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Gel-free Proteomic Analysis of Rice Planted in Acid Sulfate Soil with different Soil Amendments

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ABSTRACT

Rice is a staple crop across much of the world, particularly in Asia. However, the presence of acid sulphate soils in Malaysia is a concern, as their high acidity and elevated aluminium content make them unsuitable for rice cultivation. Application of ground magnesium limestone (GML), basalt, biochar and compost as soil amendments to acid sulphate soil to neutralize the acidity and boost rice production has been implemented. However, the molecular implications of these soil amendments on rice yield are mainly unknown. Hence, a gel-free proteomic investigation was conducted to comprehensively understand the degree of protein expression in rice plants after the application. It was found that TCA-acetone-phenol was the most effective protein extraction technique utilizing several solvents. Rice plants cultivated in acid sulphate soil with NPK fertilizer and GML had a maximum number of differentially expressed proteins in rice plants with amount of 1.286 mg/FWg. We found that protein expressions were downregulated in all soil amendments. Out of 769 proteins identified, 485 proteins were involved in the biological processes such as cellular process (405), metabolic process (401), response to stimulus (83), biological regulation (50), and localization (30). Further studies are needed to integrate omics approaches, especially transcriptomic and metabolomics, to better understand the molecular mechanisms of rice influenced by different soil amendments.

Keywords: Protein profiling, rice proteome, soil amendment, acid sulfate stress, biochar

INTRODUCTION

Rice is recognized as one of the most important staple crops in the world and it provides the main source of energy for more than half of the world's population. Like many other countries in Asia, rice is the staple food of Malaysia. The demands of rice are increasing year by year as the population increases. The world's population is predicted to grow up to 8.6 billion by 2030, 9.8 billion in 2050 and 11.2 billion in 2100 (UN, 2025). Malaysia is still a net importer of rice which imported 2.2% of the world's total rice. Besides yield enhancement, increasing participation in breeding research, producing sustainable and safe rice production, increasing the self-sufficiency level (SSL) (Omar *et al.*, 2019) and expansion of area for rice cultivation are among the strategies to sustain food security.

Currently, acid sulfate soil is being used for rice cultivation in Peninsular Malaysia (Shamshuddin *et al.,* 2016). But the rice yields each season is less than 2 t/ha which is below the national average of 3.8 t/ha and most often, soil amendments or liming materials need to be applied to improve and create a favorable soils condition for rice cultivation. (Halim *et al*., 2018). To understand how acidic soils affect crop yield and to develop better solutions, modern molecular tools such as proteomics are required. Proteomics involves identifying and measuring proteins produced by cells, tissues, or organisms under specific conditions (Tan *et*

al., 2013). Because cells rely on many metabolic and regulatory pathways to survive, studying proteomes helps reveal how these biological processes work and interact (Esteve *et al.*, 2013). The proteomic approach can also be applied to investigate plant stress responses, including manganese tolerance, salt stress, flooding stress, rice leaf response to blast fungus infection, and plant–virus interactions (Gao *et al*., 2019). Recent studies using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in rice have identified proteins that support plant growth and reduce aluminum (Al) toxicity in acidic soils (Panhwar *et al*., 2017). Proteomic research in rice plants is still emerging, particularly in Malaysia. Therefore, this study aimed to identify the optimal protein extraction method for rice and to evaluate the differential protein expression of rice grown under various soil amendments using a gel-free proteomic approach to better understand its adaptation mechanisms to acidic stress conditions.

MATERIALS AND METHODS

Plant materials

Rice leaves at filling stage, was collected from a paddy field in Sungai Manik, Perak. The leaves were washed under running tap water, and the leaves surface was sterilized with 70% ethanol, blotted dry and cut into smaller pieces. The samples were snap frozen in liquid nitrogen and kept in -80°C for protein extraction. A total of 0.5 g of leaf tissues were ground with mortar and pestle in liquid nitrogen for protein extraction.

Optimization of protein extraction from rice plant

TCA-Acetone precipitation

The TCA-acetone method started with precipitating 0.5 g of tissue samples in 1.5 mL of cold acetone/20% TCA/0.2% DTT at -20 °C for overnight, followed with centrifugation at 10000 ×g for 30 min at 4°C. The supernatant was discarded, and the pallet was rinsed by using cold acetone/ 0.2% DTT twice. Between the rinsing step, the mixture was incubated for 1 hour at -20°C and on the last step, the mixture was centrifuged at 10000 ×g for 10 min. The resulting pallet was air- dried for few second before mixed with lysis buffer and stored. The protein concentration was measured using the Bio-Rad Protein Assay at 480 nm.

