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ORIGINAL PAPER

Metabolites Profiling of Humid Tropic Simulated *Bungor* Soil Under Biofertilizer Application

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Abstract

Purpose Biofertilizer application in the agriculture industries is deemed sustainable in the long run given its ability to restore fertility of soil and increase crops productivity through several direct and indirect mechanisms. However, the dissolved fraction (DOM), which is made up of tiny molecules of plant and microbial origin produced by lysed cells and released metabolites as influenced directly through biofertilizer amendment is unknown.

Methods An untargeted metabolomics profiling was conducted via an in vitro rhizospheric *Bungor* soil series incubation with IBG Biofertilizer from IBG Manufacturing Sdn Bhd. In this study, a comparative analysis between Ultisols samples inoculated with IBG biofertilizer and control samples was conducted under simulated humid tropic conditions.

Results 18 mass-to-charge ratio (m/z) values with VIP (Variable Importance in Projection) scores exceeding 1 in the IBG biofertilizer-inoculated Ultisol. The annotated metabolites primarily consisted of endogenous compounds, including amino acids, organic acids, nucleic acids, fatty acids, and amines. Notably, a signaling compound, homoserine lactone (m/z 270), exhibited the highest fold changes in response to IBG biofertilizer inoculation on the simulated Ultisol. Furthermore, key metabolic pathways such as Glycerophospholipid metabolism, Glycine, serine, and threonine metabolism, Cysteine and methionine metabolism, and Alanine, aspartate, and glutamate metabolism were notably affected by IBG biofertilizer inoculation on the simulated soil model.

Conclusions These findings emphasized the metabolic responses induced by IBG biofertilizer in Ultisols under the simulated humid tropic conditions., which suggests that biofertilizers application have some significant changes on soil metabolites that overall soil productivity could be affected by these potential biomarkers. Understanding these metabolic shifts not only enhances crop productivity but also addresses broader questions of soil health and ecosystem sustainability in the face of climate change and agricultural intensification.

Keywords Untargeted Metabolomics · Ultisol · Biofertilizer · *Bungor* · Humid Tropic

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1 Introduction

Engineering the soil microbiome to promote nutrient availability needed for the plants' initial development and succession is a topic of considerable interest. Biofertilizers have emerged as a sustainable tool in alleviating soil fertility and improving the yield of the crops (Ajeng et al., 2020). These organic biofertilizers may improve soil fertility and encourage plant development since they are derived from living beneficial microbes. Biofertilizers provide a more cost-effective and ecologically benign way to fulfill the increasing needs of food production than chemical fertilizers, which are hazardous to both the environment and human health (Pandey et al., 2024). Recent advancements in biofertilizer research

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emphasize the development of tailored formulations to address these challenges. For instance, microbial consortia enriched with specific strains have been designed to enhance nitrogen fixation, phosphorus solubilization, and other essential nutrient processes (Ajeng et al. 2023; Behera et al. 2021).

One of the critical limitations of biofertilizers is their inability to provide nutrients synchronously with plant requirements, which can affect crop growth and yield. This issue is being tackled through innovations in biofertilizer formulation and application strategies, focusing on improving nutrient availability and efficiency (Misra et al. 2020). These formulations are engineered using state-of-the-art molecular biotechnology techniques to overcome many physical, chemical, and mineralogical factors governing the nutrient release and uptake dynamics in agricultural soils (Sharma et al. 2023; Yadav et al., 2016). Tropic soil microbial populations play a crucial role in controlling the global carbon (C) cycle and biogeochemical processes underneath including exogenously producing soil metabolites, plants phytohormones and signaling compounds within in the rhizospheric region.

Ultisols (from the Latin ultimus, "last") are acidic forest soils with little inherent fertility. They are mostly found in humid temperate and tropical climates, usually on older, stable landscapes. Ultisols are the soil type that makes up most Peninsular Malaysia's highland regions. Ultisols typically have finer textures and lower organic matter levels, limiting substrate availability and potentially suppressing enzyme activity involved in carbon cycling. Specifically, enzymes such as β-glucosidase activity was reported to be notably lower in Ultisols which could be attributed to differences in soil texture and organic matter content (Acosta-Martínez et al. 2007). Moreover, acid phosphatase activity, crucial for phosphorus cycling, showed variable responses across different land uses within Ultisols. The activity was generally higher in Ultisols, indicating better phosphorus availability in these soils. However, under agricultural land use, acid phosphatase activity in Ultisols decreased significantly compared to forest and pasture lands. This decline likely reflects the impact of intensive agricultural practices such as tillage and chemical inputs, which can disrupt soil structure and organic matter decomposition, thus affecting enzyme-mediated nutrient cycling processes. The low soil productivity on these types of soils typically limits the amount of permanent crops such as oil palm (*Elaeis guineensis*) that may be produced considering more than of the permanent crops are planted on Ultisol. However, successful plantation on such soils can be achieved when appropriate soil management techniques are used, (Von-Uexkull and Mutert 1995; Ng 2002). Ultisol's key difficulties are acid reaction and high Al content, both of which limit P solubilization; hence, Ultisols need soil treatment such as calcification or fertilizing. Several studies have indicated that the use of organic fertilizers, inorganic fertilizers, and biofertilizers could alleviate the acidity of the Ultisol, therefore resulting in the success of planting better yield crops (Zhang et al. 2023; Liu et al. 2018; Okebalama et al. 2020; Khamis et al. 2017; Cui et al. 2020). However, the impacts of long-term fertilization using inorganic compounds and chemical fertilizers may impose unknown and dangerous threats to the ecosystems especially on the soil metabolites productions and could potentially reduce the metabolic pathways activities.

Allelopathy is a phenomenon in chemical ecology whereby donor plants affect other recipient organisms by releasing their chemicals, known as allelochemicals, into the environment through processes like leaching, volatilization, secretion, and residual degradation (Rice 1984). Metabolites produced by either plant roots or soil bacteria have an indirect role in the mechanisms by which plants alter their root microbiomes and enhance soil structures and aggregates. There has been evidence that both polar and non-polar substances can affect rhizosphere interactions. More complex non-polar secondary metabolites like flavonoids, coumarins, and benzoxazinoids, as well as polar primary metabolites like organic and amino acids (Rudrappa et al., 2008; Neal et al. 2012; Ziegler et al. 2015; van Dam and Bouwmeester 2016), have been reported to play a significant role in influencing rhizosphere microbes. For instance, the rhizobacterial strain *Pseudomonas putida* KT2440, which primes the host's defenses against herbivores (Neal and Ton 2013), is attracted to the benzoxazinoid DIMBOA, which is released by the roots of maize seedlings.

