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Article

Functional Diversity of Microbial Communities in the Soybean (*Glycine max* L.) Rhizosphere from Free State, South Africa

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Abstract: The plant microbiome is involved in enhancing nutrient acquisition, plant growth, stress tolerance and reduces chemical inputs. The identification of microbial functional diversity offers the chance to comprehend and engineer them for various agricultural processes. Using a shotgun metagenomics technique, this study examined the functional diversity and metabolic potentials of microbial communities in the rhizosphere soybean. 18 genera were selected out of which six are prominent in sample AB, the prominent genera are Geobacter, Nitrobacter, Burkholderia, Candidatus, Bradyrhizobium and Streptomyces. Twenty-one functional categories were present with 14 of the functions being dominant. The dominant functions include carbohydrates, fatty acids, lipids and isoprenoids, amino acids and derivatives, sulfur metabolism, and nitrogen metabolism. Kruskal-Wallis test was used to test samples' diversity differences. There was a significant difference in the diversity with p-value of 0.04. ANOSIM was used to analyse the similarities of the samples, p-values and R-values of the samples were 0.01 and 0.5835 respectively. Phosphorus with p-value of0.718 and 64.3% contribution was more prominent among the soil properties that have influence on functional diversity of the samples. Given the functional groups reported in this study, it is clear that soil characteristics had an impact on on the functions role of the rhizospheric microbiome of soybean

Keywords: microbial communities; plant-microbe interractions; rhizodeposition; SEED subsystem; shotgun metagenomics

1. Introduction

Leguminous plants, such as soybean (Glycine max L), provide high-protein and high-oil minerals for human use. Because of its high-quality plant-based protein and oil content, soybean is one of the world's most significant crops (Nakei et al., 2022). The crop is grown on around 6% of arable land and 50% of legume-growing areas around the world (Hartman et al., 2011). The United States of America, Brazil, and Argentina are the world's top soybean producers (Fernandez-Gnecco et al., 2021). Soybeans are grown in a number of African countries, especially in Sub-Saharan Africa (SSA), and the crop is one of the most prevalent legumes in the region (Nakei et al., 2022; Santos, 2019). Soybeans are used in a variety of purposes, including non-food applications like biodiesel production as well as human and animal nutrition. Because of their agricultural relevance and propensity to create symbiotic relationships with rhizobia, legume plants have been used to study plant-microbe interactions in the rhizosphere (Sugiyama et al., 2014).

Plants are associated with diverse microbial populations that are taxonomically organised (Trivedi et al., 2022). These organisms interact with their host plant in a complex and dynamic way and the environment has a big influence on these interactions (Singh et al., 2020; Trivedi et al., 2022). Within the soil system, rhizosphere or immediate environs of the plant root is a microbial hotspot that is regarded one of the most active interfaces on the planet where various interactions occur (Sugiyama, 2019). Additionally, mutual interaction between plants and microbiomes occurs in the region around the roots of soy-

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bean plants due to high variation of microbes in the rhizosphere. The rhizosphere's microbial community is part of a complex food web that relies on nutrients (mainly exudates) supplied by the plant to regulate microbial activity and diversity in the rhizosphere (Mendes et al., 2014). This community is an important component of sustainable agriculture because they reduce fertilizer and pesticide consumption (Sugiyama et al., 2014). This microbiome is critical for plant growth, nitrogen fixation via nodulation, and environmental stress protection (Yang et al., 2020).

Despite increasing importance of the microbiome in plant health and development, leveraging microbial interactions and functions to improve plant resilience to biotic and abiotic stress remains a challenge (Trivedi et al., 2022). To address these challenges and ensure sustainable crop production, an understanding of the functional role of the microbial communities that colonize the plant rhizosphere is required. The microbiome functional diversity will aid in the discovery of appropriate and improved methods for increasing plant yield, particularly in nutrient-deficient and semi-arid regions of the world, where chemical inputs are needed to supplement plant growth (Chukwuneme et al., 2021). Rhizosphere microbial communities have been widely investigated using both culture-dependent and culture-independent approaches due to their importance in plant growth and performance (Odelade & Babalola, 2019; Sugiyama et al., 2014). Advances in next-generation sequencing techniques through the use of amplicon and shogun sequencing have enabled in-depth investigations of rhizosphere microbial community.

Metagenomics approaches have simplified the process of taxonomic and functional classification of uncultured microbial populations. This have facilitated a better understanding of microbial behaviours and processes in their environments (Chukwuneme et al., 2021; Ranjan et al., 2016). The technique has been used to obtain detailed information of the specific rhizosphere microorganisms (Lagos et al., 2015), roles, number, and compositions (LeBlanc et al., 2015) ;(Spence et al., 2014). In this study, shotgun metagenomics approach, which enables the functional profiling of microbial communities inhabiting an environment was used to unravel the microbial and functional diversities in the soybean rhizosphere.

2. Material and Method

2.1. Soil Sample collection

In March 2021 the sample collection was done using soil auger. Soybean rhizosphere soil samples were collected from a soybean farm in the Free-State Province of South Africa (27.28 °S and 26.72 °E) at a depth of 0 - 15cm in triplicate as described by Chen et al. (2006). The bulk soil was sampled from a point that is approximately 10 m from the soybean field. Rhizosphere soil samples were collected after careful uproot of the soybean plant at fruiting stage. The soil samples were collected in triplicates and replicate samples were labelled (AA, AB and CA) and bulk soil (BC). The samples were placed in a sterile zip-lock bag, kept in box containing ice packs, and was taken to the laboratory. The samples were stored at -20 °C.

2.2. Physicochemical Analysis of the soil samples

The samples were grinded and sieved using a 2mm sieve and used for physicochemical analysis. Sáez-Plaza et al. (2013) method was used to determine the total Nitrogen (N) present in the soil (Sáez-Plaza et al., 2013), pH meter was used to determine the pH of the samples in distilled water at 1:2.5 soil to water ratio.

Ammonium acetate at concentration of 1mM with neutral pH of 7 was used determine the exchangeable potassium, while the sulfur was determined using HCl after the extraction was done. Organic matter was determined using the method used by Hoogsteen et al. (2015), the amount of phosphorus in the soil sample was determined with the method explained by Madaras and Koubová (2015), the organic carbon was deter-

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mined from the soil sample with the method explained by Walkley and Black (1934). Spectrophotometry absorbance of 2 M KCl was used to determine the nitrate and ammonium content of the soil samples.

2.3. Extraction of DNA and sample sequencing

Extraction of DNA was carried out from rhizosphere soil samples and the bulk soil. Samples were allowed to thaw; 0.25 g was weighed from each soil samples for extraction. All the data set used in this study are from shotgun method of whole–metagenomes sequencing. This was done at MR DNA, Shallowater, USA. Life technologies assay kit - Qubit® dsDNA HS was used to obtain samples' DNA concentration. The libraries were made according to the manufacturer's instructions using the Illumina DNA Prep, (M) Tagmentation library preparation kit. The libraries were made with 20-50 ng of DNA. Samples were fragmented and adapter sequences were added. These adapters were used in a limited-cycle PCR in which the material was supplemented with unique indices. The ultimate concentration of the libraries follows the library preparation. After library preparation, the libraries' final concentrations were assessed using the Life Technologies Qubit® dsDNA HS Assay Kit, and the average library size was estimated using the Agilent 2100 Bioanalyzer (Agilent Technologies). After that, the libraries were pooled at 0.6 nM equimolar ratios and sequenced paired end for 300 cycles on the NovaSeq 6000 system (Illumina).

2.4. Metagenomics data annotation and data analysis

The sequences were uploaded on MG-RAST server (Meyer et al., 2008) at https://www.mg-rast.org. The quality control steps involve dereplication, which removes artificial sequences, ambiguous base-filtering, and host species-specific sequences. After quality control process, annotation of sequences was done using BLAT algorithm (Kent, 2002). This BLAST-like alignment tool was used against M5nr database, which result in non-redundant alignment of several databases (Wilke et al., 2012). Taxonomic classification was carried out with the use of RDP database while functional categories from level 1, level 2, and level 3 was assigned using SEED subsystem database using default settings. Sequences that failed annotation were discarded and no further analysis was performed on them. Unclassified reads were maintained for statistical purposes while the functional table was constructed, abundances were then transformed to percentages, and the functional table was assembled according to each functional level. The raw sequences have been deposited with NCBI under the BioProject accession number PRJNA763981.

Environmental variables that determine microbial composition was gotten using forward type CCA (canonical correspondence analysis). Soil physicochemical properties differences were established between rhizosphere soil and bulk soil with ANOVA (A one-way analysis of variance) using turkey's pairwise comparison test. The Shannon, Simpsons and the Pielou Evenness of all the samples were determined as a measure of diversity indices of each sample. Kruskal-Wallis test were used to compare the resulting indices of the samples. Monte Carlo permutation test using 9999 random permutations was used to get the test of significance. PAST version 3.20 (Hammer et al., 2001) was used for statistical analysis and to determine the differences that exist in the community composition within the same sample group. PCoA (Principal coordinate analysis) based on Euclidean distance matrix together with one-way analysis of similarities (ANOSIM) through 9999 permutations were used to calculate the beta diversity variations. Shiny heat map together with z-score were used to draw the Heat maps (Khomtchouk et al., 2017). The variable data used for CCA analysis was gotten from the environmental variables as shown in Table 1 Finally, CCA, PCoA and PCA were plotted with CANOCO software (ter Braak & Smilauer, 2012).