Sucrose extraction

Total soluble proteins were extracted following a sucrose-based protocol. Fresh plant tissue (0.5 g) was homogenized and precipitated in 1.5 mL of sucrose extraction buffer consisting of 5% sucrose, 5% β-mercaptoethanol, 4% CHAPS, and 0.05 g PVPP. The mixture was incubated at room temperature for 10 min, followed by centrifugation at 10,000 ×g for 5 min at 4 °C. The resulting supernatant was heated at 95 °C for 3 min and allowed to cool to room temperature. Protein precipitation was induced by adding five volumes of ice-cold acetone containing 0.07% DTT and incubating at −20 °C for 1 h. The mixture was centrifuged at 10,000 ×g for 10 min, and the resulting pellet was resuspended in extraction buffer (without PVPP) and centrifuged again at 10,000 ×g for 5 min. After centrifugation at 10,000 ×g for 5 min, the pellet was washed with four volumes of ice-cold 80% acetone/0.07% DTT, vortexed, incubated for 1 h at −20 °C, and centrifuged. The pellet was briefly air-dried,dissolved in lysis buffer, and stored at −80 °C until further analysis. Protein concentration was quantified using the Bio-Rad Protein Assay and measured at 480 nm using a microplate reader.

TCA- Acetone-phenol

Total protein was also extracted using the TCA–acetone–phenol method. Tissue samples (0.5 g) were homogenized with 2 mL of 10% TCA in acetone, vortexed briefly, and incubated at −20 °C for at least 16 h. The mixture was centrifuged at 10,000 ×g for 15 min, and the supernatant discarded. The pellet was washed three times with ice-cold acetone containing 0.07% DTT. For the first two washes, samples were incubated at −20 °C for 1 h and centrifuged at 10,000 ×g for 15 min. The final wash consisted of vortex and centrifugation without incubation. The pellet was air-dried and resuspended in 0.5 mL lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 10 mM Tris, 75 mM DTT), followed by incubation at 20–30 °C for 15 min in a water bath. After centrifugation at 15,000 ×g for 3 min at 24 °C, the supernatant was mixed with an equal volume of dense SDS buffer (0.1 M Tris-HCl pH 8, 30% sucrose, 2% SDS, 5% β-mercaptoethanol) and phenol (pH 8.0). The mixture was centrifuged at 15,000 ×g for 3 min to collect the phenolic phase. This phenolic phase was re-extracted with dense SDS buffer, centrifuged, and precipitated by adding five volumes of cold 0.1 M ammonium acetate in methanol and incubating at −20 °C for 1 h. Samples were centrifuged at 15,000 ×g for 5 min, and the pellet was washed once with cold methanol and twice with ice-cold acetone/0.07% DTT. After a brief air drying, the pellet was dissolved in lysis buffer and stored at −80 °C.
The protein concentration was measured using the Bio-Rad Protein Assay at 480 nm.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Protein extracts were separated using two-dimensional polyacrylamide gel electrophoresis (2D PAGE). Proteins were passively rehydrated in 24-cm, pH 3-10 ReadyStrip IPG strip. A total of 100 µg protein extract was mixed with rehydration buffer and brought to a final volume of 450 µL. The protein solution was loaded into strip holders with gel facing down and passively rehydrated for 16 h. Isoelectric focusing (IEF) was carried out using an Ettan IPGphor system. Following IEF, IPG strips were equilibrated for 15 min in reducing buffer (6 M urea, 75 mMTris-HCl pH 8.8, 2% SDS, 29.3% glycerol, 0.002% bromophenol blue, 1% DTT) followed by 15 min in alkylation buffer (6 M urea, 2% SDS, 75 mMTris-HCl pH 8.8, 29.3% glycerol 0.002% bromophenol blue, 2.5% iodoacetamide (IAA)). Proteins were then resolved on 1-mm 12.5% polyacrylamide gel. Gels (450mL) were prepared in 1.5 M Tris-HCl, pH 8.8, polymerized with 2.5 mL 10% APS and 0.125 mL TEMED. Strips were rinsed in 1x SDS-PAGE running buffer, positioned onto the second-dimension gel, and sealed with agarose solution (25 mMTris base, 192 mM glycine, 0.1% SDS, 0.5% agarose, 0.002% bromophenol blue). Electrophoresis was run at P1 (80V, 10mA/gel, 1W/gel) for 1 h and P2 (500V, 40mA/gel, 13W/gel) for 4-5 h.

Gel washing, image, and data analysis

Gel was stained with silver nitrate according to Blum (Blum, 1987). Briefly, proteins were fixed (40% ethanol, 10% acetic acid) for 1 h, rinsed with distilled water, and sensitized (30% ethanol, 25% glutaraldehyde, 0.2% sodium thiosulfate, 0.68% sodium acetate) for 20 minutes, rinsed with distilled water for 3 time and soaked for 5 minutes on rotary shaker. Then, gels were incubated with silver nitrate solution (0.02% silver nitrate, 20% formaldehyde) on rotary shaker for 20 min. After that, the gel was rinsed with distilled water and soaked in developing solution (3% sodium carbonate,0.05% sodium thiosulfate, 0.05 % formaldehyde) for 10 min and stopped with 0.5% EDTA were added. Gels were scanned using ImageScanner III (GE Healthcare) and protein spot analysis was performed using Progenesis QI proteomic software for data discovery.

Protein extraction and digestion

Sample preparation

Rice plants were collected at 45 days after transplanting from glasshouse experiment comprising six treatments: control (no amendment), NPK fertilizer, NPK fertilizer + ground magnesium limestone (GML), NPK + empty-fruit-bunch (EFB) biochar, NPK + compost (EFB). Each amendment was applied at 4 t ha^{-1} into the soil (Halim *et al*., 2018). Four biological replicates per treatment were sampled, surface-sterilized with 70% ethanol, cut into smaller pieces, snap-frozen in liquid nitrogen, and stored at -80°C. Approximately 0.5 g ground tissue was used per extraction. The total proteins were extracted from rice leaves using the method of Sah *et al*. (2022) and Wang *et al*. (2006), with slight modification.

