DEVELOPMENT AND VALIDATION OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR THE EARLY DETECTION OF BASAL STEM ROT (BSR) CAUSED BY *Ganoderma boninense*

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JUDUL: **DEVELOPMENT AND VALIDATION OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR THE EARLY DETECTION OF BASAL STEM ROT (BSR) CAUSED BY *Ganoderma boninense***

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DEGREE	:	**MASTER OF SCIENCE (BIOTECHNOLOGY)**
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ABSTRACT

Ganoderma boninense is a pathogenic plant fungus that is associated with the Basal Stem Rot (BSR) disease frequently found in oil palm plantation. Conventional detection of *G. boninense* is usually achieved through visible symptoms, culturing on *Ganoderma* selective media (GSM), ergosterol quantification that is proportional to fungal biomass, immunoassay test or through molecular detection such as Polymerase Chain reaction (PCR). However, all of these methods require skilled personnel, is time consuming, may not specific and maybe also expensive. As such, this study aims to develop a rapid, simple yet sensitive and specific detection method based on the use of loop-mediated isothermal amplification (LAMP) of DNA. The basidiocarps of *G. boninense* were obtained from Langkon estate in Kota Marudu, cultured on GSM and subcultured onto PDA to obtain pure cultures of *G. boninense*. Genomic DNA of *G. boninense* was then isolated, ITS gene amplification using PCR was performed and verified through Sanger sequencing. The rapid detection of *G. boninense* was accomplished through a set of novel outer, inner and loop primers of the Manganese Superoxide Dismutase (MnSOD) gene, specific to *G. boninense.* The optimization of LAMP condition was performed to find the suitable concentration of $MgSo_4$ and the primer ratio of outer and inner primers. The loop primers was added to the LAMP reaction and it was proven that the addition of loop primers able to accelerate the time of LAMP reaction. The signal detection of LAMP products was done in two ways which are in gel electrophoresis viewed under UV light and using SYBR Green I. For sensitivity test, two sets of *G. boninense* recombinant plasmid samples were prepared; Set A: from 60 ng to 0.6 fg, and Set B: from 10^7 to 5 copies, and were subjected to PCR, LAMP-UV analysis and LAMP-SYBR Green. The finding from the sensitivity test showed the LAMP-UV and LAMP-SYBR Green is greater in sensitivity than that obtained using conventional PCR. The detection limit of LAMP-SYBR and LAMP-UV analysis was at 0.6 fg and 10^3 copies of recombinant plasmid while conventional PCR was only at 0.6 pg and 10^4 copies. The specificity test was performed with genomic DNA of *G. boninense* and *G. australe,* and it was negative for *G. australe* while positive for *G. boninense*. Another specificity test was done using 10 unknown fruiting bodies and all these samples were subjected to DNA extraction, identification using PCR and LAMP. The LAMP assay was negative for all the samples. The evaluation of LAMP assay on trunk tissue samples to detect *G. boninense* was tested in 20 genomic DNA samples extracted from infected trunk tissues. The results showed 17 out of 20 genomic DNA samples produced LAMP ladder-like bands. In addition, the other three were negative for LAMP, GSM and ergosterol quantification respectively. This indicates that the LAMP assay was highly sensitive compared to GSM and ergosterol detection. In conclusion, the LAMP assay developed through this study was found to be useful in the detection of *G. boninense* with high sensitivity and specificity in biological and trunk tissue samples.

