IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS IN OIL PALM SEEDLINGS ARTIFICIALLY INFECTED WITH *Ganoderma*: A PROTEOMICS APPROACH

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ABSTRACT

Basal stem rot disease in oil palm is caused by the fungus, Ganoderma boninense. *Proteomics approaches using two-dimensional gel electrophoresis (2-DE) were adopted to identify proteins from oil palm that were differentially expressed in response to the fungus. Total proteins were extracted from oil palm roots after Day 3 and Day 7 post-infection and resolved in 2-DE gels. Differentially expressed protein spots between the infected and uninfected palms were determined using PD-Quest Software. Seven protein spots were differentially expressed in the infected root at Day 3 post-infection. At Day 7, the number of differentially expressed protein spots increased to 25, with a molecular weight less than 50 kDa. All protein spots were analysed by MALDI TOF/TOF mass spectrometry. Twelve candidate proteins were identified by a comparison of differentially expressed proteins at Day 7 with the database. One of the candidate proteins is glucanase, which is widely known to induce during fungal response in other plants. Other candidate proteins that were identified were also involved in defence and stress responses in other plants, suggesting that these proteins play a significant role in oil palm root pathogenicity response towards* G. boninense *infection.*

Keywords: *Ganoderma boninense*, two-dimensional gel electrophoresis, proteomics, oil palm root.

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INTRODUCTION

Ganoderma boninense, which is a basidiomycete fungus and a major pathogen of oil palm in Malaysia, is the cause of basal stem rot (BSR) disease (Idris *et al*., 2011). The impact of the disease has driven the oil palm industry players to search for effective ways to combat this problem. The fungus attacks palms by root contact. One of the symptoms of the infection is the presence of a number of fully elongated but unopened spear leaves at the centre of the crown (Corley and Tinker, 2003). In older palms, the disease can be detected by the collapse of lower leaves which hang vertically downwards, from the point of attachment to the trunk. Symptoms including blackened stems and the presence of fruiting bodies at the stem base, frond bases or roots are only seen once the disease is firmly established in the infected palms. By the time these symptoms become visible, it would be too late for the infected palm to be fully cured.

Plants have the ability to respond to pathogen invasion through the activation of a variety of defense strategies. In plant-pathogen interactions, the pathogen (fungi) secretes various enzymes

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that degrade wood tissue, primarily lignin and cellulose and the xylem will be eventually affected. The infection restricts water uptake and causes extensive damage to the stem structure. Generally, biochemical and physiological responses, such as the production of secondary metabolites, anti-fungal and pathogenesis-related (PR) proteins, are triggered by the host plant to limit the pathogen attack (Hammerschmidt, 1999). A key feature underlying defense against pathogen invasion is the synthesis and accumulation of PR proteins, which can be distinguished by specific biochemical properties.

PR proteins are low-molecular weight proteins (6-43 kDa) that are extractable and stable at low pH. Currently, PR proteins are grouped into 17 dependent families (Campo *et al*., 2004). Activities for most of the PR families are known or can be inferred. For instance, the PR-2 family consists of endo β-1,3-glucanase, whereas the PR-3, PR-4, PR-8 and PR-11 families are all classified as endochitinases. In tomato, PR-7 was found to act as an endoproteinase. Meanwhile, peroxidase belongs to the PR-9 family and its function is likely to strengthen the plant cell wall by catalysing lignin deposition due to microbial attack (Van Loon *et al.*, 1994). PR-10 family is related to ribonucleases and may be active during viral infection of plant. In addition, anti-fungal and anti-bacterial activities are exhibited by the PR-12 type defensins, PR-13 type thionins and PR-14 type lipid transfer proteins, LTP (Van Loon and Van Strien, 1999). Germin and Germin-like proteins (GLP) are classified as PR-15 and PR-16, respectively (Park *et al*., 2004).

Modes of action of these proteins vary from fungal cell wall polymer degradation, membrane channel and pore formation, damages to cellular ribosomes, inhibition of DNA synthesis and inhibition of the cell cycle. However, the mode of action of PR-1 proteins remains unknown. Despite the extensive information available on the accumulation of defense proteins in plants, the expression and accumulation of these proteins in oil palm during *Ganoderma* infection remain unknown. Therefore, time course and cellular localisation studies will provide essential information on biochemical events that are involved during *Ganoderma* attack on oil palm.