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3. Results

3.1. Physicochemical analysis of soybean soil

The results of soil properties are presented in Table 1. The soil samples were generally sandy in nature with pH that are close to neutral. The rhizosphere samples (AA, CA and AB) contain uniform amount of clay contents and had lower clay contents compared to the bulk soil (BC). All samples have low amount of total carbon and nitrogen, organic carbon, and organic matter. The bulk soil is rich (BC)in carbon, organic matter, potassium and total nitrogen than the rhizosphere samples. In contrast, the rhizosphere soils are rich in sulfur, phosphorus, and nitrogen in form of ammonium (N-NH_4^+) than the bulk soil (Table 1).

Table 1. The physical and chemical properties of the rhizosphere and bulk soil samples.

Sample	AA	AB	CA	BC
Sand (%)	88.00±1.53	86.00±2.00	88.30±0.58	84.00±1.00
Silt (%)	2.00±1.00	4.00±1.00	2.00±1.00	2.00±1.00
Clay (%)	10.00±2.00	10.00±1.00	10.00±1.00	14.00±2.00
pH	6.94±0.04	6.80±0.03	6.58±0.03	6.63±0.01
S (mg/kg)	543±3.00	563±0.01	501±1.00	496.3±0.58
Org C (%)	0.36±0.03	0.24±0.02	0.30±0.02	0.63±0.01
Org M (%)	1.62±0.02	1.44±0.01	1.40±0.02	2.66±0.01
P (mg/kg)	46.68±0.01	41.57±0.03	48.59±0.01	7.40±0.01
K (mg/kg)	81.46±0.02	93.14±0.02	97.48±0.02	106.58±0.03
Na (cmol(+)/kg	10.23±0.03	9.74±0.04	8.49±0.00	8.52±0.03
N-NO_3^- (mg/kg)	2.29±0.03	3.48±0.01	2.22±0.01	2.01±0.01
N-NH_4^+ (mg/kg)	9.83± 0.01	12.64±0.03	10.33±0.01	5.90±0.01
Total C (%)	0.38±0.005	0.26±0.005	0.32±0.00	0.64±0.00
Total N (%)	0.04±0.002	0.03±0.003	0.03±0.00	0.06±0.00

Values are triplicate mean ± standard error. Samples AA, AB, and CA are rhizosphere soils while BC is bulk soil.

3.2. Sequence Processes

The sequences uploaded for sample AA were 5,090,756, which contained 905,850,636 base pairs, with 179 bp average length. Sample AB was made up of 5,684,005 sequences, with 973,653,572 bp at 175 bp average length, while sample CA contained 7,188,831 sequences counts, 1,114,054,964 bp at an average sequence length of 161 bp. The bulk soil (sample BC) was made up of 3,869,313 sequences, which contained 451,569,818 bp at average length of 176 bp. After QC has been executed, the retained sequences in samples AA, AB, CA and BC were 4,502,125, 5,042,523, 6,406,314, and 3,452,986 sequences respectively. The predicted protein features in the samples are 4,007,282 for sample AA, 4,383,253 in sample AB, 5,716,457 in Sample CA and Sample BC contained 3,045,886 while sample AA contained 2,337,594 (55.85%), unknown protein, 2,698,188 (57.61%) unknown protein was present in sample AB and the later rhizosphere soil (Sample CA) is made up of 3,468,264 (58.07%) unknown protein but bulk soil has 1,826,886 (57.18%) unknown protein.

3.3. Taxonomy of the microbiome in both rhizosphere and bulk sample

The Phyla in the soil samples are; Proteobacteria, Actinobacteria, Firmicutes, Acidobacteria, Bacteroidetes, Chloroflexi, Planctomycetes, Cyanobacteria, Verrucomicrobia, Basidiomycota, Ascomycota, Euryarchaeota, Crenarchaeota, Thaumarchaeota, Korarchaeota, Gemmatimonadetes, and Chlorobi, but Proteobacteria predominates in rhizosphere samples. The phylum Actinobacteria predominate in the bulk soil samples. The most

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prominent archaeal is Euryarchaeota and dominant in all samples while Korarchaeota is less dominant in the both rhizosphere and bulk soil. (Figure 1)

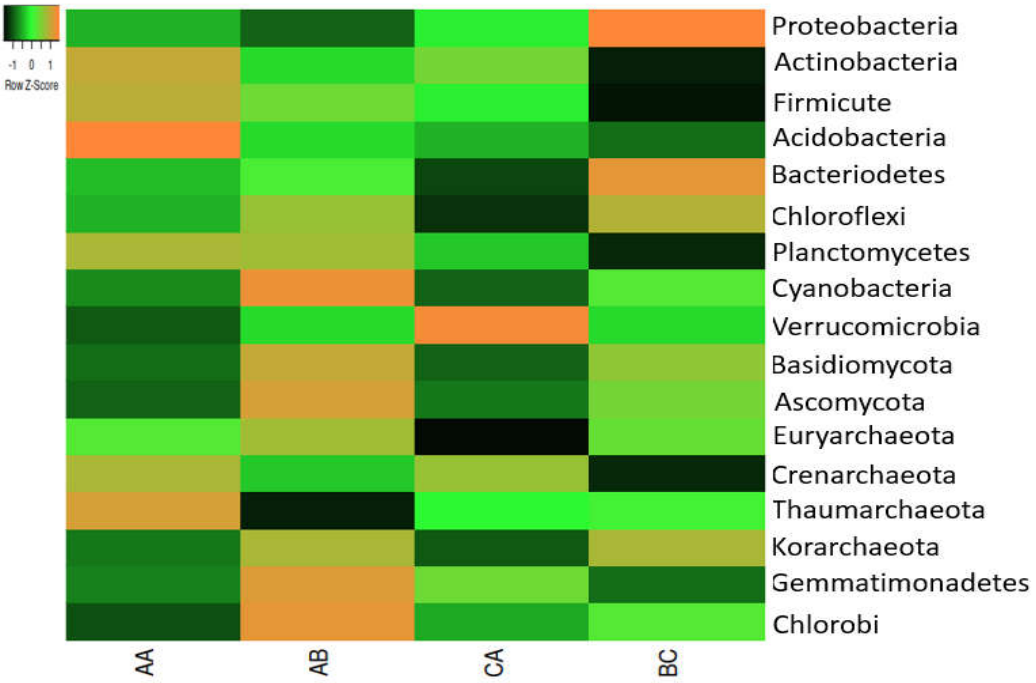

Figure 1. The heatmap representation of the relative abundances of the Phyla in the samples. The scale bar depicts a color saturation gradient based on relative abundances of the Phyla that have been z-score converted. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

The following genera were found in all samples as shown in figure 2 Bradyrhizobium, Streptomyces, Arthrobacter, Saccharopolyspora, Mesorhizobium, Geobacter, Rubrobacter, Frankia, and Burkholderia. Six genera- Geobacter, Nitrobacter, Burkholderia, Candidatus, Bradyrhizobium and Streptomyces are prominent in Sample AB while Sample CA has five prominent genera, such as Pseudomonas, Saccharopolyspora, Rhodopseudomonas, Methanothermococcus, and Saccharomyces. Rubrobacter and Frankia are prominent in both sample AB and the bulk soil (sample BC) but Methylobacterium is prominent in sample AA and the bulk soil while Methanothermococcus is less prominent in all samples (figure 2). The distribution of the genera from various sampling sites is presented in figure 4.

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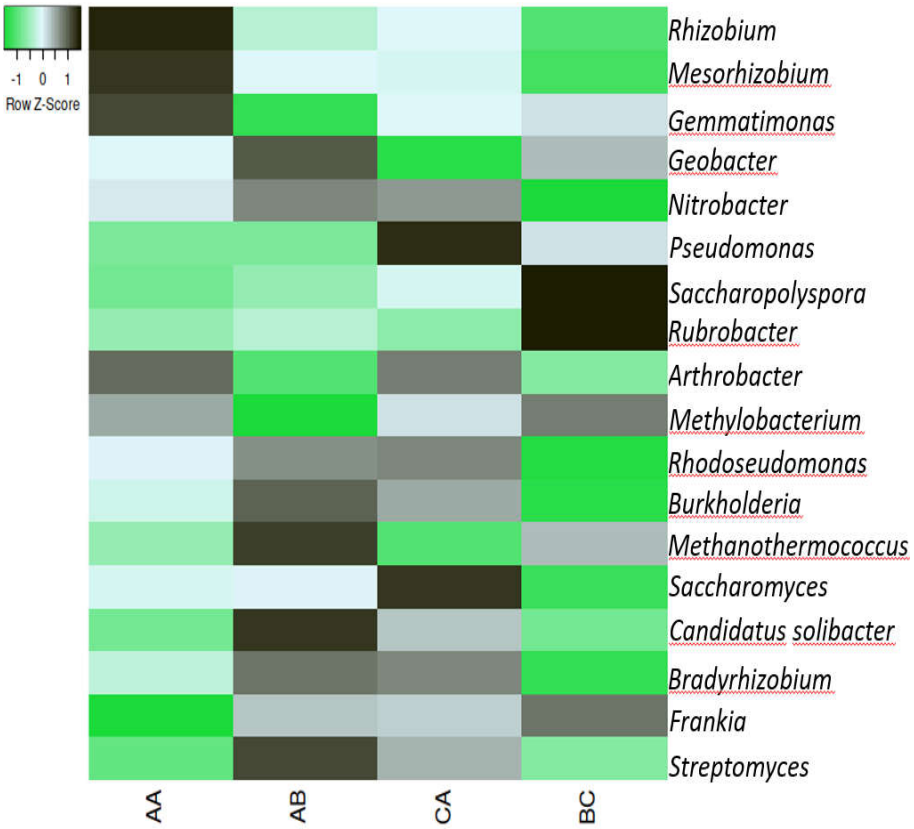

