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Original article

# Metabolic analysis of the CAZy class glycosyltransferases in rhizospheric soil fungiome of the plant species *Moringa oleifera*

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ABSTRACT

The target of the present work is to study the most abundant carbohydrate-active enzymes (CAZymes) of glycosyltransferase (GT) class, which are encoded by fungiome genes present in the rhizospheric soil of the plant species *Moringa oleifera*. The datasets of this CAZy class were recovered using metagenomic whole shotgun genome sequencing approach, and the resultant CAZymes were searched against the KEGG pathway database to identify function. High emphasis was given to the two GT families, GT4 and GT2, which were the highest within GT class in the number and abundance of gene queries in this soil compartment. These two GT families harbor CAZymes playing crucial roles in cell membrane and cell wall processes. These CAZymes are responsible for synthesizing essential structural components such as cellulose and chitin, which contribute to the integrity of cell walls in plants and fungi. The CAZyme beta-1,3-glucan synthase of GT2 family accumulates 1,3-β-glucan, which provides elasticity as well as tensile strength to the fungal cell wall. Other GT CAZymes contribute to the biosynthesis of several compounds crucial for cell membrane and wall integrity, including lipopolysaccharide, e.g., lipopolysaccharide N-acetylglucosaminyltransferase, cell wall teichoic acid, e.g., alpha-glucosyltransferase, and cellulose, e.g., cellulose synthase. These compounds also play pivotal roles in ion homeostasis, organic carbon mineralization, and osmoprotection against abiotic stresses in plants. This study emphasizes the major roles of these two CAZy GT families in connecting the structure and function of cell membranes and cell walls of fungal and plant cells. The study also sheds light on the potential occurrence of tripartite symbiotic relationships involving the plant, rhizospheric bacteriome, and fungiome via the action of CAZymes of GT4 and GT2 families. These findings provide valuable insights towards the generation of innovative agricultural practices to enhance the performance of crop plants in the future.

## 1. Introduction

*Moringa oleifera*, a resilient and rapidly growing wild plant species belonging to the Moringaceae family, thrives in various Asian countries (Gupta and Ahmed, 2020). Referred to by common names such as drumstick, horseradish tree, and malunggay (Serafico et al., 2015; Palada, 2019), different parts of this plant have demonstrated remarkable economic benefits in terms of nutrition, pharmaceutical applications, traditional herbal medicine, industry, and agriculture (Kalibbala et al., 2009; Gopalakrishnan et al., 2016). Numerous studies have revealed that this wild plant contains the vitamins C and A, as well as the minerals Ca, K, and F and the antioxidants flavonoids, polyphenols, and ascorbic acid (Rockwood et al., 2013; Milla et al., 2021). Additionally, this plant species provides pharmacological benefits by producing essential compounds such as glycosides, β-sitosterol, and N-α-rhamnophyranosyl vincosamide, which help regulate cholesterol and blood pressure (Fahey, 2005; Panda et al., 2013). Traditional medicinal applications of this plant are effective in treating chronic diseases, e.g., cancer, cardiovascular ailments, diabetes, as well as providing liver protection against oxidation and toxicity Kumar (Kumar et al., 2016). Furthermore, *M. oleifera* has been utilized for water purification and the production of various bioproducts, including cattle fodder, soil fertilizers, biofuels, soaps, and perfumes, besides its applications in the cosmetics (Folkard and Sutherland, 1996; Ashfaq et al., 2012; Rockwood et al., 2013; Gómez and Angulo, 2014). Recent research has highlighted the stability and skincare benefits of *M. oleifera* seed oil (Zouboulis et al., 2023).

Metagenomic whole shotgun genome sequencing has emerged as a valuable tool to investigate the activities of soil rhizospheric microbiomes, including archaeomes, bacteriomes, phageomes, and fungiomes, associated with both wild and domesticated plants (Borrel et al., 2020; Ashy et al., 2023; Tashkandi and Baz, 2023). The association between the rhizospheric fungiome and plant roots is facilitated by