In the context of infertile Ultisols, little is known about the impact of contemporary biofertilizers on problematic soils like Ultisols. Such research questions can be answered or at least studied using omics tools such as metabolomics, or the study of metabolites in biological matrix, including soil matrix (Baharum et al. 2023). Soil metabolomics should be thoroughly investigated in relation to the addition of any amendments such as organic, inorganic, and biological fertilizer (Pétriacq et al. 2017), where the mechanisms and soil metabolic networks can be explored by monitoring the presence of metabolites in the soil matrix. Metabolite changes, including species and quantity, are the ultimate reactions of biological systems to internal or external stimuli like gene mutations or environmental stress (Fiehn et al., 2002). To investigate metabolic pathways or metabolic networks, to compare and analyze metabolic differences in macroscopic

phenotypic phenomena among different biological individuals, and to study the metabolic response mechanism of substances after different induction and stress, qualitative and quantitative analyses of low-molecular weight metabolites have been conducted (Allywood et al., 2021). Metabolomics is typically utilized in conjunction with transcriptome and proteome research to investigate how alterations in the physiological pathway of DNA → mRNA → protein → metabolite elicit responses to diverse environmental stimuli. The incorporation of statistical analysis of difference metabolites has allowed accurate mining due to the "high-dimensional and massive" nature of metabolomics data (Fiehn et al., 2002).

Furthermore, challenges may arise especially during the preparation, calibration and evaluation of humid tropic soil simulation models where these should include crop variables as well as weather, soil, and management data required to simulate pristine-rhizospheric crop-specific conditions in the lab (in vitro) in which this study focuses on the oil palms cultivations. To ensure ideal development of the simulated humid tropic, the simulated soil should be subjected to at least 85% following oil palm farming relative humidity requirement, an average of 5 h of sunshine each day, and at least 2000 mm annual rainfall dispersed consistently throughout the year with little or no dry season. Furthermore, constant average temperatures between 24 °C and 28 °C appear to have ideal circumstances, with seasonal changes of less than 6 °C. However, even in controlled experimental plots, it is difficult to ensure near-optimal conditions due to the multitude of factors and the difficulties in removing every single limiting factor across space and time (Cassman et al. 2003). These limiting factors include (i) the prevalence of insect pests and pathogens, which is particularly problematic in the humid tropics. Therefore, a soil simulation model was used to simulate all the humid tropics conditions subjected to Ultisols to test the potential of biofertilizer on soil metabolic activity in vitro, where our work takes a first step in bridging this knowledge gap.

2 Materials and Methods

2.1 Experimental Setup

The study was conducted at the Molecular Bacteriology and Toxicology Lab, Level 2, Block J12, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. Samples of acidic soil from the *Bungor* series were collected in Gemas, Negeri Sembilan, Malaysia. Each treatment was replicated three times. The treatments comprised [T1]: No Inoculation (Room Temperature), [T2]: Control Simulated Tropical Soil, and [T3]: IBG Inoculated Simulated Tropical Soil. The collected soil was air-dried for seven days at Rimba Ilmu, Universiti Malaya, Kuala Lumpur, Malaysia. Afterward, it was sieved to eliminate any leaves, stones, and weeds, resulting in a finer soil with a mesh size of 500 µm. The soil was then apportioned into four jars, each containing 50 g of soil. Autoclaving at 121 °C for 30 min was employed to eliminate microorganisms. Subsequently, the soil was allowed to acclimate for a period of 17 days. A 5 ml portion of IBG biofertilizer was diluted by combining it with 1.5 L of distilled water. Ten milliliters of this mixture were then added to each jar of soil, which was subsequently incubated for 14 weeks in an aerobic environment. The entire experimental duration spanned four months.

2.2 Microbial Metabolomes Extraction

Following the incubation time, three samples of 2 g soil from each treatment jar were measured and deposited in a tube. A glass serological pipette was used to apply eight milliliters of nonpolar extractant (50% ethyl acetate + 50% water) to each 2 g sample. To achieve appropriate sample mobility and extraction, the samples were oriented on a tube rack at about 45 °C on a chilled orbital shaker and shaken at 150 rpm for 1.5 h. The samples were then centrifuged for 15 min at 3200 g. The top ethyl acetate layer was then removed with a glass Pasteur pipette and transferred to 1.5 mL centrifuge tubes. The metabolite-containing ethyl acetate layer was nitrogen-dried. The dried ethyl acetate extracts were resuspended in 200 µL of a C18 internal standard solution (caffeic acid) after being mixed with 1 mL of acetonitrile.

2.3 Liquid Chromatography-Mass Spectrometry Analysis

The chromatographic separation was performed using a Thermo Scientific C18 column (AcclaimTM Polar Advantage II, 3 150 mm, 3 m particle size) on an UltiMate 3000 UHPLC (Dionex) system. The gradient elution was carried out for 22 min using (A) water containing 0.1% formic acid and (B) 100% acetonitrile as the mobile phase and 0.4 mL/min flow rate at 40 °C. The gradient was begun at 5% solvent B for 3 min (0–3 min), then raised to 80% solvent B for 7 min (3–10 min) and maintained at 80% solvent B for 5 min (10–15 min). Finally, the gradient was restored to 5% solvent B in 7 min (15–22 min). The injection volume for each sample was 1 L, and the samples were produced by dissolving 10 µL of sample extracts in LC-grade methanol (990 L) supplemented with internal standards do not present in

the examined sample. The Bruker Daltonics MicrOTOF-Q III was used for high-resolution mass spectrometry analysis, with the following settings: capillary voltage at 4,500 V, nebulizer pressure at 1.2 bar, drying gas flow at 8 µL/min, source temperature at 200 °C, and m/z range of 50 to 1,000 Da. Prior to the metabolite profiling LC-MS analysis, the electrospray ionization (ESI) method was compared. Most of the metabolites detected in the negative mode were also present in the positive mode total ion chromatogram (data not shown). As a result of profiling a greater number of metabolites, the positive ESI mode was chosen. Tandem mass spectrometry (MS^2) analysis was carried out for each treatment utilizing pooled duplicates of all extracts in equal quantities and automated fragmentation settings (Auto-MS^2) spanning a mass-to-charge precursor ion range of 500 to 1,000 Da (Vargas et al., 2016).

