+	+	250
8.	AVM4F	AVM2R	-	-	-
9.	AVM5F	AVM5R	+	+	80
10.	AVM6F	AVM6R	+	+	80
11.	AVM7F	AVM7R	+	+	100
12.	AVM8F	AVM8R	+	+	200

4.3.2 Loop-mediated isothermal amplification (LAMP)

The use of the OptiGene Genie II and Genie III instruments together with their isothermal mastermix to perform the LAMP technique has eliminated the need for using gel based imaging or detection based on turbidity. Both instruments are based

on a fluorescence detection system, which allows the LAMP amplification to be monitored in real-time by producing amplification plots similar to those produced by real-time PCR (Figure 4-5). After amplification, products are analyzed by a slow annealing step; the temperature at which the product anneals or melts is consistent and characteristic for each assay (Figure 4-6). Analysis of this parameter is useful to identify the specificity of the amplification products; therefore non-specific amplification artifacts can be distinguished.

4.3.2.1 Validation of primers

Table 4-7 summarizes the results of LAMP amplifications carried out to validate the primers designed for *P. palmivora* detection. The amplification time and annealing temperature indicated positive amplification, while the assay with no amplification is indicated as negative (-ve). In the initial round of the study, primer sets PPALML, AVRL1, AVRL2, and AVRL3 showed positive amplification for all the *P. palmivora* tested and negative amplification for other outgroups. Therefore, the assay was extended to other *P. palmivora* isolates available in this study. PPALML amplified one outgroup isolate CBS358.30 originating from rubber in Sri Lanka which had previously been identified as *P. colocasiea* and AVRL1 failed to amplify an isolate from Malaysia (PPM2) and 3 isolates from Malaysia (IMI382528, IMI382544 and P3767). Primer sets AVRL2, AVRL3, and AVML2 showed species selectivity but only two sets of primers AVRL2 and AVML2 were further tested with a total of 27 *P. palmivora* and 16 outgroups consisting of 7 species of *Phytophthora*, a *Pythium* and 8 soil fungal species.

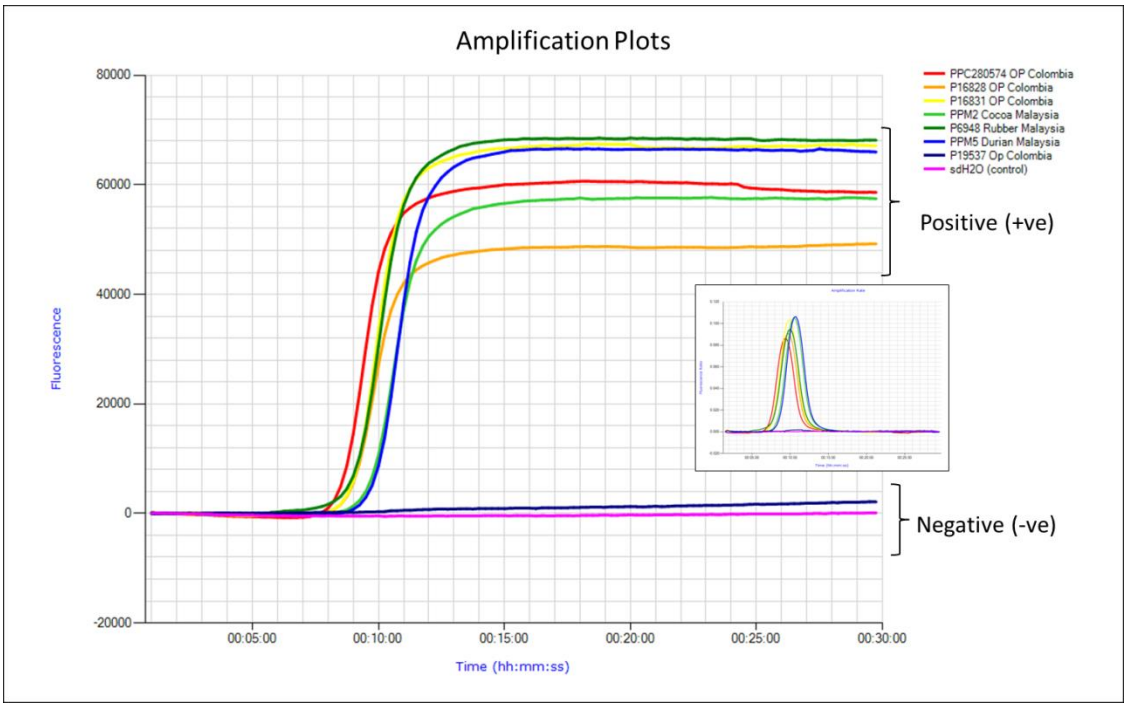


Figure 4-5 An example of real-time LAMP amplification plots using the fluorescence sensor detector system for diagnostic of* P. palmivora *with primer set PPALML

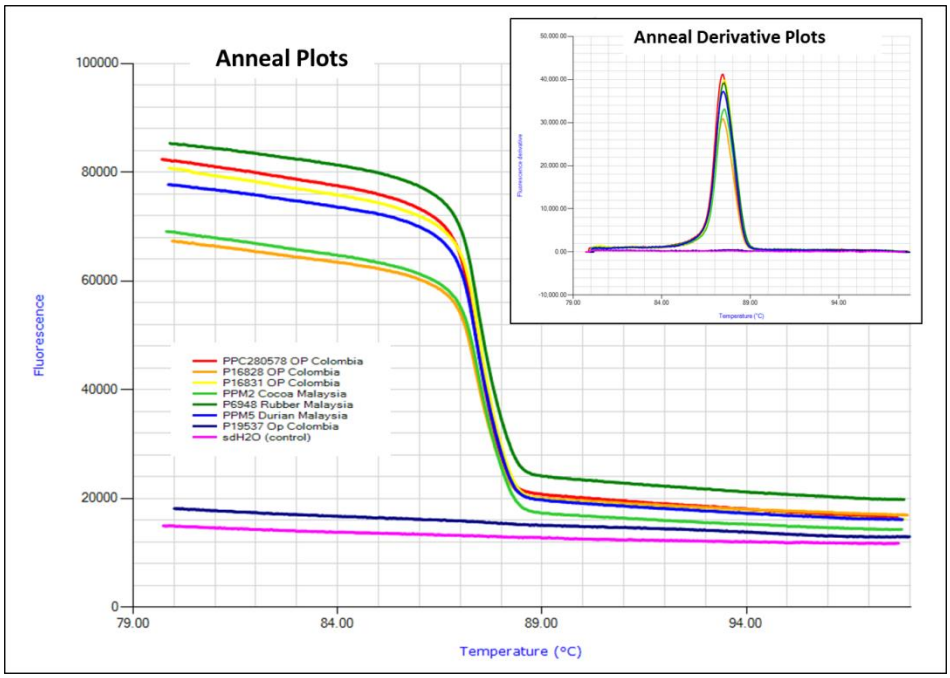


Figure 4-6 Anneal and anneal derivative plots showing the temperature of the annealing or melting of the amplification product after amplification.

Table 4-7 LAMP amplifications of different* P. palmivora *isolates using seven sets of primer

No.	Isolate	Amplification time (mm:ss) and annealing temperature (°C) in parentheses of the assay for each primer set						
		PPALML	**AVRL1**	**AVRL2**	**AVRL3**	**AVML1**	**AVML2**	**AVCL1**
1.	PPC280574 (oil palm-Colombia)	09:15 (87.5)	27:07 (90.6)	08:30 (87.1)	12:45 (87.7)	-ve	08:15 (89.2)	21:15 (89.6)
2.	PPC3614L (oil palm-Colombia)	12:00 (87.5)	23:32 (90.3)	09:15 (88.3)	12:30 (88.8)	-ve	10:00 (89.0)	n.d.
3.	PPC2614P (oil palm-Colombia)	10:45 (85.2)	25:30 (90.6)	09:00 (88.8)	10:32 (88.9)	n.d.	10:30 (89.0)	n.d.
4.	P16828 (oil palm-Colombia)	09:45 (87.5)	24:45 (90.7)	09:15 (88.9)	10:17 (89.1)	-ve	07:30 (88.7)	-ve
5.	P16831 (oil palm-Colombia)	09:45 (87.5)	02:15 (90.8)	09:15 (88.8)	10:02 (89.3)	-ve	08:30 (88.6)	-ve
6.	P8513 (cocoa-Colombia)	09:45 (85.2)	23:45 (90.9)	08:15 (88.8)	09:32 (89.2)	n.d.	08:15 (89.0)	24:15 (89.5)
7.	P0497 (cocoa-Colombia)	10:45 (85.1)	23:45 (90.7)	08:15 (87.0)	10:02 (87.3)	n.d.	12:00 (89.0)	n.d.
8.	CBS148.88 (bamboo palm-USA)	n.d.	n.d.	15:15 (89.2)	n.d.	n.d.	10:15 (90.6)	n.d.
9.	CBS298.290 (cocoa-Trinidad & Tobago)	09:30 (87.0)	25:00 (90.6)	09:00 (88.8)	10:17 (89.0)	n.d.	08:00 (89.0)	n.d.
10.	P16385 (kentia palm-California)	10:45 (85.2)	27:00 (90.8)	09:30 (89.1)	10:32 (89.2)	n.d.	08:15 (88.5)	-ve
11.	P6948 (rubber-Malaysia)	10:00 (87.5)	28:00 (89.6)	12:30 (86.8)	16:47 (87.4)	-ve	11:00 (88.8)	28:45 (88.3)
12.	PPM1 (cocoa-Malaysia MCB)	08:15 (87.0)	26:45 (90.2)	13:00 (87.2)	16:32 (87.5)	-ve	09:15 (88.9)	27:45 (88.8)
13.	PPM2 (cocoa-Malaysia)	07:45 (87.3)	-ve	14:45 (89.4)	16:17 (89.0)	n.d.	08:00 (88.8)	n.d.
14.	PPM3 (cocoa-Malaysia)	09:15 (87.5)	25:15 (90.3)	13:00 (87.3)	16:47 (87.6)	n.d.	09:30 (89.0)	n.d.
15.	PPM4 (durian-Malaysia)	n.d.	27:30 (89.9)	13:45 (87.1)	16:47 (87.4)	08:15 (86.5)	08:00 (88.7)	24:00 (88.6)
16.	PPM5 (durian-Malaysia)	10:45 (87.5)	27:00 (90.1	13:30 (87.2)	17:17 (87.5)	22:00 (84.9)	09:15 (89.0)	n.d.
17.	IMI 382528 (coconut-Indonesia)	11:45 (86.6)	-ve	13:00 (87.1)	17:17 (87.5)	-ve	08:15 (88.4)	-ve
18.	IMI 382544 (coconut-Indonesia)	10:15 (87.0)	-ve	20:15 (88.5)	21:45 (88.9)	-ve	09:00 (88.5)	n.d.
19.	P3767 (coconut-Indonesia)	12:00 (85.0)	-ve	15:00 (88.9)	17:45 (89.1)	-ve	07:45 (88.3)	-ve
20.	P11007 (Betel palm-Guam)	11:00 (85.0)	24:02 (90.6)	09:45 (88.6)	13:00 (89.2)	-ve	08:00 (88.2)	-ve
21.	CBS179.26 (cocoa-Sri Lanka)	10:00 (86.9)	23:45 (90.4)	08:00 (87.2)	10:17 (87.7)	-ve	07:00 (88.8)	27:00 (88.8)
22.	CBS236.30 (coconut-India)	10:45 (86.5)	26:02 (90.7)	11:00 (88.9)	14:30 (89.4)	n.d.	09:30 (89.2)	n.d.
23.	CBS1113346 (cymbidium-South Korea)	08:00 (87.3)	28:00 (89.5)	18:45 (88.2)	22:45 (88.5)	-ve	08:45 (88.5)	-ve
24.	PPG1 (cocoa-Ghana)	09:15 (87.4)	25:17 (90.5)	09:30 (88.3)	13:00 (88.9)	-ve	08:00 (88.3)	-ve
25.	PPG11 (cocoa-Ghana)	12:45 (87.2)	26:00 (90.4)	08:45 (87.1)	13:15 (87.6)	n.d.	09:30 (89.0)	n.d.
26.	PPG13 (cocoa-Ghana)	n.d.	25:30 (90.5)	09:30 (87.0)	13:30 (87.5)	n.d.	07:45 (89.0)	n.d.
27.	PPG8 (cocoa-Ghana)	n.d.	24:32 (90.6)	09:45 (88.4)	13:30 (89.0)	n.d.	09:00 (89.1)	n.d.

Table 4-8 Possible cross-reactivity of LAMP amplification of all tested primers

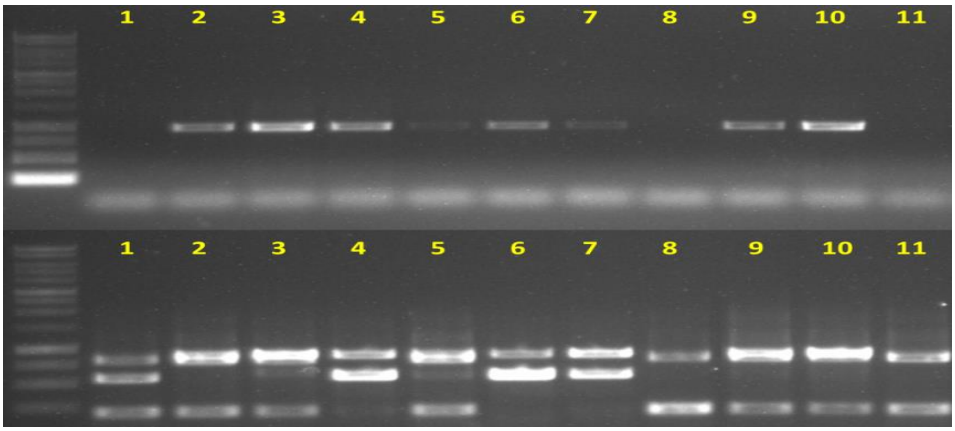
No.	Isolate	Identity	Amplification time (mm:ss) and annealing temperature in bracket*					
			PPALML	**AVRL1**	**AVRL2**	**AVRL3**	**AVML2**	**AVCL1**
28.	CBS358.30 (rubber-Sri Lanka)	*P. colocasiea*	14:45 (88.0)	-ve	-ve	-ve	-ve	-ve
29.	CBS581.48 (rubber Malaysia)	*P. citrophthora*	n.d.	n.d.	-ve	n.d.	-ve	-ve
30.	2009-7654A (potato-UK)	*P. infestans*	-ve	-ve	-ve	-ve	-ve	-ve
31.	P19537 (oil palm-Colombia)	*P. parasitica*	-ve	-ve	-ve	-ve	-ve	-ve
32.	P19538 (oil palm-Colombia)	*P. parasitica*	-ve	-ve	-ve	-ve	-ve	-ve
33.	PC01 (unknown-UK)	*P. cryptogea*	-ve	n.d.	-ve	n.d.	-ve	n.d.
34.	PPG12 (cocoa-Ghana)	*P. megakarya*	-ve	n.d.	-ve	n.d.	-ve	n.d.
35.	PY01 (unknown-UK)	*Pythium aphanidermatum*	-ve	n.d.	-ve	n.d.	-ve	n.d.
36.	MN1A (Malaysia)	*Fusarium oxysporum*	-ve	n.d.	-ve	n.d.	-ve	n.d.
37.	MN14FD3 (Malaysia)	*Fusarium sp.*	-ve	n.d.	-ve	n.d.	-ve	n.d.
38.	MN24AMD4 (Malaysia)	*Gongronella butleri*	n.d.	n.d.	-ve	n.d.	-ve	n.d.
39.	MN13AFK3 (Malaysia)	*Mortierella chlamydospora*	-ve	n.d.	-ve	n.d.	-ve	n.d.
40.	MN33MIC1 (Malaysia)	*Mortierella echinosphaera*	-ve	n.d.	-ve	n.d.	-ve	n.d.
41.	MN4MD1 (Malaysia)	*Purpureocillium lilacinum*	n.d.	n.d.	-ve	n.d.	-ve	n.d.
42.	MN32MIB1 (Malaysia)	*Rhizomucor variabilis*	n.d.	n.d.	-ve	n.d.	-ve	n.d.
43.	MN36MIF5 (Malaysia)	*Trichoderma asperellum*	-ve	n.d.	-ve	n.d.	-ve	n.d.

*Note: n.d. = not done, -ve = assay with negative result/no amplification

4.3.3 Detection of diseased samples

All diseased samples inoculated with *P. palmivora* could be amplified using primers AVR1F and AVR1R except for sample A1 (Figure 4-7). Amplification produced a single clear band of the same size (~1000 bp) as obtained from the amplification with the pure culture of *P. palmivora*. Amplification using universal primers ITS1 and ITS4 on the other hand produced multiple bands suggesting the presence of other microbes (oomycetes/fungi) in the samples including the control samples where inoculation was carried out using distilled sterile water. Negative amplification of sample A1 is probably due to the amounts of *P. palmivora* in the sample being too small or not present since the tissue was taken from the outer margins of the lesion. The presence of bands using ITS1 and ITS4 suggest that the DNA of the sample was in good condition. Negative amplifications were observed for control samples inoculated with sterile distilled water using AVR1 primers.

In addition, PCR bands from samples which underwent pre-treatment by surface sterilization with 70% ethanol and sterile distilled water were weaker than untreated. A similar finding was also observed using the LAMP assay using primers AVML2 and AVRL2. Observations from both PCR and LAMP assays using the leaf samples is summarized in Table 4-9. Integrity of genomic DNA from leaf samples was further confirmed with the positive amplification using LAMP with the plant universal primer (*Cox*). Preliminary work on using soil samples extracted with the soil DNA extraction kit and plant DNA extraction kit showed that no amplification occurred using both AVR1 and ITS universal primers. However, after further cleaning of DNA with a PVPP column, both the PpHPAVR and ITS region were successfully amplified, but only when the DNA soil extraction kit was used. LAMP was able to amplify samples using both extraction kits with or without the additional further clean up stage.



Lane 0:	1 kb ladder	Lane 4:	B2	Lane 8:	E (control)
Lane 1:	A1	Lane 5:	C1+C2	Lane 9:	74-s1
Lane 2:	A2	Lane 6:	D1	Lane 10:	74-s2
Lane 3:	B1	Lane 7:	D2	Lane 11:	Ctrl-s1 (control)

Figure 4-7 Amplification of genomic DNA extracted from leaf samples using both selective primers AVR1F/AVR1R and universal primers ITS1/ITS4

Table 4-9 Summary of preliminary study on application of PCR and LAMP using DNA extracted from leaf and soil samples

No.	DNA sample	PCR		LAMP		
		AVR1	ITS#	AVRL2	AVM2	Cox**
1.	A1 (diseased tissue)	- ve	+ve	- ve	- ve	+ve
2.	A2 (diseased tissue)	+ve	+ve	+ve	+ve	+ve
3.	B1 (diseased tissue)	+ve	+ve	+ve	+ve	+ve
4.	B2 (diseased tissue)	+ve	+ve	+ve	+ve	+ve
5.	C1+2 (diseased tissue)	+ve	+ve	+ve	+ve	+ve
6.	D1 (diseased tissue)	+ve	+ve	+ve	+ve	+ve
7.	D2 (diseased tissue)	+ve	+ve	+ve	+ve	+ve
8.	E/M11(healthy tissue/control)	- ve	+ve	- ve	- ve	+ve
9.	74-s1 (diseased tissue)	+ve	+ve	+ve	+ve	+ve
10.	74-s2 (diseased tissue)	+ve	+ve	+ve	+ve	+ve
11.	Ctrl-s1(healthy tissue/control)	- ve	+ve	- ve	- ve	+ve
12.	PPM1 (pure culture)	+ve	+ve	+ve	+ve	- ve
13.	S1 (soil extraction kit)	- ve	- ve	+ve*	n.d.	n.d.
14.	S2 (soil extraction kit+PVPP)	+ve	+ve	+ve*	n.d.	n.d.
15.	S3(Plant extraction kit)	- ve	- ve	+ve*	n.d.	n.d.
16.	S4 (Plant extraction kit +PVPP)	- ve	- ve	+ve*	n.d.	n.d.

*carried out using primer set AVRL1, ** plant Cox primer (Tomlinson et al., 2010),
#multiple bands except for PPM1 pure culture and negative amplification

4.4 Discussion

Since *Phytophthora palmivora,* the causal agent of bud rot in Colombia, is not a foreign species in Malaysia, the ultimate goal was to try and develop a detection system that could differentiate the Colombian isolate from the indigenous Malaysian or other SEA isolates. Unlike some fungal species such as *Fusarium oxysporum*, *P. palmivora* does not have obvious specialized forms or strain within the species; therefore, the task of finding good selective PCR primers is tricky but not impossible. PCR can distinguish closely related organisms at different taxonomic level (genus, species, strain or race) (Capote et al., 2012). Nonetheless the specificity and efficiency of PCR is dependent on the oligonucleotide primers (Tsai et al., 2006). The primers should be able to amplify a specific locus or target with enough variation within the species itself.