Proteomics analysis, unlike genomics, has a highly dynamic entity in terms of protein expression and the content of a given cell varies with respect to changes in the surrounding environment, physiological state of the cell, stress, health and disease. Two-dimensional gel electrophoresis is the prevailing technique for analysis of the whole protein expression profile of a given cell type under specific conditions (Wojcik and Schachter, 2000). The intensity of a protein spot is considered to be directly correlated to the level of protein expression in the particular tissue under investigation at that given time point. Typically, proteins are separated according to their isoelectric point (pI) and molecular weight (MW). Differentially expressed spots are subjected to proteolytic digestion and identified by peptide mass fingerprinting.

In view of the lack of studies on the biochemical response of oil palm to *Ganoderma* infection at early stages, we focused our efforts on examining the proteins differentially expressed in response to infection in oil palm roots. Proteomics analysis was carried out to capture distinctive proteins that are differentially secreted during *Ganoderma* infection. This information will aid in the development of early tools and subsequently control measures to protect the oil palm from BSR disease as early as possible.

MATERIALS AND METHODS

Plant Materials

A total of 108 oil palm seedlings comprising standard (Deli x AVROS), susceptible (Elmina x Elmina) and tolerant (Zaire x Cameroon) were used in this study. The selection of seedlings was based on a previous study by Idris *et al*.(2004). The germinated seeds were planted in small black polypropylene bags (6 x 9 cm) for three months in the nursery under shaded conditions and then transferred to big polypropylene bags (38 x 50 cm) containing a non-sterilised 3:2:1 mixture of soil, sand and organic matter, respectively. All soils used were non-sterilised to imitate field conditions. The bags were placed in an open space nursery at 90 cm triangular spacing. The seedlings were maintained and watered (twice daily) using a sprinkler system and fertiliser applied as appropriate. All nursery experiments were conducted at MPOB/UKM.

Oil palm seedlings were artificially infected using the root inoculation technique, as described previously by Ariffin and Idris (1990). Twelve-month old oil palm seedlings were subjected to artificial infection with *Ganoderma boninense* PER 71 and two control experiments: T1, treated with rubber wood blocks fully colonised with *Ganoderma;* T2, treated with uncolonised rubber wood block (control) and T3, oil palm seedling without wood block (absolute control). The rubber wood block preparation was previously described by Abdullah *et al*. (2001).

The three progenies were laid out in a randomised complete block design (RCBD) with six replicates for each treatment. Approximately 2 cm of infected roots at Days 3 and 7 were excised and immediately frozen.

Protein Extraction

Total protein extraction was conducted according to the revised method of Sheffield *et al*. (2006) and the *Medicago trancatula* handbook. Approximately 3.0 g of root tissue were ground into fine powder using cooled mortar and pestle. The powder was dissolved in 30 ml extraction buffer consisting of 1.5 M Tris-HCl pH 8.8, 0.5 M EDTA, 0.9 M sucrose and 0.4% 2-mercaptoethanol and 30 ml Tris-buffered phenol (pH 8.8). The extract was shaken for 30 min in the cold room and spun at 12 000 g for 30 min at 4°C. Phenol phase was removed and back-extracted with equal volume of phenol and extraction buffer. Total proteins in the phenol phase were precipitated with five volumes of ice-cold 0.1 M ammonium acetate in 100% methanol. The mixture was kept at –20°C for 16 hr and spun at 12 000 g for 30 min at 4°C. The protein pellet was washed twice in 20 ml of methanol and rinsed twice with ice-cold acetone. The pellet was then dried for 5 min and dissolved in 1 to 2 ml lysis buffer (7 M Urea, 2 M Thiourea, 4% CHAPS and 0.5% of Triton X-100), and 0.1% Immobiline pH gradient buffer (IPG) and 0.02 M DTT added prior to use. Total protein was cleaned using the 2-D Clean Up Kit (GE Healthcare, Piscataway, NJ) and quantified using the 2-D Quant Kit (GE Healthcare, Piscataway, NJ).