Proteins were extracted using the TCA-acetone-phenol which selected from the optimization method. Precipitated proteins were resuspended in 0.1 M ammonium bicarbonate containing 1 M urea, reduced with 50 mM tris (2-carboxyethyl) phosphine and alkylated with 150 mM iodoacetamide. Proteins (100 µg) were digested with 2 µg trypsin (Promega, Madison, WI, USA) at 37 °C for 16 h. Sodium deoxycholate was removed by acidification (0.5% formic acid) and centrifugation at 14 000 g (RA-300, Kubota 7820). Peptides were dried and resuspended in 100 µL of 0.1% formic acid (FA) for LC-MS/MS analysis. Clean up was performed using C18 solid-phase extraction disk which sequentially eluted with 50% acetonitrile (ACN) in 0.1% formic acid for 2.5 h. (Lau B.Y.C., and Othman A, 2019)

Liquid Chromatography-tandem Mass Spectrometry

Peptides were loaded onto an Acclaim PepMap 100 C18 column (2 µm, 0.075 ×150 mm) and separated on an EASY-nano liquid chromatography (EASY-nLC) 1200 System (Thermo Scientific, MA, USA) using 5-35% ACN gradient (0.1% FA) for 75 min at 300 nL/min. Eluted peptides were analysed on a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer system (Thermo Scientific, MA, USA) ware used to generate the peptide ions with a nanospray ionization. MS survey scan was acquired at 70,000 resolution (m/z 310-1,800). Precursors with charge states 2+ to 8+ were subjected to higher- energy collision dissociation with normalized collision energy of 28%. MS/MS spectra were acquired at 17,500 (Lau *et al*., 2020).

Data processing and protein profiling

Mass spectra were acquired using Tune (v2.11, Thermo Scientific) and processed in Proteome Discoverer (v2.3, Thermo Scientific). Peptide identification was performed using the SEQUEST HT search engine against the *Oryza sativa* ssp. *japonica* UniProt database (122,144 sequences). Search parameters included a precursor mass tolerance of 20 ppm and fragment mass tolerance of 0.5 Da. Trypsin was specified as the proteolytic enzyme, allowing up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification, while methionine oxidation and asparagine/glutamine deamidation were set as variable modifications. A target-decoy strategy was applied to estimate false discovery rate (FDR), and proteins were accepted with at least one unique peptide and ≤1% FDR. Identifications were further validated using the Percolator algorithm.

Multivariate Analysis: PCA and Volcano Plot

The groups of proteins were clustered using Principal Component Analysis and heat map generation was done with the statistical functionality incorporated in Proteome Discoverer. The significant of differential expressed protein generated between treatment and control was defined in volcano plot.

RESULTS AND DISCUSSION

Optimization protein extraction method for rice plant using 2D-PAGE

Protein preparation from rice plants is challenging due to the fibrous nature of the leaves. Grinding the tissue with liquid nitrogen helps produce a fine powder. However, protein extraction remains difficult because plant cells contain compounds such as salts, organic acids, phenolics, and pigments that interfere with 2D separation, and the cell wall composition limits protein yield (Islam *et al*., 2004). Three protein extraction methods were evaluated to determine the most effective approach for protein yield and solubilization rice leaf samples for 2-DE analysis. The quantitative comparison showed that the TCA-acetone -phenol method produced the highest protein yield in rice leaf samples (**Figure 1**).

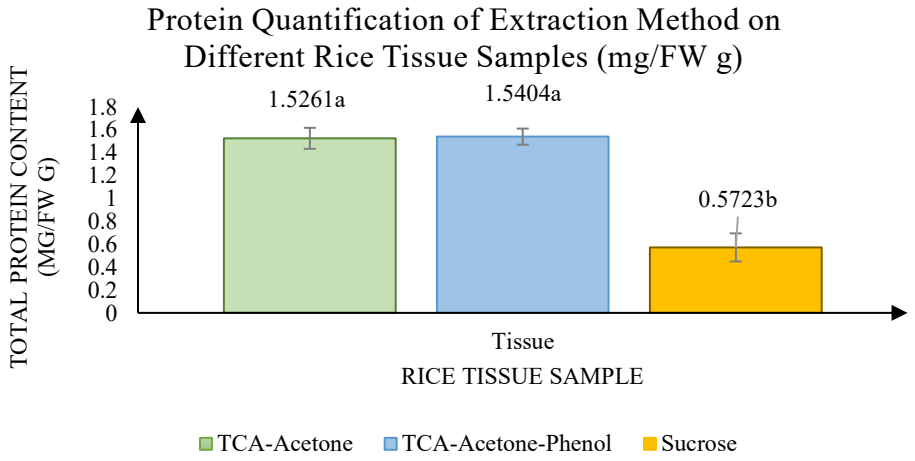

Figure 1. Total protein content in rice tissue samples with different extraction methods. Mean values with different letters are significantly different at $p < 0.05$