Many primers have been developed to identify and detect species of genus *Phytophthora* based on the internal transcribed spacer (ITS) region and intergenic spacer region (IGS) of ribosomal RNA genes for PCR (Ristaino et al., 1998; Williams et al., 2009; Mostowfizadeh-Ghalamfarsa and Mirsoleimani, 2012) and LAMP assay (Dong et al., 2015; Hansen et al., 2016). Alternatively a number of loci of nuclear (60S ribosomal protein L10, β-tubulin, enolase, HS protein 90, large subunit *rRNA*, *TigA* gene fusion, translation elongation factor 1α) and mitochondrial DNA (*Cox1, nad1, CoxII, nad9, rps10,* and *secY*) have been sequenced in phylogenetic studies of *Phytophthora* that are also useful for identification (Martin et al., 2012). Some examples of the use of alternative loci for identification are *ras*-related protein *Ypt1* (Meng and Wang, 2010) and cytochrome oxidase I (*CoxI*) and cytochrome oxidase II

(*CoxII)* (Martin et al., 2004) for PCR, whilst Chen et al. (2013) designed primers based on *ras*-related protein *Ypt1* for a LAMP assay.

PCR-based primers has been developed to detect *Phytophthora* to genus and species level such as by Ersek et al. (1994), Bonants et al. (1997), Liew et al. (1998), Cacciola et al. (2001), Jyan et al. (2002), Kong et al. (2003), Martin and Tooley (2004), Drenth et al. (2006), Huang et al. (2010), Langrell et al. (2011), Tsai et al. (2006) and Li et al. (2013). Identification and detection of *P. palmivora* has been carried out by Bowman et al. (2007) and Tsai et al. (2006). However, their work involved more complicated PCR-based techniques, the PCR-RFLP and nested PCR, respectively, therefore making the detection procedure more tedious and difficult for a large screening of samples. Conventional basic PCR is preferable, and was therefore the focus in this study.

Apart from PCR, several species-specific and genus-specific primers for LAMP assays of *Phytophthora* have been developed such as *P. infestans* (Hansen et al., 2016), *P. ramorum* (Tomlinson et al., 2007; Miles et al., 2015), *P. kernoviae* (Tomlinson et al., 2010), *P. melonis* (Chen et al., 2013), and *P. sojae* (Dai et al., 2012). Initially, the target marker genes of choice in this present study were the ITS region, β-tubulin, translation elongation factor 1 alpha, cytochrome oxidase I (*CoxI*) and *CoxII.* The characteristics of these genes such as their location, functionality, size is understood and well known to some level especially for the ITS region. However, alignments of sequences of these markers for several *P. palmivora* isolates showed a high level of homology (≥ 97%) within the species (see Chapter 3), making it difficult to design primers that are able to differentiate between Colombian and Malaysian isolates. Therefore, the new marker PpHPAVR was used as the template to design the

primers since examination of the nucleic acid sequences from this site showed some variation between isolates.

This study reports the potential of *P. palmivora* species-specific primers AVR1F/AVR1R to amplify from various hosts and origins but exclude out-group species including *P. megakarya,* which was once known as one of the *P. palmivora* complex of s-type/MF3 (Zoberi et al., 1981; Akrofi, 2015) and potential selective primers AVR3F and AVR3R that discriminate Colombian and Malaysian isolates. AVR3F and AVR3R amplify all Colombian isolates and from Trinidad & Tobago, the USA, Ghana, Guam, India and Sri Lanka but not Malaysian, Indonesian and South Korean isolates, suggesting some pattern based on origin of the isolates. There is a chance that *P. palmivora* from Malaysia, Indonesia and South Korea has a monophyletic origin. It would be interesting to know if PCR assays with *P. palmivora* from other SEA countries such as Thailand, the Philippines, Vietnam, Laos, Myanmar and Brunei would have the same results and hopefully the study can be extended in the future to answer this. Apart from primers that positively amplify Colombian and exclude Malaysian *P. palmivora*, it would also be good to design primers with the reverse action, which can be used to further confirm the diagnosis. Unfortunately, all attempts to design such primers in this study failed.

In the study of the application of LAMP assays to detect *P. palmivora*, some primer sets have shown potential as species-specific primer. Seven sets of primers were designed based on the PpHPAVR marker. Primer set PPALML, AVRL1, AVRL2 and AVRL3 were designed to pick just *P. palmivora* of all isolates; meanwhile, primers AVML1/AVML2 and AVCL were aimed to be selective primers to differentiate Malaysian and Colombian isolates. Primer sets AVRL2 and AVRL3 have species selectivity, but primers PPALML and AVRL1 show some evidence of non-selectivity to species level.

Attempts to obtain primers to discriminate Malaysian and Colombian isolates also failed. However, primer set AVM2 appears to only amplify *P. palmivora* and successfully ruled out the outgroups, as does primer set AVRL2. These two primer sets exhibit evidence of the potential use of LAMP assays as diagnostic tools for selectively diagnosing the *P. palmivora*.

In order for the assay to be good as a detection tool, especially for screening of plant samples, the method should not only work with DNA extracted from pure cultures but also from samples particularly from infected plants (specifically from leaves, in the case of bud rot disease of oil palm). Some of the concerns and challenge when working with PCR are PCR inhibitors in samples (Bürgmann et al., 2001) and the small amounts of pathogen DNA (Liew et al., 1998) that exist in the total DNA samples which can result in negative results and masking the presence of the pathogen. The inhibitors can bind and interact with polymerase, DNA template and /or primers (Opel et al., 2010). Most commercial plant DNA extraction kits eliminate most of the inhibitors and contaminants during extraction. However, different species of plants and the different plant tissues may contain different PCR inhibitors (for example plant polysaccharides) at various levels (Drenth et al., 2006). Dilution of the DNA helps to reduce the inhibitor concentration and enhance PCR efficiency but might decrease PCR sensitivity due to reducing the DNA concentration (Demeke and Adams, 1992).

PCR assays using primers AVR1F and AVR1R and LAMP assays using primers AVRL2 and AVML2 were able to amplify from the DNA extracted from the diseased oil palm leaves artificially inoculated with the zoospores of *P. palmivora* isolates P16835 and PPC280574, suggesting that the extracted DNA of oil palm leaves does not have much inhibition on PCR and LAMP, which can be overcome by diluting the DNA (1:10)

prior to assays. No cross reactivity was observed from samples without washing and surface sterilizing, suggesting a good level of specificity. Control assays with plant *Cox* primers (only carried out for LAMP) confirmed the negative results as due to the absence of *P. palmivora* in the samples, not due to other reasons such as inhibitors. More assays were performed with diseased samples from the pathogenicity study and will be reported in the next chapter. For preliminary work using soil samples, PCR was not able to amplify the DNA extracted from soil spiked with *P. palmivora* (PPC280574) using a commercial soil extraction kit, probably due to inhibition. It is often more difficult to purify microbial DNA from soil than from other environments such as water (Roose-Amsaleg et al., 2001). DNA extracted from soils usually has more inhibitors such as humic acids, lignins, carbohydrates and resin and the amount can vary depending on several factors such as soil types (Roose-Amsaleg et al., 2001; Robe et al., 2003; O'Brien et al., 2009). Positive PCR amplification was possible by cleaning up the DNA samples using PVPP spin columns prior to assays using both primer pairs AVR1 and ITS, but with low concentration (based on low intensity of the amplified band), probably due to low amounts of *P. palmivora* DNA. Cleaning the DNA using PVPP spin columns appeared to help remove inhibitory humic acid contaminants from soil extracts as demonstrated by Berthelet et al. (1996). According to Steffan et al. (1988), the removal of humic acids by PVPP greatly improved the DNA purity but lowered the DNA recovery slightly. Krsek and Wellington (1999) found that PVPP applied directly at the beginning of the lysis during DNA extraction did not show any improvement on purity. Positive amplification were observed from LAMP assays of samples with or without PVPP clean-up. LAMP assays are known to be less affected by inhibitors than conventional PCR (Kaneko et al., 2007; Tomlinson, 2013).

The sensitivity of the detection using all primers in terms of the minimum amount of DNA in the samples including the amount of *Phytophthora* (in terms of spore, mycelial or other parameters that can be quantified) was not determined since the initial focus was trying to find good specific primers. Therefore, no dedicated work on quantification has yet been carried out and determination of the amount of genomic DNA in PCR template using NanoDrop™ spectrophotometer was not convincing due to the large variation within replicates (data not shown) probably due to low DNA concentrations. The detection range of DNA concentration of the spectrophotometer is between 2 to 15,000 ng/µl dsDNA as published in *http://www.nanodrop.com/Productnd2000overview.aspx*. Work by Drenth et al. (2006) and Mostowfizadeh-Ghalamfarsa and Mirsoleimani (2012) using genus and species specific primers targeting the ITS region demonstrated a detection limit of 2 pg and 50 pg, respectively. The lower limit might be higher for other markers compared to the ITS region due to high copy number of the ITS region in *Phytophthora* genomes. Schena and Cooke (2006) mentioned the detection limit of primers targeting the *ras*-related protein (*Ypt1*) gene which has one copy number was 10 pg. Detection limit and sensitivity of LAMP is higher than conventional PCR in most assays but also depending on the primers (Parida et al., 2004). The detection limit for *P. sojae* using primers targeting the avirulence effector AVR3a promoter region by Dai et al. (2012) was 10 pg. Miles et al. (2015) demonstrated sensitivity ranging from 3 ng to 1 fg of DNA for detection of *P. ramorum* and *P. kernoviae,* whilst Tomlinson et al. (2007) reported a detection limit of between 10 pg and 50 pg for *P. ramorum* targeting the ITS region. Quantification of the amount of *Phytophthora* present in plants is a challenge and has limitations due to their biological nature (Drenth et al., 2006).

Although both PCR and LAMP techniques using the designed primers show promise as detection tools up to species level, more studies to incorporate a broader range of *Phytophthora* species and other outgroups should be carried out to make sure there is no cross reaction from other species of *Phytophthora*. It would also be beneficial to try both PCR and LAMP on different plant tissues (bark, stem, fruit/pod), plant species (such as durian, cocoa, pepper) to further verify the robustness of the primers and to carry out sensitivity tests to determine the minimum detection limit in plant samples. In addition, it would be good if the need to perform complex DNA extraction prior to PCR or LAMP assays can be eliminated. Apart from that, more studies should be carried out on the possibility to detect *P. palmivora* in soil samples using the primers to confirm the preliminary study, so that monitoring and studies of *P. palmivora* in the soil of the oil palm plantations can be conducted with ease by eliminating the need to perform biological diagnostic procedure such as selective isolation and *in vitro* culturing of *P. palmivora*. The PpHPAVR marker appears to have sufficient variation of DNA sequences to be used as a template for designing some selective primers to separate within species as demonstrated by primers AVR3F/AVR3R. However, it must be remembered that unlike other known markers such as the ITS region, the nature (for example functionality, copy number, and mapping) of the marker is somewhat unknown, hence it probably needs to be used with caution. The best guess is that the marker is related to avirulence protein genes since the primers designed to amplify the fragment were designed based on the nucleic acid sequence of the avirulence protein of *P. infestans.*

After all the necessary tests and assays have being carried out to further validate and improve the method, it is hoped that both techniques can be of great value to help protect the Malaysian oil palm industry from devastating bud rot disease

either as tools to eliminate the risk at the border or to monitor the pathogen in the local environment.

Chapter 5. Pathogenicity studies of *P. palmivora* by artificial inoculation

5.1 Introduction

Although bud rot disease has been recorded to cause a problem for the oil palm industry in South America, it was not until recently that *P. palmivora* was established as the causal agent of bud rot disease in Colombia with the confirmation of Koch's Postulates (Sarria et al., 2008b; Torres et al., 2010; Drenth et al., 2013). *Phytophthora palmivora* is known to cause disease in other tropical plants such as rubber (*Hevea brasiliensis)*, cocoa (*Theobroma cacao*), durian (*Durio zibethinus*), jackfruits (*Artocarpus heterophyllus*), coconut (*Cocos nucifera),* pepper (*Piper nigrum* L.) and papaya (*Carica papaya*). There are also reports of *P. palmivora* causing disease on olive trees (*Olea europaea* L.) in Morocco (Chliyeh et al., 2013). To manage and control the disease, a good understanding of disease epidemiology and etiology are the key. Knowing the pathogenicity of the pathogen at both the cellular and molecular levels will facilitate in development of control strategies. Hardham (2001) has provided a good review on the pathogenicity process of *Phytophthora*.

Extensive studies on the interactions between *P. palmivora* and the oil palm host, which include the infection process and colonization of the young leaf tissue, have been carried out and published recently by Sarria et al. (2016). Their study showed that the infection of oil palm tissue with *P. palmivora* was initiated with encystment of zoospores, preceding the germination and aggregation of the zoospores at the infection site of the leaflet. The preferred encystment and germination sites are

the leaflet trichome and invasion of hyphae via the trichome happens faster than invasion of intracellular spaces (Sarria et al., 2016). Initial symptoms of *P. palmivora* on immature oil palm leaves are the typical water-soaked brown lesions (Torres et al., 2010) which are also described in naturally occurring infections in the field (Martinez, 2009c; Torres et al., 2016). The initial lesions are visible after 3-4 days of infection (Martinez, 2009a).

Significant advances have been made in understanding the infection and colonization process of *P. palmivora* in oil palm in Colombia; however, why the pathogen does not cause the same devastation in other regions remains a mystery. Are the isolates from other regions and the isolates attacking other hosts such as cocoa, rubber and durian able to establish the same symptoms? If yes, does the degree of infection vary with pathogenic variation or aggressiveness between different isolates of *Phytophthora* as is known to exist in a number of other species (Turner, 1973) and can the infection develop into more severe forms of bud rot disease? In this chapter, attempts were made to re-establish Koch's postulates and infection against oil palm seedlings from Malaysia using *P. palmivora* isolates from oil palm obtained from Colombia with the help of Cenipalma. The main aim of this chapter was to study the aggressiveness of different *P. palmivora* isolates obtained from various hosts and demographic origins especially from Malaysia.

5.2 Materials and methods

5.2.1 Establishment of oil palm, rubber and durian seedlings

Oil palm germinated seeds (*Dura* x *Pisifera*) from the Malaysian Palm Oil Board (MPOB) were sown into trays filled with a mixture of soil (Levington F2 Seed & Modular Compost) and perlite at the ratio of 8:1 in a glasshouse at 28°C (day) and 22°C (night) with a photoperiod of 14-16h and watered every day during summer and on alternate days during winter. The humidity in the glasshouse was maintained by wetting the floor of the glasshouse every morning. After 3 months, the seedlings were transferred into larger pots (5 litre) filled with a soil mixture of sand based soil (John Ines No.3), perlite and vermiculite with the ratio of 8:1:1. Durian and rubber seedlings were sent by courier from Malaysia following necessary phytosanitary procedures and were planted in the 5 litre pots containing the sand based soil mixture for oil palm seedlings. All the seedlings were fed with liquid fertilizer (10% solution) containing N, P, K in the ratio of 4:2:2 and trace elements.

5.2.2 Preparation of *P. palmivora* cultures

Phytophthora palmivora was sub-cultured onto carrot agar using mycelium blocks from the actively growing region of 4-10 day old carrot agar cultures. The plates were kept in an incubator at 25°C +/- 2°C for 7-14 days. Old stock cultures were reactivated by using a pear bait technique. A small plug of mycelium of *P. palmivora* was cut out from the margin of actively growing cultures of *P. palmivora* and inserted into a triangular cut made at one side of the pear as described in section 2.2.1,

Chapter 2. The pear was then incubated at room temperature in humidity chambers for up to 8 days. A small part of the pear tissue was taken from the advancing stage near the lesion and was then sub-cultured onto selective media $P_{10}VP$ and incubated at 25°C +/- 2°C with illumination for 4-10 days.

5.2.3 Production of zoospores

Two methods were used to obtain zoospore suspensions as described by Chee (1975) and Dick et al. (2014), both with some modifications as follows;

1. Approximately 10 ml of sterile distilled water was poured into the agar plates and was chilled in the dark at 4°C for approximately 30-40 min. The culture was then dark incubated at room temperature for another 30-50 min. The zoospore suspension was then collected in a sterile beaker.
2. Four to five mycelial plugs cut from the actively growing region of agar culture plates were immersed in sterilized carrot juice in a 9 mm Petri dish and incubated at room temperature with illumination for 7-10 days. The culture dish was incubated in the dark at 4°C for approximately 30-40 min to release the spores.

The concentration of zoospores was determined microscopically using a Neubauer haemocytometer following this formula: spore counts/ml=average spore counts in each large square $x10^4$ x dilution factor. The concentration of zoospores for inoculation was set at approximately $1x10^4$ spores/ml, unless otherwise stated.

5.2.4 Establishment of pathogenicity test: Leaf detached assay of oil palm

The initial assay was conducted using mature leaves and green unopened spear leaves taken from 12 month old oil palms grown in the glasshouse, surface sterilized with 2% v/v sodium hypochlorite (NaOCl) and let to dry prior to inoculation. One hundred microlitres (µl) of zoospore suspension ($1x10^4$ zoospore/ml) was dropped onto wounded and unwounded leaves. Cotton wool was used to hold the suspension. The assay was conducted in humidity chambers as in Figure 5-2. Inoculation using mycelial plugs was also carried out.

Subsequent assays were conducted using white unopened spear leaves. The spears were divided into two parts, the upper older (greenish) part and lower younger (whitish) part. Each piece was about 14 cm in length. The spear pieces were washed with tap water and surface sterilized using 2% v/v sodium hypochlorite (NaOCl) by dipping the whole leaf into the solution for 60 seconds followed by rinsing with sterile distilled water twice and then left to completely dry on clean tissue towels. Each end of the piece was cut approximately 0.5 cm from the margin. The spear leaves were pricked/wounded twice using a sterile sharp pointed blade (no. 11) approximately 4 cm from the end on both sides. The clean 5 mm x 5 mm cotton plugs were put on top of the wounded sites. One hundred microliters (µl) of zoospore suspension (10,000 zoospore/ml) spiked with carrot juice was dropped onto the cotton pad. Distilled water (80 µl) spiked with carrot juice (20 µl) was used in control assays. The chambers were covered and incubated at room temperature with illumination for 7 days. Presence of lesions was observed and diseased leaf tissue samples were cut into small pieces, soaked in 3% KOH for five minutes and observed microscopically using a compound

microscope. Some of the pieces were also plated on the selective media with and without surface sterilization with sodium hypochlorite.

5.2.5 Inoculation of oil palm seedlings in glasshouse conditions

All inoculations were carried out in the same glasshouse where the seedlings were grown and the conditions were maintained throughout the experiment. The initial inoculation was carried out using 3-6 month old DxP oil palm seedlings according to the protocol suggested by Cenipalma (personal communication) using isolates pathogenic to oil palm (PPC280574) courtesy of Cenipalma. A piece of sterile cotton wool was placed in the middle of the seedling, near the young shoot and wrapped with Parafilm® resulting in a funnel-like shape. Approximately 1 ml of zoospores was dropped into the cotton wool so that the zoospore suspension was in contact with the shoot of the seedling. The concentration of zoospores was $1x10^4$ zoospore/ml. The zoospore suspension was replaced with water for the control assay. The whole plant was then covered with a plastic bag for 24 hours to retain humidity. Watering and fertilization was carried out as usual. All inoculations were done at least in triplicate, carried out three times. The inoculated seedlings were observed for any development of lesions. Diseased samples were collected and re-isolated by direct plating onto selective media or fruit bait. DNA extraction from diseased tissue and PCR using specific primers was also performed as discussed in Chapter 4. Subsequent inoculations were done with modifications of the above mentioned method as follows:

1. Introduce wounding at the three points in the stem
2. Flooding the seedlings in a tray containing water a week before and throughout inoculation

3. Increase the volume of inoculum by pouring up to 10 ml of zoospore (concentration of zoospores of 10^3-10^4 spores/ml) which also contain mycelia.

The inoculated seedlings were covered with plastic for 4 weeks. The seedlings were observed for any physical symptoms of bud rot disease as described by Martinez (2009c) and Drenth et al. (2012).

(a)

(b)

(c)

Figure 5-1 (a) A sterile cotton bud was put in the middle of the seedling, around the oil palm budding area (circled in red) and then (b) wrapped with Parafilm which looked like a funnel, (c) The zoospore suspension was then dropped inside the 'funnel'.

5.2.6 Cross pathogenicity

The rubber leaf samples were washed with tap water and surface sterilized using 2% v/v sodium hypochlorite (NaOCl) by dipping the whole leaf into the solution for 60 seconds followed by rinsing with sterile distilled water twice. The leaf was then allowed to dry in the laminar safety chamber on a clean tissue towel. Each leaf was assembled in a transparent humidity chamber as in Figure 5-2. A droplet of zoospores (100 µl of $1x10^4$ zoospore/ml) was dropped onto the leaf (wounded or unwounded) with or without a sterile cotton pad (approximately 5 mm x 5 mm) to help hold the suspension in place (Figure 5-3). Some inoculations were done with zoospore suspensions spiked with sterile carrot juice. Control assays were water and carrot juice. Wounding was performed with a sterile sharp-pointed scalpel blade (no. 11). The chamber was then covered and incubated at room temperature on the lab bench for 7-10 days. The inoculation was also performed with the mycelial plug with an empty agar plug as control. All assays were done in triplicate. The same assay was also carried out using durian. The isolates used in the assays with rubber and durian leaves were isolates pathogenic to oil palm (PPC280574) and several representative isolates from different host/origins; PPM1 (cocoa), PPM4 (durian), PPM5 (durian), P6948 (rubber), IMI382544 (coconut), P3767 (coconut), CBS148.88 (bamboo palm), P11007 (betel palm) and CBS1113.46 (*Cymbidium* orchid). All assays with the various isolates were conducted using mycelial plugs as inoculum source.