2.4 Mass Spectrometry Data Processing

The raw data for mass spectrometry was retrieved from Bruker DataAnalysis (version 4.1) and aligned with Bruker Compass ProfileAnalysis (version 2.1). The advanced bucketing setting was used, with the following parameters: 1 m/z = 20 mDa, 1rt = 10 s, signal-to-noise ratio threshold = 5, and smoothing width = 4. Metabolites chosen based on the highest score with a low molecular mass error tolerance range (1 ppm = 5) and manually matched to accessible public databases MassBank, MetFrag with PubChem database (Horai et al. 2010) for putative metabolite identification. Putative metabolites with molecular weights within the molecular mass error tolerance range of 1–20 ppm to the query m/z values were found from databases using positive mode adduct. Full-scan LC-MS data were collected for statistical analysis to identify molecular ions with significant differences across samples, which were then followed by independent precursor ion (PI) scans to collect MS^2 data from the PIs.

2.5 Statistical Analysis

Data Analysis software was used to collect all mass spectrum data (version 4.0, Bruker Daltonics). Profile analysis software (Bruker Daltonics) was used to process the raw data (.d) files, which included peak alignment and normalization. Metaboanalyst 5.0 (USA) to perform multivariate statistical analysis, including principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were applied to cluster samples according to the profiled metabolite features and to identify distinct metabolites that differentiate between the treatments. The data was pre-processed with Pareto (Par) scaling which was applied to the normalized data to reduce the effects of background noise interference on sample clustering to identify the response of Ultisols in different treatments. On the normalized peak intensity data, a one-way analysis of variance (ANOVA) with a significance of $P < 0.05$ was performed to distinguish the statistically significant metabolites among the treatments. The variable significance in projection (VIP) rates each variable's total contribution to the PLS-DA model, and variables with VIP > 1.0 were considered meaningful for group discrimination (Xie et al. 2008). Other data analysis, such as a heat map, was carried out using the MetaboAnalyst 5.0 software.

3 Results

3.1 Metabolites Profiling and Identification of In Vitro Simulated Humid Tropic Ultisols Under Control and IBG Biofertilizer Amendment

Figure 2 presents the multivariate analysis of the unsupervised PCA scores plot and supervised PLS-DA score plot from the metabolite profiling analysis conducted on an in vitro simulated Ultisol. The study investigated the impact of both control conditions and the introduction of biofertilizer amendment on the metabolite composition within the Ultisol. Through meticulous metabolomic analytical techniques, we aimed to elucidate the intricate metabolic responses elicited by the biofertilizer, providing valuable insights into its potential for enhancing soil nutrient dynamics and fostering sustainable agricultural practices.

To conduct a meaningful metabolomics study, the Ultisols extract must accurately replicate the original matrix; hence, the harvesting and extraction processes are crucial. Soil sampling should be standardized in terms of the sampling location, depth, rhizospheric conditions, and extraction should be reproducible and capable of keeping the compounds stable in solution. In the subsequent profiling analysis, we opted for positive ionization mode. Here, the MS^1 spectra underwent additional fragmentation to generate MS^2 mass spectrograms, which were then compared against a database of standard materials. These spectra were evaluated to obtain matching results, leading to the identification of MS^2 fragments corresponding to the metabolites. Following this process, and subsequent filtering and bucketing utilizing Bruker Compass ProfileAnalysis, we achieved successful profiling of a total of 1761 peaks, with an average of 195.7 peaks per sample.

The unsupervised PCA shown in Fig. 2 (A– C), provides a visual representation of sample relationships in a multi-dimensional space. Each point on the plot corresponds to a specific sample, and their positions reflect their proximity

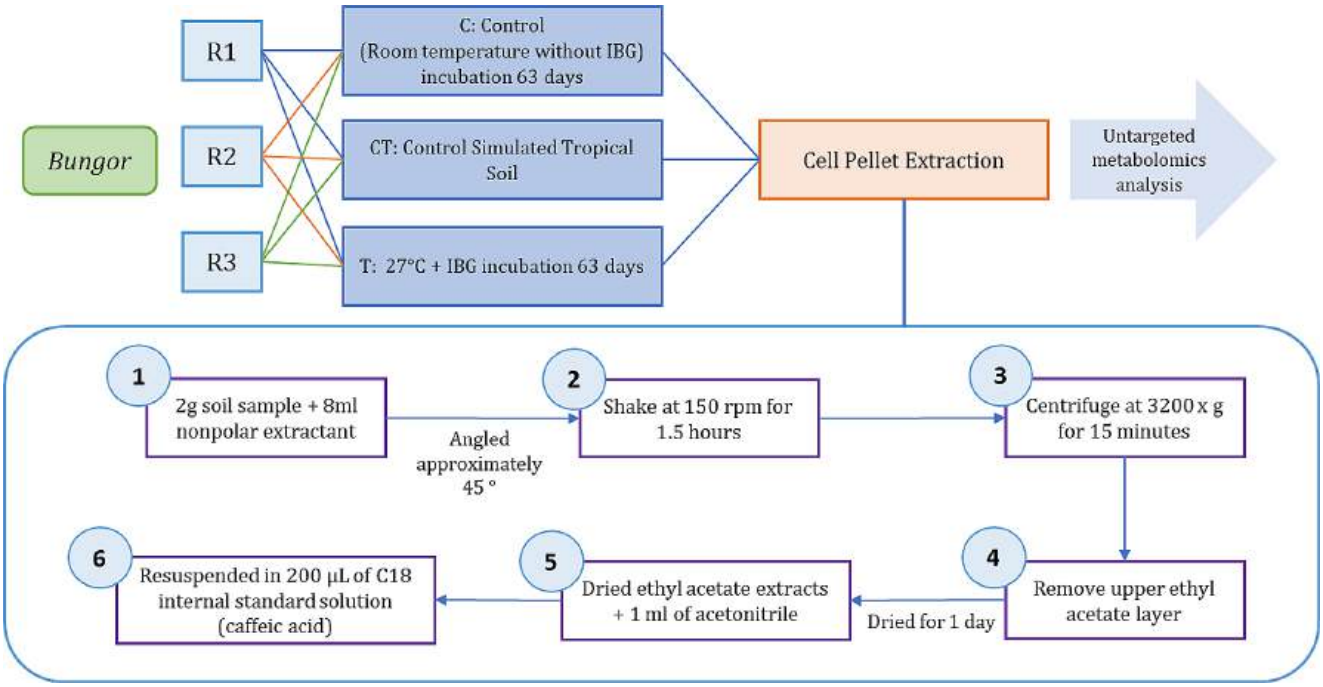

Fig. 1 Schematic diagram of the procedure used for experimental set-up and metabolome extraction

or dissimilarity to other samples. The dispersion of samples is directly linked to variations in metabolite compositions. Samples with greater similarities cluster closer together, while those with significant differences are positioned further apart on the PCA plot. This analysis offers valuable insights into the underlying patterns and relationships within the metabolite profiles of the samples under investigation. Although no outliers were observed, the separation explained only 37.5% of the variance in the first principal component, respectively. The cumulative R^2 and Q^2 values, both exceeding 0.8, indicate minimal variation between biological replicates, underscoring the effectiveness of the in vitro simulations. Conversely, lower cumulative values might be expected in field-based samplings, where environmental factors exert a more prominent influence.