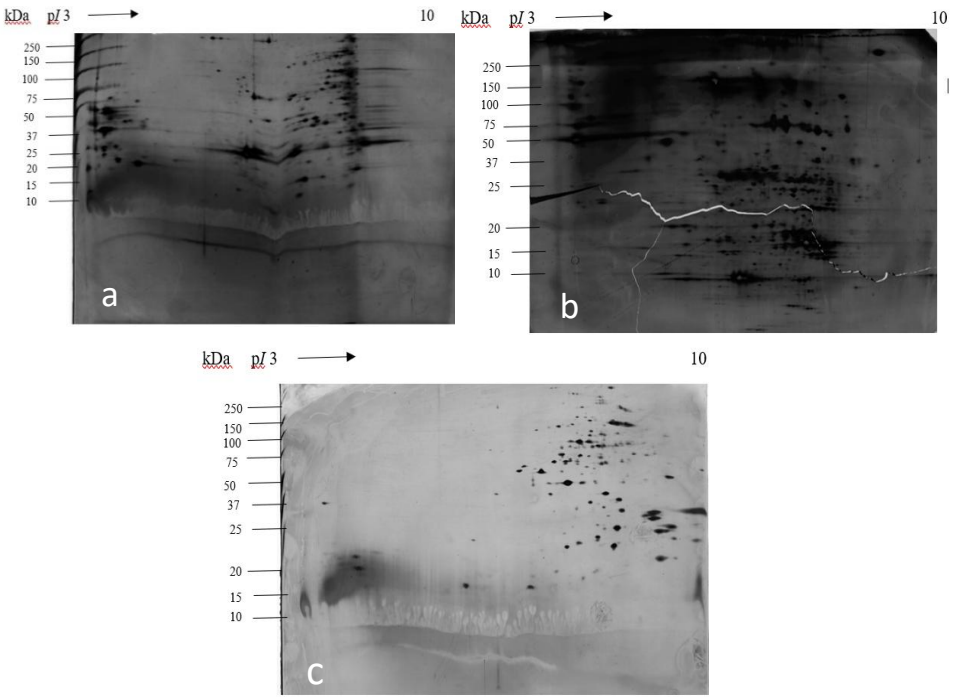

Figure 2. Two-dimensional gel electrophoresis (2D-PAGE) images of rice leaf proteins extracted using three different methods. Proteins were separated on 24 cm IPG strips (pH 3–10): (a) TCA-acetone, (b) TCA-acetone-phenol, and (c) sucrose method.

Table 1. Total number of protein spot detected using Progenesis QI software.

Sample	Treatment	Total number of protein spots
Leaf	TCA Acetone	94 ± 20
	TCA Acetone Phenol	170 ± 38
	Sucrose	74 ± 18

Based on the 2D-PAGE results shown in **Figure 2**, protein extraction using the sucrose and TCA-acetone-phenol methods produced cleaner and more focused protein spots compared to the TCA-acetone method. The TCA-acetone-phenol method detected about 170 spots, nearly twice the number observed with the other two methods (**Table 1**). Based on this study, the TCA-acetone-phenol extraction technique proved to be the most effective for protein yield, 2D gel separation, and protein spot analysis in rice leaf tissue. This efficiency is likely due to the high clean-up capacity of phenol combined with TCA-acetone washing, which enhances protein recovery from fibrous tissue (Faurobert *et al*., 2007). Therefore, this method is suitable for extracting rice leaf proteins for downstream proteomic studies (Daim *et al*., 2005).

Protein extraction and quantification from rice tissue sample

Rice leaf proteins were extracted using the TCA-acetone-phenol method, which had been previously optimized for high-quality protein recovery. This method has demonstrated broad applicability across various crop materials. Although originally developed for gel-based proteomic analysis, the extracted proteins are also suitable for gel-free approaches. The protocol has been effectively applied to a range of plant tissues, including leaves (Wang *et al*., 2006; Hu *et al*., 2010; Daim *et al*., 2015), woody stems (Xiong *et al*., 2013), fruits (Wang *et al*., 2006), seeds (Wang *et al*., 2012; Wu *et al*., 2011), and pollen (Wang *et al*., 2004; Zhu *et al*., 2011).