ABSTRAK

PENGESANAN AWAL PENYAKIT PANGKAL BATANG REPUT DISEBABKAN OLEH KULAT GANODERMA BONINENSE MENGGUNAKAN TINDAKBALAS AMPLIFIKASI GELUNG PERANTARA SUHU SEKATA (LAMP)

Ganoderma boninense adalah kulat patogenik tumbuhan yang berkait dengan penyakit pangkal batang reput di ladang kelapa sawit. Pengesanan yang konvensional selalunya dilakukan dengan mengesan simptom-simptom luaran, mengkultur di atas Ganoderma media selektif, penjumlahan ergosterol yang sejajar dengan jisim kulat, ujian immunologi atau melalui kaedah pengesanan molekular seperti tindak balas berantai polimerase (PCR). Walaubagaimanapun, kesemua kaedah pengesanan ini memerlukan pekerja yang mahir, mengambil masa yang lama, setengah kaedah tidak spesifik dan juga mahal. Tujuan kajian ini adalah untuk membina kaedah yang cepat, ringkas tetapi sensitif dan spesifik menggunakan kaedah amplifikasi gelung perantaraan suhu sekata (LAMP). Basidiokarpa daripada G. boininense diperolehi daripada Ladang Langkon di Kota Marudu, dikultur di atas GSM dan dipencilkan di atas PDA untuk mendapatkan kultur tulen. Genomik DNA daripada G. boninense telah diekstrak, gen ITS diperbanyak melalui PCR dan disahkan melalui penjujukan Sanger. Pengesanan pantas untuk G. boninense telah dicapai melalui satu set asli primer terdiri daripada primer luar,dalam dan gelung berdasarkan gen manganese-superoksida dismustase yang spesifik bagi G. boninense. Pengoptimuman keadaan LAMP telah dilakukan dengan mencari kepekatan $MgSO_4$ dan nisbah antara primer luar dan dalam yang sesuai. Primer gelung telah ditambahkan kepada tindak balas LAMP dan dibuktikan bahawa penambahan primer gelung boleh mempercepatkan masa tindak balas LAMP. Pengesanan isyarat ke atas produk LAMP telah dilakukan menggunakan dua cara iaitu dikesan di bawah lampu UV dalam proses elektroforesis gel dan juga menggunakan SYBR Hijau I. Untuk ujian kepekaan, dua set sampel rekombinan plasmid telah disediakan; Set A: daripada 60 ng to 0.6 fg, dan Set B: dari 10^7 to 5 salinan rekombinan plasmid digunakan dalam PCR, LAMP-UV analysis dan LAMP-SYBR Hijau. Keputusan daripada ujian kepekaan mendapati LAMP-UV dan LAMP-SYBR Hijau adalah lebih peka daripada PCR konvensional. Had pengesanan untuk LAMP-UV dan LAMP-SYBR ialah 0.6 fg dan 10^3 salinan rekombinan plasmid manakala konvensional PCR hanya dapat mengesan sehingga 0.6 pg dan 10^1 salinan. Ujian pengkhususan telah dijalankan menggunakan genomik DNA daripada G. boninense dan G. australe dan mendapati keputusan negatif untuk pengesanan G. australe manakala tindak balas LAMP berupaya untuk mengesan G. boninense. Ujian pengkhususan sekali lagi dilakukan menggunakan 10 sampel yang tidak diketahui. Kesemua sampel ini telah dikenalpasti menggunakan PCR dan dikesan menggunakan tindak balas LAMP. Tindak balas LAMP ke atas kesemua sampel yang tidak diketahui menunjukkan keputusan yang negatif. Penilaian lapangan untuk tindak balas LAMP diuji menggunakan 20 genomik DNA yang telah diesktrak daripada tisu batang pokok kelapa sawit. Keputusan daripada penilaian ini menunjukkan 17 daripada 20 sampel menunjukkan keputusan positif bagi tindak balas LAMP. Tambahan pula, tiga sampel yang lainnya menunjukkan keputusan negatif bagi tindak balas LAMP, GSM dan pengkuantitian ergosterol. Ini menunjukkan LAMP lebih peka bebanding GSM dan pengesanan ergosterol. Kesimpulannya, asai LAMP yang dibangunkan lebih peka dan khusus untuk pengesanan G. boninense pada sampel biologi dan tisu pokok kelapa sawit.