Subsequently, a supervised partial least squares-discriminant analysis (PLS-DA) was conducted for sample clustering (Fig. 2 (D-F)). Notably, the separation predominantly occurred along the second component, accounting for 29.3% of the variances. This model, characterized by a predictive value (Q_2) lower than R_2Y, reflects a well-balanced fit and is corroborated by a permutation test, ruling out overfitting. The PLS-DA loading plot illustrates the metabolite characteristics responsible for species discrimination. Those projecting furthest from the center exert the most influence. Additionally, alongside VIP scores, univariate statistical analysis aids in pinpointing statistically significant metabolites by assessing intensity differences among metabolite features in each sample. Only features deemed statistically significant are considered noteworthy, potentially serving as biological or chemical markers.

Based on the one-way analysis of variance (ANOVA), 50 m/z values (VIP values > 1) corresponding to metabolites present in the sample matrix are detected. Among these, 26 m/z values were associated with the treatment involving IBG biofertilizer on amended simulated Ultisols with Variable Importance in Projection (VIP) value (>) 1. These 26 m/z values could potentially serve as biomarkers or chemical indicators of biofertilizer application in infertile Ultisols conditions. Our primary focus was on metabolite features with high abundance and those identified as VIPs. We pursued putative identification by meticulously comparing accurate masses, fragmentation patterns, and MS^2 spectral data with entries in public metabolite databases like MetFrag and MassBank. This approach was chosen due to the labor-intensive nature of metabolite identification. The VIP values from the first two main components of the multivariate PLS-DA model, in conjunction with p-values and fold changes, were employed to sift through differentially expressed metabolites. Our criteria for selection were a fold change of 2, VIP score of 1, and a q-value of 0.05. The distinctive 26 VIP values associated with the IBG treatments were discerned using MetFrag. The identity of the detected compounds, along with the m/z values and retention time were listed side-by-side and shown in the heatmap visualization (Fig. 3).

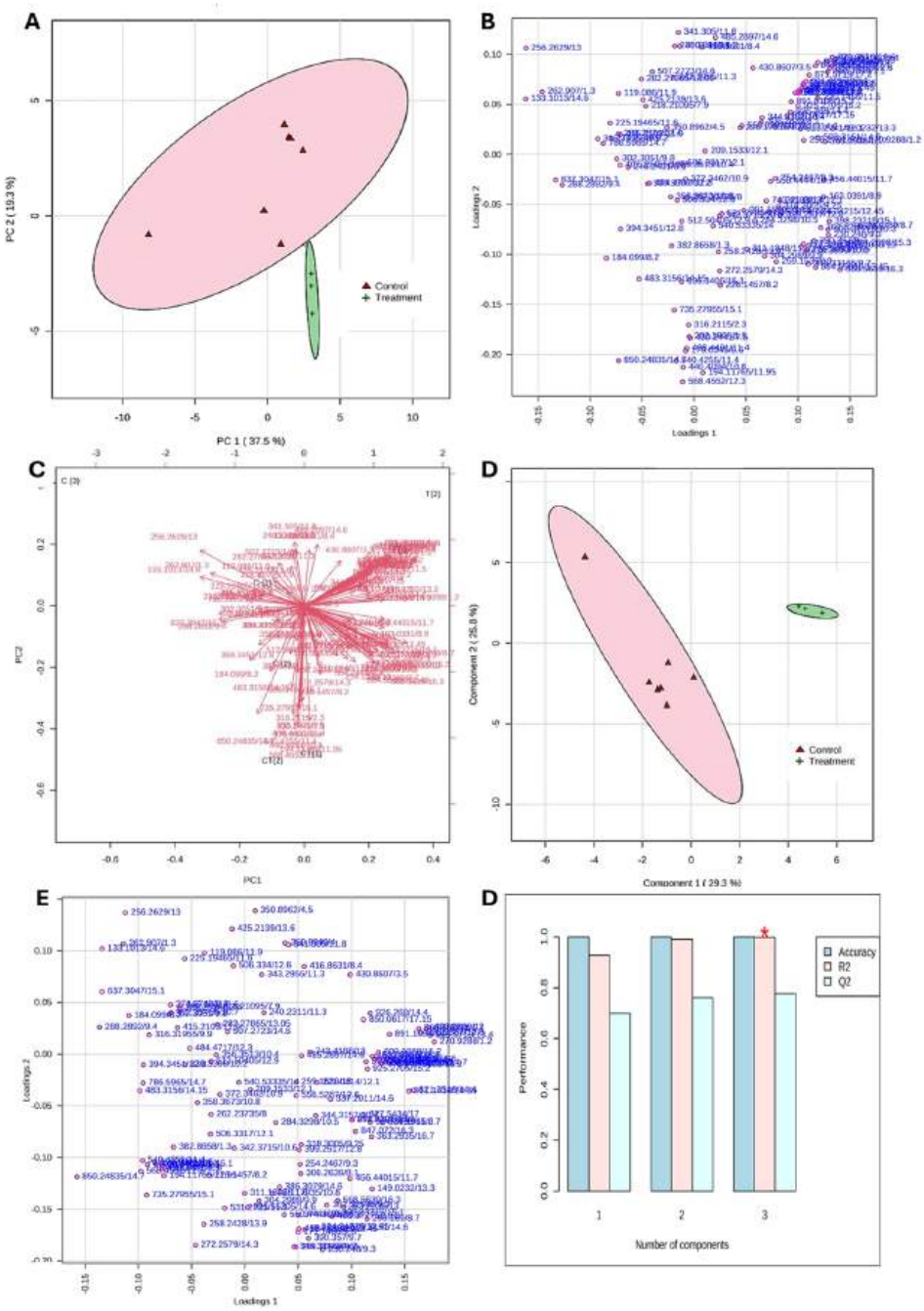

Fig. 2 Schematic diagram of the procedure used for experimental set-up and metabolome extraction