Figure 2. The heatmap representation of the relative abundances of the genera in the samples. The scale bar depicts a color saturation gradient based on relative abundances of the genera that have been z-score converted. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

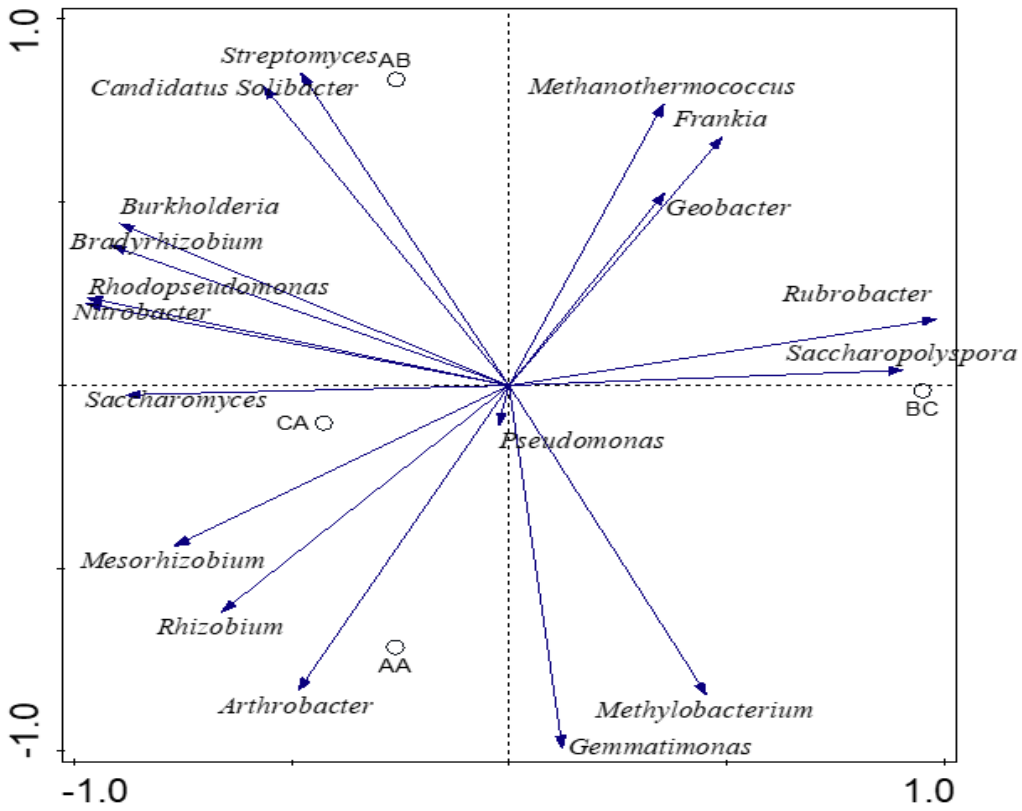

Figure 3. PCA analysis of the distribution of the genera across the sampling sites. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

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3.4. Functions of microbiomes in the soil

The functions of the microbiomes were revealed at level 1 in all the sampled soil with the help of SEED Subsystem hierarchical gene annotation. Twenty-one (21) functions were selected as shown in Figure 4. Fourteen (14) functions were prominent in sample AA and sample CA. The prominent functions were carbohydrates, vitamins, amino acid and derivatives, protein metabolism, cofactor, prosthetic groups, lipid, membrane transport, cell wall and capsule, RNA metabolism, nucleoside and nucleotides, pigments, fatty acid, and isoprenoids, phosphorus metabolism, stress response, nitrogen metabolism, motility and chemo taxis, regulation and signalling. Twelve (12) functions, such as carbohydrates, amino acid and derivatives, vitamins, protein metabolism, cofactor, prosthetic groups, pigments, lipid, membrane transport, cell wall and capsule, RNA metabolism, nucleoside and nucleotides, fatty acid, and isoprenoids, stress response, nitrogen metabolism, phosphorus metabolism were prominent in sample AB as well as the bulk soil (sample BC).

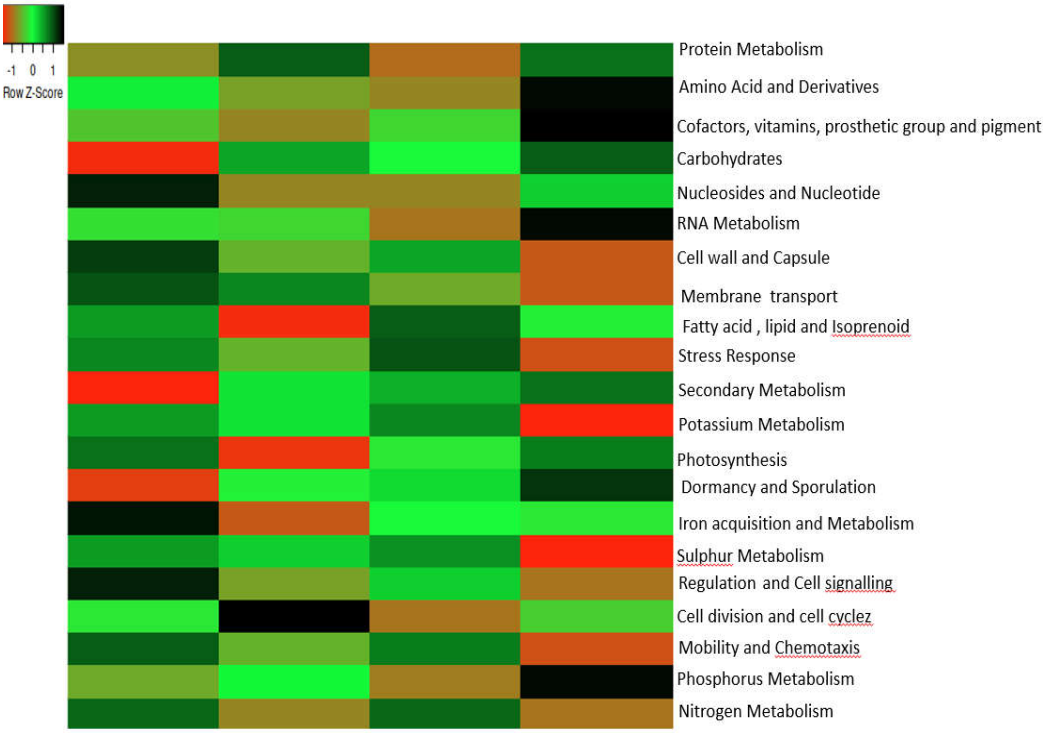

Figure 4. Metabolic functions at level 1 in the SEED subsystem. The scale bar depicts a color saturation gradient based on relative abundances of the functional categories that have been z-score converted. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

Carbohydrate is more prominent in all the samples, with sample BC being the most prominent in both carbohydrate and amino acid while sample AA is less prominent in carbohydrate and sample CA less prominent in amino acid. Protein metabolism is more prominent in sample AB but less prominent in sample CA. The four samples are almost the same in RNA metabolism, nucleotide and nucleoside with sample AB being less prominent but nucleoside and nucleotide is more prominent in sample BC. PCA (principal component analysis) was used to determine the functional categories distribution in both rhizosphere and bulk soils as shown in Figure 5.

3.5. Microbiomes indices of functions in Soybean soil samples

Alpha diversity of the functional categories for microbiomes in soybean rhizosphere for level 1 were used to determine the evenness, Simpson's and Shannon values **(Table 2).** The Shannon and Simpson's indices were higher in the rhizosphere samples compared to the bulk soil sample (Table 2). There are no significant differences obtained in the values

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in the diversity levels among the samples using the Kruskal-Wallis test at p>0.05. The relative abundances of the functional categories within the Subsystems at level 1 were visualized using the principal coordinate analysis (PCoA) plot (Fig. 4) while the R- and p-values of 0.51 and 0.01, respectively, were obtained from the analysis of similarity (ANOSIM) performed.

Table 2 - Diversity indices of functional categories in both the rhizosphere and bulk soil samples.