LIST OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

β	-	Beta
^{0}C	-	Degree Celcius
%	-	Percentage
®	-	Registered trademark
nm	-	Nanometer
cm	-	Centimetre
bp	-	Basepair
kb	-	Kilobases
µl	-	Microlitre
µg	-	Microgram
g	-	Gram
ng	-	Nanogram
pg	-	Picogram
fg	-	Femtogram
m	-	Meter
min	-	Minute
M	-	Molar
mL	-	millilitre
h	-	Hour
rpm	-	Revolution per minute
µg/ml	-	Microgram per millilitre
µg/g	-	Microgram per gram
ng/ µl	-	Nanogram per millilitre
U/ µl	-	Unit per microlitre
mM	-	Milimolar
Mg^{2+}	-	Magnesium ions
mmol/L	-	milimol per litre
sec	-	Second
mg	-	milligram

LIST OF ABBREVIATIONS

EtBr	-	Ethidium bromide
LAMP	-	Loop-mediated isothermal amplification
MnSOD	-	Manganese-superoxide dismutase
BLAST	-	Basic Local Alignment Search Tool
NCBI	-	National Centre for Biotechnology Information
DNA	-	Deoxyribonucleic acid
rDNA	-	Ribosomal deoxyribonucleic acid
rRNA	-	Ribosomal ribonucleic acid
PCR	-	Polymerase chain reaction
ITS	-	Internal transcribed spacer
EDTA	-	Ethylenediaminetetraacetic acid
$MgSO_4$	-	Magnesium sulphate
$MgCl_2$	-	Magnesium chloride
PCR-RFLP	-	Polymerase chain reaction-Restriction Fragment Length Polymorphism
PCR-RAPD	-	Polymerase chain reaction-Random Amplification of Polymorphic DNA
Bst	-	*Bacillus stearothermophilus*
Taq	-	*Thermus aquaticus*
CTAB	-	Cetyltrimethyl ammonium bromide
NaCl	-	Sodium chloride
Tris-Cl	-	Tris chloride
Tris-HCl	-	Tris hydrochloric
PVP	-	Polyvinylpyrrolidone
PCIA	-	Phenol:Chloroform:Isoamyl alcohol
dNTP	-	Deoxynucleoside triphosphate
KCl	-	Potassium chloride
UV	-	Ultraviolet
LB	-	Luria Bertani

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Oil palm (*Elaeis guineensis*) is an economically important tree crop which has been world's major sources of edible oil and a significant precursor of biodiesel fuel. The oil palm originates from West Africa but was introduced to South East Asia by the British in the early 1870s. The first commercial planting took place in Tennamaran Estate, Selangor in 1917 which then become the foundations for the vast oil palm plantations and the palm oil industry in Malaysia (Williams and Hsu, 1970). According to Ahmad (2017), Malaysia is the second largest palm oil producer in the industry which contributes 30 % from 58.31 million tonnes of world major palm producers and 37 % from 43.76 million tonnes of world major palm oil exporters as stated in Global Oils and Fats Performance 2016. However, the oil palm industry is hampered by the Basal Stem Rot (BSR) disease which is caused by *Ganoderma boninense*. It has been reported that the economic loss caused by this disease is between RM 225 million to RM 1.5 billion (up to 500 million USD) a year (Arif *et al.*, 2012). With regards to its economic importance, continuous efforts have been made to control the spread of the plant pathogen using various agronomic practises.

Currently, numerous studies have been demonstrated the development of a detection method for *Ganoderma boninense* fungus and the BSR disease, but none has shown to be promising for wide spread adoption by the industry. Typical detection methodologies currently in use are based on observation i.e. visually inspecting external symptoms such as wilting of mature leaves and falling of fronds or the presence of basidiomata of the pathogen on the tree. All these symptoms

have become an indicator for *Ganoderma* infection (Lelong *et al.*, 2010). Dependence on visual evidence implies "late stage" detection which results in considerable expense in preventing further spread of the disease. Therefore, early detection technique of this disease is essential to help planters in managing and controlling this disease at an early stage (Alexander *et al,* 2014). Early identification also enables precise targeting of the pathogen and enables the most effective treatment.