3.2 Metabolic Pathways Identification

To further assist in our understanding of the detected metabolites interactions, and selection of the best pathways detection methods, a comparison between two commonly used pathway analysis methods, Fig. 4**(A)** Topology-based method pathway analysis and Fig. 4**(B)** Over-representation analysis (ORA)-based method pathway analysis on annotated and identified metabolites (VIP value > 1) between the control and the simulated treatment groups. ORA, or Over-Representation Analysis, serves the purpose of determining whether a specific set of compounds appears more frequently in the user-uploaded compound list than would be expected by random chance. In the context of pathway analysis, ORA was the most accurate in terms of pathways matching which assessed whether the compounds detected in control and IBG biofertilizer (treatment) groups were associated with a particular pathway show an enrichment compared to what might occur by random chance alone. This evaluation was carried out using statistical tests such as Fisher's exact test or the hypergeometric test, which are the most employed methods for this type of analysis. Referencing the

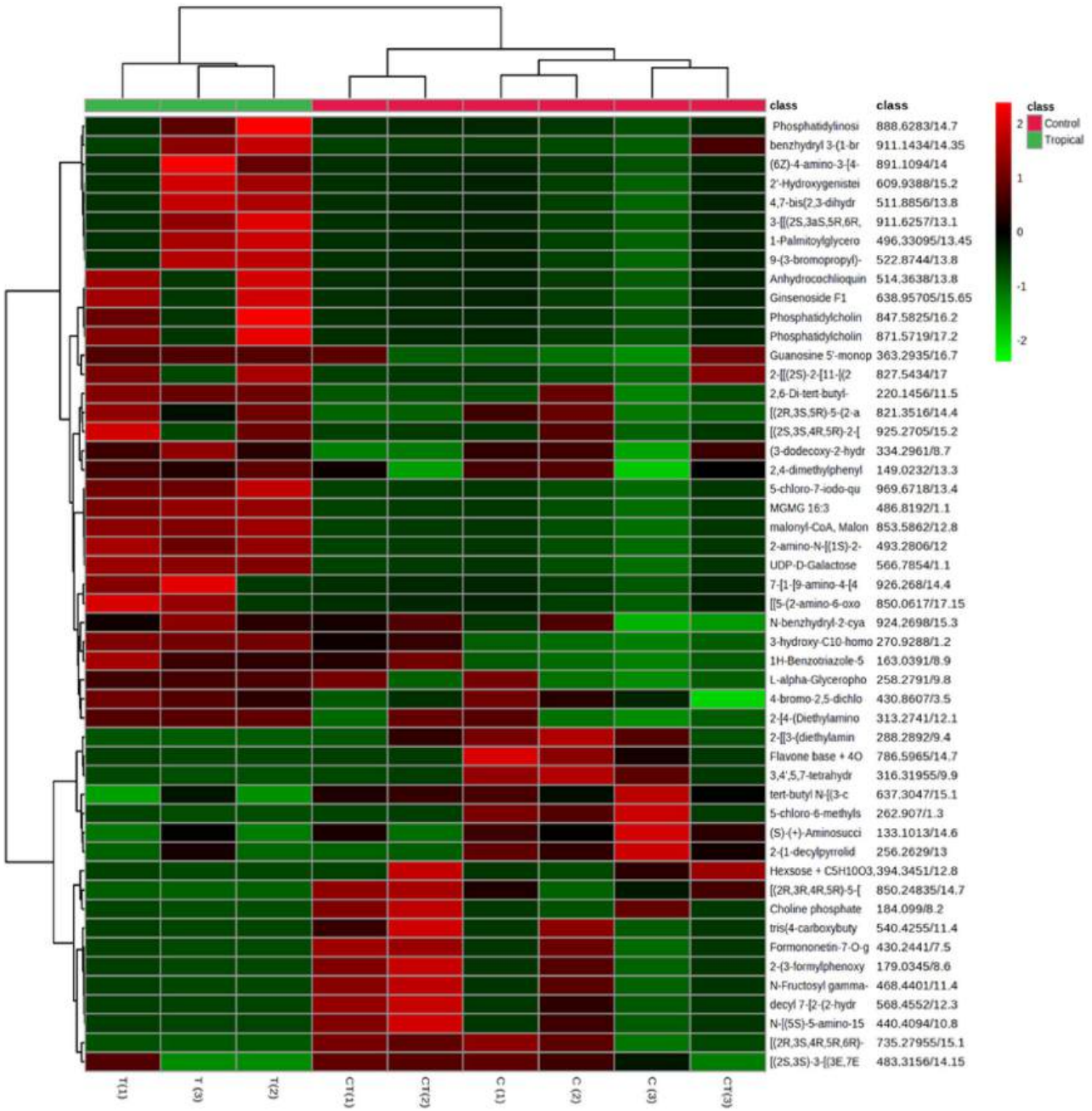

Fig. 3 The heatmap analysis of the VIP scored metabolites distribution (compound identity / m/z values / retention time) in soil under control (ambience temperature 25 °C) and tropical (~27 °C). The heatmap display is based on log^{10}-transformed metabolite concentrations (pareto scaled). The reddish hue denotes increasing metabolite concentration, whereas the greenish tint shows decreasing metabolite concentration. Where T = IBG Inoculated Simulated Tropical Soil, C = No Inoculation (Room Temperature), CT = Control Simulated Tropical Soil

Bacillus subtillis KEGG pathways, several pathways were involved/affected when IBG biofertilizer was applied under the simulated tropic Ultisols namely: Glycerophospholipid metabolism, beta-Alanine metabolism, Glycine, serine and threonine metabolism, Cysteine and methionine metabolism, Phosphonate and phosphinate metabolism, D-Glutamine and D-glutamate metabolism, Arginine biosynthesis, Glycosylphosphatidylinositol (GPI)-anchor biosynthesis, Purine metabolism, Nicotinate and nicotinamide metabolism, Histidine metabolism, Glycerolipid metabolism, Pantothenate and CoA biosynthesis, Ether lipid metabolism,

Figure 5 depicts the metabolomic pathways that were impacted in this study according to the pathway analysis conducted in Fig. 4 (A) which was based on the topological compounds database matching methods. Among the pathways that were influenced were: Glycerophospholipid