Two-dimensional Polyacrylamide Gel Electrophoresis

Total root protein extracts (500 µg) were loaded onto 18 cm IPG pH 3-10 strips (GE Healthcare, Piscataway, NJ) and rehydrated overnight. Isoelectric focusing (IEF) was performed at 0 V - 200 V for 200 VHr, 500 V gradient for 500 VHr, 4000 V for 3400 VHr and 8000 V for 13 500 VHr using the Ettan IPGphor system (GE Healthcare, Piscataway, NJ). Focused gels were equilibrated according to the manufacturer's specifications (GE Healthcare, Piscataway, NJ). Second dimension SDS-PAGE gels (13%) were run on a Ettan DALTsix Electrophoresis System (GE Healthcare, Piscataway, NJ) at 60 mA for an hour and at 72 mA for 15 hr. Gels were stained using Coomassie Brilliant Blue stain and images were acquired using a VersaDoc Imaging System (Bio-Rad, Hercules, CA). The resulting gel images were subsequently normalised with PDQuest™ 2-D Analysis Software (Bio-Rad, Hercules, CA).

Protein Identification by Matrix-assisted Laser Desorption Ionisation-time of Flight Mass Spectrometry (MALDI-TOF MS)

Differentially expressed protein spots were cored out from the gel. The spots were de-stained with 25 mM ammonium bicarbonate in 50:50 (ACN:water) three times. De-stained gel spots were vacuum-dried and kept at -20ºC prior to digestion. Dried gel spots were digested with 10 µl digested solution (12.5 µg ml^{-1} trypsin, 25 mM ammonium bicarbonate) and incubated at 37ºC, overnight. The digested peptides were extracted by two 20-min incubations with 10-20 µl ACN containing 1% TFA. The pooled extracts were dried by rotary evaporation and stored at -20ºC prior to MS analysis. MALDI TOF/TOF MS/MS was performed on a 4800 MALDI TOF/TOF TM analyser (Applied Biosystem/MDS SCIEX). The dried peptide samples were reconstituted in 2 µl standard diluent (30:70, ACN: water). The resulting solution was diluted with 10-fold of matrix solution (CHCA, 10 mg ml^{-1}) and spotted on a 384-well Opti-TOF Stainlees Steel Plate. The spotted samples were analysed using a first run of standard TOFMS. The system was set to perform a second run of MS/MS focusing on the 15 most intensive peaks of the first MS (excluding peaks known to be trypsin). The laser was set to fire 400 times per spot in MS mode and 2000 times in MS/MS mode. Laser intensity was 2800 J (MS) and 3900 J (MS/MS). A mass range of 400-4000 amu with a focus mass of 2100 amu was used. All sequences from MS/MS mass spectra were submitted for BLAST search (www.ncbi.nlm.hih.gov/BLAST) using the non-redundant SWISS-PROT database.

RESULTS AND DISCUSSION

Roots play a vital role in water and nutrient uptake from the soil. In oil palm, the roots are prone to fungal attack, particularly from *Ganoderma*. In this study, rubber wood blocks were flooded with *Ganoderma* PER 71 inoculum up to three months (*Figure 1*). Twelve-month old palms were inoculated with rubber wood blocks using the root inoculation technique (*Figure* 2) to mimic the actual *Ganoderma* infection. Root samples were taken at different time intervals until one year post-infection to monitor the biochemical activity of the infected palm seedlings. Protein expression of oil palm roots during early infection (Days 3 and 7) was studied using two-dimensional gel electrophoresis.

Total protein extraction strategy from raw materials is a very important step in 2-DE. It is crucial to prevent any protein loss at every step. Therefore, the methodology for protein extraction must be efficient to ensure that the maximum amount of proteins is extracted from the raw materials, while interfering components are eliminated. In this study, a number of extraction methods were tested for their efficiency. Preliminary studies which used the trichloroacetic acid (TCA)-based extraction method, showed very high background staining of the gel with vertical and horizontal lines. One possible component that caused this problem is carbohydrate. It blocked gel pores, causing precipitation on the top

Figure 1. Rubber wood blocks fully colonised filled with Ganoderma inoculum.