Diversity	Sample AA	Sample AB	Sample CA	Sample BC
Shannon	1.851	1.947	1.815	1.763
Simpson	0.6712	0.7152	0.6652	0.6408
Evenness	0.1027	0.113	0.08902	0.09257

AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample

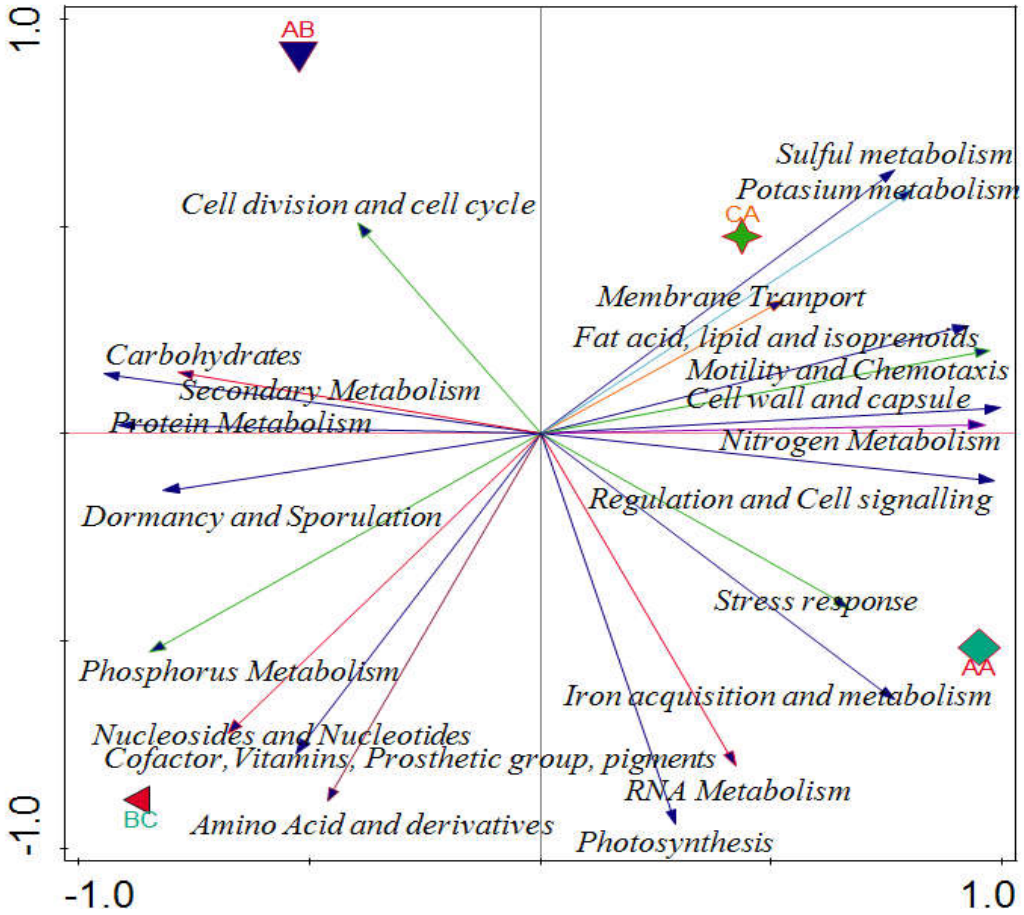

Figure 5. PCA analysis of functional categories of microbial communities in the rhizosphere and bulk soil samples showing the distribution of the microbial functions. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

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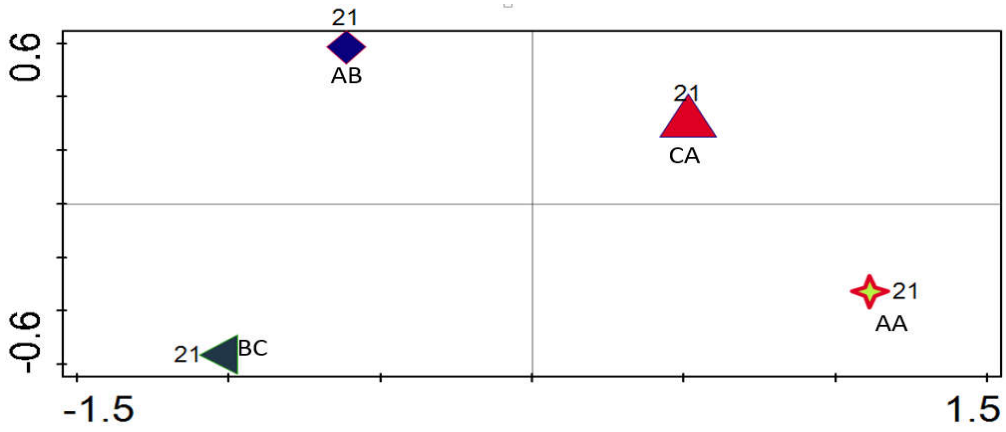

Figure 6. PCoA (Principal coordinate analysis) of functional categories of microbial communities in the rhizosphere and bulk soil samples. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

The relative abundance of the functional categories in level 2 was used to develop a bar chart (Figure 7**)** with dominant functions based on the relative abundance. Relative abundance of all the soil samples were close to one another in nearly all functions but plant-prokaryote DOE project, protein biosynthesis, lysine, threonine, methionine, and cysteine (amino acid) of the bulk soil (BC) are more pronounced, although sample AB revealed high relative abundance values of central carbohydrate metabolism, while DNA repair of sample AA, which is one of the rhizosphere soil samples was more prominent than others.

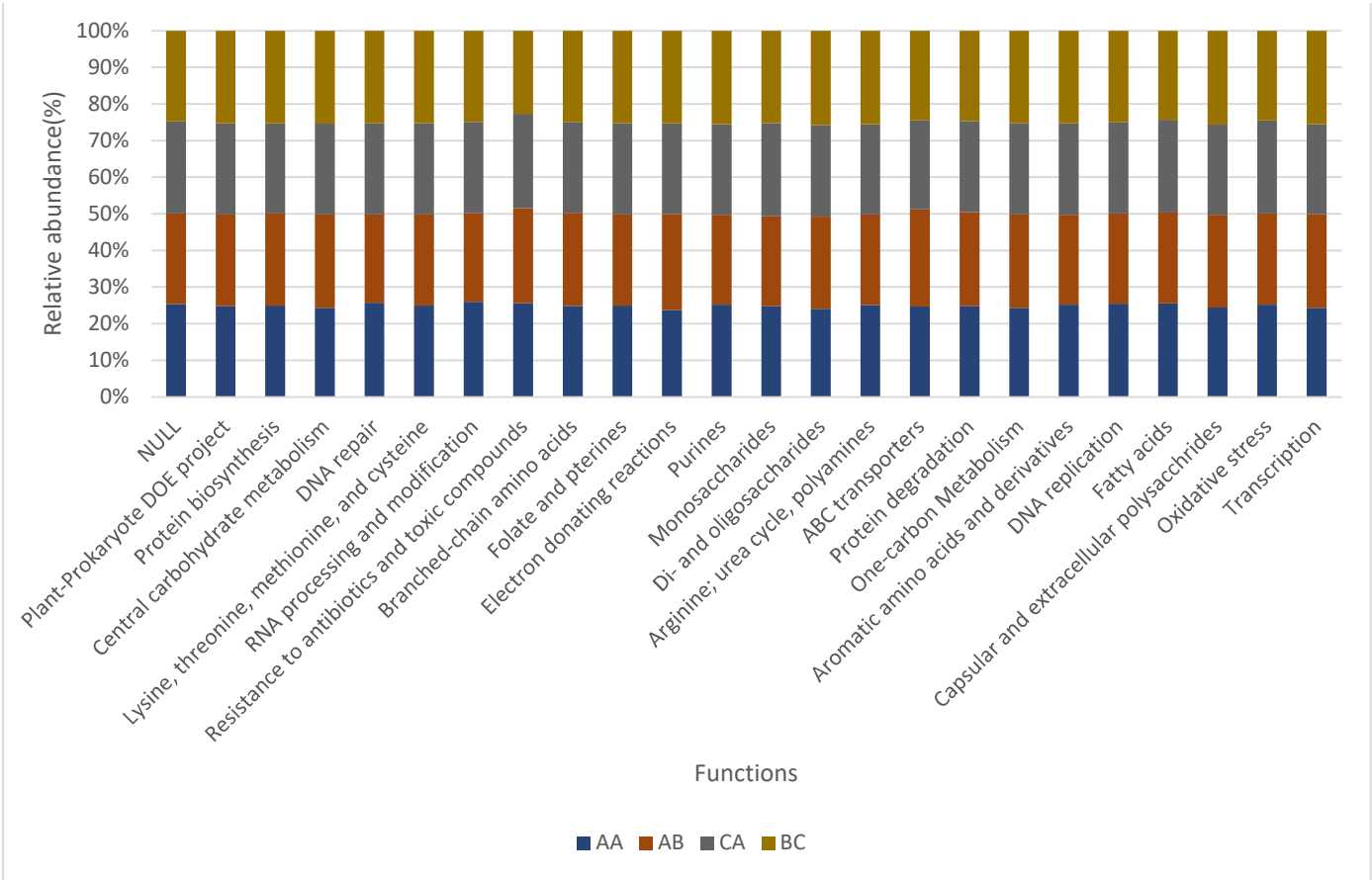

Figure 7. Bar-chart for selected functions in Level 2. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

3.6. Effects soil properties on the functional categories of the microbiome in the soil samples

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Canonical correspondence analysis (CCA) was used to evaluate the relationship that exists between the soil properties of soybean and the functional levels of the microbial communities (Figure 8). Environmental conditions of the forward selection that is good for explaining variations showed phosphorus was more significant compare to other properties. Phosphorus has 0.718 p-values with 64.3% percentage contribution in the variation, which is the same as the percentage explained (**Figure 8).** Contribution of other soil properties were gotten as stated in **Table 3 a**nd they are represented by their respective vector arrows **(Figure 8**).

