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Differentially Expressed Proteins from Roots of Healthy and Diseased *Piper nigrum*

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ABSTRACT

Introduction: Diseased *Piper nigrum* plants have affected pepper berry production in Sarawak farms, contributing to the economic loss for farmers that depend on this species for their income.

Objective: This study was performed to analyse the protein profiles of healthy and diseased *P. nigrum* roots and to identify differentially expressed proteins.

Methods: Two methods of protein extraction were compared, and the extracted proteins were quantified through two quantitative assays: the Bradford and BCA assays. The Phenol/SDS extraction method was significantly better compared to the phenol method.

Results: The SDS-PAGE analysis revealed two differently expressed unique proteins bands with the molecular size of approximately 20 kDa and 12.5 kDa present in the sample of diseased plants. These differentially expressed proteins are likely to be pathogenesis-related (PR) proteins which are produced as a response to infection.

Conclusion: These PR proteins can potentially be used as markers or be developed further to allow plants to gain systemic resistance against diseases.

Key Words: BCA assays, Pathogenesis-related proteins, Phenol extraction, *Piper nigrum*, Proteomics

INTRODUCTION

Piper nigrum plant known as black pepper is a type of woody perennial climber with dilated or swollen nodes and stipules.¹ Peppers from Indian origins are widely grown in pepper producing countries such as Thailand and Malaysia because of high quality and yield. The pepper berry has been used as a spice and seasoning due to its pungent flavour and aromatic scent. In addition to the main usage as spice and seasoning, different parts of the plant have been used for the extraction of the secondary metabolites. These metabolites were used in drugs, preservative, insecticidal and larvicidal control agents.² For example, studies have shown that *P. nigrum* affects the digestive system, where piperine increases the production of saliva and gastric secretions.^{3,4}

Through a proteomic study of *P. nigrum* roots, this study aims to identify proteins which are expressed as a response towards disease and infection. The previous study from

leaves has revealed the role of different pathogenesis-related (PR) proteins and enzymes in the disease reaction of black pepper concerning *Phytophthora capsici* foot rot infection.⁵ Nowadays, advancements in proteomic studies have allowed the quantitative and qualitative separation of complex protein mixtures, which makes it possible to monitor dynamic protein expression in plant growth and metabolism by using two-dimensional electrophoresis (2-DE) and mass spectrometry analysis.⁶⁻⁹

The study hypothesis is that there are differently expressed proteins in *P. nigrum* plants when compared between the healthy and foot rot diseased roots caused by *Phytophthora*. In this study, two different extraction protocols were evaluated. Furthermore, two protein quantification methods, the Bradford assay and Bicinchoninic acid assay were compared. Lastly, the protein profiles of the healthy and diseased samples were compared to identify any differentially expressed proteins.

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MATERIALS AND METHODS

Study site

The healthy and diseased roots of the *P. nigrum* samples were collected in Serian, Sarawak, Malaysia. The samples of healthy *P. nigrum* plants were identified by the plants producing green rounded fruit berries and healthy green leaves. Meanwhile, the diseased samples were collected from the plants that showed diseased symptoms like visibly stunted growth and some rotting on the plant. Three replicates of healthy and diseased plants were collected for sample analysis.

Plant Materials

The roots were washed and cleaned with running water to remove any attached soil. Then, the roots were rinsed with distilled water, followed by 70% ethanol to remove any remaining contamination. The samples were stored at -80 °C until used. The roots were finely powdered by grinding with liquid nitrogen using a mortar and pestle. Proteins were extracted from the healthy and diseased roots using two different extraction methods which were the phenol extraction and the phenol/SDS extraction method, with three replicates, respectively.

Protein Extraction Methods

Phenol protein extraction

This phenol extraction method is based on Faurobert et al.¹⁰ with modification as described. Firstly, 0.4 g of ground tissue was suspended in 0.8 ml of extraction buffer (500 mM Tris-HCl, pH 8.0, 50 mM EDTA, 700 mM sucrose, 100 mM KCl, 2% β -mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride) and vortexed. Then, it was incubated by shaking for 10 min on ice. After that, an equal volume of Tris-buffered phenol was added to the mixture, vortexed and incubated on a shaker for another 10 min at room temperature. The sample was centrifuged at 16 000 X g for 10 min at 4 °C. The upper phenolic phase was transferred into a new tube. Then, this phenolic phase was back-extracted with 0.8 ml of extraction buffer. The sample was vortexed and shaken at room temperature for 10 min. Centrifugation was repeated at 16 000 X g for 10 min at 4 °C. The upper phenolic phase was transferred into a new tube. Next, three volumes of cold 0.1 M ammonium acetate in methanol was added as a precipitation solution. The tube was shaken by inverting, and the sample was incubated overnight at -20 °C. The protein was pelleted by centrifugation at 16 000 X g for 10 min at 4 °C. The pellet was washed once with cold precipitation solution and once with cold 80% acetone. Finally, the pellet was air-dried and stored at -20 °C.