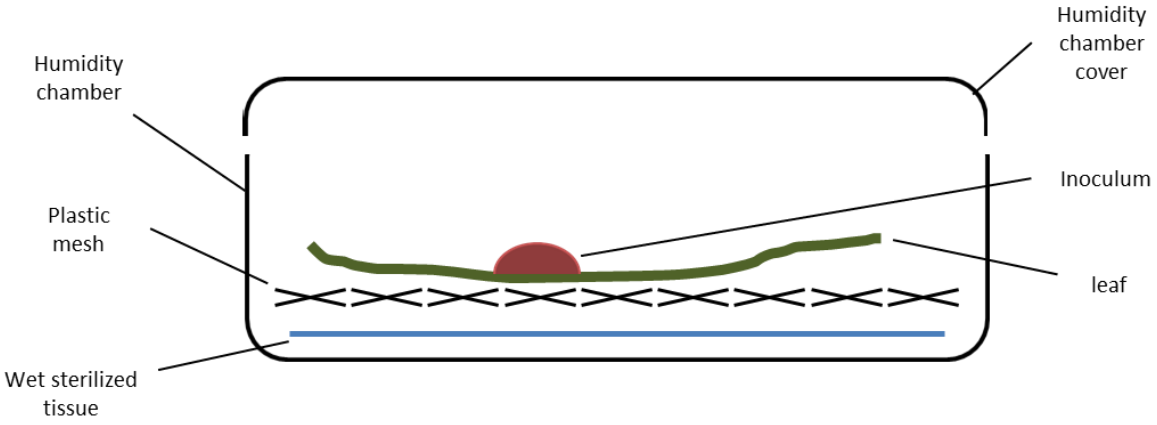


Figure 5-2 Arrangement of leaf in humidity chamber for leaf detached assay

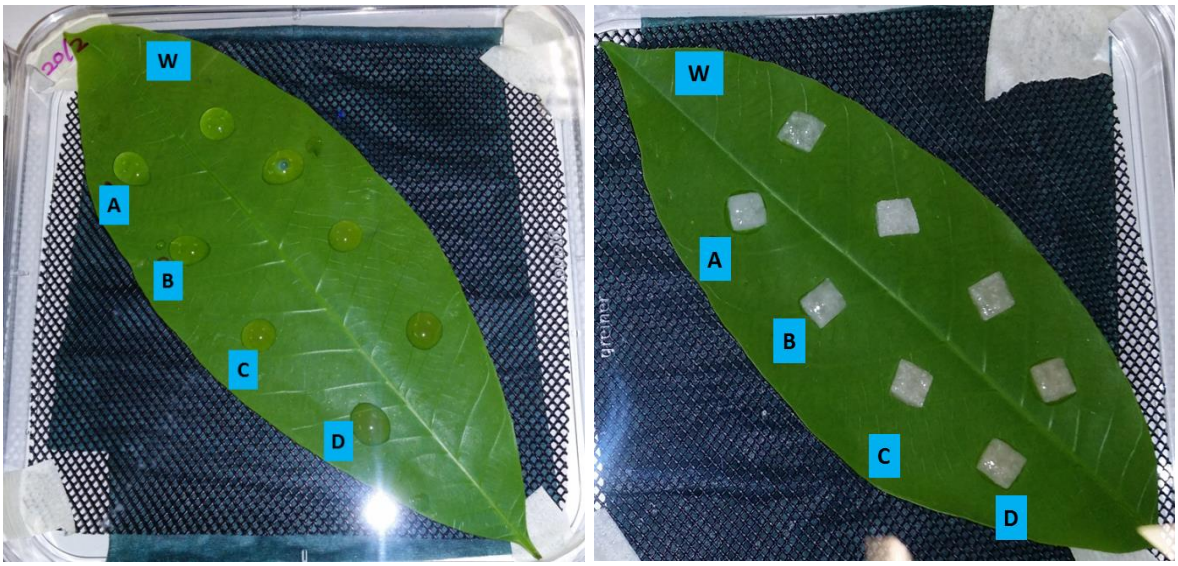


Figure 5-3 Leaf detached assay using rubber leaves using zoospore suspensions without (left) or with (right) cotton wool pads.

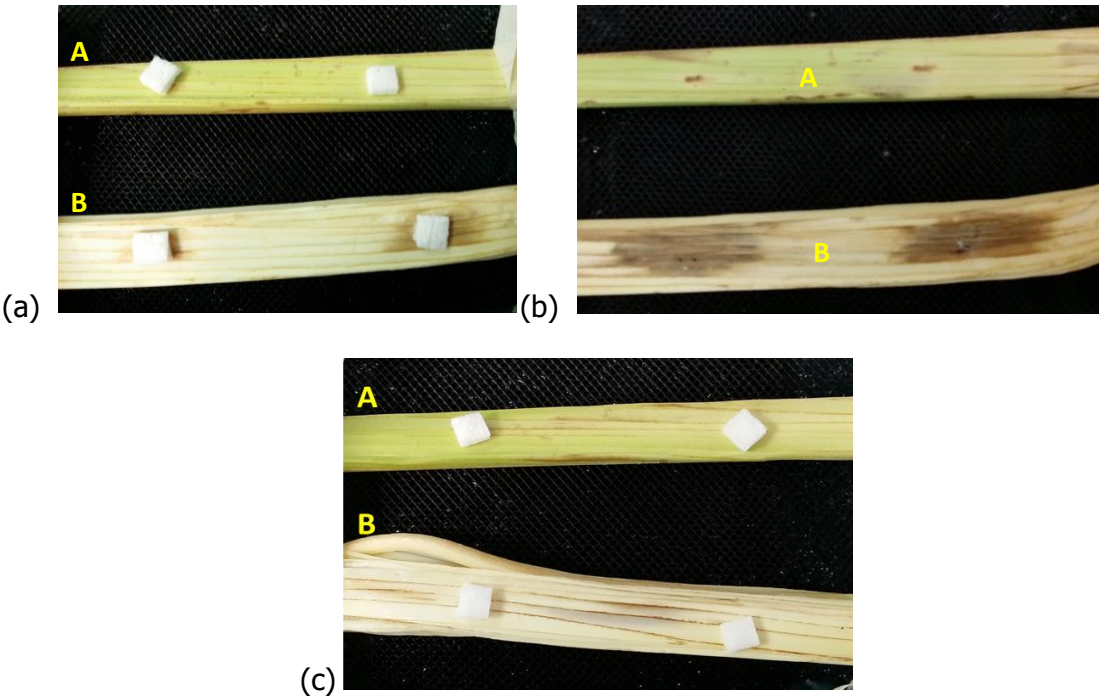
Note: The left side of the leaf was wounded and the right was un-wounded. Assays were done on the same leaf with A: 100 µl sterile distilled water, B: 90 µl zoospore suspension+10 µl sterile water, C: 90 µl sterile distilled water+10 µl carrot juice and D: 90 µl zoospore suspension+10 µl sterile carrot juice.

5.3 Results

5.3.1 Leaf detached assay

No lesions were observed in the initial trials using green mature leaves and green unopened spear leaves of 12 month old oil palms inoculated with zoospore suspensions (approximately 10^4 zoospores/ml) held with sterile cotton wool (Figure 5-4) and mycelial plugs of oil palm pathogenic isolate PPC280574, both with and without wounding, by the 5th day after inoculation. Brown lesions were observed at 4 days after inoculation using white unopened spears on the lower part (whitish) nearer to the crown/growing point but not the with the upper greenish part, but only with wounded leaves (Figure 5-5). Inoculation using young oil palm spears was not repeated with mycelial plugs or with other isolates due to the lack of material, as 12 month old oil palm seedlings require a large amount of glasshouse space, hence the number of seedlings (material) grown was limited and removing the young spear leaves was destructive. The presence of *P. palmivora* in the diseased tissue was confirmed by microscopic evaluation of the diseased tissue (Figure 5-6) and re-isolation using selective media (Figure 5-7). *Phytophthora palmivora* was not observed in control assays and there was no mycelial growth on the selective media.

Figure 5-4 Mature oil palm leaf (top) and green unopened spear leaf (bottom) inoculated with zoospore suspensions of PPC280728 held with sterile cotton wool after five days of inoculation



(a) (b) (c)

Figure 5-5 (a) Brown lesions on the inoculation site of very young oil palm spear leaves (bottom) observed at the 4th day of inoculation, (d) lesion at 5th day and (c) control assay with water

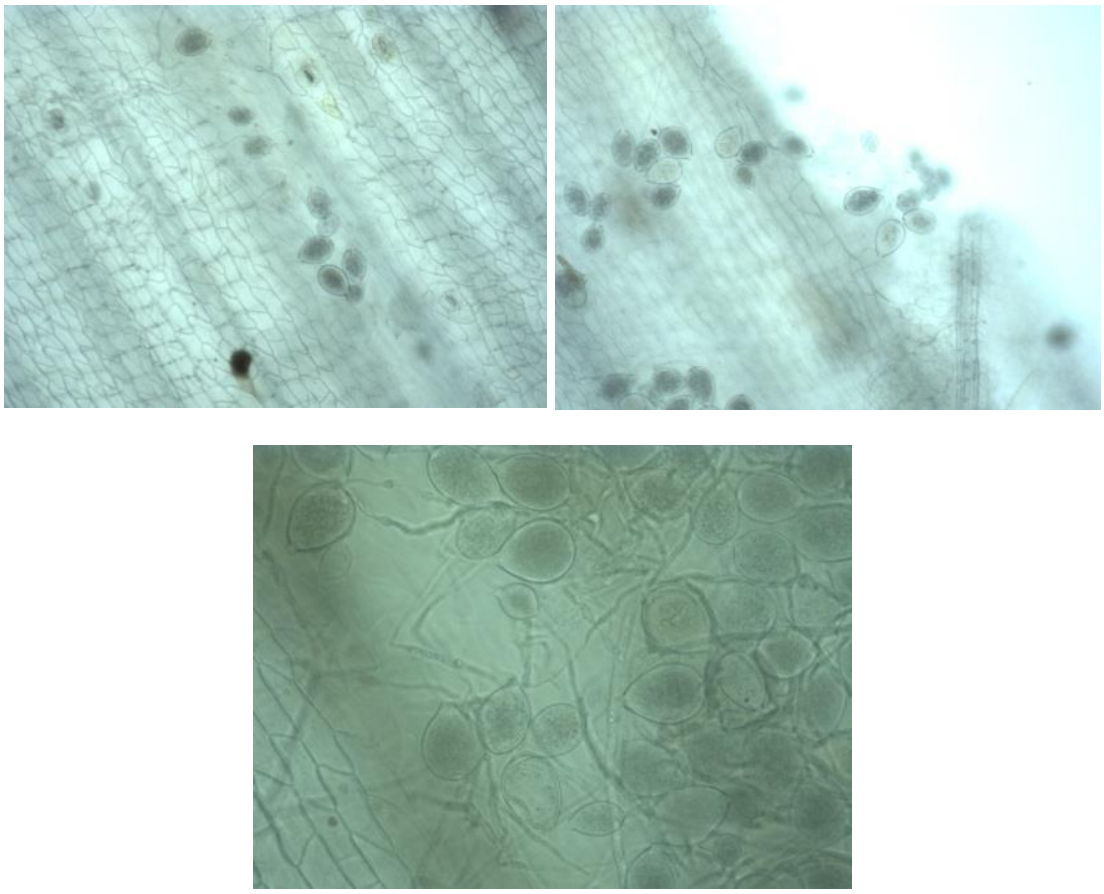


Figure 5-6 Sporangia observed on diseased tissue at the inoculation site of* P. palmivora*. Magnification: 10x10 (left and middle), 40x10 (right)

Figure 5-7 Mycelial growth from the re-isolation of the diseased tissue (brown lesion)

5.3.2 Nursery evaluation

In the early trials carried out between December and March using pathogenic isolate PPC280574, no lesions were observed on any of the inoculated seedlings, including the assays using modified methods. First trials using other isolates, P16828, P16831, P8513, P6948, PPM1, PPM4, P11007 and IMI382544, also showed no disease symptoms. However, inoculation assays using P16385, CBS1113.46 and PPG1 carried out at the end of May started to show brown lesions on the 7th day after inoculation (Figure 5-8A). Inoculation was then repeated with the same 12 isolates and the presence of brown lesions was observed on seedlings treated with all isolates 7 days after inoculation but not with all the replicates (Table 5-1, Figure 5-8B). Similar findings were shown with the seedlings in another trial repeated with only four isolates (Table 5-2) and trials with oil palm seedlings of different genetic backgrounds, (TxT) and (DxD)(Table 5-3).

Most lesions appeared to be localized on the wounded site and no further infection was observed after two weeks of inoculation at the infection site. The size of the lesion did not expand or grow. Nevertheless, there were three seedlings (inoculated with PPM4, PPM1 and CBS111346) that had bigger infection areas, where half of the young spear leaf become brown and infected. On all infected leaves, the diseased tissue become necrotic and dried out. After some time, the necrotic tissue fell out leaving a hole in the leaf, but the rest of the leaf (the healthy tissue) kept on growing (Figure 5-9), including the new shoot. No recurrent infections were observed on any inoculated seedlings.

(A-i)

Continue…

(A-ii) 

(Bi) 

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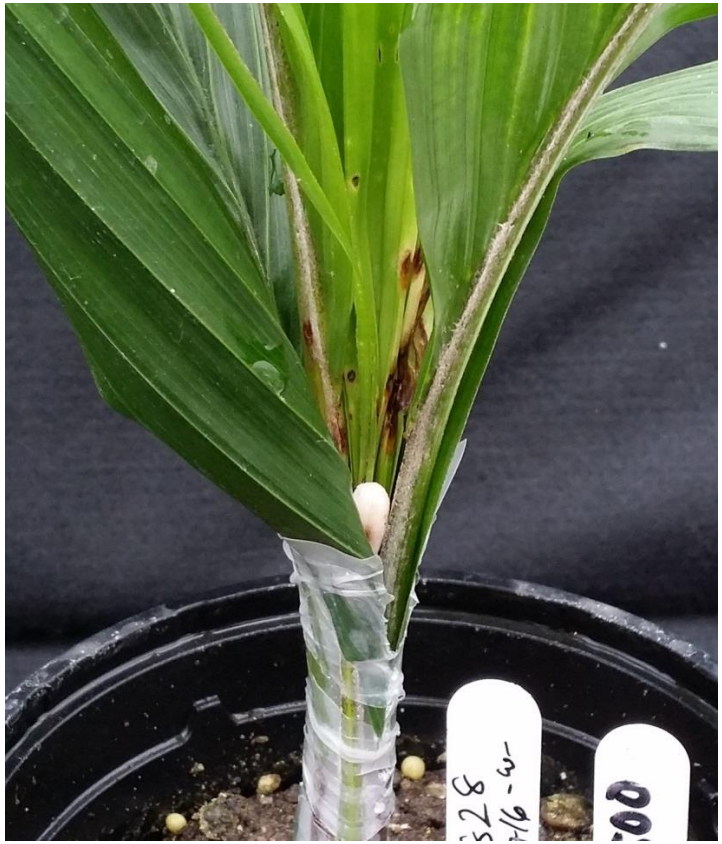
(B-ii) 

(B-iii) 

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(B-iv) 

(B-v) 

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(C) 

Figure 5-8 Lesion observed on infected oil palm seedlings inoculated with* P. palmivora *isolate P16835 (A-i) and CBS111346 (A-ii) carried out in the middle of May. Similar disease symptoms were also observed on the subsequent inoculation repeated with the same isolates as in the previous inoculation. Shown are some examples of the symptoms that appeared on the seedlings inoculated with; B-i) PPC280574, B-ii) P16828, B-iii) PPM4, B-iv) PPM1, B-v) P6896 and C) Control (distilled water spiked with carrot juice)

Table 5-1 First summer inoculation using DxP African oil palm seedlings

Isolates	Host and origin	No. of inoculated palms	No. of palms with lesions	% of seedlings with lesions	% of palms recovered after 6 months
Ctrl (dH2O)	-	5	0	0	-
PPC280574	Oil palm - Colombia	5	3	60	100
P16828	Oil palm - Colombia	5	2	40	100
P16831	Oil palm - Colombia	5	1	20	100
P8513	Cocoa - Colombia	5	1	20	100
PPM1	Cocoa - Malaysia	5	4	80	100
PPM4	Durian - Malaysia	5	3	60	100
P6948	Rubber - Malaysia	5	4	80	100
IMI382544	Coconut - Indonesia	5	2	40	100
CBS1113.46	Cymbidium - South Korea	5	4	80	100
P11007	Betel palm - Guam	5	3	60	100
P16385	Kentia palm - California	5	3	60	100
PPG1	Cocoa-Ghana	5	2	40	100

Table 5-2 Second round summer inoculation using DxP African oil palm seedlings

Isolates	Host and origin	No. of inoculated palms	No. of palms with lesions	% of seedlings with lesions	% of palms recovered after 6 months
Ctrl (dH2O)	-	10	0	0	-
PPM1	Cocoa - Malaysia	10	3	30	100
P6948	Rubber - Malaysia	10	4	40	100
PPC280574	Oil palm - Colombia	10	5	50	100
P8513	Cocoa - Colombia	10	5	50	100

Table 5-3 Inoculation of isolate PPC280574 against different crosses of African oil palm seedlings

Treatment	No. of inoculated palms	No. of palms with lesions	% of seedlings with lesions	% of palms recovered after 6 months
DxP (water)	10	0	0	-
DxD (water)	10	0	0	-
TxT (water)	10	0	0	-
DxP+PPC280574	10	6	60	100
DxD+PPC280574	10	6	60	100
TxT+PPC280574	10	8	80	100

Figure 5-9 Infected seedlings at 6 months after inoculation with* P. palmivora *zoospores.

Note: The seedling on the bottom right is a control seedling (wounded+carrot juice)

5.3.3 Cross-pathogenicity

In the preliminary work on the inoculation using zoospore suspensions of $1x10^4$ - $5x10^4$ zoospores/ml of isolate PPC280574, disease lesions were only observed when the leaves were wounded prior to inoculation (Figure 5-10). The use of cotton wool pads (Figure 5-9a) did not interfere with infection and gave similar results to droplets of zoospore inoculum (Figure 5-9b). Mycelial plugs were found to be as effective as zoospore inoculum in the infection (Figure 5-11). All isolates tested showed cross-pathogenicity against rubber and durian leaves (Table 5-4).

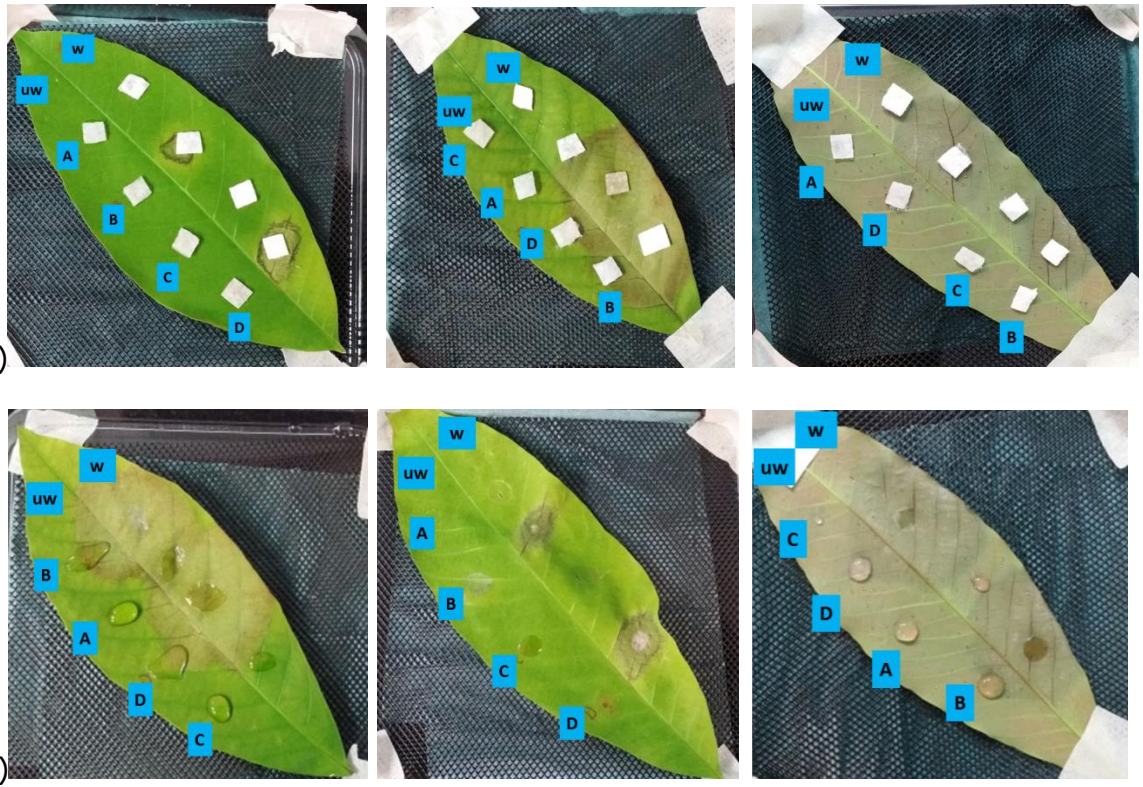


Figure 5-10 Some examples of lesions developed after 3-4 days of inoculation with zoopore suspensions of* P. palmivora *isolate PPC280574 (a) held with cotton pad (b) without cotton pad.

