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CORRELATION BETWEEN NON-RIBOSOMAL PEPTIDE SYNTHETASE (NRPS) PRODUCTION AND VIRULENCE OF *Ganoderma boninense* PER71 ON OIL PALM (*Elaeis guineensis*)

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ABSTRACT

Basal stem rot (BSR) disease caused by the white rot fungus Ganoderma boninense *is the most destructive oil palm disease leading to production losses in fresh fruit bunches (FFB). Non-ribosomal peptide synthetase (NRPS) plays an important role in fungal pathogenicity. These large multi-modular enzymes catalyse the biosynthesis of secondary metabolites (SMs) that act as fungal virulence factors. In this study, the detection of NRPS in* G. boninense *was achieved using polymerase chain reaction (PCR)-based method. Core motifs of adenylation domain of NRPS gene was identified in* G. boninense*. The deduced amino acid sequence showed similarity to the conserved core motifs (A2, A3 and A5) of the adenylation domain. Siderophores were predicted as the potential SMs synthesised by NRPS. Expression analysis of* GbNRPS *in 3-month-old oil palm artificially infected with* G. boninense *has confirmed the upregulation of* GbNRPS *at 1 month after inoculation (MAI) peaking at 4 MAI in susceptible clone but not in tolerant clone. There was a correlation between* GbNRPS *gene expression and disease severity. Susceptible clones showed significantly higher disease severity index (DSI) (62.50%) compared to tolerant clones (28.13%) at 4 MAI. This is the first putative detection of adenylation domain of NRPS (*GbNRPS*) gene and functional analysis of NRPS as a virulence factor in disease development.*

Keywords: adenylation domain, basal stem rot, non-ribosomal peptide synthetase.

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INTRODUCTION

African oil palm (*Elaeis guineensis* Jacq.) is an oil crop widely cultivated in Southeast Asia for its edible palm oil. Palm oil and palm kernel oil have become important raw materials in food and non-food industries such as pharmaceutical, cosmetics, animal feed and is a promising source of biofuel. Malaysian palm oil industry constituted 27.7% of the global palm oil production, which is equivalent to approximately USD16.14 billion annual export revenue in 2018 (Kushairi *et al.*, 2019).

Basal stem rot (BSR) caused by the soil borne *Ganoderma boninense* which is a basidiomycete white rot fungus is catastrophic to oil palm plantations resulting in a 50%-80% reduction in palm oil production following reduction of standing palms, number and weight of fresh fruit bunches (FFB) (Chong *et al.*, 2017; Corley and Tinker, 2015). The disease kills young palms within 6-24 months after the first appearance of foliar symptoms such as water stress, one-sided canopy mottling, crown flattening and multiple unopened spears followed by the sign

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of fruiting body at stem base (Paterson, 2007; Rees *et al.*, 2009). The effectiveness of current disease control measures is debatable. These processes include physical (ploughing and fallowing before planting, mechanical soil mounding and surgery removal of basidiocarps), chemical (systemic fungicides) and biological (antagonistic activity of *Trichoderma harzianum* and *Pseudomonas aeruginosa*) (Hushiarian *et al.*, 2013). The incidence of BSR is exacerbated by climate change with an estimated 0.44 million hectares (65.6 million palm trees) in untreated oil palm plantations in 2020 (Abas and Abu Seman, 2012).