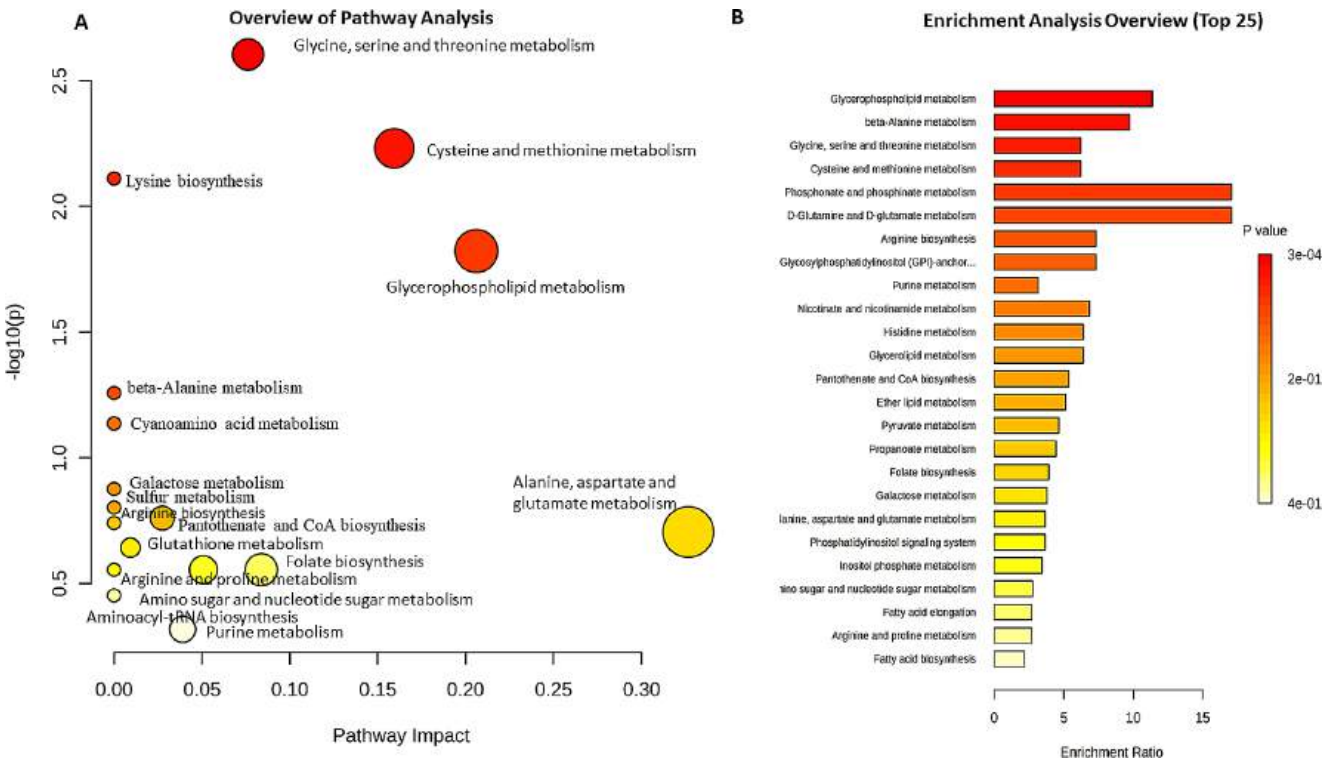

Fig. 4 Comparison between two commonly used pathway analysis methods **(A)** Topology-based method pathway analysis and **(B)** Over-representation analysis (ORA)-based method pathway analysis on annotated and identified metabolites (VIP value > 1) between the control and the simulated treatment groups where colour indications ranging from yellow to red) indicates that the metabolites are present in the data at changing degrees of significance

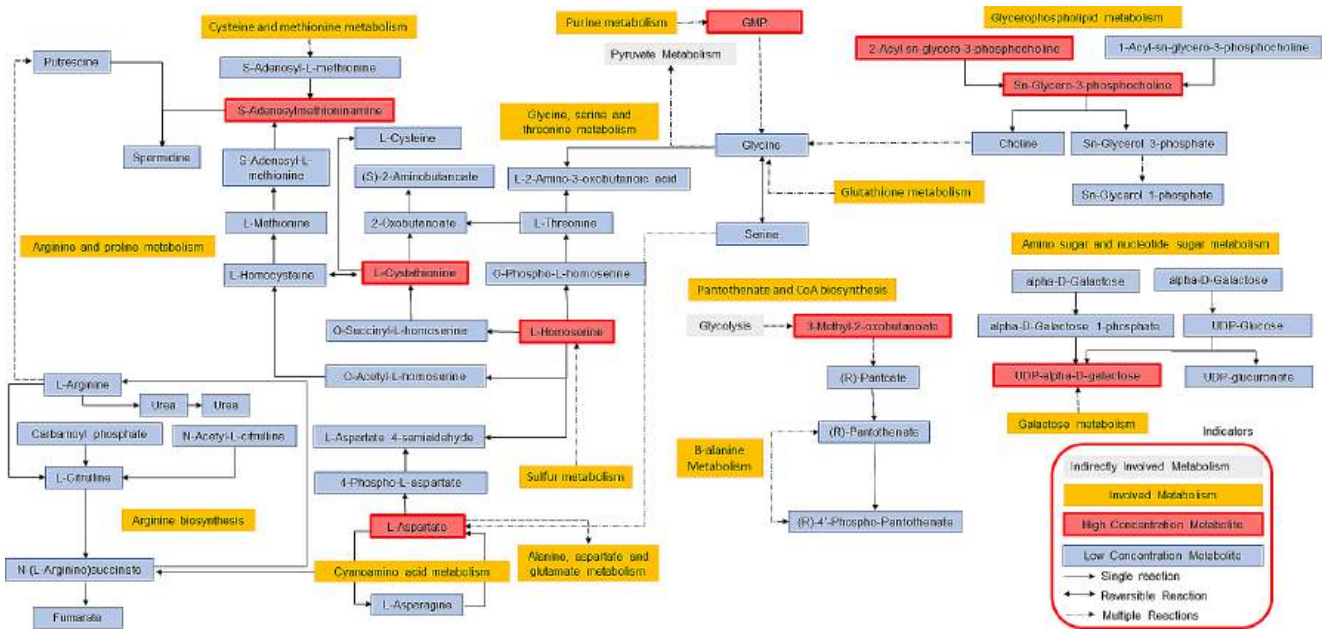

Fig. 5 Among the metabolites involved in the biosynthetic pathway of IBG biofertilizer on simulated humid tropical soil. Single arrows represent one-step enzymatic conversions, while dashed arrows represent multiple reactions. Reference KEGG library: *Bacillus subtilis*