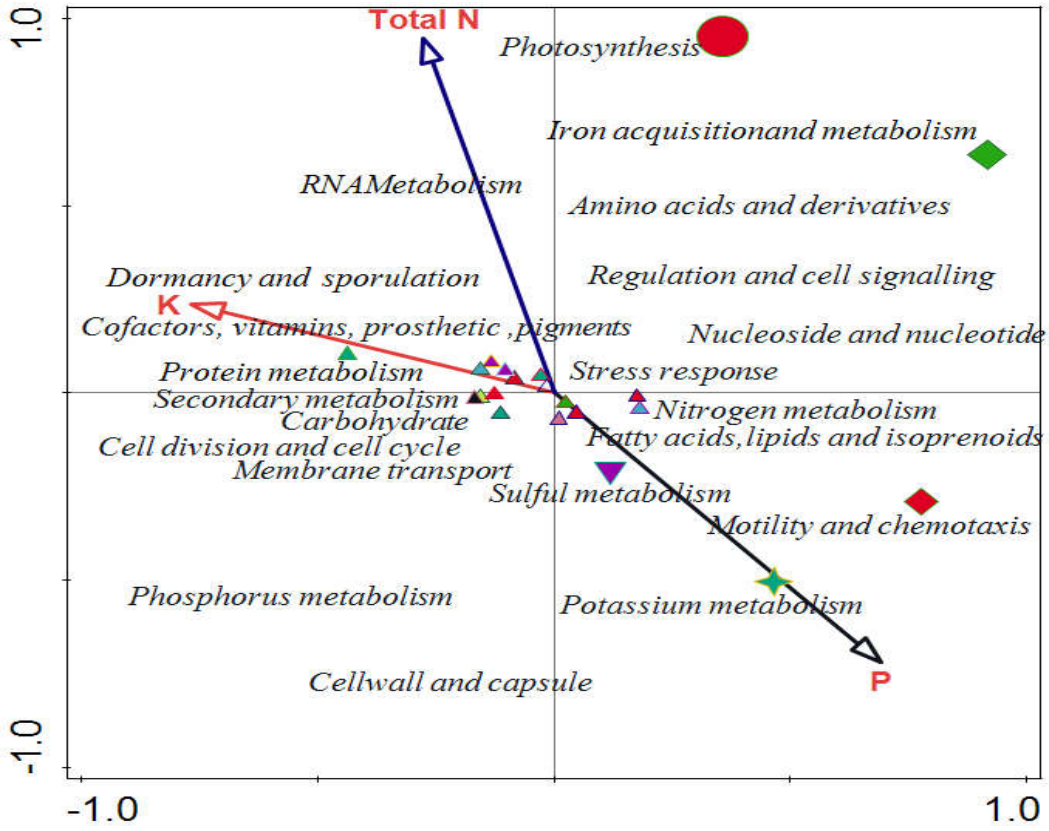

Figure 8. Canonical correspondence analysis showing the effect of physicochemical parameters on functional categories in the rhizosphere. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

Table 3. Selection of variables in the environment that best explain the differences in functional composition of the microbes' habitat using canonical correspondence analysis**.**

NAME	Explain%	Contribution%	Pseudo-F	P-value
Total N	**30.8**	**30.8**	**0.90**	**0.91**
P	**64.3**	**64.3**	**13.2**	**0.718**
K	**4.9**	**4.9**	**<0.1**	**1.**

3.6. Pathways revealing the functions of microbiomes living in the soil samples

The subsystem at level 3 revealed sequences involved in several pathway involved in fatty acid degradation regulons, serine glyoxylate cycle, DNA repair, inorganic sulphur assimilation, ammonia assimilation, bacterial chemotaxis, cobal-zinc-cadmium resistance, and peptidoglycan biosynthesis (Figure 9). cobalt-zinc-cadmium resistance, RNA polymerase bacterial, macromolecular synthesis peron, translation elongation actor G family, cAMP signaling in bacteria, terminal cytochrome C oxidases, bacterial chemotaxis, ammonia assimilation, type VI secretion systems, multidrug resistance efflux pumps, regu-

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lation of oxidative stress response, peptidoglycan biosynthesis, inorganic sulfur assimilation, staphylococcal pathogenicity islands SaPI, At3g50560 and ribonucleotide reduction. Fatty acid degradation regulons were more pronounced compare to other functions in that level with sample AA (rhizosphere soil sample) having the highest relative abundance while Bulk soil sample (BC) showed the least value.

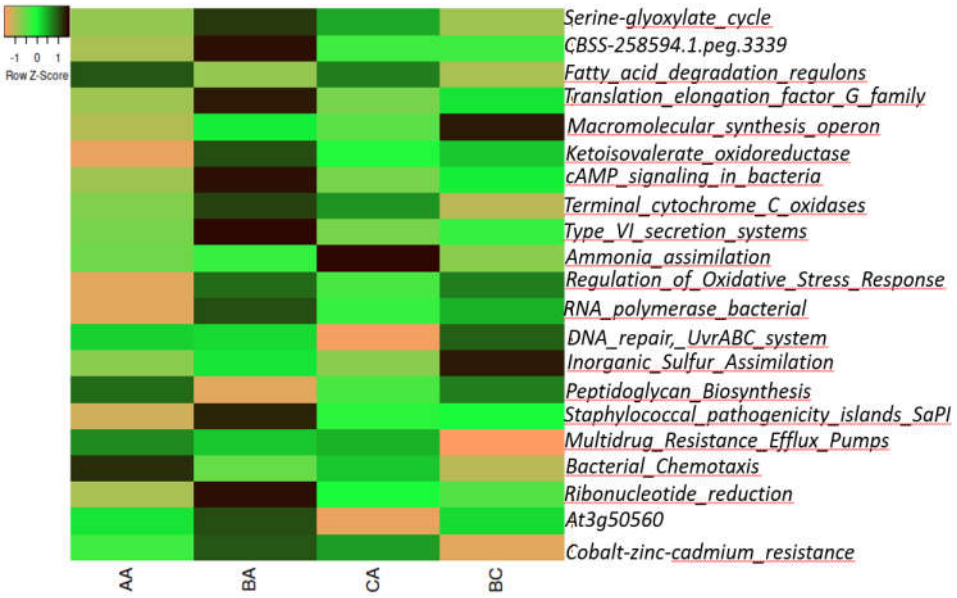

Figure 9. Pathway of selected pathways at level 3 susbsystem. The bar shows the saturated color gradient base on the relative abundance in Z-score which was gotten from metabolic pathways of the microbiomes in that habitat. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

From respiration (cell metabolism) pathway, terminal cytochrome C oxidases, formate dehydrogenase, and hydrogenases are more abundant in sample AB compared to sample AA that has the least relative abundance in formate dehydrogenase and hydrogenases. The relative values of sample AB and bulk soil (BC) were higher (0.15%) in respiratory complex I, while sample AA and CA were the same values (0.14%). The relative abundance of 0.1% was gotten from F0F1-type ATP synthase, formate dehydrogenase, respiratory dehydrogenases 1 and anaerobic respiratory reductases in all samples. However, 0.06%, 0.04 % and 0.03% were values for both the rhizospheric and bulk soil samples in biogenesis of cytochrome c oxidases, carbon monoxide dehydrogenase maturation factors and ubiquinone menaquinone cytochrome c reductase complexes accordingly. The highest value 0.03% was recorded in NiFe hydrogenase maturation for sample AB, CA, and bulk sample. Quinone oxidoreductase family, succinate dehydrogenase, and trim ethylamine N-oxide (TMAO) reductase were observed to have the same relative value of 0.01% in all soil samples. The value observed (0.004%) in formate hydrogenase metabolic pathway in all the rhizospheric soy samples was also recorded in sample AA and CA of V-Type ATP synthase metabolic pathway. However, the relative abundance of sample CA has its only highest value (0.003%) in CO dehydrogenase metabolic pathway with the lowest value (0.001%) from the bulk sample but relative abundance (0.005%) of Membrane bound hydrogenases metabolic pathway was the highest in bulk soil with least value (0.003%) in sample AB and CA (Figure 10a**).**

Fatty acids, lipids, and isoprenoids pathways showed the same relative value of 0.1% in polyprenyl synthesis, glycerolipid and glycerophospholipid metabolism in bacteria, polyhydroxybutyrate metabolism, also the same value of 0.03% were observed in isoprenoid biosynthesis and archaeal lipids while relative abundance of 0.01% was the same in triacylglycerol metabolism, polyprenyl diphosphate biosynthesis microorganisms. This same value was observed in phospholipid and fatty acid biosynthesis elated

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cluster except in bulk soil with 0.003%. The highest value (0.65%) in Fatty acid degradation regulons was observed in sample AA with least value (0.60%) in the bulk sample (BC).

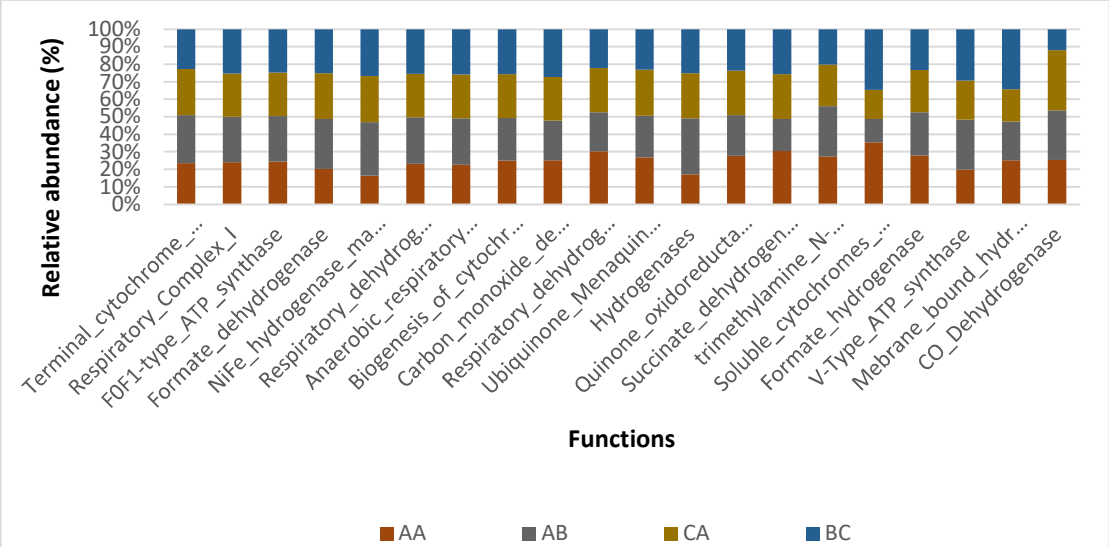

Figure 10. a: Cell metabolism (respiration) pathway at level 3. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