In the *G. boninense*-oil palm pathological system, the pathogen establishes as a biotroph in the root system and switches to hemibiotroph as the disease progresses. Necrotrophs and hemibiotrophs employ a wide variety of secondary metabolites (SMs) which are associated with cellular development, pathogenicity and tolerance to various environmental stresses (Pusztahelyi *et al.*, 2015). SMs have a wide variety of chemical structures that are normally produced via biosynthetic pathways such as non-ribosomal peptide synthetases (NRPS) (Gunatilaka and Wijeratne, 2011; Keller *et al.*, 2005). NRP are synthesised by NRPS enzyme complexes and involve a thiotemplate mechanism in which peptide synthesis is independent of ribosome-based translation processes. Instead, proteinogenic and non-proteinogenic amino acids are serially incorporated (Sayari *et al.*, 2019). These enzymes are multi-modular and most NRPS modules consist of three conserved domains: adenylation (A) (identification and substrate activation), thiolation (T) or peptidyl carrier protein (PCP) (covalent binding and transmission) and condensation (C) (formation of peptide bond) which when grouped together are referred to as a single module (Martínez Núñez and López, 2016; Soukup *et al.*, 2016). Each module is responsible for recognition and incorporation of amino acids into the extending peptide backbone depending on the complexity of the peptide. These modules may also occur with other accessory domains such as thioesterase (TE) (release of newly synthesised peptide from enzyme), epimerisation (E) (L to D configuration) and N-methyl transferase (MT) (transfer of methyl group) to further modify the peptide products (Sayari *et al.*, 2019). The final peptide products may be further modified by additional tailoring enzymes which catalyse glycosylation, hydroxylation, acylation or halogenation; thereby resulting in the structural and chemical diversity of NRP (Bushley and Turgeon, 2010). The peptides produced might be of linear type, cyclic or branched. So far, 600 peptides or peptide metabolites have been described from various taxa of bacteria and fungi (Welker and von Döhren, 2006). The size of the peptides produced non-ribosomally ranged from two to 40 amino acids (more in depth information can be obtained from Finking and Marahiel, 2004; Kleinkauf and von Döhren, 1996; Schwarzer *et al.*, 2003; Schwarzer and Marahiel, 2001; Stachelhaus and Marahiel, 1995; von Döhren, 2004). General guidelines of NRPSs lie in the modular structure assembly where the modules are organised in domains: adenylation, thiolation and condensation domains. Examples of enzymatically purified NRPS are HC-toxin synthetase, enniatin synthetase, AM-toxin synthetase and bassianolide synthetase (*Table 1*) (Eisfeld, 2009). One of the most studied functions of NRPS-produced peptide is the role as siderophore in forming Fe^{3+} complex. Two types of siderophores (ferrichrome and coprogen) have been reported in several fungi such as *Aspergillus fumigatus, A. nidulans, Ustilago maydis, Omphalotus olearius, Schizosaccharomyces pombe* and phytopathogenic fungi (*Alternaria brassicicola, Cochliobolus heterostrophus, Fusarium graminearum, Magnaporthe grisea, etc.*) (Philpott, 2006).

Adenylation domain (A-domain) is the most important determinant in NRPS modules as each activity begins with A-domain that loads amino acid substrate onto the carrier protein cofactor. There are around 550 amino acids in A-domain with two purpose which are to select and to activate the related substrate (Eisfeld, 2009). The substrate can be an amino acid, an imino-, a hydroxyl- or a carboxy- acid. The activation will start after that and later on added to the peptide chain as it grows (von Döhren, 2004). The characteristic for A-domains is to recognise and bind the substrates with a set of conserved sequence motifs as shown in *Table 2* (Konz and Marahiel, 1999). These highly conserved motifs are situated mostly around active areas in which the substrates are bound at (Marahiel *et al.*, 1997). *Figure 1* shows the position of the core motifs (A1-A10) in A-domain with their functions and relationship of one another.

To reveal more information on the A-domains, a sequence alignments method was used and the alignments showed an adenylate (AMP) binding motif which is conserved in adenylate forming enzymes superfamily which includes 4-coumerate-CoA ligases, oxidoreductases and acetyl-CoA synthetases (Turgay *et al.*, 1992). There is a homologous structure observed in these family members despite of the low similarity in the sequences. The catalysis by the A-domain is parallel to the initial step done by aminoacyl-tRNA synthetases (Turgay *et al.*, 1992), even though they displayed no sequence or structural similarities (Arnez and Moras, 1997; Weber and Marahiel, 2001).

Siderophores (a class of iron specific chelators) are one of the examples of NRP that are important fungal effectors that supress host immunity. Siderophores' role in fungus (pathogen)-plant interactions has not been clearly established.

TABLE 1. EXAMPLES OF CHARACTERISED NON-RIBOSOMAL PEPTIDE SYNTHETASES (NRPSs), THEIR PRODUCTS AND PROPERTIES

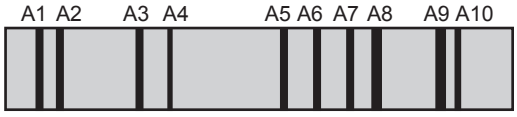
Producer	NRPS/Structure	Product	Properties
Fusarium oxysporum	Enniatin synthetase CATCAMTTC	Cyclo-hexadepsipeptide	Ionophore; inhibitor of acyl-CoA-cholestro lacyltransferase and of phosphodiesterase; potential anticancer compound
Cochlibolus carbonum	HC-toxin synthethase (HTS1) ATEC(ATC)[3]	Cyclo-tetrapeptide	Inhibitor of histone-deacetylases; maize-pathogen
Alternaria alternata	AM-toxin synthetase (ATC)[4]	4-peptidolactone	Phytotoxic
Xylaria sp. BCC1067	Bassianolide synthetase CATCAMTTCR	Cyclooctadepsipeptide	Pathogenic to insects

Source: Eisfeld (2009).