Phenol/SDS protein extraction

The phenol/SDS extraction method is based on Rodrigues et al.¹¹ with modification as described. Firstly, 0.4 g of ground

tissue was suspended in 0.8 ml of Tris-buffered phenol, pH 8.0 and 0.8 ml of SDS buffer (0.1 M Tris-HCl, pH 8.0, 2% SDS, 5% β -mercaptoethanol, 30% sucrose, and 1 mM phenylmethylsulfonyl fluoride). The mixture was vigorously vortexed and incubated for 10 min. Then, the mixture was centrifuged at 16,000 X g for 5 min at 4 °C. The upper phenolic phase was transferred into a new tube. Three volumes of cold 0.1 M ammonium acetate in methanol were then added to the tube. The tube was shaken by inverting, and the sample was incubated overnight at -20 °C. The protein was pelleted by centrifugation at 16,000 g for 5 min at 4 °C and washed in the same way as the phenol extraction method; once with cold 0.1 M ammonium acetate in methanol and once with cold 80% acetone. Finally, the pellet was air-dried and stored at -20 °C.

Protein Quantification

Bradford assay

The protocol was adapted from Bradford¹² and Stoscheck¹³. The protein standards using bovine serum albumin (BSA) were prepared with different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml). Each of the volumes was pipetted into a 1.5 ml Eppendorf tube and the volume was adjusted to 20 μ l by adding a buffer solution, 0.05 M Tris-HCl. On the other hand, the sample proteins were prepared with a range of 1, 1:1, 1:9 dilutions with a total of 20 μ l and pipetted into a 1.5 ml Eppendorf tube. After that, all of the tubes were added with 1 ml of Bradford reagent including the blank tube (20 μ l 0.05 M Tris-HCl), vortexed and incubated at room temperature for 5 min. A spectrophotometer (OPTIMA SP-300) was used to analyse the BSA standard solution and protein samples at the absorbance of 595 nm.

Bicinchoninic acid (BCA) assay

The BSA protein standards and samples were prepared as described for the Bradford assay.^{14,15} 1 ml of standard working solution was added to each sample and mixed using a vortex. The samples were then incubated for 30 min at 60 °C. Next, the samples were cooled before measurement using a spectrophotometer (OPTIMA SP-300) at 562 nm.

SDS PAGE

Protein extracts were separated using SDS-PAGE according to the protocol from Laemmli¹⁶. The gels were stained with Coomassie Brilliant Blue R (CBB-R) staining solution. The gels were then destained with 10% acetic acid for 2 hours.

Image analysis and MALDI-ToF/ToF mass spectrometry

The gel image was analysed using ImageJ software and the selected protein bands were subjected to MALDI-ToF/ ToF analysis for protein identification. The gel bands excised from 1-D gels were de-stained and proceeded with in-gel trypsin digestion based on the in-house method developed

from Shevchenko et al.^{17,18} The extracted peptides were analysed using MALDI ToF/ToF 5800 mass spectrometer (Sciex, Framingham, USA). Spectra obtained were analysed to identify the proteins of interest using Mascot search engine.¹⁹

RESULTS

Protein quantification for *Piper nigrum*

Quantification of protein was done using two assays which were Bradford assay and BCA assay. Table 1 shows the average concentration of proteins from the roots of healthy and diseased *P. nigrum*, as quantified using the Bradford and BCA assays.

Protein profiles using SDS-PAGE

From the results of SDS-PAGE stained using Coomassie Brilliant Blue R (CBB-R), there were some differences observed between the protein profiles of healthy and diseased *P. nigrum* roots, where two additional protein bands were present in diseased roots. Diseased roots (Figure 1; lane b and d) showed two distinct bands estimated at ~20 kDa and ~12.5 kDa respectively. These bands were only faintly observed in healthy roots (Figure 1; lane a and c), compared to other protein bands in both healthy and diseased samples that were of equal intensity. Analysis of variance (ANOVA) for protein concentration was conducted to identify any differences in protein concentration between the extracted samples. From Table 2 and Table 3, the F-values were smaller than F-critical, $2.175 < 4.066$ and $0.742 < 4.066$ respectively, to determine significant differences of protein concentration between the healthy and diseased samples from the two different extraction methods.