Note: The left side of the leaf was wounded and the right was un-wounded. All assays were done on one leaf with A: 100 ul sterile distilled water, B: 90 ul zoospore suspension+10ul sterile water, C: 90 ul sterile distilled water+10ul carrot juice and D: 90 ul zoospore suspension+10 ul sterile carrot juice.

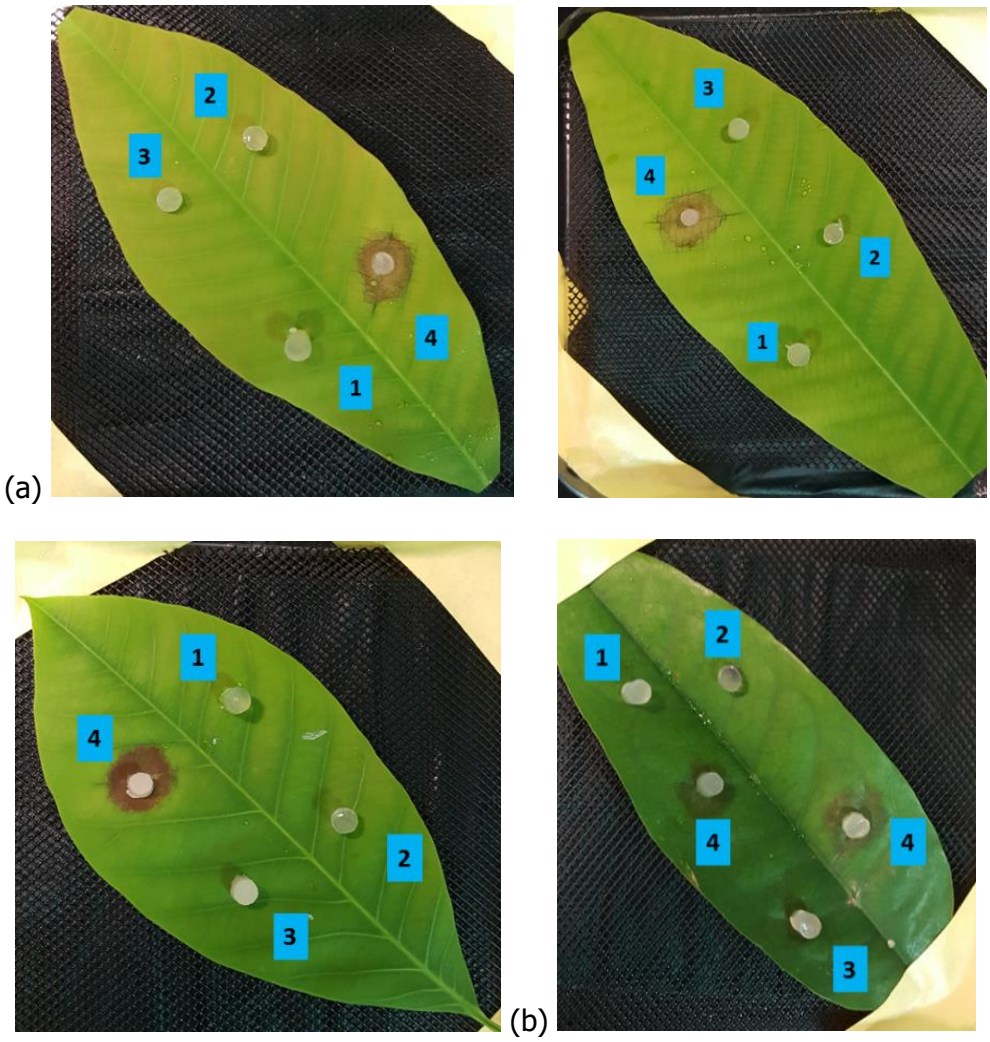


Figure 5-11 Lesions observed on the (a) rubber leaf inoculated with mycelial plug of P11007 (upper left), CBS111346 (upper right) and P148.88 (bottom left), (b) durian leaf with P6948 (bottom right)

Note: 1: unwounded+(carrot agar plug/carrot agar juice), 2: wounded+control (carrot agar plug/carrot agar juice), 3: unwounded+inoculum (mycelial plug/zoospore suspension), 4: wounded+inoculum (mycelial plug/zoospore suspension)

Table 5-4 Cross pathogenicity of various* P. palmivora *isolates on rubber and durian

Isolate	Ex-host	Cross pathogenicity*	
		Rubber	Durian
PPC280574	African oil palm	+	+
PPM1	cocoa	+	+
PPM4	durian	+	+
PPM5	durian	+	+
P6948	rubber	+	+
IMI382544	coconut	+	+
P3767	coconut	+	+
CBS148.88	bamboo palm	+	+
P11007	betel palm	+	+
CBS1113.46	*Cymbidium* orchid	+	+

Note* based on the observation of lesions in leaf detached assay,+=presence of lesion

5.4 Discussion

The initial stage of this study was to re-establish infection using the *P. palmivora* isolate PPC280574, pathogenic to oil palm obtained from Colombia. The study involved artificial inoculation of leaflets in the lab and in the glasshouse at Nottingham set up to mimic tropical conditions, to establish the standard positive baseline. Infection was successfully established on the very young spear leaves. The initial symptoms of small brown lesions with water-soaking at the edge were observed at 3-4 days after inoculation, which coincides with the symptoms described in several reviews such as Martinez (2009c), Sarria (2013) and Torres et al. (2016). Similar water-soaked symptoms was also described by Van Tri et al. (2015) on the jackfruit leaf inoculation using the same species. Turner (1969) reported that the water-soaked margin was only observed on inoculated immature leaves of pepper piper betel and both upper and lower leaf surfaces can be inoculated.

In our study, it was observed that wounding of the spear was required for the infection to occur in contrast with the findings by Sarria et al. (2016), where the infection readily occurred without wounding. However, Sarria et al. (2016) used individual leaflets of the young spear instead of direct inoculation on the un-opened spear. Although assays using Malaysian isolates were not performed in this study due to the shortage of oil palm materials, the assay has been conducted in Malaysia using isolates obtained from diseased tissue of cocoa and durian where the brown discoloration was observed after 3 days of inoculation with both isolates (Mohamed Azni et al., 2016). The re-isolation of the diseased tissue and microscopic evaluation confirmed the presence of *P. palmivora*. In addition, PCR amplification using extracted DNA of the diseased tissue samples using our *P. palmivora* specific primers (discussed

in previous chapter) also came back positive and no amplification was observed for samples in the control assays.

Apart from inoculation of oil palm young spear leaves, cross pathogenicity of the oil palm isolate against other hosts susceptible to *P. palmivora* such as cocoa, durian and rubber will give us some information on the host specificity and compatibility of the isolates. However, attempts to established cocoa seedlings in the greenhouse in the UK as sources of leaf materials failed; therefore, the assay was conducted only on rubber and durian. Isolates from oil palm were observed to cause infection in rubber and durian leaves. Both inoculum sources of zoospores and mycelial plugs have the potential to infect the leaves and the pathogenicity levels in terms of lesion growth varied in each assay using the same isolates which might reflect the influence of many factors such as humidity in the inoculation chamber, age/condition of the leaf and inoculum potential.

Initially, we could not establish infection in the glasshouse through artificial inoculation of *P. palmivora* on oil palm seedlings using the isolate originated from oil palm in Colombia believed to be pathogenic to oil palm. Several trials were conducted including trials with modifications of inoculation methods including increasing the inoculum (in term of volume, zoospore counts, combinations of mycelium, sporangium, chlamydospores and zoospores), introduction of wounding at the stem base of the seedlings and waterlogging the seedlings before and after inoculation. In order to avoid loss of virulence during sub-culturing, the isolate was reactivated in the fruit (apple/pear) and re-isolated onto selective media prior to production of zoospores. Positive infection on rubber leaves suggested the continued existence of the pathogenic nature of the isolate. The artificial inoculation was than extended to other isolates originating from oil palm and cocoa in Colombia, cocoa, durian, rubber

in Malaysia, *Cymbidium* orchid (South Korea, betel palm (Guam), kentia palm (California) and cocoa (Ghana), regardless of the failure to established infection using the isolate from the oil palm as positive reference. It is believed that the infection of *P. palmivora* to the seedlings is affected by the temperature because eventually, infections were observed at the end of May, which was the beginning of spring in the UK, and subsequent inoculations with the same isolates as tested before (with no infection), showed positive infections when retested during the summer months.

The initial symptoms of brown lesions with water-soaked margins (observed on the seedlings inoculated with Colombian and other isolates) coincided with the previous detached leaf assay and observations from Sarria et al. (2016). However, the lesions appeared to be localized in our study as reported by Mohamed Azni et al. (2016) with work using Malaysian isolates in Malaysia. The infection did not grow further in most infected seedlings. In other words the Colombian, Malaysian and other isolates from different hosts all caused mild symptoms and the disease did not progress to a severe form with the typical aggressive symptoms that had been found to occur in inoculation tests in Colombia (Torres et al., 2010; Sarria, 2013). Torres et al. (2010) reported 15% of the seedlings inoculated with 40,000 zoospores developed into typical bud rot symptoms but none in our study even though we used up to 180,000 zoospores per seedling. The disease cycle of *Phytophthora* often involves primary and secondary inoculum. Primary inoculum initiates the infection and upon successful infection, a second generation of secondary inoculum is produced. The rate of propagation of secondary inoculum determines the severity of the next infection (Drenth and Guest, 2004a). In the case of our infection in the glasshouse, there were some factors affecting the propagation of secondary inoculum including environmental conditions such as temperature, humidity and maybe the presence or absence of other

microbes as secondary invaders that are different in the UK and Malaysia compared to Latin America.

In term of disease incidence, not all seedlings inoculated with each isolate were infected. The incidence observed on the inoculated seedlings was variable between and within isolates. We had some difficulty in producing zoospores for each trial, such that the inoculum strength in terms of zoospore could not be exactly standardized and was in the range of 1-9 x 10^4 zoospore/ml for the first summer inoculation and 5-8 x 10^3 zoospore/ml for the second round summer inoculation, and this may have affected the incidence scores between tests. However, the incidence data is useful in providing information on the cross pathogenicity between isolates against oil palm seedlings even though it may not be appropriate for showing the virulence levels of each isolate.

Cross pathogenicity of isolates from different hosts; coconut, cocoa, durian, rubber, bamboo palm, betel palm and orchid on both durian and rubber leaves suggested that *P. palmivora* does not have specific strains adapted for each host as observed for *Fusarium* sp., supporting the hypothesis of a broad host range for *P. palmivora* (Drenth and Guest, 2004a). Pongpisutta and Sangchote (2004) showed cross pathogenicity of *P. palmivora* isolates from durian against black pepper and rubber leaves. However, not all *Phytophthora* species have broad host ranges. Different species of *Phytophthora* may have different degrees of host specificity. Some species such as *P. havea* have narrow ranges and *P. colocasiae* is very host specific to taro (*Colocasia esculenta*)(Drenth and Guest, 2004a). Some species of *Phytophthora* seem to have both non-host and host-specific receptor-based recognition systems for induction of encystment of zoospores by host surface components, therefore enabling general and host specific pathogenicity, which enables them to invade compromised plants in the absence of preferred hosts (Raftoyannis and Dick, 2006a). However,

there is also the possibility that the specificity of host selection arises during the attempts at penetration and invasion of plant tissue and that the zoospore stage is non-specific (Van West et al., 2002). Several reports with root diseases show that zoospores of *Phytophthora* species are attracted to and encyst similarly on roots of susceptible and resistant seedlings of plants (Raftoyannis and Dick, 2006a). Raftoyannis and Dick (2006b) found that the relationship between encystment of zoospores and disease development depends on the oomycete–plant combination.

Similar to the inoculation of oil palm young spear leaves, the inoculation on durian and rubber leaves in the cross-pathogenicity assays using several isolates from various hosts including oil palm conducted in this study also failed to established infection without wounding. Introduction of wounding in the artificial inoculation is not new in pathogenicity studies of *Phytophthora* spp. using stems to facilitate infection and has been shown by others especially when working with stem rots. Nevertheless, most studies with leaves usually do not involve wounding and infections on the leaves become established without wounding such as in citrus (Ann, 1984), durian (Lim and Chan, 1986) and jackfruit (Van Tri et al., 2015). Meanwhile, other researchers have introduced wounding prior to inoculation such as Pongpisutta and Sangchote (2004). O'Gara et al. (2004a) reported that *P. palmivora* was attracted to fresh wounds on the durian leaf and rapidly colonized the entire leaf lamina when infection happened through the fresh wound and non-wounded durian leaves did not develop disease symptoms reliably (O'Gara et al., 2004b). In contrast, Brooks (2008) found that there was no different in the infection of *P. colocasiae* on taro leaves.

It is hoped that more studies can be conducted to understand more on the pathogenicity and aggressiveness of *P. palmivora* against oil palm. Artificial inoculation of oil palm seedlings using different *P. palmivora* isolates should be repeated but with

the same inoculum size for each isolate so that the aggressiveness of the different isolates originating from different hosts and geographical regions can be assessed without prejudice and probably can be correlated with the molecular characterization to see if the isolates belonging to same clade have similar levels of aggressiveness against oil palm. It would also be good if the assay can be conducted in a tropical environment; however, due to biosecurity constraints, it is difficult to carry out such experiments in countries such as Malaysia and Colombia as it would involve introducing the foreign isolates to the areas. But the evaluation of the local isolates obtained from different hosts against oil palm is possible.

Chapter 6. *In vitro* evaluation of chemicals against *P. palmivora*

6.1 Introduction

Effective plant disease management consists of integration of several approaches amongst which are management of cultural practices, the use of chemical control, and biological control, and these can also be applied to diseases caused by *Phytophthora* and other oomycetes. Good cultural practices such as proper irrigation and drainage to remove excess water in nurseries, and improving soil aeration are important to manage *Phytophthora* diseases (Portales et al., 2004). The use of fungicides has also been accepted and used for control of *Phytophthora*. Drenth and Guest (2004b) listed some fungicides frequently used for management of *Phytophthora*. One of the oldest fungicides used is a copper based protectant called Bordeaux mixture (a mixture of copper sulphate with calcium hydroxide and water), initially developed to control the oomycete downy mildew of grapevine (*Plasmapora viticola)* but now used to control many other *Phytophthora* diseases (Drenth and Guest, 2004b), such as fruit rot of areca nut caused by *Phytophthora arecae* (Nayaka et al. (2005) as cited in Mathew et al. (2015)).

In the management of bud rot disease of coconut, the used of fungicides is one of the strategies recommended alongside other practices such as good maintenance of plantation hygiene by removal of diseased and dead palms and their debris, control of weed infestation, maintaining drainage systems and also good integrated nutrient

management which needs to be conducted throughout the year especially in endemic areas for effective management of the disease. Fungicides such as Bordeaux mixture are suggested to be sprayed to the crowns of the trees as prophylactic measures before the onset of monsoons, or to place one or two sachets containing mancozeb in the two innermost leaf axils (ICAR, 2013). The effectiveness of the application of both chemicals has been demonstrated by Sharadraj and Mohanan (2012). However, superior results were obtained using potassium phosphanate (0.5% @ 300 ml/palm). In addition to application of the fungicides by pouring on the palm crown and soil drenching, the use of trunk injections to the palm can be explored as another method to introduce the chemical to the infected site. This method has been explored using fungicides such as phosphonate (Guest et al., 1995), potassium phosphite (Gentile et al., 2009), fosethyl-Al (Darvas et al., 1983) and metalaxyl (Matheron and Mircetich, 1985) to control *Phytophthora* diseases. In Colombia, measures to control the bud rot disease of oil palm are similar to the strategies adopted in the coconut diseased area. Management requires at least the improvement of agronomic practices, including drainage and balanced fertilization, removal of affected tissue and the use of pesticides to control *P. palmivora* and other secondary microorganisms (Torres et al., 2016).

A number of fungicides have been successfully used to control *Phytophthora* diseases from several fungicide groups, mainly the phenylamides (acyanilides) and alkyl phosphonates or phosphites (which refers to the salts of phosphonic acid (H_3PO_3)). Phenylamides inhibit oomycetes/fungal ribosomal RNA (rRNA) biosynthesis (Davidse et al., 1983; Taylor et al., 2002) whilst phosphonates have a complex mode of action (Silva et al., 2016).

Among many fungicides in the phenylamides group, metalaxyl is extensively used and has been tested in many *Phytophthora* species such as *P. parasitica* (Timmer and Castle, 1985), *P. infestans* (Zhu et al., 2008)*, P. cactorum* and *P. citrophthora* (Thomidis and Tsipouridis, 2001). Other example of fungicides with good efficacy to control oomycetes pathogens are the phenylamides mefenoxam (metalaxyl-M)(Taylor et al., 2002; Hu et al., 2008), furalaxyl, benalaxyl, ofurace and oxadixyl (Gisi and Ziegler, 2003), and the phosphonates fosetyl-aluminium (fosetyl-Al) (Chase et al., 1985; Erkilic and Canihos, 1999), potassium phosphonate (Vawdrey et al., 2004), dimethomorph and cymoxanil (Ziogas and Davidse, 1987). The carbamates, prothiocarb and promamocarb are also effective but at high concentrations (Cohen and Coffey, 1986). Although not much work has been reported on fungicide efficacy for control of *P. palmivora* on oil palm apart from Aya et al. (2011), several studies have been conducted on other hosts such as cocoa (Tey and Wood, 1983; Holderness, 1992; Opoku et al., 2006), durian (Chan and Kwee, 1986), papaya (Vawdrey et al., 2004) and orchid (Lim and Lam, 1983).

In this chapter, we investigate the efficacy of some fungicides and two liquid fertilizers, on the growth of *P. palmivora* isolated from diseased oil palm using *in vitro* method to indicate the potential for extending these studies *in planta*. Most of these fungicides including the liquid fertilizers have been recommended for controlling potato leaf blight caused by *Phytophthora infestans* and are already known to be beneficial in management of *Phytophthora* disease, such as cymoxanil, mancozeb and chlorothanil. The liquid fertilizer was chosen based on the presence of phosphate and copper components both of which have been known to inhibit some *Phytophthora* species.

6.2 Material and methods

6.2.1 Preparation of *P. palmivora* cultures

Phytophthora palmivora isolates PPC280574 (oil palm Colombia), PPM1 (cocoa Malaysia), PPM4 (durian Malaysia) and IMI382544 (coconut Indonesia) were sub-cultured onto carrot agar using mycelium blocks from actively growing regions of 4-10 days old carrot agar cultures. The plates were kept in an incubator at 25°C +/- 2°C for 7-14 days.

6.2.2 Preparation of Poison Agar Plates

Carrot agar without antibiotics was prepared as in Appendix 1. The agar mixture was then sterilized and allowed to cool down to approximately 60°C. The fungicides were added into the molten agar to final active ingredient (a.i.) concentrations of 0.001µg/ml, 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml 10 µg/ml and 100 µg/ml of the main active ingredient (Table 6-1). About 20 ml of the mixtures were poured into 9 cm Petri plates and then left to solidify.

6.2.3 *In vitro* evaluation of potential chemicals - Poison agar tests

The *in vitro* evaluation was carried out using poison agar tests (poison food tests) essentially as described in Sinclair and Dhingra (1995) and Adams and Wong (1991). Mycelial agar plugs of about 5 mm in diameter excised from the margins of actively growing cultures of *P. palmivora* (isolates PPC280574, PPM1, PPM4 and IMI382544)

were placed (mycelial side down) onto the centres of the carrot agar amended with the fungicide. Unamended carrot agar inoculated with the mycelial plug acted as the control plates. The assays were done in at least 4 replicates. The plates were sealed with Parafilm® and incubated in the dark at 25±2°C. Radial growths of mycelial on the plates were monitored and measured every day in four perpendicular directions (r1, r2, r3, r4) until the 5th day of incubation (Figure 6-1). Percentage inhibition of the mycelial growth expressed as percentage inhibition of radial growth (PIRG) was then calculated based on the formula, PIRG = $(R_a–R_b /R_a)*100$, where R_a is the mean radial growth of *P. palmivora* on untreated/control plates, and R_b is the mean radial growth of *P. palmivora* on treated plates as described by Navi et al. (2016). EC_{50}, which is the fungicide concentration that inhibits mycelial growth by 50%, was calculated based on the dose response curve, by subjecting the PIRG values to probit values (y-axis) and fungicide concentration values to $\log_{10}$ (x-axis) as described by Finney (1952) as cited in Rekanović et al. (2012). Overall mycelial growth reductions from the 1st to 5th day after incubation were also expressed as Area Under Mycelial Growth Curve (AUMGC) calculated using the trapezium method as described by Simko and Piepho (2012).