Carotenoids metabolic pathways has same relative abundance of 0.08% in all the rhizosphere soil samples but it is more abundant in bulk soil. However, bulk soil sample showed least value in cholesterol catabolic operon and polyunsaturated fatty acids synthesis but the values of rhizosphere sample were the same for all samples. Sample AA showed highest relative value (0.002%) in fatty acid biosynthesis FASI while other samples have the same value of 0.001% **(**Figure 10b**).**

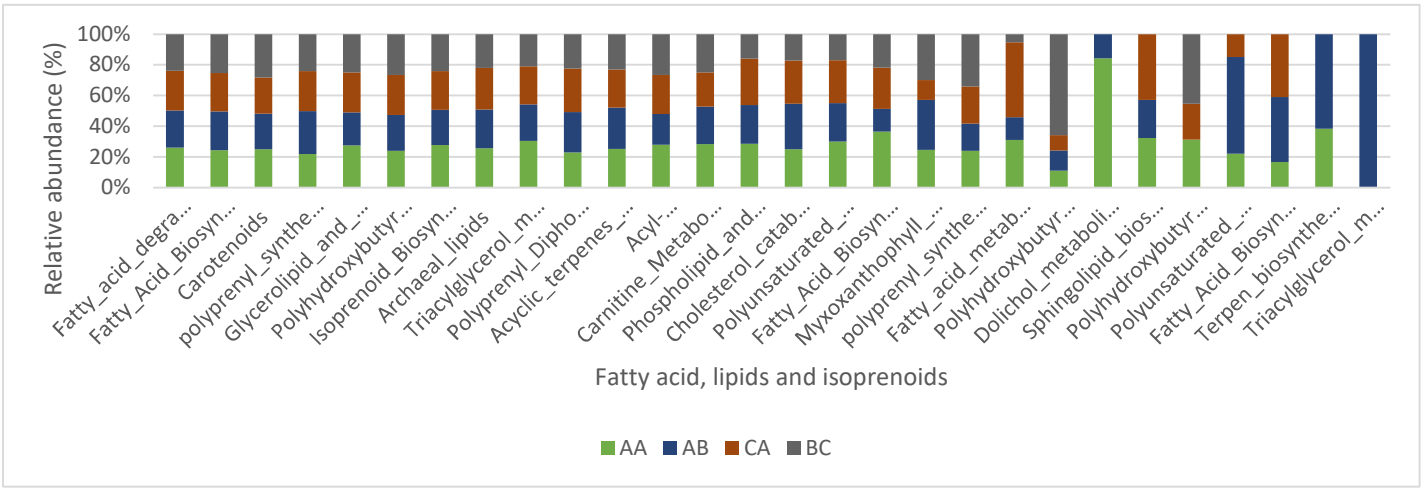

Figure 10. b: Pathway for selected fatty acid, lipids and isoprenoids metabolism. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

The pathways needed in sulfur metabolism showed that alkanesulfonates utilization, thioredoxin-disulfide reductase and release of dimethyl Sulphide (DMS) from dimethyl sulfonic propionate (DMSP) have 0.01%,0.04% and 0.001% respectively in all soil samples both rhizosphere samples and bulk samples. Bulk soil sample showed highest value (0.26%) in inorganic sulfur assimilation pathway while sample AA and CA were observed to have the least relative value (0.23%). However, it was observed that DMSP breakdown pathway has least value in sample AA while sample BC (bulk soil) has the highest. Galactosylceramide and sulfatide metabolism, sulfur oxidation, utilization of glutathione as

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sulfur source, sulphate reduction-associated complexes and DMSP breakdown were higher in sample AB. In galactosylceramide and sulfatide metabolism sample CA and BC were said to have the same relative value of 0.03% and the least value from sample AA but in sulfur oxidation and utilization of glutathione as a sulfur source sample AA, CA and BC have least relative value 0f 0.05% and 0.04 % respectively. Sample AA and BC have the highest value (0.003%) in L-Cystine Uptake and Metabolism and the least (0.002%) from sample BA and CA. Alkane sulfonate assimilation and taurine utilization pathways have their highest values (0.04% and 0.03% respectively) in samples AA, AB and CA. F420-dependent sulphite reductase pathway displayed lowest relative values in all samples (Figure 10c).

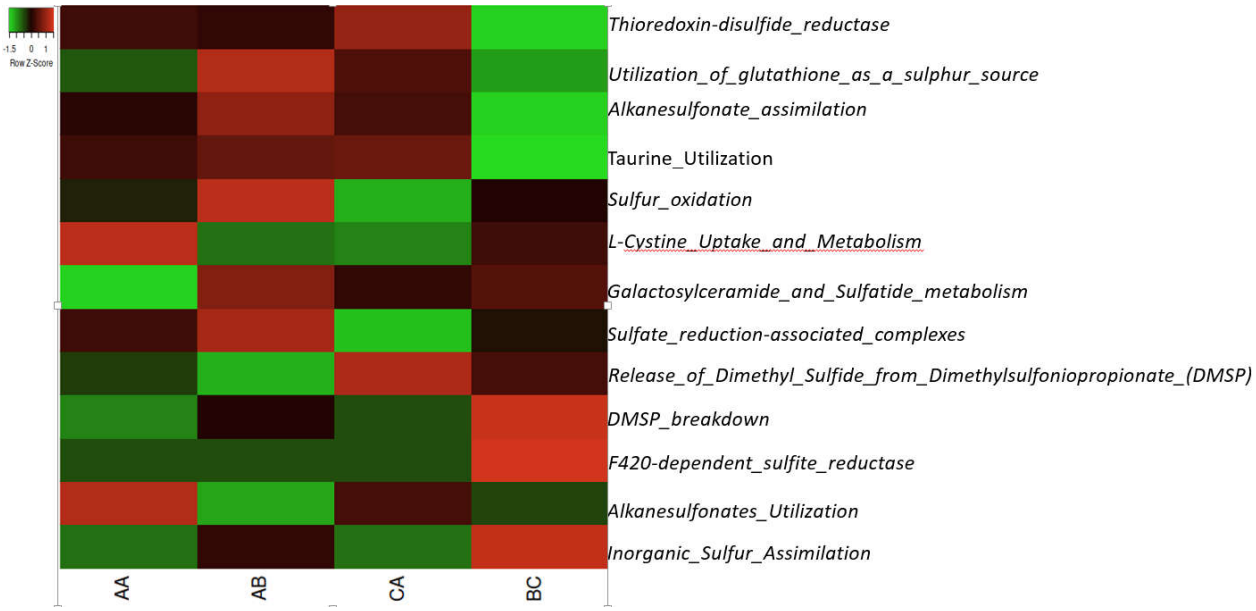

Figure 10. c: The sulfur metabolic pathway at subsystem level 3. The bar shows the saturated color gradient base on the relative abundance in Z-score which was gotten from metabolic pathways of the microbiomes in that habitat. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

Nitrogen metabolism pathway unveiled common relative abundance values (0.01%) in amidase clustered with urea and nitrile hydratase functions and nitrosative stress but ammonia assimilation, denitrification, cyanate hydrolysis, and nitrilase (0.27%, 0.02%, 0.003% and 0.001% respectively) in all soil samples (Figure 10d). Sample AA and AB showed the same relative values of 0.15% in nitric oxide synthase pathway with sample CA having the highest value of (0.16%). Nitrate and nitrite ammonification showed highest value in Sample AA and CA (0.12%) but the least from sample BA (0.10%). The least relative values (0.03%) for allatioin utilization metabolism was revealed in the rhizosphere samples (AA, AB and CA). However, high nitrogen fixation was observed in the samples of soil attached to the root (rhizosphere). Sample AA and AB were the highest relative value (0.003%) in CBSS-280355.3.peg.2835 while sample CA and bulk soil were the least value (0.002%).