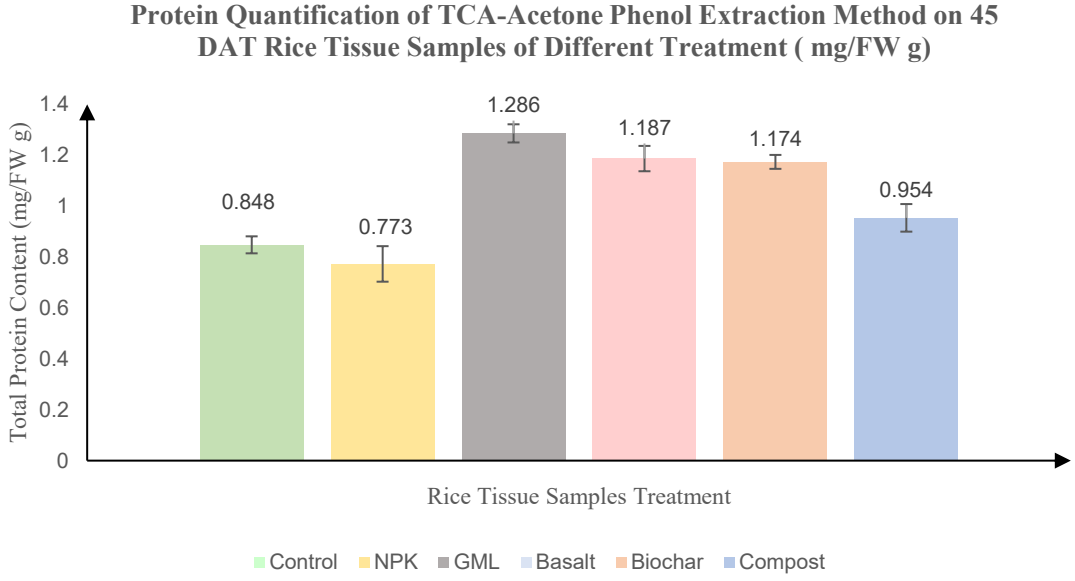

Figure 3. Total protein content in different rice tissue samples with different treatment. Mean values with different letters are significantly different at $p < 0.05$.

It was found that extracted protein from each treatment gives a different protein yield where untreated rice plant samples were found with low amount compared to treated samples.

Among the treatments, GML produced the highest protein yields (1.286 mg/FWg), followed by basalt (1.187 mg/FW g), biochar (1.174 mg/FW g), and compost (0.594 mg/FW g**)** in **Figure 3**. These differences may be attributed to the effects of soil amendments, which alter the plant's growth environment and influence protein expression, antioxidant mechanism and profiles (Viger *et al.*, 2015; Islam *et al*., 2004).

Protein identification and profiling

Each protein sample was digested by trypsin and the peptide identification was performed using liquid chromatography – mass spectrometry (LCMS). Each treatment was individually analysed (Figure 4).

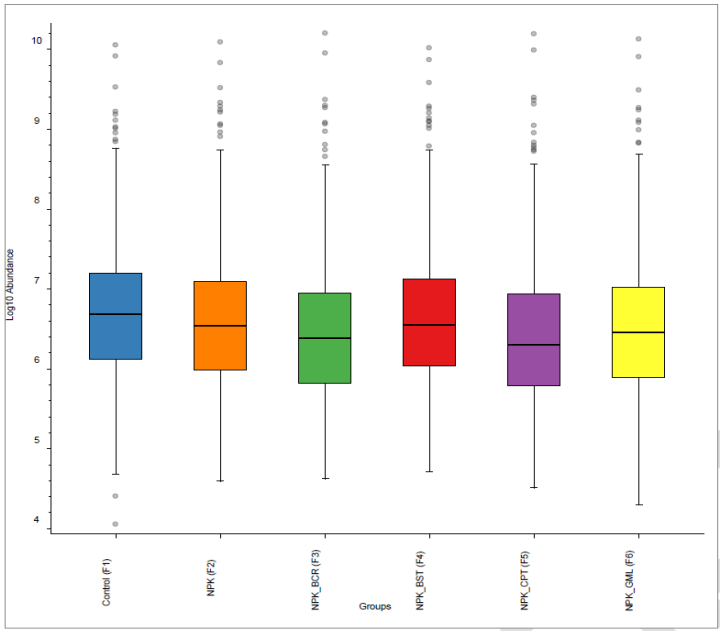

Figure 4. Variability of replicates of protein samples content in different samples with different treatments.

In this study, a total of 769 proteins was identified, which the data can be categorized into few categories such as gene ontology, enzyme class, keyword, and pathway from UniProtKB data resources. Gene ontology (GO) allow researcher to describe a gene product in detail (Ashburner *et al*., 2000) which from this study, 591 protein was identified by molecular function, 462 proteins for cellular component ad 485 protein involved in biological process.
The second categories of protein identified is based on their enzyme class. An enzyme is a protein or RNA produced by living cells, which is highly specific and highly catalytic to its substrates. Enzymes are a very important type of macromolecular biological catalysts. Due to the action of enzymes, chemical reactions in organisms can also be carried out efficiently and specifically under mild conditions (creative-enzymes.com, 2021). All seven enzymes class was identified from the protein Id detected. 85 proteins identified as Oxidoreductases, 40 protein id of Transferases, 27 proteins involved in Hydrolases, 32 proteins of Lyases, 35 proteins on Isomerases and 7 proteins on Ligases and catalysing the translocation of hydron. Other categories of proteins involved was found based on their key word and pathway (**Table 2**).