Identification of differentially expressed proteins by MALDI-ToF/ToF MS

The differentially expressed proteins by MALDI-ToF/ToF mass spectrometry showed Mascot scores for the Protein Band 1 at 44 and 30, indicating close but not exact or matched identity to the identified proteins (Table 4). The Protein Band 1 showed peptide with the highest score of 44 matched a mannose-binding lectin (MBL) or the score of 30 to match with agglutinin protein. Result of the Protein Band 2 analysis showed low hit Mascot scores in the protein database (Table 4). The peptide with the highest score-matched and AAA-type ATPase family protein, as well as a hypothetical protein OsI_11535.

DISCUSSION

Analysis of protein quantification for *Piper nigrum*

The phenol/SDS extraction method yielded a higher protein concentration than the phenol extraction method. Compared

to the original phenol/SDS extraction method²⁰, the modified phenol/SDS extraction method by Rodrigues et al.¹¹ avoided the preliminary washing step. It was suitable both for removing interfering compounds and minimising protein degradation in extracts.

The analysis of variance (ANOVA) for protein concentration to identify differences in protein concentration between the extracted samples has indicated that there was no difference in terms of protein concentration between the healthy and diseased samples from the two different extraction methods. However, a t-test conducted on proteins quantified with the Bradford assay showed significant differences in terms of protein concentration between healthy and diseased samples with p-value is less than 0.05 (Table 5). This indicates that different quantity of protein was extracted from healthy and diseased roots of *P. nigrum*.

Meanwhile, a comparison of the two quantification methods resulted in an almost ten-fold difference in protein concentration. The BCA assay yielded higher protein concentration than the Bradford assay. A t-test was conducted to test if there was any statistical difference in protein concentration between the two methods. The p-value was 0.0000421 (Table 6), lower than 0.05. Therefore, this value indicates a significant difference in the number of proteins between the two methods. The differences may be caused by the distinct sensitivities of the Bradford and BCA assays or due to interfering compounds that exist in the samples.¹⁵ The BCA assay is very sensitive towards interference from reducing sugars and lipids, which cause over-estimation of the protein concentration.^{14,21} Meanwhile, the Bradford assay is sensitive towards detergents, ampholytes and polyphenolics.²²

Relationships between quantification analysis and SDS-PAGE

The phenol/SDS extraction method yielded only slightly higher protein concentration compared to the phenol method for both healthy and diseased roots. Nevertheless, statistical analysis showed there was no significant difference. From a previous study, the phenol/SDS extraction method had given maximised protein yields from olive leaves, displayed a good 2-DE resolution, and gave more spots with increased intensity, compared to the phenol method¹⁰. Furthermore, this method was faster than the phenol method, which is time-consuming. Qualitatively, the phenol/SDS extraction method gave sharper more distinct bands compared to the phenol extraction method. Thus, band 1 and band 2 in the diseased sample could be distinguished from the healthy sample using the phenol/SDS extraction method. This modified method of phenol/SDS extraction had been proven to be efficient, simple, economical and appropriate for proteomics studies with soybean roots, and likely apply to the root tissues of other plants.¹¹

The protein concentration of the diseased sample was significantly higher than the healthy sample. Since the amount of starting materials and the amount of extracted protein samples loaded onto the SDS-PAGE gel was standardised, this could be attributed to the presence of the additional protein bands observed in the diseased sample. Although this study was based only on one-dimension gel analysis, distinct bands were observed, showing that proteomics differential expression can be performed based on SDS-PAGE as a preliminary study to discover plant responses to infections. Observation of the protein profile differences for healthy and diseased roots suggest that the distinct proteins in the diseased roots were induced as a response to infection or microbial activity surrounding the plant's roots.^{22,23}