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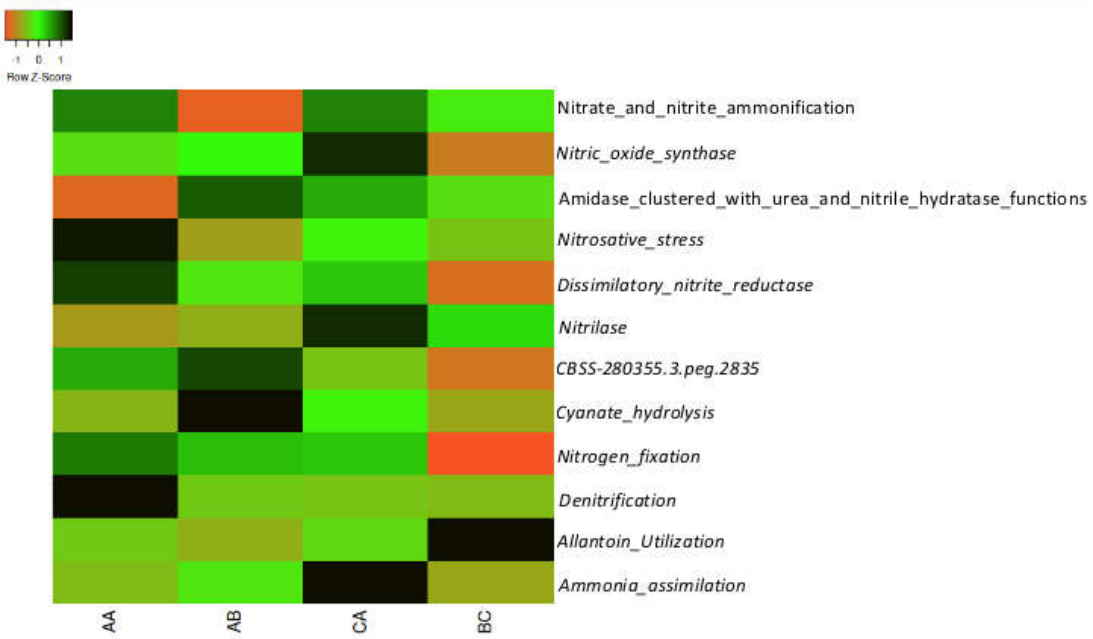

Figure 10. d; Pathway for nitrogen metabolism at level 3. The bar shows the saturated color gradient base on the relative abundance in Z-score which was gotten from metabolic pathways of the microbiomes in that habitat. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

In metabolism pathway for amino acids in Figure 10e, 9 were selected from branched amino acids and 10 from aromatic amino acids- histidine degradation, histidine biosynthesis, ketoisovalerate oxidoreductase, branched-chain amino acid biosynthesis, isoleucine degradation, branched chain amino acid degradation regulons, leucine degradation and HMG CoA metabolism, HMG CoA Synthesis. valine degradation, isoleucine degradation, leucine biosynthesis, chorismate: intermediate for synthesis of tryptophan, PAPA antibiotics, PABA, 3-hydroxyanthranilate and more, pathway for synthesis of aromatic compounds (DAHP synthase to chorismate), phenylalanine and tyrosine branches from chorismate, chorismate synthesis, tryptophan synthesis, aromatic amino acid degradation, tryptophan catabolism, aromatic amino acid interconversions with aryl acids, bacilysin biosynthesis, and indole-pyruvate oxidoreductase complex. 0.1% was observed in all the samples in the following pathways- ketoisovalerate oxidoreductase, branched-chain amino acid biosynthesis, isoleucine degradation, branched chain amino acid degradation regulons, histidine degradation, branched chain amino acid degradation regulons, leucine degradation and HMG-CoA metabolism, HMG CoA synthesis, histidine biosynthesis, leucine biosynthesis and chorismate: intermediate for synthesis of tryptophan, PAPA antibiotics, PABA, 3- hydroxyanthranilate and more while 0.02% was in all the samples for both aromatic amino acid degradation and isoleucine degradation.

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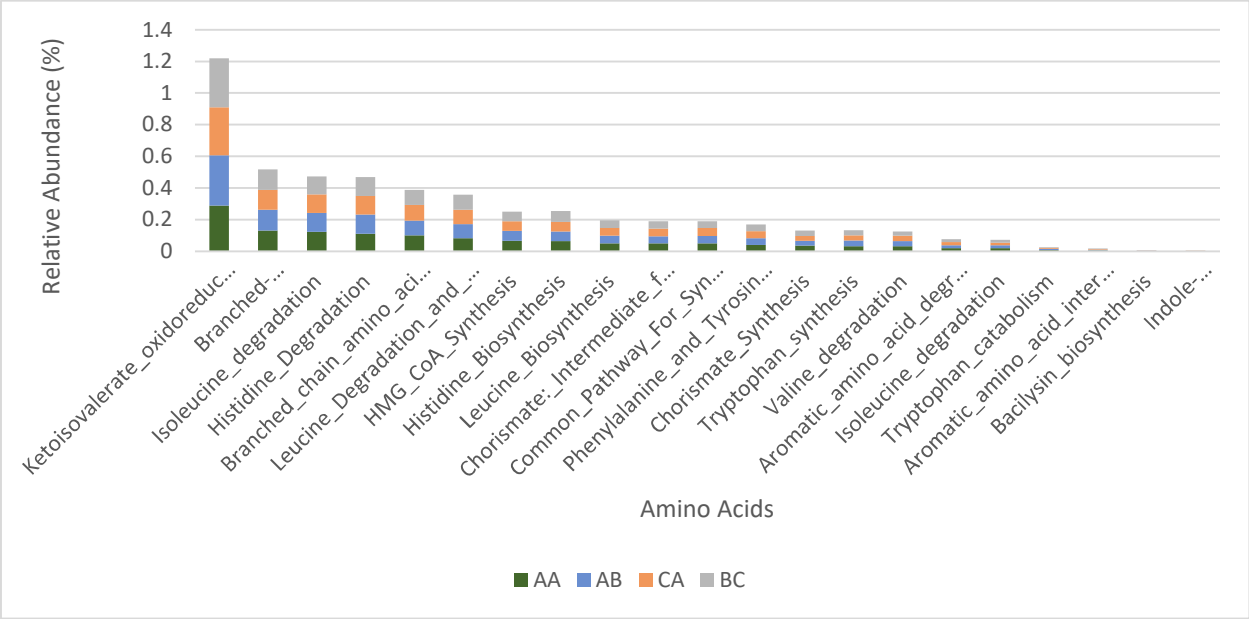

Figure 10. e: Bar-chart showing the pathway for selected histidine, aromatic and branched amino acids. AA, AB, and CA are rhizosphere soil samples while BC is bulk soil sample.

The highest percentage (0.3%) in amino acid metabolism pathway was observed in all the samples under Ketoisovalerate oxidoreductase pathway but Phenylalanine and Tyrosine Branches from Chorismate pathway showed 0.04% in both rhizosphere and bulk soil samples, which is the least percentage in common pathway for synthesis of aromatic compounds (DAHP synthase to chorismate) in bulk soil (sample BC) while the rhizosphere samples (AA, AB and CA) with 0.05%. had higher percentages. Chorismate synthesis, tryptophan synthesis and valine_ degradation revealed the higher percentage (0.04%) in sample AA, BA and CA respectively with lower value of 0.03% in sample BA, CA and BC in chorismate Synthesis, sample AA, CA and bulk soil in tryptophan synthesis and AA, AB and BC.

4. Discussion

Rhizosphere microbial communities, which are crucial for crop growth and yield, have been found to be influenced by plants. The rhizosphere is home to microbial community that play critical role in plant growth and productivity(Meena et al., 2018). In the current study, the functional profile of the microbial communities from the rhizosphere of soybean were examined. In this method of classification, the genes found in a given sample are organized into subsystems while the metabolic pathways are constructed hierarchically (Overbeek et al., 2005). Specific pathways make up the lowest organizational levels, whereas catabolic and anabolic microbial functions define the highest organizational levels. In this study, Proteobacteria, Acidobacteria, Bacteroidetes, and Actinobacteria were the dominant microbial community in soybean rhizosphere, which is consistent with earlier reports about the soybean rhizosphere microbiome (Mendes et al., 2014; Sugiyama et al., 2014). Proteobacteria were consistently enriched in the rhizosphere of soybean in all the samples. This agrees with the idea that copiotrophs, such as Proteobacteria and Actinobacteria are more abundant in nutrient-rich environment, such as the rhizosphere (Pérez-Jaramillo et al., 2016). This indicate that the soybean rhizosphere is a rich environment for the growth and activity of microbial group.

Sequence alignment to a curated, sizable database of annotated sequences is typically required for functional profiling of metagenomics sequences in order to find similar matches (Mendes et al., 2017). Functional investigation of the rhizosphere microbiome along with compositional characterization may be more instructive for understanding mi-

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crobiome assembly and boosting applications for sustainable agriculture, taking into account the functional redundancy between various bacteria community (Liu et al., 2019). Alpha diversity from Shannon, Evenness and Simpson obtained from the samples and confirmed with Kruskal-Wallis test with p-value 0.042. Index values for Shannon in all the sample locations were in line with the 2.81 theoretical limits as established by Dinsdale et al. (2008) but lower than that gotten by Li et al. (2010). Since, dominant functions are few in level 1 and it caused the evenness indices to be lower such as vitamins, amino acid and derivatives, protein metabolism, cofactor, prosthetic groups, lipid, membrane transport, cell wall and capsule, RNA metabolism, nucleoside and nucleotides, pigments, fatty acid, and isoprenoids, phosphorus metabolism, stress response, nitrogen metabolism, motility and chemotaxis, regulation and signaling. Dominant pattern's difference showed the importance of some functions over the others in the metagenomes. Beta diversity of the study was examined with PCoA usingANOSIM to establish the difference within the samples as well as composition results in 0.58 and 0.01 values for R and pvalue respectively. The differences in microbial functions between the rhizosphere and bulk samples showed the variations that existed between samples. The variation cumulative value between functional categories of 72.55% and 94.04% and Eigen values of 72.55% and 21.48%in axis 1 and 2 respectively (Fig 6). The variation cumulative value between functional categories in this study is lower than 73.2% gotten by Kumar et al. (2017)., Principal component analysis was used to test the variation between functions and metabolic potential in the samples.. Total variation of 0.029%, explanatory variables of 47%, and adjusted explained variation of 20.50%, which shows how omics can determine metabolic function of the microbiomes in a particular sample. The point that each field was shown in figure 6 shows the combined sequence in conjunction with the functions of the subsystem. The pointed arrows reflect the main distribution of the metabolic function in each sample. The metabolic function that is of high importance to the microorganisms in the specified field can be seen clearly in figure 6. Such as the difference demarcated by some metabolic functions found in field CA, AB and AA include- sulfur metabolism, membrane transport, fatty acids, lipids and isoprenoids, motility and chemotaxis, cell wall and capsules, nitrogen metabolism, regulation and cell signaling, stress response, iron acquisition and metabolism, RNA metabolism and photosynthesis from those found in field BC (bulk soil) in figure 4.