Figure 2. Root inoculation of a twelve-month old palm.

of the gels or extended focusing times, resulting in streaking and loss of samples. Another method for protein extraction using urea/thiourea in the lysis buffer was conducted according to Molly *et al*. (1998). The addition of thiourea raised the solvent capacity of the buffer. However, this method resulted in streaking on the two-dimensional gel image.

Finally, we also tested the method developed by Sheffield *et al*. (2006) based on phenol extraction. Despite having minimal activity in dissolving nucleic acid and polysaccharides, phenol served as a strong protein solvent (Carpentier *et al*., 2005). Sucrose was added to the extraction buffer to create a phase inversion. The bottom phase (aqueous) contained carbohydrates, nucleic acid and insoluble debris. The upper phase (phenol) contained cytosolic and membrane proteins together with lipids and pigments. Utilisation of phenol at pH 8.8 assured nucleic acids reside in the aqueous phase, not in the phenol rich phase (Pusztai, 1966). By using this method, many spots were obtained, while streaking and high background problems were successfully resolved. This method, with minor modification at the washing step, was found to be the best method to extract proteins from oil palm roots.

We have conducted preliminary analysis of oil palm root proteins using high resolution 2-DE. Our previous results revealed that oil palm root proteins were resolved by 2-DE in the 3-10 pH range (Syahanim *et al*., 2011). Therefore, this range was used throughout this study. The same amount of protein samples (500 μg) was used in the 2-DE. Analysis of the gel images was done by PDQuest™ 2-D Analysis Software to determine the differential protein spots in infected and uninfected palms at Days 3 and 7. This software provides both qualitative and quantitative information corresponding to induced proteins in root. Only proteins that were observed to be differentially expressed were selected for further analysis. This analysis identified a total of 32 protein spots that appeared to be differentially expressed between infected and control palm.

From the total protein extract at Day 3, only 7 protein spots were found to be differentially expressed (*Figure 3*). Meanwhile, at Day 7 post-infection, 25 protein spots were found to be differentially expressed (*Figure 4*). These spots were excised from the gel and subjected to in-gel tryptic digestion. MALDI-TOF/TOF MS/MS analysis was carried out and the mass spectra were searched against protein sequence database. The identification output was a list of possible identifications sorted according to their Molecular Weight Search (MOWSE) scores. All candidates with MOWSE scores greater than 1 x 10^6 were selected as significant assignments. Of the 32 digested peptides, only 12 protein spots at Day 7 gave significant identities - six protein spots from standard palm (denoted as E1, E2, E4, E11, E13 and E14), four protein spots from tolerant palm (denoted as E17, E18, E19 and E20) and only two protein spots from the susceptible palm (denoted as E22 and E25).

The identity of these proteins is listed in *Table 1*. These 12 protein spots represent eight proteins, due to the fact that some protein spots had the same identity (*i.e*., spots E1 and E2, glucanase; spots E11, E17, E8, E19 and E20, early flowering protein 1 and spots E13 and E25, nucleoside diphosphate kinase), suggesting possible post transcriptional modification of these proteins. Among the differentially expressed candidate proteins identified in this study, protein spots E1 and E2 matched to beta-1,3-glucanase. Beta-1,3-glucanase is induced as part of the hypersensitive response to pathogen infection (Menu-Bouaouiche *et al*., 2003). Glucanase is a candidate protein of interest, since it is classified as pathogenesis-related protein class 2. Previous studies reported β-1,3-glucanase is induced in wheat during *Fusarium graminearum* infection (Pritsch *et al*., 2000; 2001). On other hand, Keat-Ai *et al*. (2012) reported low expression of glucanase (putative β-D glucan exohydrolase) in infected oil palm roots at week 3, 6 and 12 post-infection. In our study, glucanase appeared at Day 7 post-infection.