TABLE 2. CORE MOTIFS OF ADENYLATION (A), THIOLATION (T)/PEPTIDYL CARRIER PROTEIN (PCP) AND CONDENSATION (C) WITHIN THE DOMAINS OF NON-RIBOSOMAL PEPTIDE SYNTHETASE (NRPS)

Domain	Core	Consensus protein sequence
Adenylation	A1	L(TS)YxEL
	A2 (core 1)	LKAGxAY(VL)P(LI)D
	A3 (core 2)	LAYxxYTSG(ST)TGxPKG
	A4	FDxS
	A5	NxYGPTE
	A6 (core 3)	GELxIxGxG(VL)ARGYL
	A7 (core 4)	Y(RK)TGDL
	A8 (core 5)	GRxDxQVKIRGxRIELGEIE
	A9	LPxYM(IV)P
	A10	NGK(VL)DR
Thiolation or peptidyl carrier protein	T (core 6)	DxFFxxLGG(HD)S(LI)
Condensation	C1	SxAQxR(LM)(WY)Xl
	C2	RHExLRTxF
	C3 (His)	MHHxISDG(WV)S
	C4	YxD(FY)AVW
	C5	(IV)GxFVNT(QL)(~)Xr
	C6	(HN)QD(YV)PFE
	C7	RDxSRNPL

Source: Konz and Marahiel (1999).



A3 interaction with pyrophosphate leaving group
A4 interaction with amino group
A7 interaction via hydrogen bonds with oxygen atoms of nucleoside ribose moiety
A8 essential for adenylation
A10 binds to substrate and ribose oxygens

Source: Eisfeld (2009).

Figure 1. Position and function of conserved motifs of the adenylation domain.

Nonetheless, over the time, investigation on siderophores have shown pathogenicity in several plant-pathogenic fungi and it was hypothesised that they play essential roles in supplying fungal pathogens with iron, which is a limiting factor within host environments (Lee *et al.*, 2005; Oide *et al.*, 2006; Schrettl *et al.*, 2007). Siderophores are reported to be essential for pathogenicity of most fungal pathogens and associated with the development of disease symptoms (Gerwien *et al.*, 2018).

There were few NRPS genes reported in other basidiomycetes. Synthesis of siderophore was

investigated in the ustilaginomycete *Ustilago maydis* and in the homobasidiomycete *Omphalotus olearius*. Production of ferrichrome and ferrichrome A were found in *U. maydis* (Budde and Leong, 1989). Siderophore synthetases that encode the ferrichrome synthetase is SID2 and the ferrichrome A synthetase is FER3 (Eichhorn *et al.*, 2006; Yuan *et al.*, 2001). A putative ferrichrome A synthetase gene was detected in *O. olearius*. The NRPS gene encoding the ferrichrome A synthetase in this fungus (FSO1) was the first NRPS gene described in a higher basidiomycete. Siderophore synthetase of ferrichome-type from the basidiomycete has high similarity to the siderophore synthetases of ascomycetes. It is interesting to find that there are 48 introns interruption in FSO1 (Welzel *et al.*, 2005). Even though high number of introns is not common in NRPS genes, it is reported that basidiomycete's genes are basically interrupted by a number of introns (Larrondo *et al.*, 2004; Martinez *et al.*, 2004).

Hence, a complete understanding of NRPS as a pathogenicity factor of *G. boninense* may provide insights to the fungal counter defence against innate plant defence system. The goals of this study were to i) identify a putative NRPS gene (A-domain) in *G. boninense*, ii) deduce the possible products of the said gene and iii) correlate between NRPS gene expression and disease severity in oil palm.