Differentially expressed proteins by MALDI-ToF/ToF MS

Based on the analysis of differentially expressed proteins by MALDI-ToF/ToF mass spectrometry, results based on Mascot score of more than 46 indicates identity or extensive homology ($p < 0.05$) for the analysed protein fragments. All Mascot scores for the Protein Band 1 are below 46, indicating close but not exact or matched identity to the identified proteins (Table 4). This is likely because *P. nigrum* proteins are not well represented in the database with only 241 hits on Uniprot (as of 12 September 2020). Nevertheless, for Protein Band 1, the peptide with the highest score of 44 matched a mannose-binding lectin (MBL) or agglutinin from *Morus nigra*. The size of this MBL was 161 amino acids, with a predicted molecular weight of 17 kDa. This matched quite closely to the estimated band size of the Protein Band 1, which was ~20 kDa. The size differences are expected when comparing homologous proteins from different species. Furthermore, it has been reported that PR proteins from different plant genera showed similar but different mobilities during SDS-PAGE separation.²⁴ From Table 4, the second-highest scoring peptide also matched an agglutinin isoform. Based on this, it is likely that the first differentially expressed protein is an MBL. Thus far, only two isoforms of MBLs have been identified in *P. nigrum*.²⁵ In contrast, at least 49 lectins are belonging to the GNA (MBL) family in *Arabidopsis thaliana*.²⁶ Therefore, there is a high possibility that the first differentially expressed protein is an MBL which has yet to be identified and sequenced.

MBLs or agglutinins belong to a family of mannose-binding proteins which are believed to recognise and bind high mannose type glycans in plant pathogens.^{26,27} These lectins play an important role in plant defence signalling by recognising the specific glycans on pathogens during a pathogen attack. In the normal state, lectins are generally expressed at low levels but get induced to a higher level following a pathogenic attack or stress.²⁸ This triggers the cell-wall defence response, such as the reinforcement of plant cell walls, produc-

tion of antimicrobial metabolites and Pathogenesis Related (PR) proteins, and the plant hypersensitive response.²⁸ There have been several reports of MBLs acting as PR proteins.²⁹⁻³¹ MBLs were overexpressed during infection of *Capsicum annuum* with a pathogen.²⁸ Suppression of this gene led to increased susceptibility towards infection while overexpression of this MBL in *Arabidopsis* has been shown to increase plant resistance towards pathogens.

The study of plants using proteomic analysis has facilitated the discovery of PR proteins that act as a defence mechanism.⁵ Diseased plants usually synthesise a specific group of PR proteins which function as systemic acquired resistance.³² An induced infection study of *P. nigrum* leaves infected by *Phytophthora* showed that infected leaves expressed two additional proteins estimated at 16.5 kDa and 8 kDa.⁵ In this artificial induction study, the relatively tolerant *P. nigrum* 'Kalluvally' variety showed delayed symptom development, where PR proteins were expressed after inoculation of *Phytophthora*. A study by Anh et al.³³ reported similar findings with two overexpressed PR proteins of approximately 30 kDa and <10 kDa, as well as overexpression of glucanase.

Analysis of Protein Band 2 resulted in low hit Mascot scores in the protein database (Table 4). The peptide with the highest score-matched and AAA-type ATPase family protein, as well as a hypothetical protein OsI_11535. The molecular size of the 12.5 kDa protein in this study most closely matched the hypothetical protein OsI_11535 which was estimated to be 16.5 kDa. The difference in molecular weight between the two matches can be explained by a high possibility of the hypothetical protein being a fragment of the AAA-type ATPase family protein sequence. The hypothetical protein sequence matched the number 1 to 165 sequence of the AAA-type ATPase family protein sequence. Hence, it is predicted that the 12.5 kDa differentially expressed protein was likely to be an AAA-type ATPase family PR protein.

An AAA-type ATPase protein has been reported as an additional novel mutant lesion mimic resembling (LMR) prediction gene in rice. LMR encodes this gene and enhanced resistance to rice blast and bacterial blight that is associated with the activation of two disease-related genes, PBZ1 and PR1.³⁴ In another study on putative pathogenesis-related gene expression, the protein was found to be likely involved in the necrotic stage of *Rhizoctania solani* pathogenesis along with pyruvate carboxylase (PC), glutathione-S-transferase (GLU) and major facilitator superfamily protein (MFS).³⁵ With diverse functions, it is involved in detoxification of host efflux in multiple plant pathogenic fungi especially in defending the pathogen against reactive oxygen species.^{36,37}

Based on this preliminary study on differentially expressed proteins in normal and diseased pepper plants, the proteins expressed by the diseased roots in this study are likely to be PR proteins acting in response to pathogenesis. This suggests

that PR proteins were also expressed by diseased *P. nigrum* when naturally infected. However, these proteins could differ from the ones expressed by leaves under artificial induction, because under natural conditions the roots interact with the soil microbiome and are also related to the disease affecting the plant.