6.2.4 Data analysis

All data calculations were done in Microsoft Excel. The statistical evaluations such as mean separation, analysis of variance (ANOVA), post hoc ANOVA (Fisher's least significant difference (LSD)) were carried out using SPPS Software, Version 23.0 (IBM Corp., USA).

***Table 6-1 Chemical (fungicides and fertilizers) used in the* in vitro *study against* P. palmivora**

Fungicides	reference a.i. *	Trademark name	Active ingredient	Trademark company
cymoxanil	cymoxanil	CURZATE 60DF®	cymoxanil 60% w/w	E.I. du Pont
azoxystrobin	azoxystrobin	AMISTAR®	azoxystrobin 23.1% w/w (250 g/l)	Syngenta Group
mancozeb	mancozeb	PENNCOZEB WDG®	mancozeb 75% w/w	Cerexagri BV
mancozeb +benthiavalicarb -isopropyl	mancozeb	VALBON®	mancozeb (700g/kg) and benthiavalicarb-isopropyl (17.5g/kg)	Kumiai Chemical Industry Co. Ltd
chlorothalonil+ tebuconazole	chlorothaloni	TIMPANI®	chlorothalonil 22.0% w/w (250 g/l) and tebuconazole 7.92% w/w (90 g/l)	Nufarm
phosphate+ potassium+ nitrogen	phosphate	OMEX DP98®	phosphate (P2O5) 38.0% w/v, potassium (K2O) 17.5% w/v nitrogen (N) 4.0% w/v,	Omex Agriculture Ltd
Zinc+ sulphur+ copper	zinc	OMEX ZiCu®	zinc 4.74% w/v, sulphur 3.34% w/v, copper 2.67% w/v,	Omex Agriculture Ltd

*used as reference in calculation of final concentration

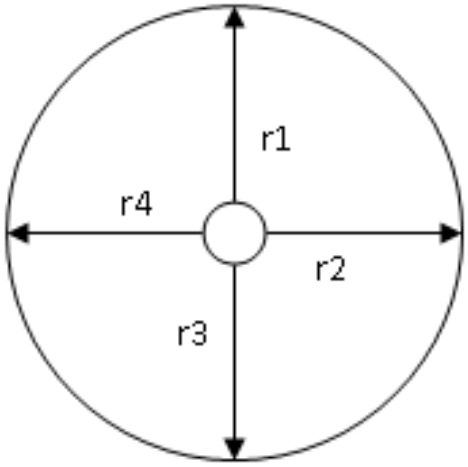


Figure 6-1 Mycelial radial growth measurements

6.3 Results

6.3.1 Assay with isolate PPC280574, an isolate pathogenic to oil palm

Overall, the mycelial growth of PPC280574 was reduced when grown on media with all fungicides/chemicals tested compared to the control plates without fungicides at certain concentrations of active ingredient (a.i.) and the greatest reduction was observed from the second day after incubation. The growth of mycelia in the control plates reached a maximum on the 9 mm Petri dish at 5 days after incubation of the mycelial plug onto the media plate. The percentage of inhibition of the mycelial radial growth (PIRG) was calculated based on the radial growth of the culture on amended and unamended (control) media on the 5^{th} day of incubation (Figure 6-2).

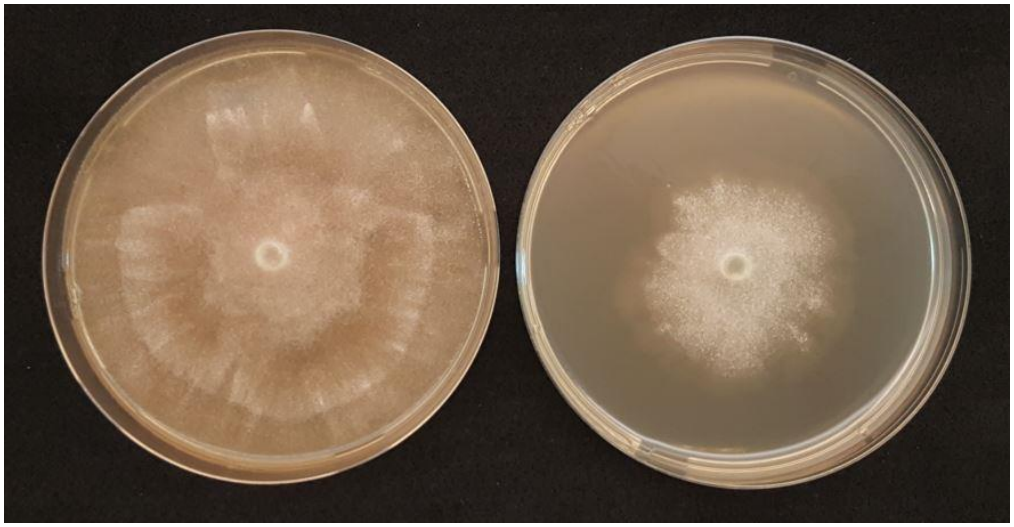


Figure 6-2 Example of mycelial growth of* P. palmivora *isolate PPC280574 on control plate (left) and amended plate (cymoxanil 100 µg/ml) on 5th day after incubation

The mycelial growth of the culture grown on media amended with 0.001 to 1 µg/ml of cymoxanil agar was not significantly different from the control on the unamended plate and a significant reduction was only observed at 10 µg/ml a.i. (Table 6-2). The PIRG using cymoxanil at 10 µg/ml a.i. and 100 µg/ml a.i. were calculated as 28.05% and 75.8%, respectively (Table 6-3). The overall reductions from the 1st day to 5th from the mycelial growth curve expressed as Area Under Mycelial Growth Curve (AUMGC) were also found to be significantly lower than controls at these concentrations (Figure 6-3). Cymoxanil inhibits half of the *P. palmivora* growth at the high concentration of 100 µg/ml.

Table 6-2 Mycelial radial growth of* P. palmivora *isolate PPC280574 in amended carrot agar after 5 days of incubation

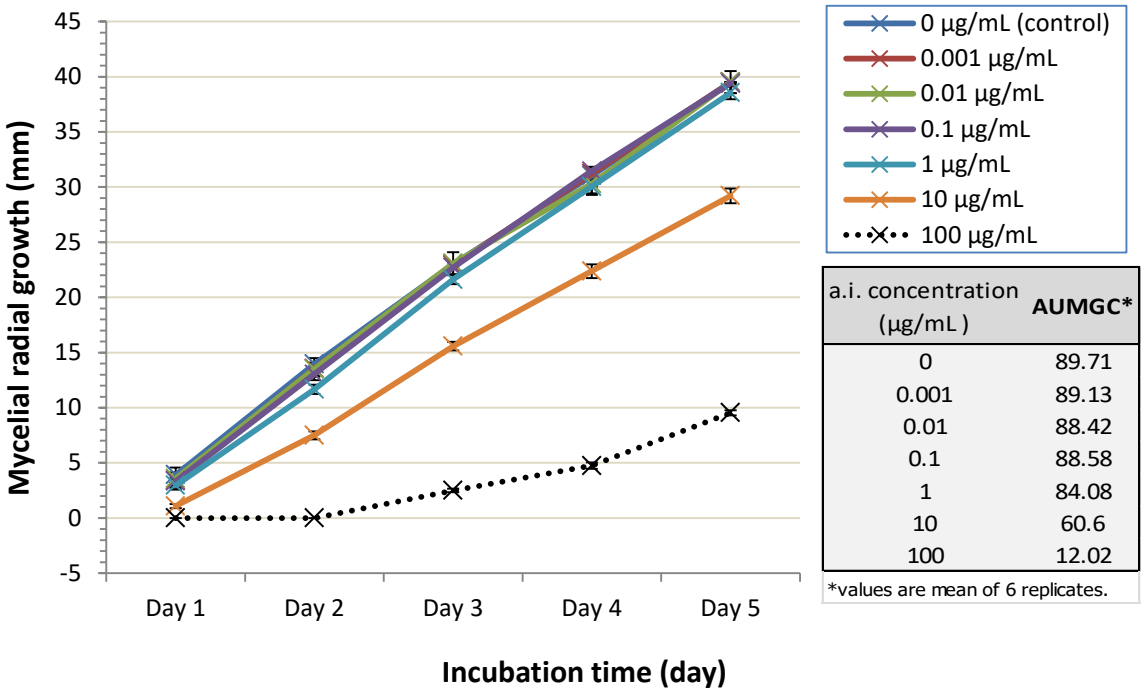
Final concentration a.i. in the agar plate (µg/ml)	Mycelial radial growth (mm) at 5 days after incubation*						
	cymoxanil	azoxystrobin	mancozeb	mancozeb+ benthiavalicarb -isopropyl	chlorothaloni+ tebuconazole	Zinc+ sulphur+ copper	phosphate+ potassium+ nitrogen
0 (control)	39.50a	36.21a	39.42a	39.50a	39.50a	39.50a	39.50a
0.001	39.50a	37.25a	36.71ab	38.95a	38.17ab	39.50a	39.50a
0.01	39.50a	34.50b	37.83ab	38.85a	38.10ab	39.50a	38.67ab
0.1	39.42a	31.58c	36.96b	37.28b	37.80b	39.50a	38.63ab
1	38.54a	31.67c	14.83c	1.48c	35.83c	38.18b	34.83c
10	29.21b	23.50d	0.00d	0.00d	12.54d	32.0c	26.25d
100	9.54b	12.38e	0.00d	0.00d	6.38e	6.96d	21.25e

*values are mean of 6 replicates and values with the same letter in the same column are not significant based on Fisher's Least Significant Difference Test at P=0.05

Table 6-3 Percentage of radial inhibition (PIRG) of isolate PPC280574 at 5 days after incubation on different fungicide/chemical

Final concentration a.i. in the agar plate (µg/ml)	% PIRG at 5 days after incubation*						
	cymoxanil	azoxystrobin	mancozeb	mancozeb+ benthiavalicar b-isopropyl	chlorothaloni+ tebuconazole	Zinc+ sulphur+ copper	phosphate+ potassium+ nitrogen
0 (control)	0.00c	0.00d	0.00d	0.00d	0.00e	0.00d	0.00d
0.001	0.00c	2.36d	5.52cd	1.7d	3.57de	0.00d	0.00d
0.01	0.00c	4.19d	4.02cd	1.47d	3.58de	0.00d	2.11d
0.1	0.21c	12.36c	6.26c	5.72c	4.32d	0.00d	2.22d
1	2.43c	12.08c	62.35b	96.32b	9.28c	3.37c	11.81b
10	26.05b	34.70b	100.00a	100.00a	68.25b	18.58b	33.54b
100	75.8a	65.7a	100.00a	100.00a	83.86a	82.38a	46.20a

*values are mean of 6 replicates and values with the same letter in the same column are not significant based on Fisher's Least Significant Difference Test at P=0.05

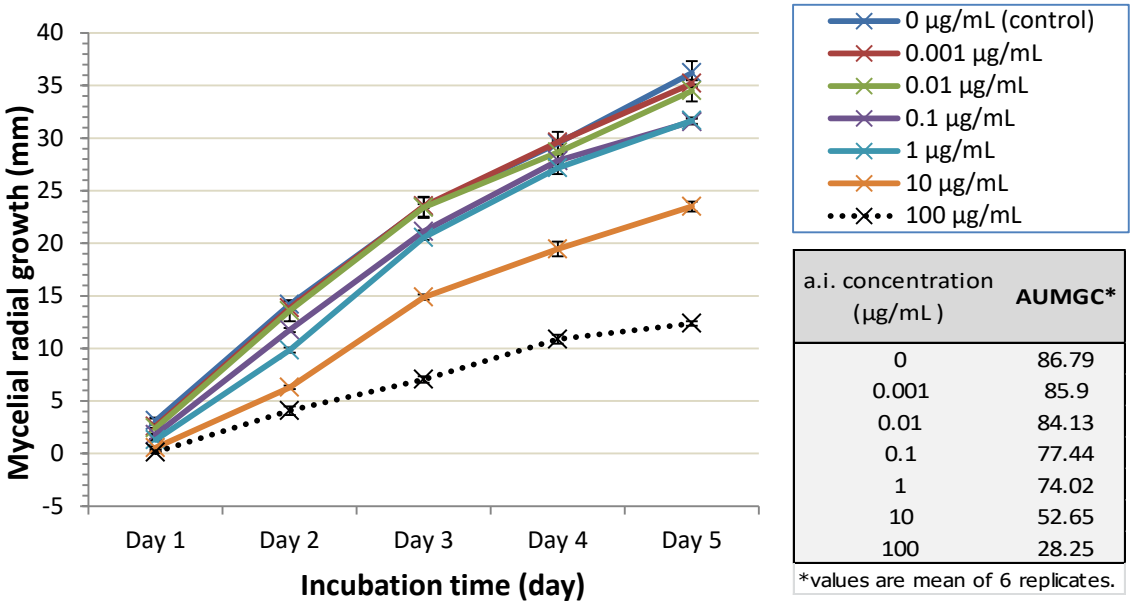


a.i. concentration (µg/mL)	AUMGC*
0	89.71
0.001	89.13
0.01	88.42
0.1	88.58
1	84.08
10	60.6
100	12.02

*values are mean of 6 replicates.

Figure 6-3 Mean of mycelial radial growth of* P. palmivora *on cymoxanil amended media.

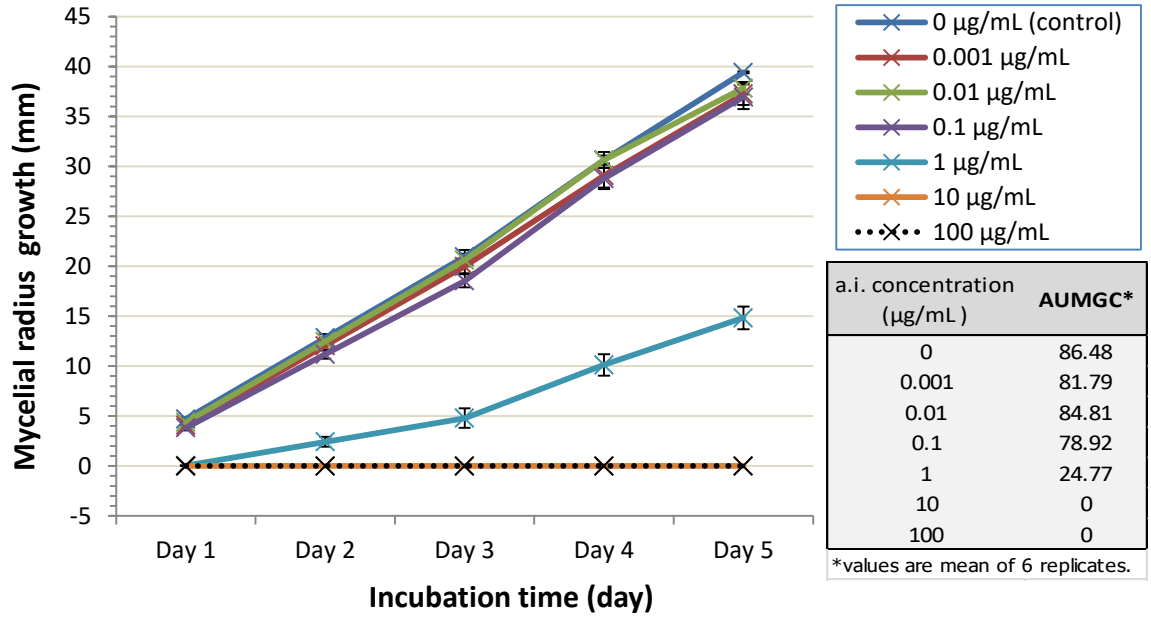
For azoxystrobin, a significant reduction was observed with a concentration of 0.01 µg/ml and higher, but more than 50% reduction was only observed with at least 100 µg/ml (Table 6-3) with the AUMGC of 28.25 (Figure 6-4). Meanwhile, two fungicides containing mancozed completely inhibited *P. palmivora* at concentrations of 10 µg/ml and 100 µg/ml; no growth of *P. palmivora* was observed on amended media from day 1 after incubation (Figure 6-5 and Figure 6-6). Mean of radial growth of the mycelia on media amended with both mancozed fungicides at a concentration of 0.1 µg/ml was found to be significantly lower than control plates with AUMGC values of 78.92 and 87.83, respectively. A mixture of chlorothalonil+tebuconazole showed significant reduction in mycelial growth compared to controls at 0.1 µg/ml a.i. (chlorothalonil) but with a small percentage of inhibition (4.32%). Half reduction was only observed on plates at 10 µg/ml a.i. with the AUMGC value of 26.69 (Figure 6-7). Two types of liquid fertilizer/growth enhancer containing a mixture of zinc+sulphur+copper and mixture of phosphate+potassium+nitrogen also significantly reduced mycelial growth of *P. palmivora* at a concentration of 1 µg/ml a.i. and higher. The PIRG at a concentration of 1 µg/ml a.i. was calculated at 3.37% for mixture solution of zinc+sulphur+copper and 11.81% for mixture solution of phosphate+potassium+nitrogen. Higher inhibition (≥50%) was only observed at the high concentration of 100 µg/ml a.i. with AUMGC of 13.23 and 46.56, respectively (Figure 6-8 and Figure 6-9).



a.i. concentration (µg/mL)	AUMGC*
0	86.79
0.001	85.9
0.01	84.13
0.1	77.44
1	74.02
10	52.65
100	28.25

*values are mean of 6 replicates.

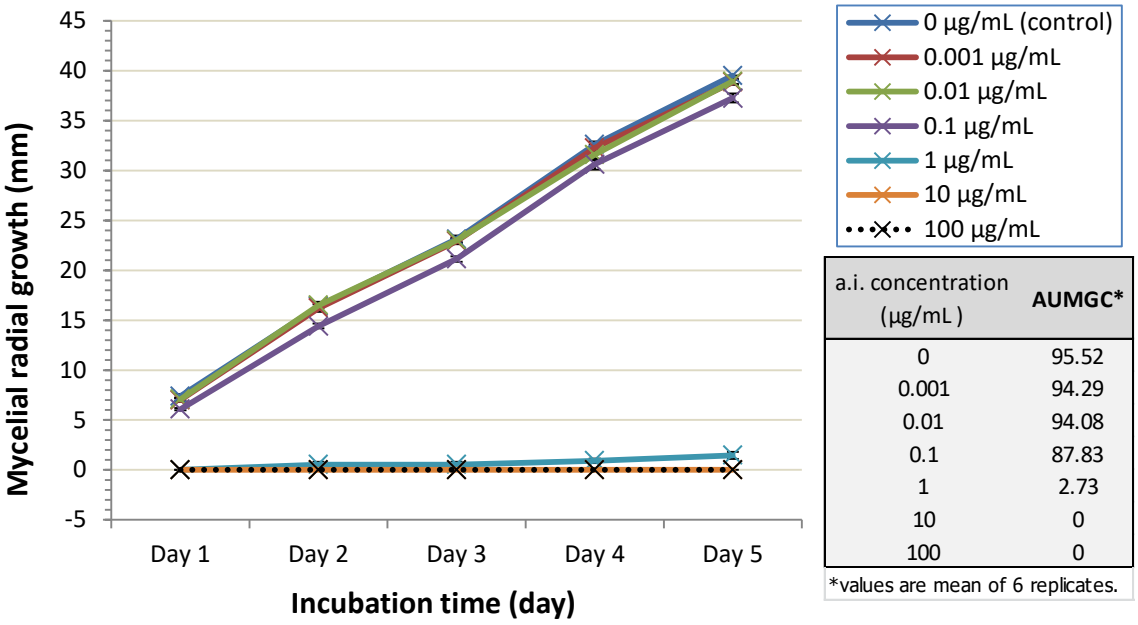
Figure 6-4 Mean of mycelial radial growth of* P. palmivora *on azoxystrobin amended carrot agar media



a.i. concentration (µg/mL)	AUMGC*
0	86.48
0.001	81.79
0.01	84.81
0.1	78.92
1	24.77
10	0
100	0

*values are mean of 6 replicates.

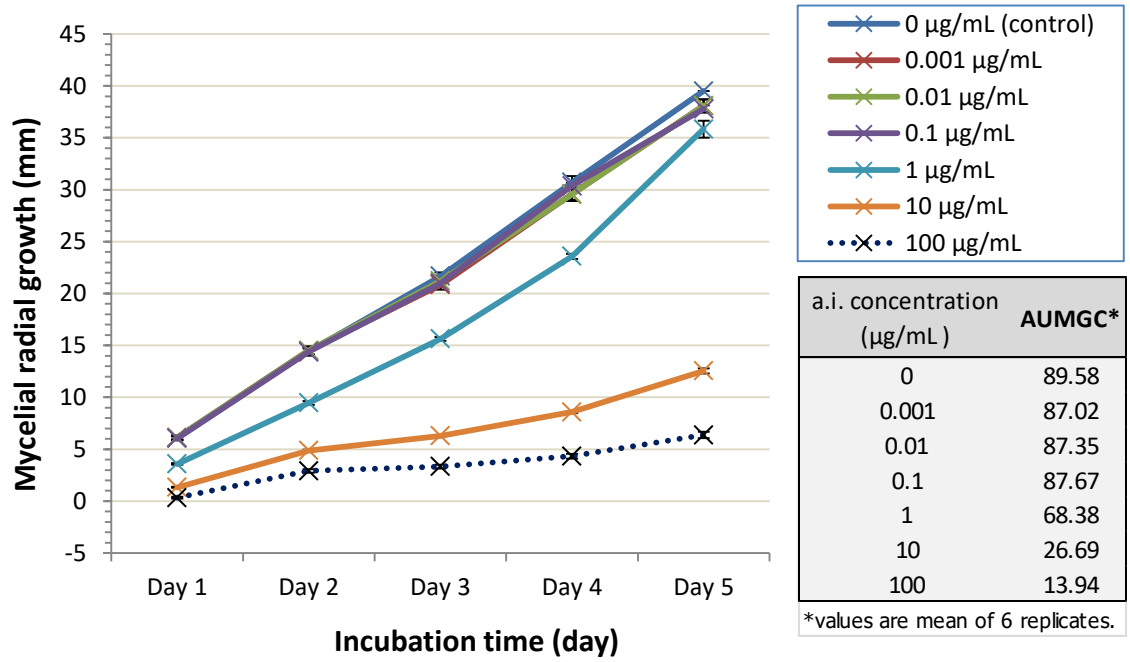
Figure 6-5 Mean of mycelial radial growth of* P. palmivora *on mancozeb amended carrot agar media



a.i. concentration (µg/mL)	AUMGC*
0	95.52
0.001	94.29
0.01	94.08
0.1	87.83
1	2.73
10	0
100	0

*values are mean of 6 replicates.