Some conventional methods have been used in the early detection of *G. boninense* such as *Ganoderma* selective media (GSM) developed by Ariffin and Idris, (1992). This media contains are fungicides and antibiotics which functions to control the growth of bacteria and other contaminating fungi and allows *Ganoderma* to thrive. Despite that, the method is less accurate as some fungi can also grow on the media (Chong *et al.*, 2014). Concern on the inaccuracy of current methods, a more accurate alternative diagnostic technique based on molecular based approaches is needed to detect *G. boninense* infection. The earliest molecular approach is based on immunoassay, which utilises antibodies in culture media to detect *Ganoderma* (Reddy and Ananthanarayanan 1984; Darmono *et al.* 1993; Darmono and Suharyanto 1995). However, the lack of taxonomy information and confusion between the pathogenic nature of some species of the genus *Ganoderma* are the disadvantages of this method (Paterson, 2006). Other molecular and immunological method such as polymerase chain reaction (PCR) and enzyme-linked immunosorbet assay (ELISA) have been used to detect the infection of *Ganoderma sp* (Utomo and Niepold, 2000; Utomo *et al.*, 2005) but this method cannot be done routinely because of protocol complexities, costly reagents, sensitive to contamination, time consuming and requires highly skilled personnel. Thus, a simple, rapid and cost-effective detection method is urgently needed.

Nowadays, several isothermal amplification techniques have been developed to detect pathogen without using thermocycler machine. One example of these isothermal amplification techniques is loop-mediated isothermal amplification or LAMP. LAMP was first developed by Notomi *et al.*, (2000) for the detection of viruses. LAMP is a one-step amplification reaction that amplifies a target DNA sequence under isothermal conditions (Mori & Notomi, 2009). The amplification

was basically an auto cycling strand displacement DNA synthesis performed by *Bacillus stearothermophilus* DNA Polymerase (*Bst* polymerase) with a set of four specifically designed primers hybridizing to six different parts of the target DNA sequence (Notomi *et al.*, 2000). LAMP reaction allows the synthesis of large amounts of DNA in short time proving that this method is a highly efficient amplification method. The LAMP method has been used to diagnose viruses, bacteria, protozoa even fungus that cause big impact on the health of humans, animals and plants. The detection of the amplification products usually depends on complex and expensive tools such as electrophoresis and may require the use of harmful chemical like ethidium bromide (EtBr) to reveal the signal. Other than that, there will be a risk of cross contamination of samples due to massive production of DNA during LAMP. Various different methods for in-tube detection of DNA amplification have been developed which are using fluorogenic intercalating dyes, for example SYBR Green I (Vaagt *et al.*, 2013), PicoGreen (Tomlinson *et al.*, 2007), ethidium bromide (Moradi *et al.*, 2014), SYTO9 (Pu *et al.*, 2014) and others. in-tube detection of LAMP products can be done and viewed in that instance, which is very convenient in application

1.2 Objectives of the study

The hypothesis of this study is the designed LAMP primers will be able to rapidly detect and amplify the target gene of *G. boninense* with high sensitivity and specificity and able to detect the present of *G. boninense* on trunk tissue samples. To prove the hypothesis, three objectives listed below must be achieved. The objectives of this study are;

a. To develop a protocol for the rapid detection of *G. boninense* based on loop-mediated isothermal amplification (LAMP) utilizing manganese superoxide dismutase (MnSOD) gene.

b. To evaluate the sensitivity and specificity of loop-mediated isothermal amplification (LAMP) in detecting *G. boninense* infection.

c. To verify the ability of loop-mediated isothermal amplification (LAMP) of DNA in detecting *G. boninense* infection on trunk tissue samples.