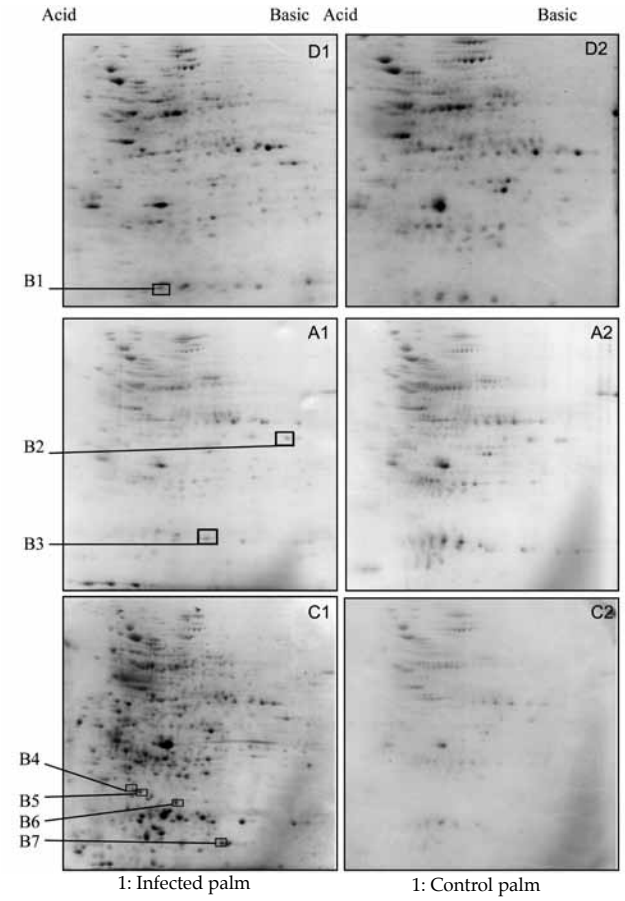

Figure 3. Proteome map of protein extract of standard (D), tolerant (A) and susceptible (C) palm at Day 3 after infection (B). A1, C1 and D1; oil palm infected with Ganoderma. *A2, C2 and D2; oil palm without block. The squared area represents spots that were cut for MALDI-TOF MS analysis. The separation of oil palm root proteins was performed on 18 cm strips pH 3-10.*

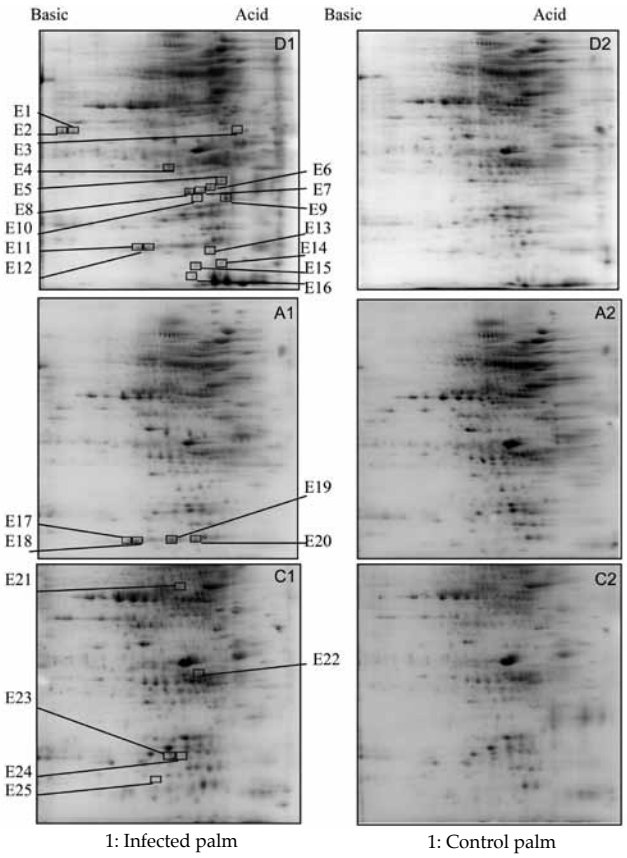

Figure 4. Proteome map of protein extract of standard (D), tolerant (A) and susceptible (C) palm at Day 7 after infection (E). A1, C1 and D1; oil palm infected with Ganoderma. *A2, C2 and D2; oil palm without block. The squared area represents spots that were cut for MALDI-TOF MS analysis. The separation of oil palm root proteins was performed on 18 cm strips pH 3-10.*

It can be concluded that glucanase do plays a role during early infection of *G. boninense* in oil palm.