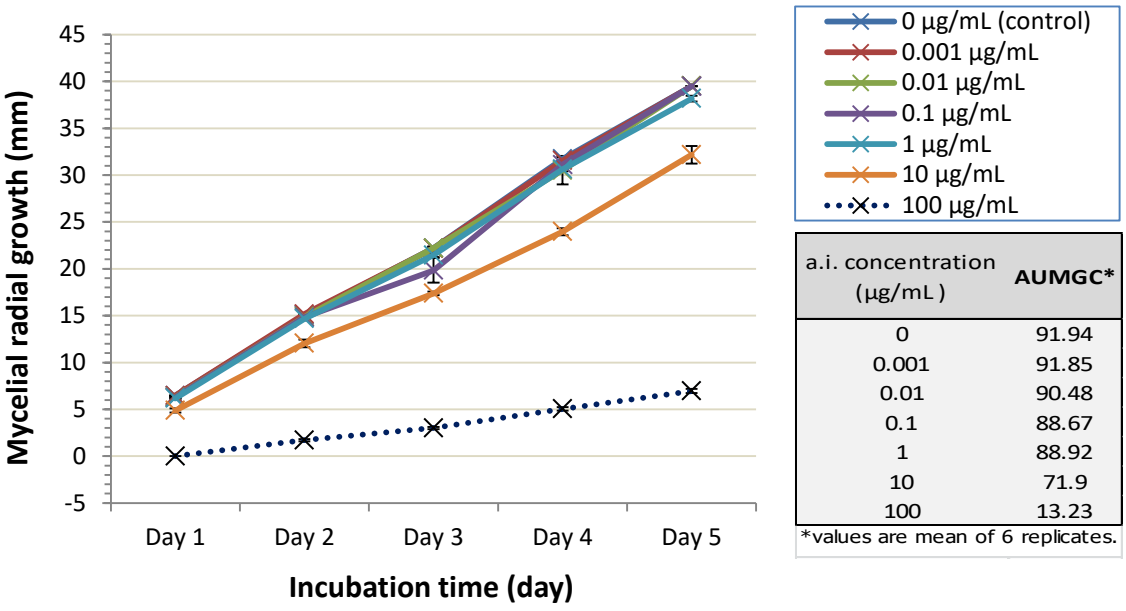
Figure 6-6 Mean of mycelial radial growth of* P. palmivora *on mancozeb+benthiavalicarb-isopropyl amended media



a.i. concentration (µg/mL)	AUMGC*
0	89.58
0.001	87.02
0.01	87.35
0.1	87.67
1	68.38
10	26.69
100	13.94

*values are mean of 6 replicates.

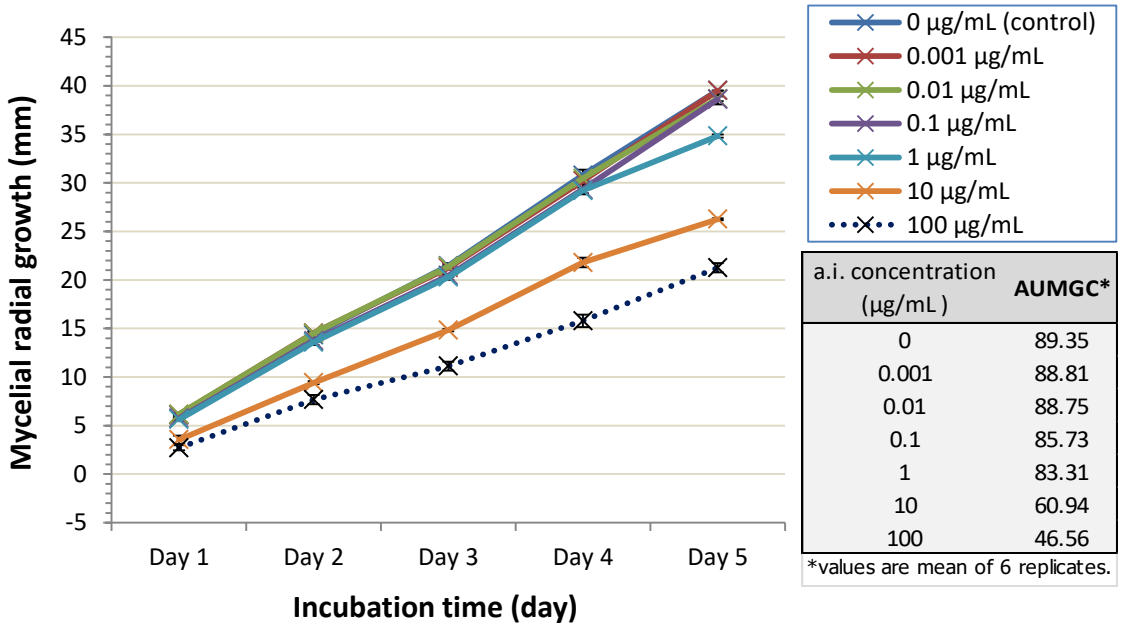
Figure 6-7 Mean of mycelial radial growth of* P. palmivora *on chlorothalonil+ tebuconazole amended media



a.i. concentration (µg/mL)	AUMGC*
0	91.94
0.001	91.85
0.01	90.48
0.1	88.67
1	88.92
10	71.9
100	13.23

*values are mean of 6 replicates.

Figure 6-8 Mean of mycelial radial growth of* P. palmivora *on liquid fertilizer containing phosphate+potassium+nitrogen amended media



a.i. concentration (µg/mL)	AUMGC*
0	89.35
0.001	88.81
0.01	88.75
0.1	85.73
1	83.31
10	60.94
100	46.56

*values are mean of 6 replicates.

Figure 6-9 Mean of mycelial radial growth of* P. palmivora *on liquid fertilizer containing zinc+sulphur+copper amended media

6.3.2 Isolates PPM1, PPM4 and IMI382544

For the assay with PPM1 with a concentration of a.i. from 1 to 100 µg/ml, all fungicides/chemicals showed a significant reduction of mycelial growth compared to controls except with mixture solution of phosphate+potassium+nitrogen which only showed significant reduction at a concentration of 10 µg/ml (Table 6-4). The half reduction was observed at 1 µg/ml for azoxystrobin, mancozeb+benthiavalicarb-isopropyl and chlorothalonil+tebuconazole and 10 µg/ml for cymoxanil and mancozeb. The liquid fertilizers, zinc+sulphur+copper and phosphate+potassium+nitrogen only reduce growth to half at the higher concentration of 100 µg/ml (Table 6-5). Complete inhibition of PPM1 was observed with mancozeb and mancozeb+benthiavalicarb-isopropyl at 10 µg/ml but only with fungicide mixture mancozeb+benthiavalicarb-isopropyl for isolate PPM4 (Table 6-6 and Table 6-7) and nearly 100% for isolate IMI382544 (Table 6-8 and Table 6-9). In general, *in vitro* assays with 1 µg/ml and 10 µg/ml a.i. of all tested fungicides showed significant differences in mycelial growth inhibition among isolates PPC280574, PPM1, PPM4 and IMI382544 (Figure 6-10 and Figure 6-11). Cymoxanil, azoxystrobin and chlorothalonil+tebuconazole showed higher inhibition of PPM1 compared to other isolates; meanwhile, mancozeb, mancozeb+benthiavalicarb-isopropyl and phosphate+potassium+nitrogen showed significantly higher inhibition on isolate PPC280574 at 1 µg/ml a.i., meanwhile zinc+sulphur+copper appeared to have higher suppression on isolate IMI382544, which originated from coconut in Indonesia.

Table 6-4 Mycelial radial growth of* P. palmivora *isolate PPM1 in amended carrot agar after 5 days of incubation

Final concentration in the agar plate (µg/ml)	Mycelial radial growth (mm) at 5 days after incubation (DAI)*						
	cymoxanil	azoxystrobin	mancozeb	mancozeb+ benthiavalicarb -isopropyl	chlorothaloni+ tebuconazole	Zinc+ sulphur+ copper	phosphate+ potassium+ nitrogen
0 (control)	30.88a	33.19a	36.00a	23.81a	32.94a	31.37a	28.1a
1	24.56b	17.00b	27.94b	3.25b	14.13b	28.42b	26.9a
10	11.06c	15.19b	0.00c	0.00c	5.63c	26.01c	22.2b
100	0.00d	2.13c	0.00c	0.00c	0.31d	17.80d	9.9c

*values are mean of 4 replicates and values with the same letter in the same column are not significant based on Fisher's Least Significant Difference Test at P=0.05

Table 6-5 Percentage of radial inhibition (PIRG) of isolate PPM1 at 5 days after incubation on different fungicide/chemical

Final concentration in the agar plate (µg/ml)	% PIRG at 5 days after incubation*						
	cymoxanil	azoxystrobin	mancozeb	mancozeb+ benthiavalicarb -isopropyl	chlorothaloni+ tebuconazole	Zinc+ sulphur+ copper	phosphate+ potassium+ nitrogen
0 (control)	0.00d	0.00c	0.00c	0.00c	0.00d	0.00d	0.0c
1	20.47c	48.77b	22.36b	86.37b	57.12c	9.32c	3.9c
10	66.56b	54.22b	100.00a	100.00a	82.95b	17.06b	21.1b
100	100.00a	93.59a	100.00a	100.00a	99.03a	43.28a	64.6a

*values are mean of 4 replicates and values with the same letter in the same column are not significant based on Fisher's Least Significant Difference Test at P=0.05

Table 6-6 Mycelial radial growth of* P. palmivora *isolate PPM4 in amended carrot agar after 5 days of incubation

Final concentration in the agar plate (µg/ml)	Mycelial radial growth (mm) at 5 days after incubation (DAI)*						
	cymoxanil	azoxystrobin	mancozeb	mancozeb+ benthiavalicarb -isopropyl	chlorothaloni+ tebuconazole	Zinc+ sulphur+ copper	phosphate+ potassium+ nitrogen
0 (control)	34.56a	37.31a	39.88a	31.31a	36.63a	38.94a	35.41a
1	32.38a	35.94a	31.44b	6.81b	23.69b	34.25b	35.15a
10	25.81b	30.44c	8.69c	0.00c	8.75c	33.44b	33.06b
100	0.00c	16.88c	0.00d	0.00c	0.31d	30.19c	25.44c

*values are mean of 4 replicates and values with the same letter in the same column are not significant based on Fisher's Least Significant Difference Test at P=0.05

Table 6-7 Percentage of radial inhibition (PIRG) of isolate PPM4 at 5 days after incubation on different fungicide/chemical

Final concentration in the agar plate (µg/ml)	% PIRG at 5 days after incubation*						
	cymoxanil	azoxystrobin	mancozeb	mancozeb+ benthiavalicarb -isopropyl	chlorothaloni+ tebuconazole	Zinc+ sulphur+ copper	phosphate+ potassium+ nitrogen
0 (control)	0.00c	0.00c	0.00d	0.00c	0.00d	0.00c	0.00c
1	6.33c	3.65c	21.08c	78.25b	35.33c	12.03b	0.72c
10	25.39c	18.42b	78.21b	100.00a	76.11b	14.12b	6.64b
100	100.00a	54.76a	100.00a	100.00a	99.15a	22.48a	28.14a

*values are mean of 4 replicates and values with the same letter in the same column are not significant based on Fisher's Least Significant Difference Test at P=0.05

Table 6-8 Mycelial radial growth of* P. palmivora *isolate IMI382544 in amended carrot agar after 5 days of incubation

Final concentration in the agar plate (µg/ml)	Mycelial radial growth (mm) at 5 days after incubation (DAI)*						
	cymoxanil	azoxystrobin	mancozeb	mancozeb+ benthiavalicarb -isopropyl	chlorothaloni+ tebuconazole	Zinc+ sulphur+ copper	phosphate+ potassium+ nitrogen
0 (control)	32.11a	29.44a	35.88a	30.63a	31.38a	38.94a	32.19a
1	29.40b	27.88a	29.81b	8.44b	19.38b	29.13b	29.50b
10	19.40c	23.63b	0.31c	0.00c	10.31c	26.94c	29.50b
100	0.89d	17.44b	0.00c	0.00c	6.44d	16.69d	17.13c

*values are mean of 4 replicates and values with the same letter in the same column are not significant based on Fisher's Least Significant Difference Test at P=0.05

Table 6-9 Table 5 11 Percentage of radial inhibition (PIRG) of isolate IMI382544 at 5 days after incubation on different fungicide/chemical

Final concentration in the agar plate (µg/ml)	% PIRG at 5 days after incubation*						
	cymoxanil	azoxystrobin	mancozeb	mancozeb+ benthiavalicarb -isopropyl	chlorothaloni+ tebuconazole	Zinc+ sulphur+ copper	phosphate+ potassium+ nitrogen
0 (control)	0.00d	0.00c	0.00c	0.00c	0.00d	0.00d	0.00c
1	8.36c	5.13c	17.04b	72.50b	38.28c	25.18c	8.21b
10	39.57b	19.76b	99.13a	100.00a	67.15b	30.81b	8.21b
100	97.22a	40.61a	100.00a	100.00a	79.51a	57.15a	46.82a

*values are mean of 4 replicates and values with the same letter in the same column are not significant based on Fisher's Least Significant Difference Test at P=0.05

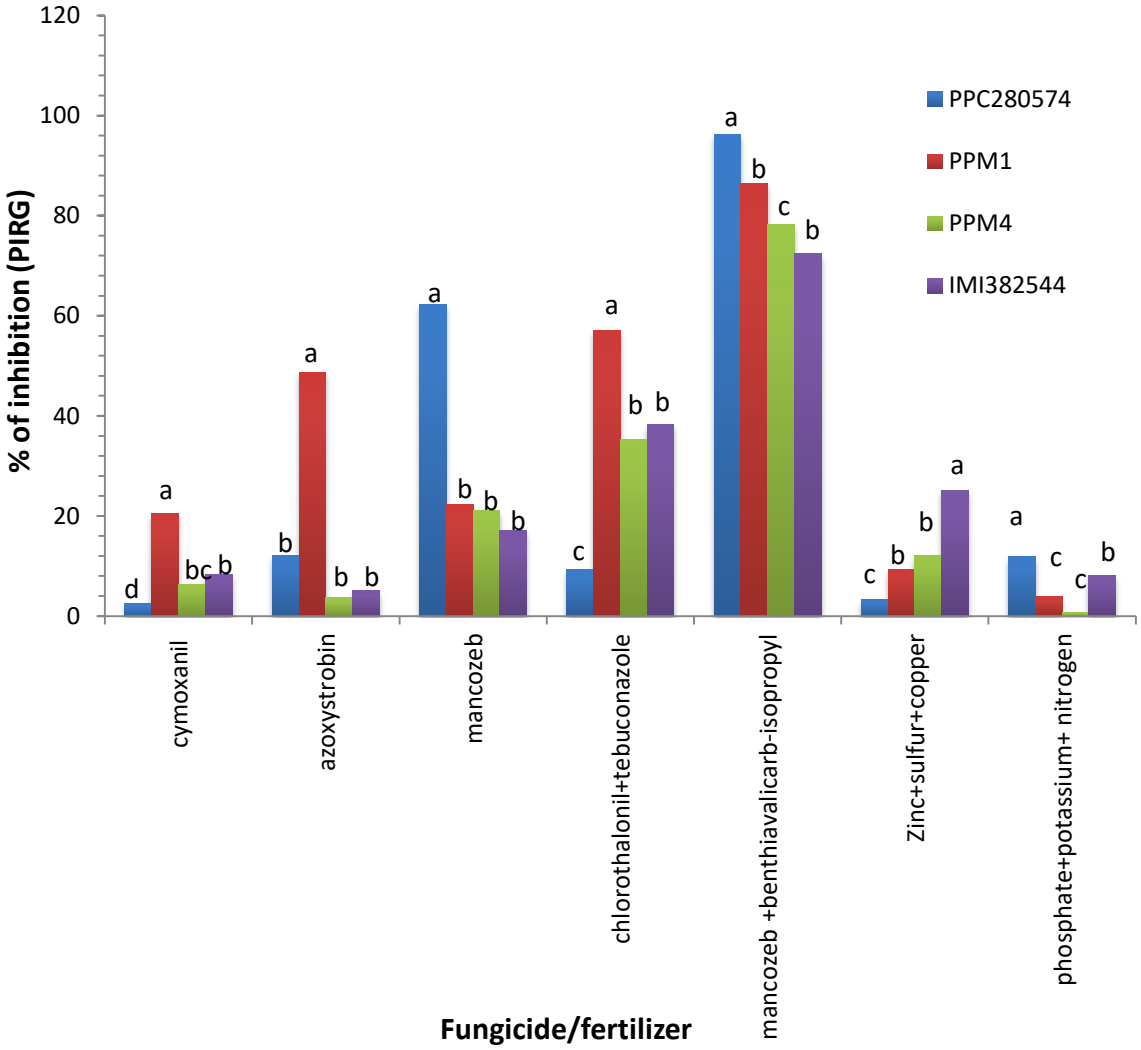


Figure 6-10 Percentage of inhibition (PIRG) of four different* P. palmivora *isolates using various type of fungicide/fertilizer at concentration a.i. of 1 µg/ml calculated based on radial growth measured at 5 days after incubation.

Note: Plotted values are mean of 4 replicates and values with the same letter between isolates for each chemical not significant based on Fisher's Least Significant Difference Test at P=0. 05

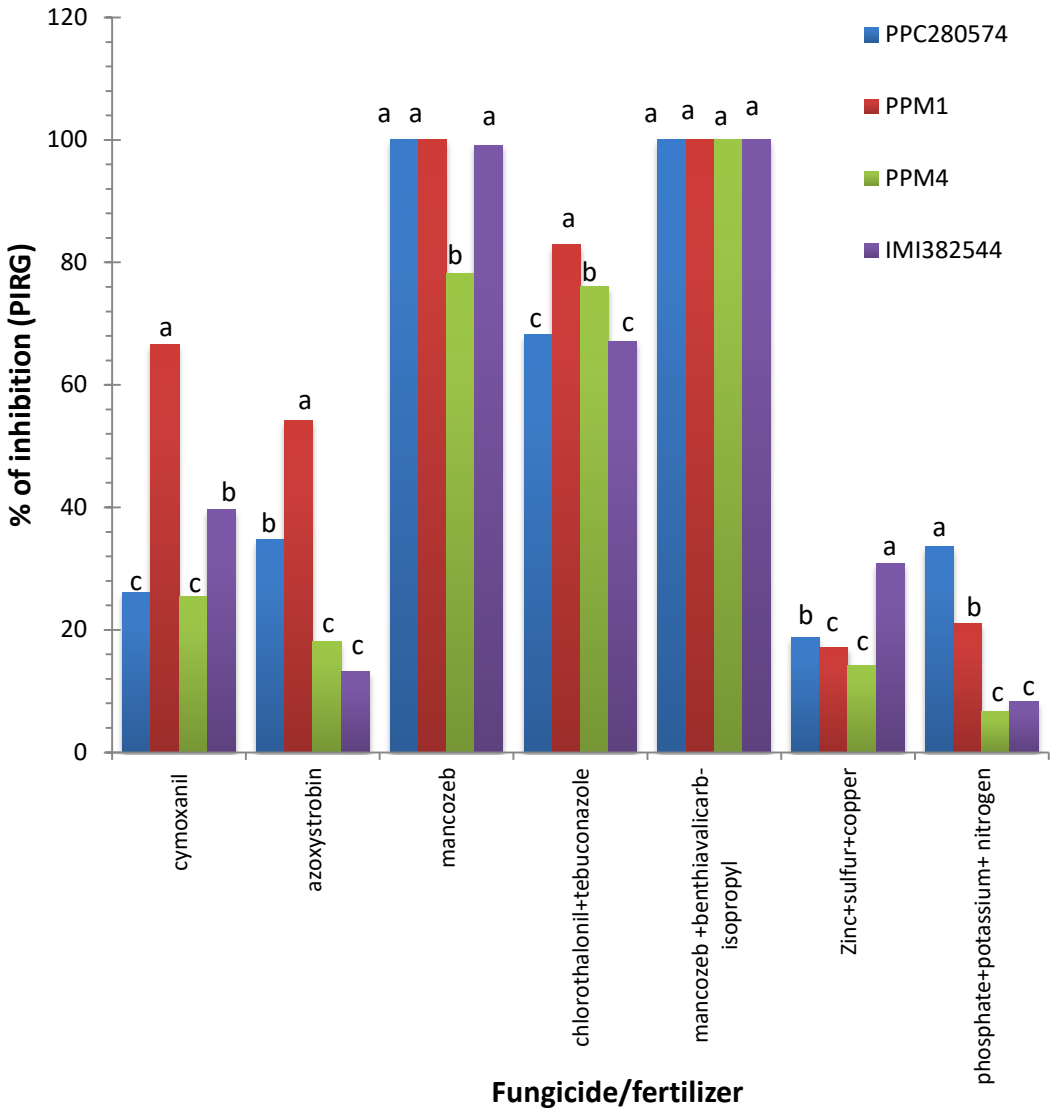


Figure 6-11 Percentage of inhibition (PIRG) of four different* P. palmivora *isolates using various type of fungicide/fertilizer at concentration a.i. of 10 µg/ml calculated based on radial growth measured at 5 days after incubation.