Higher relative abundance was observed in the sequences associated with carbohydrate, amino acids and derivatives, protein metabolism, cofactor, vitamins, prosthetic group, pigments, DNA metabolism, and respiration in subsystem level 1 (Figure 4). Carbohydrate contains carbon, hydrogen and oxygen, the released energy is always very important to the microbiomes for survival. Since carbohydrate breaks down to disaccharides, monosaccharides and polysaccharides. Soil microorganisms depend on various carbon sources like glucose, maltose, rhamnose, and inositol for development and survival since they use carbon as a source of energy for their metabolism and growth. The abundance of functional categories related to carbon dioxide fixation, organic acids, sugars alcohols, amino sugars and glycoside hyrolases at level 2 and the presence of metabolic pathways for the serine glyoxylate cycle, isobutyryl-CoA to propionyl-CoA module, methylglyoxal metabolism, acetone butanol ethanol synthesis and, glycolysis and gluconeogenesis in the samples are evidence of carbon utilization from different sources. Mendes et aland Dubey et al. reported high relative abundance of carbohydrate in soybean rhizosphere (Dubey et al., 2022; Mendes et al., 2014). The findings showed that the microbial communities in our samples help plants in carbon acquisition through various metabolic pathways (Prabha et al., 2019). Amino acids are also used by microbes as source of energy for survival in nutrient-poor conditions and in soils with little organic matter (Gianoulis et al., 2009). The abundance of pathways related to motility, chemotaxis, dormancy, and sporulation are needed as response mechanisms of microbial survival in nutrient-deprived soils in order to survive environments with low nutrients. Amino acid as source of energy is needed in symbiotic interaction that occurs in the root of the plant. The

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effects of microbial metabolism of amino acid and some nutrients (phosphorus, nitrogen and sulfur) cannot be underrated in symbiotic interaction that occurs in the rhizosphere (Rigobelo & Baron, 2021).

The process of fixing nitrogen in the symbiosis of soybean and rhizobia increases soybean yield (Ohyama et al., 2017). In this study, the relative abundance of amino acid metabolism was relatively the same for the four samples, just as it has been reported by Liang et al. (2014). Xu et al. reported the availability of amino acid to microbes in plants' rhizosphere for enhanced plant- microbes relationship (Xu et al., 2018). Higher nitrogen metabolism was noted in the rhizosphere samples compared to the bulk soil. This metabolism is being regulated by gram positive bacteria in the nodules of legumes through bacterial fixation (Jacoby et al., 2020).

Mineral elements, such as phosphorus, iron acquisition, nitrogen, potassium and sulfur are essential for plant growth (Fatima et al., 2007;Kathpalia, 2018) and they were discovered in both bulk and rhizosphere soils metagenomes. These elements are essential for the growth and health of plants. As a result, our findings imply that the microbial communities that are present in the samples assist in the availability of crucial nutrients for the growth and development of soybean.

All the samples show dominance of these sulfur metabolic pathways; - inorganic sulfur assimilation, galactosylceramide and sulfatide metabolism, alkanesulfonates utilization, sulfur oxidation, utilization of glutathione as a sulfur source, thioredoxin disulphide reductase, alkane sulfonate assimilation, taurine utilization, L-cystine uptake and metabolism, release of dimethyl sulphide (DMS) from dimethyl sulfoniopropionate (DMSP), and sulphate reduction-associated complexes. Numerous roles that microorganisms from various environments play in the enzymatic metabolism of sulfur have been described in studies (Yin et al., 2014; Zhang et al., 2016). This study shows that the microbiomes can produce sulfur metabolic genes or protein enzymes like sulfur carrier proteins adenylyl transferase *ThiF* as cofactors, to prevent soybean plants during biosynthesis of thiamine in both rhizosphere and bulk soil samples, as well as sulphate and thiosulfate import ATP-binding protein *CysA* (EC 3.6.3.25) to protect soybean plants from stress response during the uptake of selenite. The findings in this study imply that the microbes living in the rhizosphere thioredoxin and use glutathione, a peptide containing L-cysteine, as a cofactor to provide defence against oxidative stress in plants (Takagi & Ohtsu, 2016).

The physicochemical result of soil samples showed that all the samples contain sulfur in a good amount, which work together with the microbiomes to make enough sulfur needed by the plant., This established that microbiomes in the rhizosphere promote plant growth as well as cycling of nutrient present in the rhizosphere as reported by Anandham et al. (2007). We discovered that the chemical characteristics of the soil had an impact on microbial functions using the canonical correspondence analysis plot. According to the graph plot, total N and K were positively correlated with RNA metabolism, dormancy and sporulation, and protein metabolism while P positively correlated with nitrogen metabolism, fatty acids, lipids and isoprenoids, sulphur metabolism, and motility and chemotaxis. Potassium was identified as the soil parameters with the greatest influence on the composition of the microbial functional diversity in the CCA plot utilizing SEED subsystems at level 1, providing 64% of the variability in the functions. According to studies, the characteristics of soils are the primary causes of the variations seen in the structural diversity of soil microbial communities (Hanson et al., 2012), whereas the physical and chemical characteristics of soils also influence the functional diversity of microbial communities (Klimek et al., 2016). It is clear from the findings in this study that the chemical properties of the soil played a role in determining the relative abundance of microbial functions in the sampling area.

The sequences of respiration pathway were observed as well in figure 10a. Respiration in level 2 was revealed in functions like electron donation, reverse electron transport, synthases of ATP and sodium ion coupled energetics. While at subsystem level 3, terminal cytochromes C oxidases, respiratory complex I, F0F1 type ATP synthase, *NiFe* hydrogen-

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ase maturation, respiratory dehydrogenases 1, anaerobic respiratory reductases, biogenesis of cytochrome c oxidases, carbon monoxide dehydrogenase maturation factors, respiratory dehydrogenases 1, ubiquinone menaquinone-cytochrome c reductase complexes, hydrogenases, Quinone oxidoreductase family, Succinate dehydrogenase, trimethylamine N-oxide (TMAO) reductase, soluble cytochromes and functionally related electron carriers, formate hydrogenase, V-Type ATP synthase, Membrane bound hydrogenases and CO dehydrogenase some of these pathways are present in equal amount in all samples while others are predominant in sample AB.

The microbes associated with nitrogen metabolism were observed at level 1 and it was discovered to be in abundant in sample CA compared to other samples. Nitrogen metabolism described how nitrogen is cycled in the rhizosphere. Microbiomes in the soil helps to improve the yield of crop with the help of nitrogen cycling (Wang et al., 2021). At level 2, nitrogen metabolism was not found while at level 3- ammonia assimilation, nitric oxide synthase, nitrate and nitrite ammonification, allantoin utilization, denitrification, nitrosative stress, amidase clustered with urea and nitrile hydratase functions, nitrogen fixation, cyanate hydrolysis, nitrilase and dissimilatory nitrite reductase were observed. These metabolic pathways are very important in the formation of amino acid and protein metabolism. The Amino acids were observed in subsystem level 1, level 2 but at level 3 the relative abundance in aromatic amino acids, branched acids amino acids and histidine were considered (Fig 10e). Amino acids are proteins subunit that are important in biosynthesis pathways. When single amino acid or combination of many acids are deficient it can lead to stunted growth in plants.

5. Conclusion

Shogun sequencing provides information about the function roles of different microbial populations in the rhizosphere in fostering plant development and health. Through the manipulation of microbes, metagenomics offers new prospects for the development of effective and environmentally acceptable methods of enhancing crop productivity. Finding from previous studies have demonstrated that the rhizosphere of plants is home to a variety of microbial population that aid growth and development of plants. The rhizosphere soils and bulk soil were dominated by identical microbial functional categories using shotgun analysis of the microbial functions in the soybean soils. This study revealed the functions of all the microbiota in the soil attached to the root part of soybean and the bulk soil. The pathway with the highest relative abundance at level 1 is carbohydrate metabolism, clustering-based subsystems (pathway with no established function), and amino acids and derivatives. It is anticipated that the microbial communities in these soils will assist plant growth, development, and survival in their diverse habitats due to the availability of these services in the soils. We also found a large number of metabolic pathways, including those for sulphur, nitrogen and secondary metabolism. The discovery of novel culturable methods to isolate these soil microorganisms with strong functions for biotechnological and agricultural use could result from a complete understanding of these functions and harvesting such special functional advantages for increasing crop productivity. The results of this investigation showed that the chemical properties of the soils were responsible for controlling the microbial functional diversity in the rhizosphere and bulk soils with P being the most significant. The existence of these functional categories that are connected to many biological processes explains how the microorganisms respond to and adapt to their microenvironment as well as how their metabolic abilities can improve the growth and development of soybean.

Author Contributions: T.T.A. managed the literature searches, carried out the laboratory work, interpreted the results, and wrote the manuscript's first draft. A.S.A. assisted in genome analysis, reviewed the drafts, and proofread the manuscript while O.O.B., the principal investigator, provided academic input, thoroughly critiqued the manuscript, proofread the draft, and secured funds for the research. All authors have read and agreed to the published version of the manuscript.

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