MATERIALS AND METHODS

Fungal Strain and Culture Conditions

Ganoderma boninense PER71 culture was obtained from Malaysian Palm Oil Board (MPOB). The culture was maintained on malt extract agar (MEA) and incubated in the dark at 25°C. Mycelial agar discs (5 mm diameter) were obtained from the edge of a 7-day-old culture and inoculated onto malt extract broth (MEB) on a rotary shaker at 150 rpm. Fungal mycelium was harvested at 14 days after inoculation.

Total RNA Extraction

Total RNA was extracted from 100 mg fungal mycelium using Norgen Total RNA Purification Kit (Norgen Biotek Corporation, Canada) according to the manufacturer's instruction. On-column DNase I (Fermentas, Lithuania) digestion was performed at 25°C for 15 min to eliminate traces of genomic deoxyribonucleic acid (DNA). Ribonucleic acid (RNA) concentration was quantified using NanoDrop™ ND-1000 Spectrophotometer and total RNA integrity was determined using 1% agarose gel electrophoresis.

Complimentary DNA (cDNA) Synthesis and Polymerase Chain Reaction (PCR) Amplification

A total of 1 μg DNase-treated RNA was reverse-transcribed using Omniscript Reverse Transcription Kit (Qiagen, German) and oligo (dT) primer at 37°C for 60 min. Degenerate primers were designed from the conserved A-domain of NRPS. Internal transcribed spacer (ITS) was used as an internal control (Utomo *et al.*, 2005). All primer sequences are as listed in *Table 3*. PCR amplification was performed in a total volume of 25 μL consisting 200 ng μL^{-1} cDNA template, 0.5 μM forward and reverse primer each and DreamTaq DNA Polymerase (Fermentas, Lithuania). PCR programs were as follows: initial denaturation at 95°C for 3 min; 40 cycles of 95°C for 30 s, 57°C annealing for 30 s, 72°C extension for 1 min and 7 min of final extension at 72°C. PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) according to the instructions from the manufacturer. The purified PCR products were cloned into pDrive cloning vector (Qiagen, Germany) and transformed into the competent Qiagen EZ bacterial cells. The positive recombinant colonies were screened using indicator plates incorporated with 80 μg mL^{-1} X-gal, 50 μM isopropyl-β-d-thiogalactopyranoside (IPTG) and 100 μg mL^{-1} ampicillin. Positive clones were harvested for plasmid purification

TABLE 3. LIST OF PRIMERS USED FOR POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION

Primer	Primer sequence (5′-3′)	Protein sequence (adenylation domain)
A3-4F	CACCACCGGCAAGCCNAARGGNGT	TTGKPKGV (A3)
A8-1R	CCNCKDATYTTNACYTG	QVKIRG (A8)
ITS1	AGCTCGTTCGTTTGACGA	
ITS1	AGCTCGTTCGTTTGACGA	
ITS3	CGATCAATAAAAGACCGA	
GADPH-F	ACTGCTACTCAGAAGACTGTTGATG	
GADPH-R	TGCTGCTAGGAATGATGTTAAAGCT	

using the QIAprep Spin Miniprep Plasmid Kit (Qiagen, Germany). Inserts were verified using *Eco*RI restriction enzyme digestion (Fermentas, Lithuania) and were analysed using 1% agarose gel electrophoresis. DNA fragments were then sequenced and the obtained DNA sequences were analysed using homology-based searches for A-domain in Basic Local Alignment Search Tool (BLAST).

Chrome Azurol S (CAS) Assay

Fungal siderophore producing ability was tested according to a modified chrome azurol S (CAS) assay outlined by Milagres *et al.* (1999). All glassware was treated with 6 M hydrogen chloride (HCl) and rinsed with distilled water to remove all traces of iron. Briefly, 60.5 mg CAS was dissolved in 50 mL distilled water and mixed with 10 mL iron (III) solution (1 mM $FeCl_3.6H_20$ in 10 mM HCl). The mixture was added to 40 mL of hexadecyltrimethylammonium bromide (CTAB) while stirring. CTAB solution was prepared by dissolving 72.9 mg CTAB in 40 mL distilled water. The resulting dark blue solution was kept at 50°C. Agar solution was prepared by dissolving 30.24 g piperazine-N,N'-bis(2-ethanesulphonic acid) (PIPES) and 15 g agar in 750 mL distilled water and, the final pH of the solution was raised to 6.7 using sodium hydroxide (NaOH). CAS agar was prepared by adding the dye solution to the agar solution and autoclaved at 121°C for 15 min. Petri dishes (9 cm diameter) containing potato dextrose agar (PDA) was cut into half in a sterile condition, one of which was replaced by CAS agar. Fungal agar discs were inoculated far from the borderline between the two media. The plates were incubated at 26 ± 2°C in the dark for two weeks. Colour of the CAS agar changed from blue to orange when siderophore was produced. Siderophore production was determined by measuring the distance of the orange zone in the CAS agar. All CAS reactions were performed in triplicates.