Table 2. Categories of 769 identified protein by using UniProtKB data resources

Categories	No of results	List of categories (number of protein)
Gene ontology	3	Molecular function (591) Cellular component (462) Biological process (485)
Enzyme class	7	Oxidoreductases (85) Transferases (40) Hydrolases (27) Lyases (32) Isomerases (35) Ligases (7) Catalyzing the hydron translocation (7)
Key word	8	Technical term (395) PTM (104) Molecular function (421) Ligand (229) Domain (288) Coding sequence diversity (11) Cellular component (282) Biological process (234)
Pathway	23	Amine and polyamine biosynthesis; putrescine biosynthesis via agmatine pathway; putrescine from N carbamoylputrescine (amidase route) Amino-acid biosynthesis (10) Amino-acid degradation (2) Aromatic compound metabolism; phenylpropanoid biosynthesis (1) Carbohydrate biosynthesis (4) Carbohydrate degradation (16) Carbohydrate metabolism; tricarboxylic acid cycle (3) Cofactor biosynthesis (6) Energy metabolism (4) Glycan biosynthesis; starchbiosynthesis(3) Isoprenoid biosynthesis (4) Lipid metabolism (1) Metabolic intermediate biosynthesis; chorismate biosynthesis; chorismate from D-erythrose 4-phosphate and phosphoenolpyruvate (2) Nitrogen metabolism (1) Nucleotide-sugar biosynthesis; UDP-alpha-D-glucuronate biosynthesis; UDP-One-carbonmetabolism; tetrahydrofolate interconversion (3)

From the 485 identified proteins associated with biological process, five main categories were involved: cellular process (405 proteins) metabolic process (401), response to stimulus (83), biological regulation (50), and localization (30) (**Figure 5**). Proteins involved in biological processes represent specific functions that the organism is genetically programmed to perform, defined by their outcomes and ending states (EMBL-ELB, 2021).

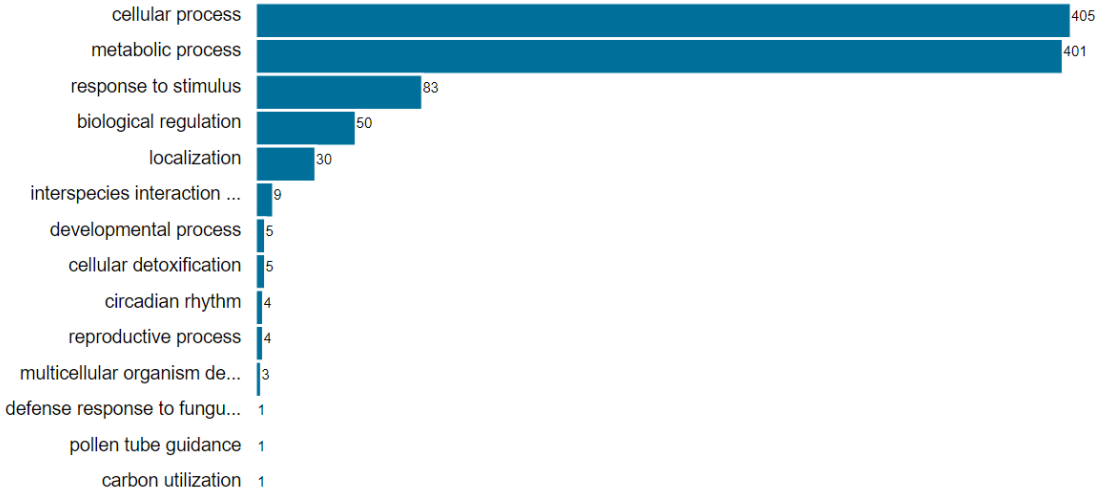

Figure 5. List of process and number of proteins involved in biological process of rice plant protein samples in this research

Differentially expressed protein in rice planted with different soil amendment

To further evaluate the gene list, Principal Component Analysis (PCA), heat map clustering, and volcano plot analyses were performed using the same dataset based on uniquely expressed genes across treatments. Principle Component Analysis (PCA) based on the differentially expressed unique genes of the treatment samples is shown in Figure 6. The analysis revealed four distinct clusters: compost and biochar treatments grouped together; basalt and ground magnesium limestone formed another cluster, while control and NPK fertilizer each formed separate clusters. These results indicate that organic treatments produced distinct effects on the observed parameters.

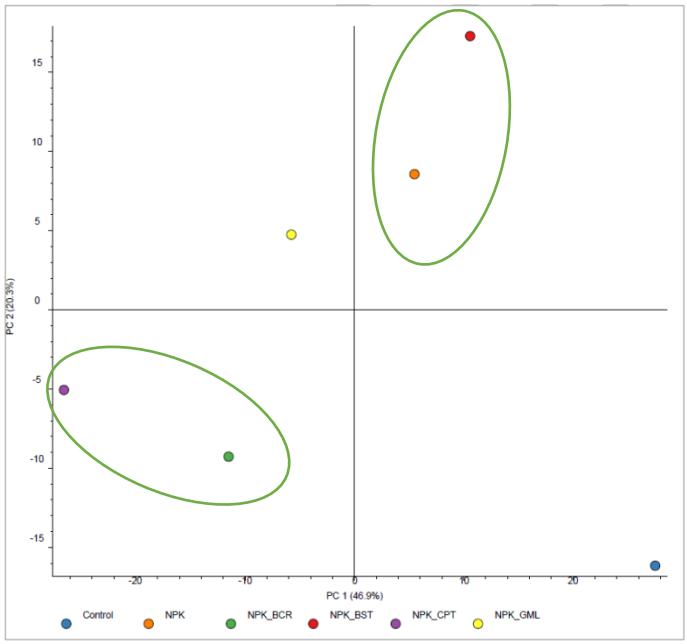

Figure 6. PCA clustering of samples of protein samples content in different samples with different treatment

A biological heat map was generated to illustrate the clustering of rice protein expression under different soil amendment treatments: control, NPK fertilizer, NPK + ground magnesium limestone (GML), NPK + biochar (BCR), NPK + basalt (BST) and NPK + compost (CPT) was generated (**Figure 7**). The heat map displays the clustered distribution of rice proteins based on their expression levels, providing an overview of the variation among treatments. Rows and columns in the heat map were organized using hierarchical clustering (Vacanti, 2019).