metabolism, Lysine biosynthesis, Glycine, serine and threonine metabolism, Cysteine and methionine metabolism, beta-Alanine metabolism, Monobactam biosynthesis, Cyanoamino acid metabolism, Nicotinate and nicotinamide metabolism, Sulfur metabolism, Arginine biosynthesis, Pantothenate and CoA biosynthesis, Alanine, aspartate and glutamate metabolism, Galactose metabolism, Amino sugar and nucleotide sugar metabolism, Folate biosynthesis, Aminoacyl-tRNA biosynthesis, Purine metabolism. According to the overview, 4 pathways were prominent namely: Glycine, serine and threonine metabolism, Cysteine and methionine metabolism, Glycerophospholipid metabolism and Alanine, aspartate and glutamate metabolism. Meanwhile, the ORA analysis (Table 1) revealed that Glycerophospholipid metabolism, beta-Alanine metabolism, Phosphonate and phosphinate metabolism, D-Glutamine and D-glutamate metabolism might be heavily influenced following the IBG biofertilizer amendment (T) as compared to the C and CT treatments.

4 Discussion

As metabolomics evolves as an area of research with a wide range of applications across disciplines, drawing meaningful conclusions from such data becomes increasingly vital. ORA is one of the most often utilized methods for deriving functional interpretations from metabolomics data. The list of compounds of interest (typically corresponding to metabolites that change between conditions in experiments) is an important input for ORA, and we have shown that how these compounds are chosen has a significant influence on pathway analysis findings. The m/z value 270.928 had the highest VIP value of 2.148 and was putatively identified in the IBG biofertilizer amended simulated humid tropic Ultisols (T treatment), as well as minute concentrations detected within the uninoculated simulated humid tropic Ultisols (Fig. 3). Meanwhile the compound was not detected in the control sample. The m/z value 270.928 was identified as 3-hydroxy-C10-homoserine lactone, a N-acylhomoserine lactone (AHL) family which is categorized as diffusible signal molecules employed by mainly gram-negative bacteria (Ortori et al. 2007).

Homoserine lactones are involved in a cell-to-cell communication mechanism known as quorum sensing, which allows a bacterial population to determine its numerical

Table 1 Pathways analysis based on over-representation analysis (ORA) on the annotated metabolites (VIP value > 1)

	Total	expected	hits	Raw *p*	Holm *p*	False Discovery Rate (FDR)
Glycerophospholipid metabolism	36	0.352	4	0.000289	0.0243	0.0243
beta-Alanine metabolism	21	0.205	2	0.0168	1	0.706
Glycine, serine and threonine metabolism	33	0.322	2	0.0395	1	0.802
Cysteine and methionine metabolism	33	0.322	2	0.0395	1	0.802
Phosphonate and phosphinate metabolism	6	0.0586	1	0.0573	1	0.802
D-Glutamine and D-glutamate metabolism	6	0.0586	1	0.0573	1	0.802
Arginine biosynthesis	14	0.137	1	0.129	1	1
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	14	0.137	1	0.129	1	1
Purine metabolism	65	0.635	2	0.13	1	1
Nicotinate and nicotinamide metabolism	15	0.146	1	0.137	1	1
Histidine metabolism	16	0.156	1	0.146	1	1
Glycerolipid metabolism	16	0.156	1	0.146	1	1
Pantothenate and CoA biosynthesis	19	0.186	1	0.171	1	1
Ether lipid metabolism	20	0.195	1	0.179	1	1
Pyruvate metabolism	22	0.215	1	0.195	1	1
Propanoate metabolism	23	0.225	1	0.203	1	1
Folate biosynthesis	26	0.254	1	0.227	1	1
Galactose metabolism	27	0.264	1	0.235	1	1
Alanine, aspartate and glutamate metabolism	28	0.273	1	0.242	1	1
Phosphatidylinositol signaling system	28	0.273	1	0.242	1	1
Inositol phosphate metabolism	30	0.293	1	0.257	1	1
Amino sugar and nucleotide sugar metabolism	37	0.361	1	0.308	1	1
Fatty acid elongation	38	0.371	1	0.314	1	1
Arginine and proline metabolism	38	0.371	1	0.314	1	1
Fatty acid biosynthesis	47	0.459	1	0.374	1	1
Aminoacyl-tRNA biosynthesis	48	0.469	1	0.38	1	1

size (or density). AHLs are created and accumulate as the bacterial culture expands. Once a certain concentration of the chemical (and hence a certain population density) is reached, a coordinated shift in bacterial behavior occurs. Bioluminescence, conjugation, secondary metabolite synthesis, biofilm formation, swimming and swarming motility, and pathogenicity are all controlled by AHL-dependent quorum-sensing mechanisms. The detection and active expression of AHLs in the IBG inoculated simulated humid tropic Ultisols could positively alter the soil chemical properties and microbial metabolisms, which translates into healthier and more productive soil.

The AHL family differs in acyl chain length, substitution at carbon 3 (and degree of saturation), but all have the homoserine lactone ring structure. This finding was corroborated by Ortori et al. (2017) where the author revealed that enhanced product ion (EPI) spectra of 3-oxo- C6, C7, C8 and C10 AHLs eluted sequentially at 1.7, 2.4, 3.3 and 4.7 min, had nominal m/z values of 214, 228, 242 and 270 respectively. The absence of this signaling chemical in the control plot might indicate that the microbial communities or pathogens formerly present in the soil sample matrix were effectively eliminated by autoclaving. However, microorganisms that had previously reached a dormant state in the soil matrix or pores may grow when favorable growing conditions such as humidity are introduced, as in the control simulated humid tropic (without IBG inoculation) treatment could be the reason behind the minute concentration of the AHLs detected within the treatment without IBG biofertilizer inoculation. The metabolite was then matched to the existing pathway database (*Bacillus subtilis*) and it was revealed that 3-hydroxy-C10-homoserine lactone is involved in the Glycine, serine and threonine metabolism as well as Cysteine and methionine metabolism. Methionine is produced from homoserine through a series of four steps.