Plant Materials and Treatments

Three-month-old susceptible clone PK5407 and tolerant clone PK5505 oil palm seedlings (45 seedlings per clone) were obtained from MPOB Kluang Research Station, Johor, Malaysia. Thirty-six seedlings of each clone were inoculated with one-month-old *G. boninense*- colonised rubber wood block (RWB) (12 cm x 6 cm x 6 cm) using direct sitting technique (Sariah *et al.*, 1994). RWBs were used as carrier medium to provide nutrients for *G. boninense* under host-free condition. Nine seedlings of each clone were used as control. The seedlings were arranged in a completely randomised design (CRD) in the green house and maintained for six months.

Disease Assessment

Disease severity of the inoculated seedlings was assessed at 0, 1, 2, 4, 6 months after inoculation (MAI) according to external disease symptoms and rated based on 0-4 disease severity value. Disease severity index (DSI) was calculated using the Equation: Σ (A x B) x 100 / Σ (n x 4), of which:

A = disease class (0, 1, 2, 3 or 4)
B = number of plants showing that disease class per treatment
n = total number of plants per treatment
(Nur Ain Izzati and Faridah, 2008)

Gene Expression by Reverse Transcription (RT-PCR)

Nine seedlings each were harvested from control, susceptible and tolerant progenies at 0, 1, 2, 4 and 6 MAI, respectively. Root samples were collected, snapped frozen in liquid nitrogen and were stored at -80°C until extraction. Prescott and Martin (1987) method was employed to extract total RNA with the addition of aurintricarboxylic acid in RNA extraction buffer and also using the Norgen Total RNA Purification Kit (Norgen Biotek Corporation, Canada). RNA was analysed on 1% agarose gel to determine its quality. All the extracted RNA root samples of control and infected plants from both progenies were reverse-transcribed using the Omniscript® Reverse Transcription Kit (Qiagen, Germany). The RNA samples were thawed on ice and 4-6 µL (1 µg) of RNA root samples was added to master mix containing 2 µL 10x buffer RT, 2 µL dNTP (5 mM of each dNTP), 2 µL oligo-dT primers (10 µM), 0.3 µL RNase inhibitor (10 units μL^{-1}), 1 µL Omniscript Reverse Transcriptase and 6.7-8.7 µL RNase-free water in 0.2 mL PCR tube. The mixture was incubated for 1 hr at 37°C on a thermocycler to reverse transcribe the template RNA to cDNA. The cDNA was then diluted with nuclease-free water and standardised to a final concentration of 200 ng μL^{-1} to be used for PCR analysis right away or stored at -20°C for later use. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) primers served as PCR positive control. The GADPH primers were designed with PCR product around 100 bp. Primer pair of A3(4)F / A8(1)R were tested using cDNA of root samples. Total RNA from fungal mycelium was extracted using Norgen Total RNA Purification Kit (Norgen Biotek Corporation, Canada). RNA concentration and quality were examined using NanoDrop™ ND-1000 Spectrophotometer and 1% gel electrophoresis. A total of 1 µg DNase-treated RNA was reverse-transcribed using the Omniscript® Reverse Transcription Kit (Qiagen, German) and dT primer at 37°C for 60 min. The concentration of cDNA was quantified and diluted using nuclease-free water to a final concentration of 200 ng μL^{-1}.

PCR amplification was performed according to the condition as mentioned in the previous section. GADPH was used as internal control. All PCR reactions were performed in triplicates.