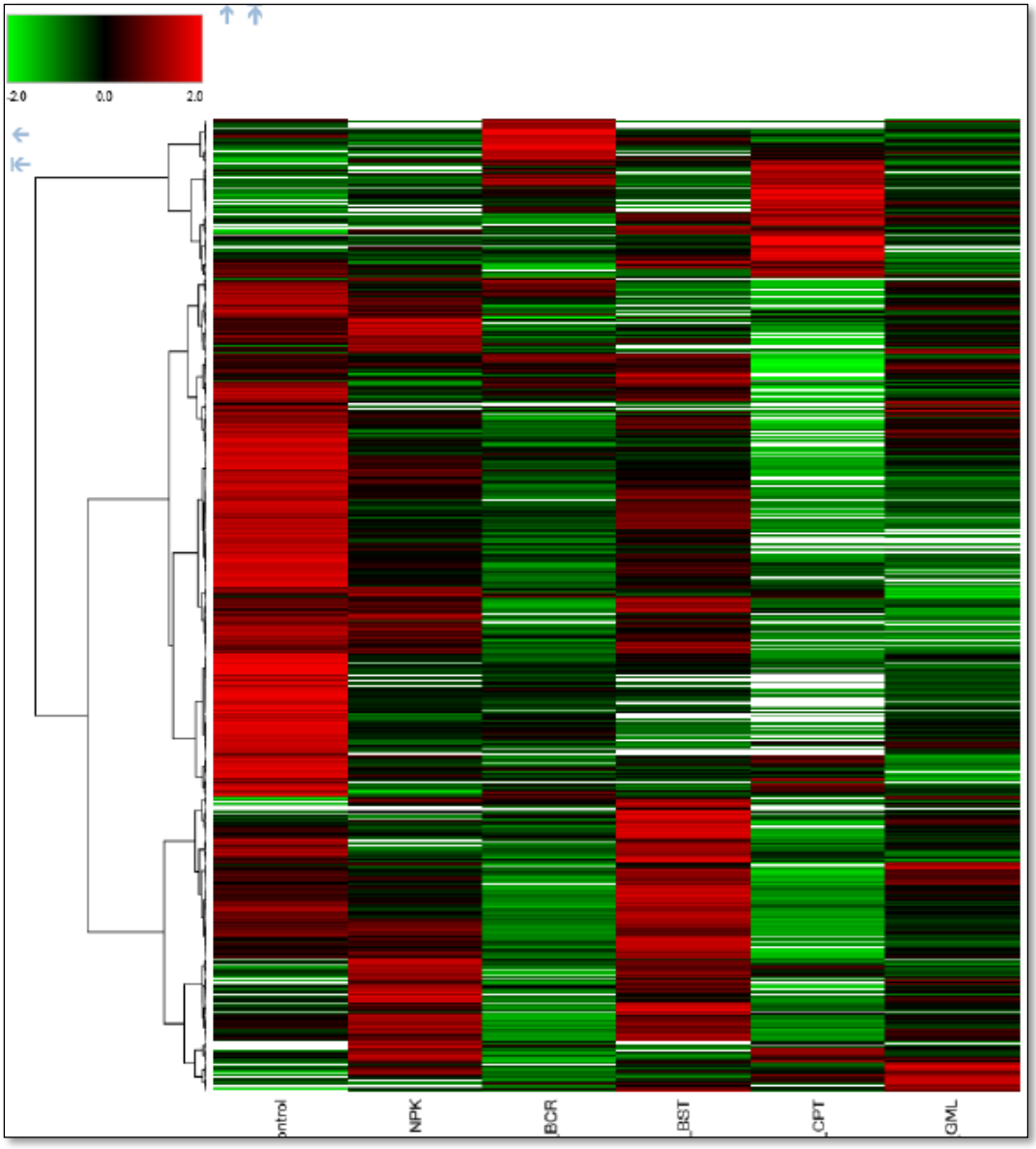

Figure 7. Heat map of differentially expressed proteins in rice under different soil amendments. The data sources; Abundances (Normalized), distance function; Pearson, linkage method; Ward's and scaling; Scale after clustering.