Note: Plotted values are mean of 4 replicates and values with the same letter between isolates for each chemical not significant based on Fisher's Least Significant Difference Test at P=0.05

6.4 Discussion

Poison agar tests, also known as poison food tests, are one of the methods used to evaluate chemicals and compounds with antifungal potential, along with other *in vitro* methods such as the agar-well diffusion technique (Magaldi et al., 2004), spore germination tests (Everett et al., 2005), dual culture plug technique (Navi et al., 2016) and paper disc-agar diffusion technique (Conner, 1983). *In vitro* evaluation expedites the screening process for evaluating potential compounds to control plant pathogens. In this study, an *in vitro* assay was tested with seven commercial fungicides. Three of them, cymoxanil, mancozeb 75% w/w and mancozeb+benthiavalicarb-isopropyl consist of at least one active ingredients (a.i.) known to be effective on *Phytophthora* species, cymoxanil and mancozeb. Two liquid fertilizers, which consist of growth promoting elements such as phosphate, zinc, sulphate and potassium, were also included as comparison.

Using isolate PPC280574 from oil palm in Colombia, it was found that the highest mycelial growth inhibition (%) was achieved using fungicide mancozeb +benthiavalicarb-isopropyl followed by mancozeb 75% w/w which effectively inhibit more than 50% of growth at 1 µg/ml and completely inhibit growth at 10 µg/ml. Mancozeb is a protectant fungicide that belongs to the dithiocarbamate group, more specifically to a class of compounds known as ethylene bisdithiocarbamates (EBDCs)(Gullino et al., 2010). Wagner et al. (2008) observed complete inhibition of mycelial growth of *P. ramorum* with the fungicide Dithane Ultra® WP (with mancozeb as a.i.) at a final concentration of >1000 µg/ml, whilst reported complete inhibition of *P. ramorum* at 100 µg/ml. Inhibition of zoospore germination was observed at a lower concentration of 1 µg/ml a.i. (Wagner et al., 2008). Meanwhile, Tey and Wood (1983)

reported an EC_{50} at 24 µg/ml Dithane 945® (mancozeb 80% w/w) against *P. palmivora* isolated from cocoa, which was a higher dosage than our estimations, which were calculated at 0.36 µg/ml a.i. for mancozeb 75% w/w and 0.24 µg/ml a.i. mancozeb +benthiavalicarb-isopropyl; however, different product formulations have slightly different efficacies especially if the fungicide consists of more than one a.i. with different modes of action (Cohen and Levy, 1990; Gisi, 1991). In addition, mixtures of more than one a.i. may also increase the efficacy. In our case, a mixture of mancozeb+benthiavalicarb-isopropyl showed superior inhibition to single mancozeb 75% w/w at the same a.i. level.

Benthiavalicarb-isopropyl belongs to a new chemical class, the amino acid amide carbamates, and was shown to have a high activity on mycelial growth, sporulation and sporangia/zoospore germination of *P. infestans* (LC_{90} of mycelial growth at 0.07 µg/ml a.i.) (Hofman and Van Oudheusen, 2004). This fungicide also showed 100% inhibition of mycelial growth of *P. ramorum* at 0.1 µg/ml (Heungens et al., 2006). Nevertheless, the efficacy mixture of mancozeb+benthiavalicarb-isopropyl in our study against *P. palmivora* (PPC280574) was lower.

Efficacy of the other two fungicides containing a.i. known to have potential against *Phytophthora* species, cymoxanil and chlorothalonil +tebuconazole indicated that these were less effective than the mancozeb against *P. palmivora* in this study. Their efficacy was shown to be nearly the same level as azoxystrobin, and two liquid fertilizers mixture zinc+sulphur+copper and mixture phosphate+potassium+nitrogen, where complete inhibition was not achieved even at the high concentration of 100 µg/ml a.i.. Chlorothalonil was also reported to not completely inhibit *P. ramorum* at the same concentration (Heungens et al., 2006). In this study, the ranking of effectiveness *in vitro* against *P. palmivora* (with the exception of mancozeb fungicides)

at 10 µg/ml was chlorothalonil+tebuconazole) followed by azoxystrobin, phosphate fertilizer (phosphate+potassium+ nitrogen) cymoxanil and zinc fertilizer (Zinc+ sulphur+copper) based on overall mycelial growth expressed as AUMGC and growth inhibition (PIRG). This is interesting since cymoxanil has been reported to be effective on oomycetes including some species of *Phytophthora* (Schwinn and Staub, 1987). For example, it was reported to completely inhibit *P. ramorum* at 100 µg/ml (Heungens et al., 2006) and the EC_{50} of cymoxanil against several isolates of *P. infestans* was reported at between 0.27-0.57 µg/ml by Rekanović et al. (2012) and at 1 µg/ml by Ziogas and Davidse (1987). Cymoxanil was reported to have low efficacy against *P. citrophora* (Thomidis and Tsipouridis, 2001), but not many evaluations of cymoxanil against *P. palmivora* have been reported. The most similar is the evaluation of cymoxanil (8% w/w)+mancozeb (64% w/w) by Sharadraj and Mohanan (2014a) using *P. palmivora* isolated from bud rot disease of coconut which showed complete inhibition of mycelial growth at 250 µg/ml.

It was found that the inhibition of mycelial growth using some fungicides were variable for different isolates of *P. palmivora*. In some assays, there were significant differences between isolates but not always. For example, the percentage of inhibition using isolates PPM1, PPM4 and IMI3825 with both mancozeb 75% w/w and mancozeb +benthiavalicarb-isopropyl appeared to be significantly lower ($P<0.05$, LSD) than for isolate PPC280574 at 1 µg/ml a.i., but not different at 10 µg/ml where all isolates (except PPM4 on mancozeb 75% w/w amended media) showed a complete inhibition. On the assay with cymoxanil, whilst less than 50% of inhibition was recorded for other isolates at 10 µg/ml, PPM1 showed good inhibition with more than 50% inhibition. Zhu et al. (2008) and Rekanović et al. (2012) in their fungicide evaluations of several fungicides including cymoxanil and mancozeb reported variation in mycelial growth

inhibition for different isolates of *P. infestans.* The variability might be due to inevitable experimental variation such as nutrient content of the media, incubation temperature and the condition of inoculum, despite the efforts to minimize variability in these factors.

Overall, the efficacy data obtained from the *in vitro* tests is valuable in giving us initial insight on the effectiveness of the tested compounds. However, they do not show exactly what would work *in planta* since some fungicides and compounds has complex modes of action in inhibition of the pathogen. Therefore, *in vitro* evaluations are usually followed up with *in planta* evaluations, which are more likely to resemble field, or natural conditions. However, *in planta* evaluations could not be carried out in this study because of materials and time constraints. In future, more chemicals or fungicides can be tested against *P. palmivora in vitro* and *in planta* as one of the efforts to improve control and management of the bud rot disease in oil palm and other crops. The fungicides with good efficacy against *P. palmivora* can be used to reduce the inoculum potential in soil and infected plant tissue by several application methods such as soil drenches, sprays, trunk/stem paint and also trunk injection. For example, Vawdrey et al. (2004) have tested the possibility of managing the foliar disease of papaya caused by *P. palmivora* using metalaxyl and potassium phosphonate applied by soil drenching. In the case of bud rot disease infection in the oil palm, the use of trunk injection can also be explored since spraying will be a problem due to the plant height. The choice of the fungicide application method should consider several aspects such as the nature of the infection, the plant physiology, mode of action of the fungicides and practicality in the field. The safety aspect in term of fungicides residue in the edible crop should not be neglected, and this should also include the possible hazard to humans and other ecosystem components. The effectiveness of the

fungicides might also depend on the stage of the infection. The opportunity to control and reduce damage caused by the disease is usually higher if the disease is managed in the earlier stages. Therefore, the ability to detect the disease in the earlier development is crucial to mitigate the losses.

Chapter 7. General discussion

Bud rot disease has been a threat to the oil palm industry in South America for the past few decades, and recent advances in the study of this disease have started to fill in some knowledge gaps about the causal agent, which in Colombia has been identified as a species of the oomycetes, *Phytophthora palmivora* (Torres et al., 2010). This *Phytophthora* species is also known to be responsible for several tropical diseases on other plant species in Malaysia and other countries in South East Asia (SEA) but has never been reported in oil palm in SEA until now. However, the same species has been identified as one of the possible causes of bud rot disease of other palm species in SEA, including the coconut in Indonesia (Blaha et al., 1994; Purwantara et al., 2004), the Philippines (Concibido-Manohar, 2004) and India (Sharadraj and Mohanan, 2013a). *Phytophthora palmivora* is the most commonly found *Phytophthora* species in the tropics and was first described by Butler in 1919 (Drenth and Guest, 2004a). Recently, *P. palmivora* has also been reported to cause bud rot disease on Bactris/Palmito palm (*Bactris gasipaes*) in Ecuador (Ordoñez et al., 2016).

The objectives of this present study were to understand why the disease occurs in oil palm in South America, such as in Colombia, but not in South East Asia, by looking at aspects of the genetic diversity of the causal agent, *P. palmivora.* Our aim was to establish whether the genomic sequences of *P. palmivora* pathogenic to oil palm in South America were similar to the genomic sequences of the isolates originating from other regions and hosts, in particularly the isolates from Malaysia. To do this, we collected 26 isolates of *P. palmivora,* including one isolate pathogenic to oil

palm, with the help of Cenipalma, Colombia, and a further 11 isolates of different *Phytophthora* species.

Isolation of *P. palmivora* from cocoa pods and diseased leaves of cocoa seedlings can be carried out by plating the diseased tissue from the margins of the lesion directly onto the selective media $P_{10}VP$ containing CMA without any serious contamination from unwanted fungi and bacteria, and the use of $P_{10}VP$ has been shown to work successfully for other isolates of *Phytophthora* and eliminate most fungi except *Pythium* and *Mortierella* (Brodrick et al., 1975). However, to isolate *P. palmivora* from diseased oil palm in the field, a baiting method is preferred as demonstrated by Torres et al. (2010), as direct isolation onto the selective media was shown to usually fail (Martinez, 2014, *personal communication*). However, re-isolation from diseased tissue of oil palm following artificial inoculation carried out in the lab is possible, probably due to low contamination by other opportunistic microbes on the tissue samples inoculated in the lab since this is done in a controlled environment. Attempts to isolate *P. palmivora* from soil taken from cocoa fields and oil palm plantations in Malaysia were unsuccessful, using both direct plating onto selective media and baiting using apple and cocoa pods. This might be due to the wrong sampling and isolation methods.

In order to confirm the identity of the isolates, identification of all isolates, including the isolates obtained from culture collection centres, were carried out using molecular techniques. It was found that identification of the *P. palmivora* isolates using the Basic Local Alignment Search Tool (BLAST) of internal transcribed spacer (ITS) and cytochrome c oxidase subunit I (*CoxI*) sequences with the GenBank® database can be achieved with a high score and identity value (ident value ≥ 99%). The ITS region has been sequenced and used extensively as a genetic marker for

identification and to address research questions relating to systematics and phylogeny of oomycetes up to species level (Diaz et al., 2012). Meanwhile the *CoxI* gene has been accepted by GenBank® and the Consortium for the Barcode of Life (CBOL) as the default DNA barcode of the oomycetes genus *Phytophthora* and has proven useful in phylogenetic studies of them (Robideau et al., 2011). The morphological characteristics of all the *P. palmivora* isolates were the same as described by Waterhouse (1963). The hyphae of *P. palmivora* was smooth and very thin on corn meal agar (CMA) for all isolates but looked denser on carrot agar and V8 agar with stellate to rosaceous growth patterns, but the patterns were not consistently shown for each isolate. Although we did not conduct a comprehensive study on the morphological characteristics, colony morphology and sporangia shape and size were observed to vary from one plate to another for the same isolate. According to Duncan and Cooke (2002) some morphological characteristics of *Phytophthora* are not constantly expressed in cultures and may vary even within isolates.

In Chapter 3, the genetic variation of all the isolates collected was studied using two methods. These were DNA sequence analysis of several DNA regions and the AFLP fingerprinting method. Analysis of DNA sequences of target regions or genes has been used to study the diversity, phylogenetics and polymorphisms among oomycetes, particularly *Phytophthora*. In the present study, several nuclear and mitochondrial DNA genes were selected as genetic markers including a non-coding region, the rDNA internal transcribed spacer (ITS). The selected genes; beta-tubulin (*β-tubulin*), translation elongation factor 1 alpha (*EF-1α*), cytochrome c oxidase subunit I (*CoxI*) and subunit II (*CoxII*) are housekeeping genes that have been previously studied in research on diversity, phylogenetic evolutionary and relationships of various *Phytophthora* species (Martin and Tooley, 2003a; Villa et al., 2006; Blair et

al., 2008). Housekeeping genes are genes that are required for the maintenance of basic cellular functions (Eisenberg and Levanon, 2013). In this study, the variation among DNA sequence in ITS region among 26 different isolates of *P. palmivora* was between 0-3%. Phylogenetic differences based on the ITS of 26 isolates of *P. palmivora* from various host and demographic origins was not clearly observed. However, good variations and phylogenetic separation was achieved between *P. palmivora* and other reference species (*P. parasitica, P. cryptogea*, *P. infestans, P. colocasiae* and *P. megakarya*).

Phylogenetic trees reconstructed using sequence data for *CoxI*, *CoxII*, *β-tubulin* and *EF-1α*, along with one additional marker, the *ras*-related protein gene (*Ypt1*) demonstrated similar findings to the ITS, with low intraspecific variations in DNA sequences among the *P. palmivora* isolates including the multi-locus tree reconstructed from the concatenated partial sequences of the five genes. All trees did not exhibit consistent similarities in grouping based on demographic and host origin. Malaysian and Colombian isolates were not separated into different clusters and the sub-clusters did not show any meaningful characteristics based on host and origin of the isolates. It was concluded that the ITS region, *CoxI, CoxII*, *β-tubulin* and *EF-1α* genes are more suitable for inter-specific studies between species but not for intra-specific evaluation within species of *P. palmivora*. Previous studies also show that a high resolution at interspecific levels was achieved using the ITS marker such as demonstrated by Lee and Taylor (1992) and Cooke and Duncan (1997). Intraspecific variations using this region are rarely encountered and limited (Sorensen et al., 1998) but not impossible for some species of *Phytophthora* and fungi.

Since the evolution of one (or several specific) gene (s) may not represent the entire genome (Villa et al., 2006), another approach using a fingerprinting method

was included in this study in order to look at a broader perspective. Amplified fragment length polymorphism (AFLP) was chosen based on the ability of this method to simultaneously screen many DNA regions distributed randomly throughout the genome. Hence the analyses involve the whole genome rather than specific loci. The analyses were carried out using some representative isolates of *P. palmivora* from Colombia and Malaysia. The phylogenetic tree based on the fragment analysis data of the CE of three primer markers, *Eco*RI-A/*Mse*I-AG, *Eco*RI-AC/*Mse*I-AG and *Eco*RI-TA/*Mse*I-AG, showed a separation of Colombian and Malaysian isolates into distinct clades, which indicates that there is genomic variation within *P. palmivora* isolates. It would have been useful to extend the AFLP analyses using these primers to other *P. palmivora* isolates to see the phylogenetic relationship of all isolates. In addition, it would be interesting to study the genetic variation of *P. palmivora* using additional molecular markers as some of the housekeeping genes are highly conserved and whilst they might be useful to study interspecific variation, they are less suitable for use in the intraspecific study within the same species.

The AFLP data in this current study triggered the idea of trying to find other regions or genes that might have variation in DNA sequences between isolates, since the other objective of this study was to develop a detection method that can identify the presence of *P. palmivora* in the plant samples, and hopefully can distinguish between Malaysian and Colombian isolates. Amongst the regions/genes of interest are the genes that encode the proteins involved in the infection processes of the *Phytophthora* such as genes encoding effector proteins, the *Avr* gene. We found no references on any *Avr* genes of *P. palmivora* during the study but studies on *P. infestans,* the causal agent of potato late blight responsible for the Irish potato famine in the 1840s, are much more extensive and a number of *Avr* genes have been

identified. In this study, attempts were made to sequence the DNA encoding an effector protein of *P. palmivora*. One avirulence (*Avr4*) gene encoded to the effector protein of *P. infestans* identified as an RXLR-dEER effector (van Poppel et al., 2008; van Poppel, 2009) was chosen based on the availability of the sequence in the GenBank®. A set of primers (ARP1F and ARP1R) were designed based on this DNA sequence. It was possible to sequence a fragment from the PCR amplification of *P. palmivora* isolates originated from the diseased oil palm from Colombia (PPC280574) using these primers (conducted at a low annealing temperature). This sequence is assumed to be a putative *Avr* gene of *P. palmivora*, hence the sequence fragment was called *Phytophthora palmivora* hypothetical avirulence protein gene/region (PpHPAVR). Phylogenetic evaluation using the PpHPAVR sequences showed some level of intraspesific variation among isolates of *P. palmivora,* particularly of Colombian and Malaysian isolates. This might be related to the level of aggressiveness of the isolates, particularly if this sequence is related to the avirulence gene, but further studies on the characteristics and functionality of the gene need to be conducted. It is also hoped that more studies can/will be conducted on other avirulence genes (e.g. *Avr3*, *Avr2*) encoding for avirulence effector proteins of *P. palmivora*, which will hopefully provide more information on the PpHPAVR fragment observed in this study. Furthermore, it will be interesting to include more isolates of *P. palmivora* from Malaysia, Indonesia, Thailand and other South East Asia countries in the future studies of genetic variation, particularly using the PpHPAVR marker.

Since the PpHPAVR region showed some level of DNA variation, the development of primers for detection of *P. palmivora,* particularly the Colombian isolates, was then based on this region, because the main objective of Chapter 4 was to establish a diagnostic method that can distinguish Colombian and Malaysian

isolates. Several primers were designed from this region but the most promising are the PCR primers AVR1F/R and AVR3F/R designed from the PpHPAVR region of isolate PPC280574. Primers AVR1F and AVR1R were species specific to *P. palmivora* allowing direct detection of *P. palmivora* by PCR. Although many primers have been designed to identify *P. palmivora* previously using PCR such as by Bowman et al. (2007) and Tsai et al. (2006), their work involved more complicated PCR-based techniques (PCR-RFLP and nested PCR), which involve tedious and difficult protocols that are not suitable for a large screening of samples and can only be carried out by skilled persons. The AVR1F/AVR1R pair was shown to amplify from various hosts and origins but exclude out-group species including *P. megakarya,* which was once known as one of the *P. palmivora* complex of s-type/MF3 (Zoberi et al., 1981; Akrofi, 2015). Another interesting primer set is AVR3F and AVR3R, which have potential as selective primers that discriminate Colombian and Malaysian isolates. These primers do not amplify Malaysian, Indonesian and South Korean isolates of *P. palmivora* but amplify all Colombian isolates and isolates from Trinidad & Tobago, the USA, Ghana, Guam, India and Sri Lanka suggesting some pattern based on origin of the isolates. Isolates from Malaysia, Indonesia and South Korea probably have a monophyletic origin. Unfortunately, the PCR validation could not be carried out using other SEA countries such as Thailand, the Philippines, Vietnam, Laos, Myanmar and Brunei due to difficulties in obtaining cultures.