RESULTS AND DISCUSSION

Detection and Molecular Characterisation of NRPS cDNA

Total RNA extracted from the root samples showed two distinct rRNA bands (28S and 18S). The $A_{260/280}$ and $A_{260/230}$ ratios ranged from 1.89-1.97 and 1.9-2.10, respectively indicating that the RNA samples were of good purity with minimal protein and organic compound contamination. PCR amplification products of 835 bp were obtained. BLAST analysis against NRPS sequences indicated sequence similarity to the A-domain core motifs of NRPS gene. The sequence was identified to contain the putative A2 (LKAGxAYL(VL)P(LI)D, A3 (TSG(TS)TGxPKxV) and A5 (NxYGPxE) motifs of the A-domain. Thus, the sequence was designated as *GbNRPS*. The low sequence similarity of the A-domain motif in *G. boninense* could suggest its novelty.

Siderophore Production in *G. boninense*

The formation of orange-coloured zone on CAS agar indicated the production of siderophores by *G. boninense* (*Figure 2*). Fungal mycelia grew rapidly on PDA agar but did not grow at all on CAS agar. Siderophores were produced and diffused through the CAS agar to cause colour change. Colour change on CAS agar was first observed at seven days after inoculation (DAI) when fungal mycelia started to cover the PDA. CAS reaction rate was calculated at 2 mm per day by measuring radial diameter of the orange-coloured zone. The colour change reaction was the result of iron removal from CAS dye by the siderophores secreted by *G. boninense*. Siderophores are iron-binding compounds produced by various organisms including pathogenic fungi to acquire iron, an important cofactor for iron-dependent enzymes required for various cellular processes (Sayari *et al.*, 2019). In addition, the preclusion of iron from the host plant is an important strategy to weaken the defense mechanism due to nutritional deficiencies (Haas, 2014).

Disease Severity Index (DSI)

Destructive sampling was carried out 1 MAI to examine the external and internal disease symptoms for each clone. DSI was determined based on the disease symptoms observed. The earliest disease symptoms were first observed in both susceptible and tolerant progenies at 4 MAI. Artificial infection of oil palm using direct sitting technique caused successful disease incidence (DI) in both susceptible and tolerant clones. Infection was confirmed by visual observation of white mycelia on root tissues. Uninoculated seedlings showed no sign of BSR foliar symptom, healthy stem and no fungal mycelia on root tissues. At 4 MAI, the tolerant clones showed lower DSI (28.13%) compared to the susceptible clone (62.50%). The disease symptoms observed in both clones followed the typical BSR disease progression patterns in oil palm. The infected

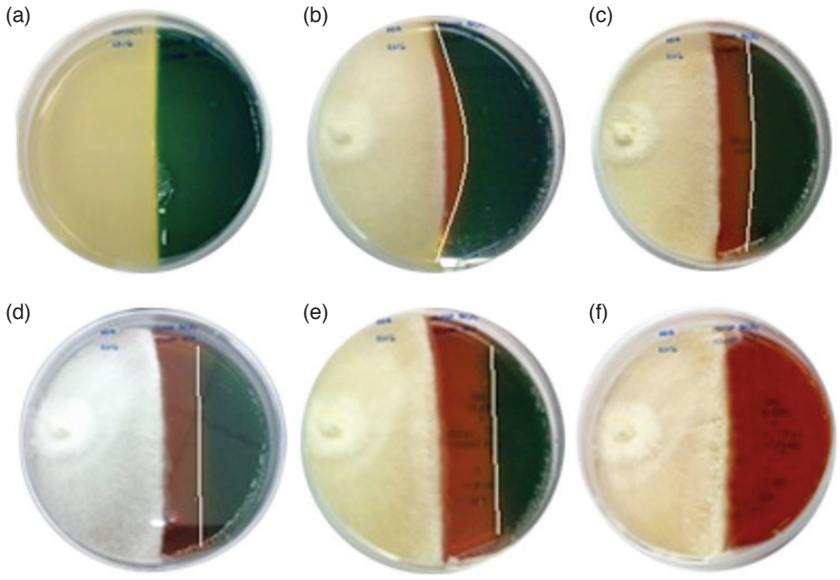


Figure 2. Development of orange-coloured zone on blue chrome azurol S (CAS) agar inoculated with G. boninense. *(a) Uninoculated control, (b) seven days after inoculation (DAI), (c) 10 DAI, (d) 11 DAI, (e) 12 DAI, and (f) 14 DAI.*