The process involves three intricate reactions that substitute the hydroxyl group of homoserine with -SH, leading to the formation of homocysteine. In *E. coli*, succinylation of the hydroxyl group with succinyl-CoA initiates these reactions, while in gram-positive organisms, acetylation is the triggering mechanism. Cysteine is introduced in the second step and serves as the source of reduced sulfur. The third enzyme in this pathway facilitates the hydrolysis of the intermediate cystathionine. Interestingly, this enzyme also has the capability to hydrolyze cysteine and serine. However, the presence of serine inhibits this reaction, and the addition of exogenous serine can lead to a requirement for methionine. In the fourth and final step, the donation of a methyl group to the sulfur of homocysteine results in the production of methionine. This methyl group is provided by N5-methyltetrahydrofolate, which is generated through the reduction of N5, N10-methylene tetrahydrofolate. The last crucial step involves two methionine synthases that facilitate the final transfer of the methyl group to homocysteine: one of these enzymes relies on cobalamin (vitamin B12), whereas the other does not. Notably, the latter enzyme is sensitive to oxidative stress. Furthermore, S-adenosylmethionine (SAM) is synthesized in a single step from methionine and ATP.

The acyl side chains of AHLs, which are thought to be derived from fatty acid biosynthesis, in which the AHL compound was matched to in the ORA analysis (Table 1) are composed of 4 to 18 carbons, generally in increments of two carbon units (C4, C6, C8, etc.) (Della et al., 2019). The majority of the acyl side chains are unbranched, saturated or monounsaturated, and even-numbered, matching fatty acids found in microbial cells. Most of the metabolites present in the Ultisols matrix could be endogenous metabolites such as the amino acids, organic acids, nucleic acids, fatty acids, amines where these metabolites are assessable to another biota within the natural environment and are most likely to accumulate as SOM as a result of interacting with minerals and negatively charged clay particles (Swenson et al. 2015).

Glutamate metabolism (GM) is among the metabolite pathway influenced due to the amendment of IBG biofertilizer where the metabolism is essential for amino acid metabolism, orchestrates crucial metabolic activities, and plays a role in pathogen defense, particularly L-glutamate, a protein-building component (Baharum et al. 2023). It is worth noting that glutamate is a critical metabolite in all organisms because it connects carbon and nitrogen metabolism. The interaction between N metabolism with the dynamics of other nutrients such as phosphorus and potassium would lead to an improved physiological development of crops such as the roots, as well as other plant functions that influence yield and quality (Farhan et al. 2024). Nitrogen is a primary component of amino acids, proteins, and enzymes essential for plant growth and development. Its availability and efficient utilization are closely intertwined with the uptake and utilization of phosphorus and potassium. Phosphorus is integral to energy transfer processes in plants, DNA and RNA synthesis, and root development. Potassium, on the other hand, regulates water uptake, enzyme activation, and overall plant turgor pressure, influencing stomatal function and nutrient transport within plants (Khan et al. 2023).

Optimal nitrogen metabolism supports these functions by enhancing nutrient uptake and translocation. For instance, nitrogen stimulates root growth, thereby increasing the surface area for phosphorus and potassium absorption. Moreover, nitrogen plays a pivotal role in maintaining the balance between shoot and root growth, which is critical for the plant's overall biomass accumulation and resilience to environmental stresses (Hao et al. 2023). The synergistic interaction between nitrogen, phosphorus, and potassium

metabolism not only promotes robust root development but also enhances photosynthesis, nutrient translocation, the production of phytohormones and protection against diseases (Ortel et al. 2024). This coordinated physiological response ultimately leads to improved crop productivity, better nutrient use efficiency, and enhanced crop quality traits such as yield, protein content, and disease resistance.

This observation suggests that the incorporation of IBG biofertilizer into challenging and less fertile soils, specifically Ultisols simulated under a humid tropical environment as examined in this study, promotes the production of essential metabolites, notably L-aspartate, within the soil matrix most likely due to the effective microbes in the soil following the biofertilizer application.

This, in turn, potentially triggers the initiation of carbon and nitrogen metabolic processes within the soil. Initiation of the nitrogen metabolism will further signal nitrogen transport through the glutamine synthetase and glutamine-oxoglutarate aminotransferase cycles, cellular redox regulation, and the reprogramming of energy via the tricarboxylic acid cycle are three key activities in the soil microbes. The initiation of the nitrogen transport could be seen as either supporting the ongoing defense strategy against pathogenic attacks or disease resistance which ultimately shape an effective resistance response, or they are exploited by the effective microbes in the soil to enhance and facilitate primary biological processes in the soil (Baharum et al. 2023; Seifi et al. 2013). Furthermore, ether lipid metabolism that was involved during the IBG biofertilizer treatment is said to be a class of lipids that include glycerol ethers. Microbial activity involved in lipid metabolism can influence the turnover of organic matter and the release of nutrients (Schouten et al. 2013). Meanwhile, amino acids related pathways such as the purines, cysteine, Glycine, Serine, and Threonine Metabolism are the components or building blocks of nucleic acids (DNA and RNA) and protein (Reitzer 2000). Microbial activity involved in the decomposition of organic matter contributes to the release of purines, which can impact nitrogen cycling in the soil where the transformation of nutrients affected by the release of these compounds will greatly affect the biogeochemistry in the soil.

Furthermore, glycerophospholipids metabolisms are involved in synthesis key constituents of cell membranes of microbes. When these molecules are digested, they can produce phosphorus-containing chemicals. Phosphorus is an essential ingredient for plant development, although its availability in soil is generally limited. The release of phosphorus from glycerophospholipid metabolism may help to increase soil fertility by delivering a vital mineral for plants (López-Lara et al., 2017; Vance 1991).

5 Conclusion

In conclusion, this study illuminates the promising potential of biofertilizer as a viable solution to address soil infertility issues and elevate overall agricultural productivity using Ultisol. Through comprehensive untargeted metabolomics profiling in the context of Ultisols incubation with IBG Biofertilizer, we have identified key metabolites and pathways that respond to this intervention. The distinct shifts observed in metabolite profiles, particularly the remarkable changes in homoserine lactone levels, highlight the dynamic impact of IBG biofertilizer on Ultisols in simulated humid tropic conditions. Additionally, the significant alterations in essential metabolic pathways underscore the intricate interplay between biofertilizer application and soil biochemistry. These findings not only provide valuable insights into the biochemical intricacies of biofertilizer-induced soil enhancement but also offer practical implications for sustainable agriculture practices in challenging environments. By understanding and harnessing the potential of biofertilizers, we can pave the way for more efficient and eco-friendly approaches to soil management, ultimately contributing to global food security.

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