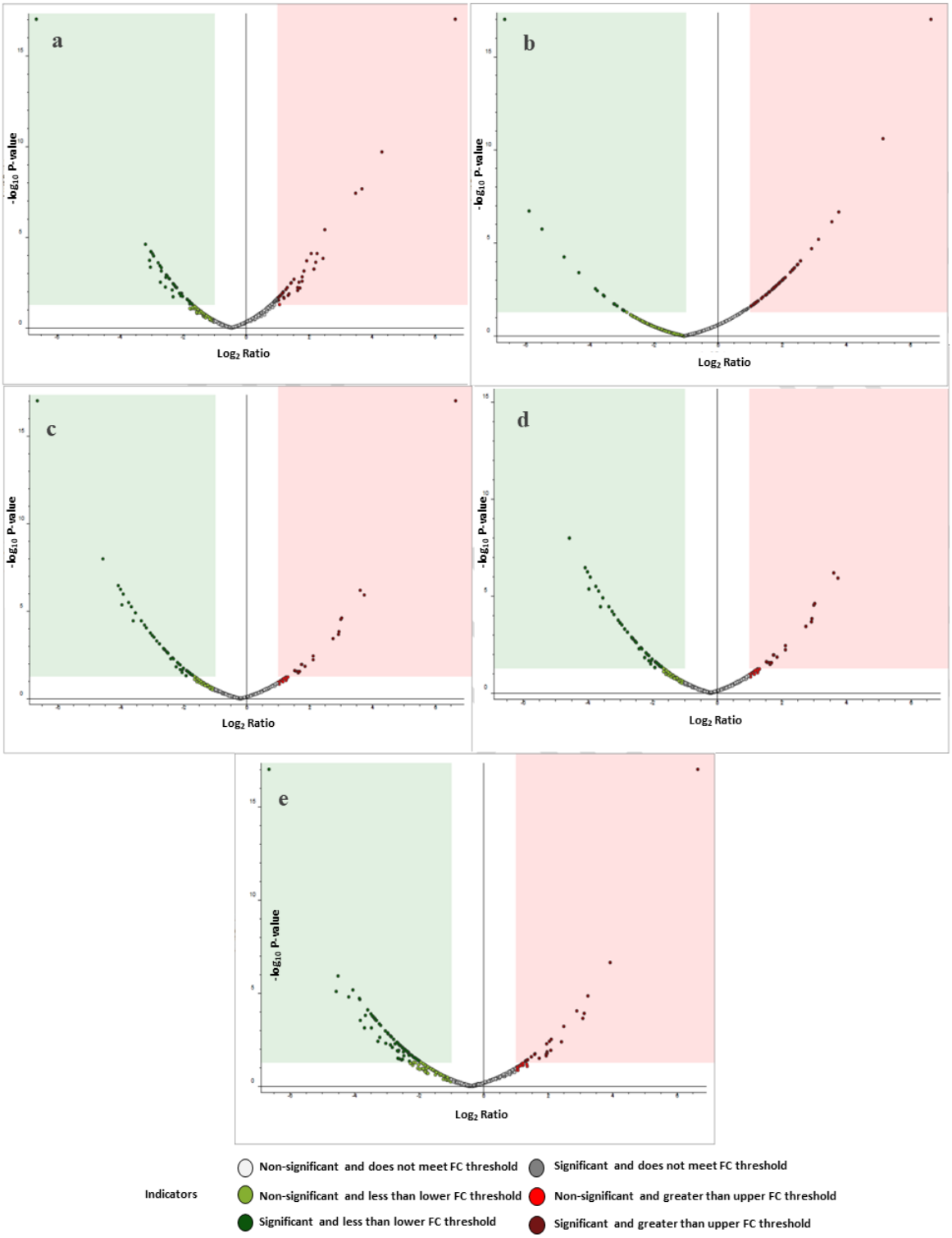

Figure 8. Differentially expressed proteins between (a) NPK Fertilizer treatment and Control treatment (b) Biochar and Control (c) Basalt treatment and Control (d) Compost treatment and Control (e) Ground Magnesium Limestone treatment and Control

The heat map (**Figure 7**) depicted the datasets as clustered patterns which show an overview of the distribution of rice proteins represented according to their expression were further classified according to their molecular functions based on the UniProt information available. The heat map shows a different cluster of protein in each treatment. The colours in the heat map represent the relative abundance of proteins in each dosage level in comparison to $\log_2$ transformed spectral count data in controls (red = up-regulation, green = down-regulation). High abundance proteins with little fold change cause a cluster of proteins with very faint hues. We found that the numbers of up-regulated proteins decreased, and down-regulated proteins increased as amendment by GML, basalt and biochar. From the volcano plot obtained, it can be identified that treatment of ranking from GML, basalt, compost and biochar give a significant and greater than lower FC threshold (**Figure 8**). These results demonstrated that some proteins expression levels first decreased in the control studies and then increased in rice leaves in response to organic amendment, such as spots seen in **Figure 2**.

CONCLUSION

Identification of protein in plants is crucial and begins with proper sample preparation, which is often the most challenging in proteomic studies. Selecting and optimizing an appropriate extraction method is essential for obtaining reliable results. From this study, TCA-acetone-phenol method showed the best performance for rice plant protein extraction and was suitable for both gel-based and gel free protein analysis. A total of 769 protein profiles were identified from rice plant, with 485 proteins involved in biological processes. The five major biological processes identified were cellular process (405) metabolic process (401), response to stimulus (83), biological regulation (50), and localization (30). Among the treatment, rice plants grown on acid sulfate soil with NPK fertilizer and GML exhibited the highest number of significant values of protein in the differential expression of proteins. These findings were supported by protein quantification, PCA, and volcano plot analyses. Overall, protein profiling provides valuable insights into the responsive proteins that play key roles in improving plant growth under acid sulfate soil conditions.

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