seedlings were observed with progressive chlorosis and necrosis from the older to the younger leaves. Fungal mycelia were observed on root tissues and followed by progressive stem necrosis and the formation of basidioma at basal stem. At 6 MAI, DSI of tolerant and susceptible clones were increased to 31.25% and 71.88%, respectively. The symptoms of leaf chlorosis and desiccation in infected palms were caused by restricted water and nutrient uptake due to damaged stem and root tissues (Hushiarian *et al.*, 2013). The differential responses of the tolerant and susceptible clones to *G. boninense* reflect the distinct molecular regulation of defense related genes between both clones. The higher expression of defense related genes such as chitinases, isoflavone reductase, metallothionine-like protein and serine protease inhibitor in the root tissues of the tolerant clone likely contributed to its higher tolerance to *G. boninense* infection (Riza *et al.*, 2016).

NRPS Expression in Relation to DSI

The expression of NRPS gene potentially involved in the invasion of *G. boninense* was examined at 1, 2, 4 and 6 MAI in both tolerant and susceptible clones. The expression levels of the housekeeping gene GAPDH were consistent in all tested samples. NRPS gene was detected at 1 MAI in both tolerant and susceptible clones despite the lack of observable disease symptoms. At 1 MAI, the PCR band intensity of NRPS gene was higher in the susceptible clones compared to the tolerant clones. The expression of NRPS gene has progressively upregulated from 1 to 4 MAI and sustained to 6 MAI in the susceptible clones, whereas in the tolerant clone the expression level has upregulated from 1 to 2 MAI and sustained to 6 MAI (*Figure 3*). NRPS gene expression shows a potential correlation with the higher DSI observed in the susceptible clones compared to the tolerant clones. The induced expression of the NRPS gene in both tolerant and susceptible clones indicates that it might play an important role in the infection process. NRPS is identified as a virulent factor for several pathogenic fungi as it plays an essential role in the biosynthesis of SMs such as siderophores and cyclic peptides with phytotoxic activities (Pusztahelyi *et al.*, 2015). The siderophore-mediated iron acquisition is also required for the synthesis of iron-dependent antioxidants (superoxide dismutase and catalase) in fungal pathogens in response to hydrogen peroxide stresses induced by the host plant defense mechanism (Chung, 2012; Lee *et al.*, 2005; O'Hanlon *et al.*, 2012). The increased expression of NRPS gene in the susceptible clones might suggest its involvement in fungal survival and cell proliferation in the host plant. Gene silencing studies are recommended to verify the function of the NRPS gene in *G. boninense*.

CONCLUSION

A partial cDNA encoding adenylation core motifs of NRPS gene was successfully isolated from *G. boninense*. *GbNRPS* shared a similarity to other fungal NRPS A-domain. Siderophores are mostly produced via NRPS pathway. From this study, *G. boninense* was found to produce siderophore *in vitro* on CAS agar. However, the virulence factor is yet to be determined. There was a correlation between the expression of *GbNRPS* gene and disease severity during *Ganoderma*-oil palm interaction. These findings may contribute to the development of appropriate strategies for BSR disease management. Further studies are recommended to determine the modular structure and other domains of NRPS in *G. boninense*, the role of NRPS to the fungus and during plant-pathogen interaction.

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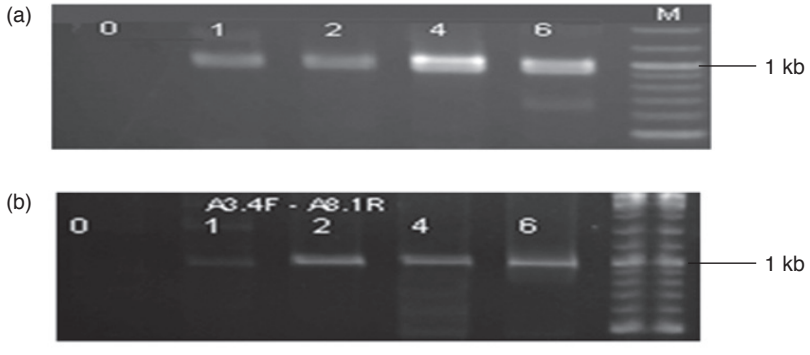

Figure 3. GbNRPS *expression analysis in susceptible and tolerant oil palm clones at 1, 2, 4 and 6 months after inoculation (MAI). (a) root samples of susceptible clones, (b) root samples of tolerant clones, Lane M: GeneRuler DNA Ladder Mix (Fermentas, Lithuania).*

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