Protein spot E4 was identified as glutathione-S-transferase. This enzyme is classified as an antioxidant enzyme and plays an important role in detoxifying contaminants through conjugation (Campo *et al*., 2004). A study in barley responding to *Fusarium graminearum* at Day 3 post-infection showed that glutathione transferase is in abundance during infection (Geddes *et al*., 2008). Protein spots E13 and E25 showed high homology with a nucleoside diphosphate kinase (NDPK). In wheat, kinase genes are highly expressed during fungal infection (Foroud *et al*., 2012). The induction of this enzyme is a cellular response to various stresses, such as wounding, heat shock and UV, suggesting NDPK may have a regulatory role in addition to their primary metabolic function in plants (Sweetlove *et al*., 2001). Kinases are also involved in early signalling events in response to various stimuli (Foroud *et al*., 2012). Protein spot E14 was identified as thioredoxin H2 protein. This protein exhibits antioxidant activity against free radicals in sweet potato storage roots (Huang *et al*., 2004). Five protein spots had significant identity with early flowering protein 1 (EFP) (protein spots E11, E17, E18, E19 and E20). Early flowering protein is classified in Bet v 1 superfamily in the plant food allergen protein family (Radauer and Breiteneder, 2007). Protein spot E22 observed in the infected palm was shown to share identity to ferritin. This protein plays a role in storing iron, during plant development and under stress conditions (Briat, 1996).

CONCLUSION

We report here the application of proteomics towards understanding the oil palm root and fungus *G. boninense* interactions. Twelve protein spots were identified as β-1,3-glucanase, NDPK, glutathione-S-transferase, early flowering protein 1, ferritin and thioredoxin H2, in the infected palm at Day 7 post-infection. These proteins have been reported previously to be involved in plant defence and stress responses. This finding, coupled with functional validation analysis, may provide new insights into pathogenicity of *G. boninense* in oil palm.

TABLE 1. IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS FROM OIL PALM ROOTS INFECTED WITH *Ganoderma boninense* AT DAY 7 POST-INFECTION BY MALDI TOF/TOF MS/MS

Spot	Mass (kDa)/pI	MOWSE Score	Peptides matched	Protein name	Species
E1	36.2/9.2	315	4	Beta -1,3-glucanase	*Elaeis guineensis* var. *tenera* (B3TLW8)
E2	36.2/9.2	135	5	Beta -1,3-glucanase	*Elaeis guineensis* var. *tenera* (B3TLW8)
E4	24.3/6.3	50	1	Glutathione S-transferase	*Arabidopsis lyrata* (D7LHB8)
E11	17.1/5.8	47	1	Early flowering protein 1	*Elaeis guineensis* var. *tenera* (B3TLX2)
E13	12.8/5.6	106	2	Nucleoside diphosphate kinase	*Musa auminata* (Q8RV01)
E14	3.0/5.5	76	-	Thioredoxin H2	*Spinacia oleracea* (Q9S880)
E17	17.1/5.9	89	3	Early flowering protein 1	*Elaeis guineensis* var. *tenera* (B3TLX2)
E18	17.1/5.9	154	3	Early flowering protein 1	*Elaeis guineensis* var. *tenera* (B3TLX2)
E19	17.1/5.9	137	4	Early flowering protein 1	*Elaeis guineensis* var. *tenera* (B3TLX2)
E20	17.1/5.9	104	4	Early flowering protein 1	*Elaeis guineensis* var. *tenera* (B3TLX2)
E22	29.5/5.7	46	1	Ferritin	*Populus trichocarpa* x *Populus deltoides* (A9PK81)
E25	16.4/6.5	252	2	Nucleoside diphosphate kinase	*Camelia sinensis* (F4YFB4)

Note: *Individual ions scores >46 indicate identity or extensive homology ($p<0.5$).
MALDI TOF/TOF - matrix-assisted laser desorption ionisation-time of flight.
MOWSE – molecular weight search.

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