CONCLUSION

In conclusion, the extraction of proteins from healthy and diseased roots of *P. nigrum* using the phenol/SDS extraction method yielded high-quality extraction where distinct protein bands could be observed between samples. The differentially expressed proteins bands, overexpressed in the diseased roots were identified through MALDI-ToF. However, due to the lack of database entries for *P. nigrum*, their identity could not be confirmed. Nevertheless, these proteins are likely to be PR proteins which could be used as invaluable markers for detection and management of plant pathogens. Furthermore, the induction of PR proteins before the infection could help plants gain resistance against disease. This, in turn, would help farmers to protect and improve the quality of the *P. nigrum* plants on their farm.

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Conflict of Interest

Nil

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Author's Contribution

HH, AAH and SL designed the study. NEH and NFMC performed the experiments, and together with HH written the draft manuscript with input from all authors. AAH and SL helped to interpret the protein analysis data. All authors discussed the results and commented on the manuscript.

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Table 1: Concentration of proteins extracted from the roots of *P. nigrum* using Bradford and BCA assays

Type of extraction	Condition of the roots	Average protein concentration using Bradford assay (mg/ml)	Average protein concentration using BCA assay (mg/ml)
Phenol	Healthy roots <i>P. nigrum</i>	0.849	16.916
	Disease roots <i>P. nigrum</i>	1.280	16.876
Phenol/SDS	Healthy roots <i>P. nigrum</i>	1.311	20.298
	Disease roots <i>P. nigrum</i>	1.325	20.087

Table 2: Analysis of variance of protein concentration using Bradford assay

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.471	3	0.157	2.175	0.168	4.066
Within Groups	0.577	8	0.072			
Total	1.049	11				

Table 3: Analysis of variance of protein concentration using BCA assay

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	32.669	3	10.889	0.742	0.556	4.066
Within Groups	117.292	8	14.661			
Total	149.961	11				

Table 4: Identification of predicted proteins

No.	Fragment Sequence	Score	Accession no.	Protein ID	Molecular Size
Protein Band 1					
1.	KTYGPGYK	44	AAL10685.1	mannose-binding lectin [<i>Morus nigra</i>]	17 kDa
			XP_024018763.1	agglutinin alpha chain [<i>Morus notabilis</i>]	17 kDa
2.	SGAYLDAIGVHMEYF	30	ABC70328.1	agglutinin isoform [<i>Castanea crenata</i>]	31 kDa
Protein Band 2					
1.	AVAVEVDSGK	20	ABF95891.1	AAA-type ATPase family protein, puta- tive, expressed [<i>Oryza sativa</i> Japonica Group]	110 kDa
			EAY89974.1	hypothetical protein OsI_11535 [<i>Oryza sativa</i> Indica Group]	16.5 kDa

Table 5: t-test between healthy and diseased samples assuming two equal variances from Bradford assays

	Variable 1	Variable 2
Mean	0.849	1.280
Variance	0.0085	0.0799
Observations	3	3
Pooled Variance	0.044	
Hypothesized Mean Difference	0	
df	4	
t Stat	-2.505	
P(T<=t) one-tail	0.033	
t Critical one-tail	2.131	
P(T<=t) two-tail	0.066	
t Critical two-tail	2.776	

Table 6: t-test for Bradford assays and BCA assays using two samples assuming two equal variances

	Variable 1	Variable 2
Mean	1.325	16.876
Variance	0.04	2.712
Observations	3	3
Pooled Variance	1.376	
Hypothesized Mean Difference	0	
df	4	
t Stat	-16.236	
P(T<=t) one-tail	0.0000421	
t Critical one-tail	2.131	
P(T<=t) two-tail	0.0000842	
t Critical two-tail	2.776

Figure 1: Band separation of proteins extracts, (1 mg/mL) by SDS-PAGE using Coomassie Brilliant Blue R (CBB-R) staining. (a) Phenol extraction method- Healthy roots of *P. nigrum* (b) Phenol extraction method- Diseased roots of *P. nigrum* (c) Phenol/SDS extraction method- Healthy roots of *P. nigrum* (d) Phenol/SDS extraction method- Diseased roots of *P. nigrum*. The molecular weight of the protein standard as indicated on the left.