CHAPTER 2

LITERATURE REVIEW

2.1 Oil palm

Oil palm tree (*Elaeis guneensis* jacq.) originated from Central and West Africa near Gulf of Guinea. It was introduced to South East Asia by the British in the early 1870s (Hushiarian *et al.*, 2013) as an ornamental plant. The word '*Elaeis*' is derived from Greek meaning oil while '*guneensis*' refers to the name for the area named Guinea that located in West Africa. The oil palm tree is a member of the family *Palmae*, subfamily *Cocosideae* (also includes the coconut) and *Elaies* is the genus. Oil palm structure resembling coconut palm (*Cocos nucifera*), exhibit an erect stem and markedly ringed but less spines, vary in height which from 25 to 30 m tall when growing spontaneously and 10-15 m tall when cultivated. The leaves are pinnate, having a solitary columnar stem with short internodes (Corley and Tinker, 2003). Oil palm is sowing seed for propagation while coconut palm produces off shoots. The natural distributed seeds do not germinate readily, and while awaiting favourable seasonal conditions, many of them might be destroyed by rodents or boring beetles. Seeds can be stored for at least a year at ambient temperatures. Germinated seeds, transported in polythene bags or packed in boxes, can remain in the bags for up to ten days before planting. When the seedlings reach the 4-5 leaf stage which happen in 4-5 months, they will be transferred to field nurseries or large polythene bags where they grow for 6-12 months before transplanting to the field.

Oil palm is a monoecious crop because it bears both male and female flowers on the same tree. Each tree produces compact bunches weighing between 10 to 25 kilograms with 1000 to 3000 fruitlets per bunch. These fruits are about the size of a large olive, and are naturally reddish in colour because the fruit pulps high carotenoid content. Each fruitlets consists of a hard kernel (seed) enclosed in a

shell (endocarp) which is surrounded by a fleshy mesocarp. Oil palm produces two types of oil which are palm oil (from outer mesocarp) and kernel oil (from the kernel within the hut). Palm oil and kernel oil are extracted in milling process where each ripe bunch, commonly referred to as a Fresh Fruit Bunch (FFB) is sterilized and the fruitlets stripped off. The loose fruitlets are then being digested and pressed to extract Crude Palm Oil (CPO), while the kernels are separated from the fibrous mesocarp in the press cake and later cracked to obtain Crude Palm Kernel Oil (CPKO).

Oil palm tree will start bearing fruit after 30 months of field planting and will continue to be productive for the next 20 t0 30 years (Hushiarian *et al.*, 2013). This ensures continuous and consistent supply of oils. The oil palm is the most efficient oil-bearing crop in the world which only needs 0.26 hectares of land to produce one tonne of oil (Hushiarian *et al.*, 2013) compared to sunflower and soybean that need 2 and 2.22 hectares respectively, to produce the same amount of oil.

2.2 The growth of oil palm industry in Malaysia and its economic importance

The development of oil palm industry in Malaysia was attributed to Henri Fauconnier and his associate Hallet from France in 1911. Fauconnier was visited Hallet's oil palm development in Sumatra and purchased some oil palm seeds and these seeds were planted at his Rantau Panjang Estate in Selangor. In 1912, Fauconnier returned back to Sumatra to obtained seeds that he had selected together with Hallet for further planting. With seedlings obtained from year 1911 and 1912 importation, Fauconnier established the first commercial oil palm planting at Tennamaram Estate, Selangor to replace an unsuccessful planting of coffee bushes (Teoh, 2002).

Analysis of oil palm industry in Peninsular Malaysia was classified into three distinct phases according to Hacharan Singh (1976). The first phase is the experimental phase which done from late 1800s to 1916, then development phase begin in Tennamaram estate in 1917 until about 1960. The expansion phase was response to the Government's diversification policy, in 1960s. This policy was