Another method, the loop-mediated isothermal amplification (LAMP), which offers a faster diagnostic time, was also tested. LAMP can amplify a few copies of DNA to 10^9 in less than an hour under isothermal conditions. The robustness of LAMP has been proven in various studies with outstanding results when compared to other pre-existing molecular techniques (Abdullahi et al., 2015). LAMP is carried out using a

specific DNA polymerase that has strand displacement ability that displaces and releases a single stranded DNA, together with a set of six primers which consist of two inner primers (FIP and BIP), two outer primers (F3 and B3) plus two loop primers (LoopF and LoopB) (Notomi et al., 2000; Nagamine et al., 2002; Tomlinson et al., 2010). In this study two set of primers, AVRL2 and AVRL3, were developed that show *P. palmivora* species selectivity. When used with the OptiGene LAMP system consisting of an Isothermal Mastermix and Genie II or Genie III instruments, the diagnosis of *P. palmivora* could be achieved within 9-30 minutes. The amplification could be monitored in real-time similar to real time PCR; therefore, quantitative measurement of the amplification are possible. Preliminary work with DNA of diseased tissues from the artificial inoculation trials in Chapter 5 showed good amplification. In addition, LAMP assays were able to amplify DNA of *P. palmivora* in the soil samples without further cleaning up of the DNA to remove inhibitors in soil. More testing should be conducted using different plant and soil samples to improve the assays. Another advantage of LAMP is the prospect of conducting the tests in the field. *In situ* screening in the field is possible once the method is established since the Genie II and Genie III (OptiGene, UK) are lightweight and can operate off a battery for several hours. It is hoped that both PCR and LAMP diagnostic tools could be adopted by the stakeholders in the oil palm industry (or other crops) either by government agencies such as MPOB, Department of Agriculture and Malaysian Quarantine Inspection Services (MAQIS) or non-government organisations such as plantation owners to monitor the bud rot disease and its causal agent *Phytophthora palmivora*. The primers that can discriminate the isolates from other regions particularly from the hot spot region where the bud rot disease is devastating can help eliminate potential risk of the entry of the foreign isolates that might be harmful to the local oil palm and other crops by providing screening or diagnostic tools of material imported to Malaysia.

Another aim of this work was to study the pathogenicity level or aggressiveness of various *P. palmivora* isolates against commercial oil palm. Leaf detached assays were conducted using mature and young spear leaves of oil palm grown in controlled environments in the glasshouse in the UK. The inoculation using an isolate isolated from the oil palm in bud rot hot spot region in Colombia was observed with a very young unopened spear leaf, which is the first spear leaf. The infection was only observed in the whitish lower part of the spear but not on the older upper part and mature leaf. The isolate was isolated from the infected palms and believed to be pathogenic to oil palm (Martinez, 2014, *personal communication*). Initial attempts to establish infection using the isolate from the Colombian diseased oil palms to the Malaysian DxP oil palm seedlings failed in the glasshouse conditions in the UK, but we were able to observe infection when experiments were repeated in the warmer summer months. The initial symptoms of brown lesions with water-soaked margins observed in this study were similar to those described by Martinez (2009c) and Torres et al. (2016). No severe forms were observed during the inoculations in the glasshouse in the UK, suggesting the absence of secondary infections, probably because the conditions in the glasshouse were not conducive to promote subsequent infection by the secondary inoculum. Recently, some advances in the study on the infection and pathogenicity processes of *P. palmivora* to oil palm seedlings have been reported by Sarria et al. (2016) which is the first report on the *P. palmivora*-oil palm patho-system. Their study showed that zoospores are attracted to trichomes and penetration and colonization occur at the trichomes, cuticle or stomata, without the presence of wounding. Therefore, why it was not possible to induce infection without wounding in this present study is still a question. It is also not understood why the infection did not occur in older leaves with or without inoculation. Perhaps similar studies to those of Sarria et al. (2016) could be repeated to answer these questions.

The artificial inoculation of oil palm seedlings with other isolates from different hosts (coconut, cocoa, durian, rubber, orchid, betel palm and kentia palm) could cause infection in oil palm seedlings of DxP, DxD and TxT. Unfortunately, no conclusive analysis on the aggressiveness of the isolates could be made due to difficulties in standardising the inoculum strength (zoospore count). It was also found that the *P. palmivora* isolates pathogenic to oil palm and other isolates from cocoa, durian, coconut, and orchid can cross infect rubber and durian and the infection can occur by zoospores or mycelial inoculum. Recently, Torres-Londono (2016) has studied the virulence of various *P. palmivora* isolates including from Colombia and Malaysia using apple as a biological indicator. He also carried out extensive studies on the morphological characteristics including size and shapes of various microscopic structures such as sporangia and evaluation of fungicide sensitivity of *P. palmivora in vitro* and *in planta*.

Evaluation of fungicides was also a part of this study, but using a different approach than conducted in the above study. The use of fungicides has been accepted and used for control of *Phytophthora* and several fungicides have been listed as effective in controlling *Phytophthora* diseases (Drenth and Guest, 2004b). In Chapter 6, several fungicides including liquid fertilizers were evaluated for their potential to inhibit *P. palmivora in vitro* using poison agar tests. It was found that the fungicide mancozeb showed good inhibition of *P. palmivora* mycelial growth. However, *in planta* evaluation should be conducted to further confirm the efficacy of the fungicides in suppressing *P. palmivora* infection in plants, particularly oil palm seedlings.

Overall, the findings obtained in this study help us to understand more about *P. palmivora*, the causal agent of the bud rot disease of oil palm in Latin America. Although more studies need to be conducted to understand why the disease is

devastating in Latin America but not in Malaysia and SEA, some hypotheses and speculation based on evidence gathered in this study can be made. A possible factor is the involvement of additional abiotic and biotic factors. It is suggested that the infection level of *P. palmivora* is affected by the environmental conditions, primarily the temperature and humidity, based on our observation in the glasshouse that the infections only started to manifest themselves in the UK in the warmer months of summer. However, since the infections that did occur in the summer months did not progress to the severe forms of typical bud rot disease symptoms, it is also hypothesized that other factors are involved. The absence of these factors in the glasshouse during inoculation trials presumably resulted in conditions that were not conducive for secondary infection. These could be related to weather, such as temperature, humidity and rain or other biotic components such as microbial communities (that act as secondary/opportunist pathogens or suppressants of *P. palmivora*), and/or the absence of insects that might be required for wounding or serve as vectors in South America. These factors may possibly be absent in Malaysia as SEA as well, which might also explain why diseases of oil palm are usually regional. Maybe this is why *Ganoderma* basal stem rot disease is a problem in Malaysia but not in Latin America, and *Fusarium* wilt is a significant problem in Africa but not in other parts of the world. However, the interaction of these factors and infection levels might be complicated and needs further investigation.

Another possibility is the difference in the genetic background of the oil palm in Colombia and Malaysia and/or the genetic background of the causal agent, *P. palmivora*. However, it has already been established that The DxP materials from Asia have shown the highest incidence rates (65%-85%) compared to African materials in studies in the eastern region of Colombia (Santacruz et al., 2004). In terms of genetic

background of *P. palmivora*, even though there are no differences between oil palm Colombian and Malaysian isolates in the sequences of ITS regions and several housekeeping genes, there is evidence of variation in the genome of the *P. palmivora*. PpHPAVR sequence analysis showed that there are differences in regions that may be related to avirulence of the pathogen such as genes encoding for effector proteins, which might lead to the difference in virulence or aggressiveness against oil palm. Studies on relationships of the virulence levels of these pathogenic and non-pathogenic isolates with the genetic variations in the avirulence genes could probably result in a deeper understanding of the significance of variation in such genes. To do this, more studies on the effector proteins *of P. palmivora,* such as is being carried out in *P. infestans,* is crucial.

Apart from this, differences in cultural practices in management of oil palm plantations between Latin America and SEA might also influence the disease progression, especially at the nursery level. Therefore, enhancing good cultural practice in the nurseries is one of the important steps that could be taken to reduce plant diseases. Another aspect that could be examined is the presence of *P. palmivora* in the soil in the oil palm plantations in Malaysia and in Colombia. Establishment of good isolation methods from the soil is crucial and should be use together with the molecular tools such as PCR and LAMP that have been developed in this study.

7.1 Recommendation for future studies

There are several areas that can be investigated in future research based on the findings from this study either to strengthen the findings or for taking the research to the next level. Some of the main recommendations are:

- Include more isolates from different hosts and geographical origins, particularly from South East Asian and South American regions, in several aspects of the study, particularly in the molecular characterization mainly in the use of AFLP and new PpHAVR marker analyses, since the findings in this study show some evidence of the genetic variation within Malaysian and Colombian isolates using both markers. It will be interesting to know if isolates from other SEA (i.e. Thailand, the Philippines, Indonesia) and South American countries are closely related with Malaysian and Colombian isolates, respectively. Another aspect is in the testing of PCR and LAMP primers, particularly the PCR primers for AVR3, that have shown potential to discriminate Malaysian and Colombian isolates. The extension of the validation using more isolates is crucial to confirm the specificity of the primers. It will be beneficial if the primers can separate all the isolates from the South American countries from the isolates from Malaysian or at least South East Asian isolates. Therefore the primers can be used to screen materials contaminated with potential risk isolates from the hot spot area.
- The new PpHPAVR marker should be investigated further as a putative *P. palmivora Avr* protein gene in terms of the expression of the gene and its relationship with pathogenicity to the plant host. Is this new marker an *Avr* gene of *P. palmivora*? If yes, what is the *R* gene of the host plant that complements this *Avr* gene? What is the interaction between *P. palmivora* and

the oil palm host? Are there different levels of aggressiveness between *P. palmivora* isolates? Is there a susceptible gene (S gene) involved in the interaction between *P. palmivora* and oil palm? There are so many questions that need to be addressed in future studies of *P. palmivora* and the oil palm disease caused by it!

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Appendix

Appendix 1 Media preparation

Carrot Agar

Blend 100 gm peeled carrot (cut to small pieces) with 400 ml of distilled water. Filter the juice with two layers muslin cloth. Add distilled water to make 1000 ml. Add 7.5 g agar or 8.5 g Corn Meal Agar (CMA). Boil the media in the microwave to clarify the agar. Autoclave at 121 ̊c for 15 min. *Add antibiotics after autoclave, when the media has cooled down (55-60°C) if needed.

**Antibiotics:*

2.5 ml of ampicillin (100 mg/ml), final concentration in 1 L media is 250 µg/ml)

1 ml of penicillin (50 mg/ml), final concentration in 1 L media is 50 µg/ml)

Corn Meal Agar

Add 8.5 g of Corn Meal Agar (CMA) into 500 ml distilled water. Top up distilled water to 1000 ml. Boil in microwave to clarify the agar. Autoclave at 121 ̊c for 15 min. *Add antibiotics after autoclave, when the media has cooled down (55-60°C) if needed.

Selective Media $P_{10}VP$

Stock preparation of pentachloronitrobenzene PCNB (25 mg/ml): Dissolved 1 g of PCNB in 40 ml 90% ethanol, incubate in 70°C in waterbath and vortex to dissolve.

Add 20 g of CMA and 4 ml of PCNB stock into 500 ml of distilled water. Add distilled to 1000 ml. Boil to clarify in microwave. Autoclave at 121 ̊c for 15 min. Allow the media to cool down before adding antibiotics (0.4 ml pimaricin (25 g/ml) and 2 ml Vancomycin (100 mg/ml). Antibiotics are filter sterilized prior usage. Pimaricin are sensitive to light, store plates/media in the dark.

V8 agar

Add 200 ml of V8 juice in the flask. Add 3 g of Calcium carbonate ($CaCO_3$) and 15 g of agar. Add water to 1000 ml. Adjust pH to 7.2. Boil in microwave to clarify agar.

Autoclave at 121 ℃ for 15 min. * Add antibiotics after autoclave, when the media has cooled down (55-60°C) if needed.

Appendix 2: Preparation of stock culture of *Phytophthora*

Add approximately 20 ml of distilled into a universal bottle. Autoclave at 121 ℃ for 15 min and allows to cool to the room temperature. Add 15-20 mycelial plug excises from the marginal of plate culture into the universal bottle. Keep in the dark at 20°C.

Appendix 3: DNA extraction protocol-DNeasy® Plant Mini Kit (Qiagen)

1. Disrupt samples 120 mg mycelial scrapped from the plate culture.
2. Add 400 µl Buffer AP1 and 4 µl RNase A. Vortex and incubate for 10 min at 65°C. Invert the tube 2–3 times during incubation. Note: Do not mix Buffer AP1 and RNase A before use.
3. Add 130 µl Buffer P3. Mix and incubate for 5 min on ice. Centrifuge the lysate for 5 min at 20,000x g (14,000 rpm).
4. Pipet the lysate into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuge for 2 min at 20,000x g.
5. Transfer the flow-through into a new tube without disturbing the pellet if present. Add 1.5 volumes of Buffer AW1, and mix by pipetting.
6. Transfer 650 µl of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at ≥6000 x g (≥8000 rpm). Discard the flow-through. Repeat this step with the remaining sample.
7. Place the spin column into a new 2 ml collection tube. Add 500 µl Buffer AW2, and centrifuge for 1 min at ≥6000 x g. Discard the flow through.
8. Add another 500 µl Buffer AW2. Centrifuge for 2 min at 20,000 x g.
   Note: Remove the spin column from the collection tube carefully so that the column does not come into contact with the flow-through.
9. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
10. Add 100 µl Buffer AE for elution. Incubate for 5 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x g.

Appendix 4: QIAQuick® PCR Purification Kit (Qiagen)

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix.
2. Place a QIAquick column in a provided 2 ml collection tube or into a vacuum manifold.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 0.75 ml Buffer PE to the QIAquick column. Centrifuge for 30–60 s. Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

Appendix 5: QIAquick® Gel Extraction Kit

1. Excise DNA fragment from agarose gel with a clean, sharp scalpel. A 1% regular Agarose in 1X TBE is fine.
2. Weigh the gel slice in a colourless tube. Add 3 volumes of Buffer QX1 to 1 volume gel (100 mg ~ 100 ml). The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg, use more than one column.
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortex the tube every 2-3 min during the incubation.
4. After the gel slice has dissolved completely, check that the colour of the mixture is yellow.
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2-ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.
8. Discard flow-through and place the QIAquick column back in the same collection tube. (Optional): Add 0.5 ml of Buffer QX1 to QIAquick column and centrifuge for 1 min.
9. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.
10. Discard flow-through and centrifuge the QIAquick column for an additional 1 min at ~13,000 rpm.
11. Place QIAquick column into a clean 1.5-ml microfuge tube.
12. To elute DNA, add 50 ml of 10 mM Tris-HCl, pH 8.5 of H_2O to the centre of the QIAquick column and centrifuge for 1 min at maximum speed. Alternatively, to increase DNA concentration, add 30 ml elution buffer to the centre of the QIAquick column, let stand for 1 min, and then centrifuge for 1 min.

Appendix 6: Sequences of PpHPAVR (PPC280574)

>PpHPAVR (PPC280574)

agtaaaagcggaatgacggcttctgcgtttgtaagttgtgaaccagcatcagttctgtcgactgccgcaattcccgaatctg
tgtctatttccatgacagtcgcacctcagtcaggtaagccgcttatcgaatacgctattctgtctgatacagcataaggtatcc
agttatatacatatttaactaagcattacattacccaatatcctatgcaggtgactgatatcaccgaggtccgttgaagcgac
gccgaggaggaatttaagagcgaggagggcgttgctgatgatgtgggtgtctttgactcggtctatttcatggacgcacta
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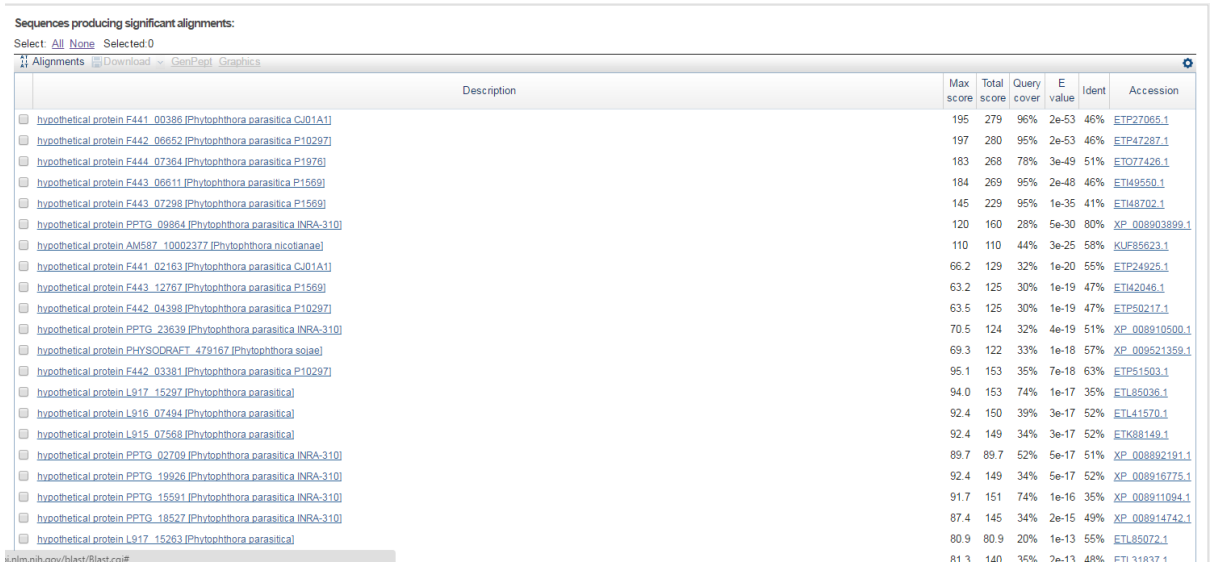
Appendix 7: BLAST results of PpHPAVR

Blastx



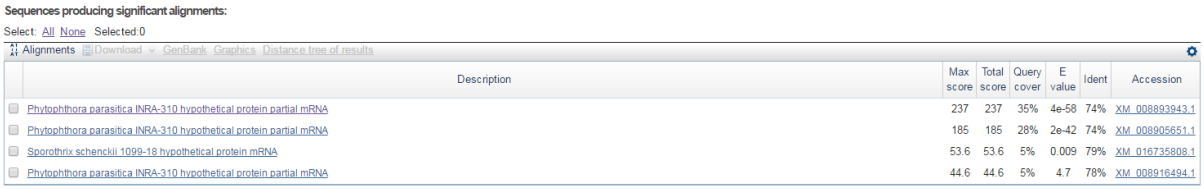
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenPept Graphics

Description	Max score	Total score	Query cover	E value	Ident	Accession
hypothetical protein F441_00386 [Phytophthora parasitica CJ01A1]	195	279	96%	2e-53	46%	ETP27065.1
hypothetical protein F442_06652 [Phytophthora parasitica P10297]	197	280	95%	2e-53	46%	ETP47287.1
hypothetical protein F444_07364 [Phytophthora parasitica P1976]	183	268	78%	3e-49	51%	ETO77426.1
hypothetical protein F443_06611 [Phytophthora parasitica P1569]	184	269	95%	2e-48	46%	ETI49550.1
hypothetical protein F443_07298 [Phytophthora parasitica P1569]	145	229	95%	1e-35	41%	ETI48702.1
hypothetical protein PPTG_09864 [Phytophthora parasitica INRA-310]	120	160	28%	5e-30	80%	XP_008903899.1
hypothetical protein AM587_10002377 [Phytophthora nicotianae]	110	110	44%	3e-25	58%	KUF85623.1
hypothetical protein F441_02163 [Phytophthora parasitica CJ01A1]	66.2	129	32%	1e-20	55%	ETP24925.1
hypothetical protein F443_12767 [Phytophthora parasitica P1569]	63.2	125	30%	1e-19	47%	ETI42046.1
hypothetical protein F442_04398 [Phytophthora parasitica P10297]	63.5	125	30%	1e-19	47%	ETP50217.1
hypothetical protein PPTG_23639 [Phytophthora parasitica INRA-310]	70.5	124	32%	4e-19	51%	XP_008910500.1
hypothetical protein PHYSODRAFT_479167 [Phytophthora sojae]	69.3	122	33%	1e-18	57%	XP_009521359.1
hypothetical protein F442_03381 [Phytophthora parasitica P10297]	95.1	153	35%	7e-18	63%	ETP51503.1
hypothetical protein L917_15297 [Phytophthora parasitica]	94.0	153	74%	1e-17	35%	ETL85036.1
hypothetical protein L916_07494 [Phytophthora parasitica]	92.4	150	39%	3e-17	52%	ETL41570.1
hypothetical protein L915_07568 [Phytophthora parasitica]	92.4	149	34%	3e-17	52%	ETK88149.1
hypothetical protein PPTG_02709 [Phytophthora parasitica INRA-310]	89.7	89.7	52%	5e-17	51%	XP_008892191.1
hypothetical protein PPTG_19926 [Phytophthora parasitica INRA-310]	92.4	149	34%	5e-17	52%	XP_008916775.1
hypothetical protein PPTG_15591 [Phytophthora parasitica INRA-310]	91.7	151	74%	1e-16	35%	XP_008911094.1
hypothetical protein PPTG_18527 [Phytophthora parasitica INRA-310]	87.4	145	34%	2e-15	49%	XP_008914742.1
hypothetical protein L917_15263 [Phytophthora parasitica]	80.9	80.9	20%	1e-13	55%	ETL85072.1

Blastn



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Phytophthora parasitica INRA-310 hypothetical protein partial mRNA	237	237	35%	4e-58	74%	XM_008893943.1
Phytophthora parasitica INRA-310 hypothetical protein partial mRNA	185	185	28%	2e-42	74%	XM_008905651.1
Sporothrix schenckii 1099-18 hypothetical protein mRNA	53.6	53.6	5%	0.009	79%	XM_016735808.1
Phytophthora parasitica INRA-310 hypothetical protein partial mRNA	44.6	44.6	5%	4.7	78%	XM_008916494.1