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fungal arbuscules, hyphae, and mycelium, forming a connected network that enables nutrient exchange, uptake, and protection against microbial diseases and adverse environmental stimuli (Jeffries et al., 2003; Lidoy et al., 2022). Notably, mycorrhizal fungi (MF) can establish a pseudo-root system that connects plant roots with the intact microhabitats within and beyond the rhizospheric soil area (Buscot and Varma, 2005; Huang et al., 2019; Awaad et al., 2021; Seleiman and Hardan, 2021). Interestingly, mycorrhizal fungi can also interact with the soil bacteriome, contributing to improved soil quality and enhanced plant fitness (Barea et al., 2005; Berrios et al., 2023; Lu et al., 2023). This tripartite interaction, mostly initiated by plant exudate secretion, benefits all partners involved, and specific responses are triggered in the soil microbiome upon recognition of these cues. For instance, phosphate solubilization, a crucial process for plant root uptake, is facilitated by phosphate-solubilizing bacteria, with mycorrhizal mycelium serving as a vector for transporting solubilized phosphate to enhance plant absorption (Barea et al., 2005; Berrios et al., 2023). Similar mechanisms also exist to absorb other nutrients, e.g., nitrogen and sulfur, in the MF-plant symbiotic relationship (Gahan and Schmalenberger, 2014; Hodge and Storer, 2015; Enebe and Erasmus, 2023).

In the current work, we studied the function of rhizospheric fungiome of *Moringa oleifera*, focusing on the genes that encode CAZymes from the most enriched families of the CAZy class glycosyltransferases (GT). Our goal was to shed light on the potential novel bi- and tripartite symbiotic interactions involving the plant root, its soil fungiome, and bacteriome.

## 2. Materials, and methods

### 2.1. Soil collection, and DNA isolation

Three replicates of the rhizospheric as well as bulk soil of *M. oleifera* plants grown individually, as well as the bulk soil samples located in close proximity (10 m apart) were collected from the Mecca region of KSA (Al-Eisawi and Al-Ruzayza, 2015). DNAs were extracted from the soil microbiomes using the CTAB/SDS method (Smith et al., 1989). Subsequently, 30 µl (10 ng/l) of extracted DNA was sent to Novogene (Hong Kong, China) for deep sequencing and construction of gene catalogues.

### 2.2. Whole shotgun genome sequencing

Before next-generation sequencing (NGS), the DNAs underwent physical fractionation, and data pre-processing steps. Short reads and reads containing an N nucleotide above a 10-bp threshold were eliminated, along with low-quality reads (with threshold ≥40-bp). NGS was conducted using Illumina HiSeq platform (San Diego, CA, USA) and the generated sequencing datasets were submitted to the European Nucleotide Archive (ENA) and received acc. nos. of ERR10100770-74 and ERR10100781.

### 2.3. Data analysis

The clean read datasets were assembled using MEGAHIT with a K-*mer* size of 55. Scaffolds containing highly abundant genes were retrieved, and chimeric DNAs were eliminated as previously described (Mende et al., 2012). The unassembled reads from less-abundant genes across different datasets were combined to construct NOVO_MIX scaffolds, which were subsequently trimmed at a given "N" base to generate scaftigs for further annotation (Mende et al., 2012). Gene prediction and dereplication were performed using MetaGeneMark, and CD-HIT methods, respectively, employing assembled DNAs from individual soil samples and NOVO_MIX scaftigs as targets (Fu et al., 2012; Nielsen et al., 2014). Gene catalogues were then defined as described (Li et al., 2014). Functional prediction was carried out via the use of MEGAN categorization approach (Huson et al., 2016), while abundance was detected utilizing the eggNOG database (Huerta-Cepas et al., 2017) and deduced amino acid sequences were annotated following Diamond approach (Buchfink et al., 2015). Information generated after annotation was further aligned to the CAZy database (Lombard et al., 2014), and the resulting CAZymes were assigned to the respective families of different classes. To determine the functional pathways, the generated CAZymes were searched against the KEGG pathway database.

## 3. Results

### 3.1. Validation of high throughput sequencing datasets

The metagenomic datasets obtained from bulk and rhizospheric soils of *M. oleifera* were validated using PCA (principle component analysis), as depicted in Fig. 1. The results revealed a substantial genetic distinction between the microbiomes of rhizospheric and bulk soils. The microbiome entities in the rhizosphere soil samples were predominantly positioned on the negative side of PC1 dimension across the three CAZy levels, while the microbiomes of the bulk soil were located on the positive side. Moreover, PC2 dimension indicated higher level of diversity among the bulk soil microbiome samples compared to that among the rhizospheric soil microbiome samples. This discrepancy in diversity levels could be attributed to the homogeneous nature of the habitat surrounding rhizospheric soil samples, which receive similar exudates or signals from the plant root cells, leading to specific microbial actions. In contrast, the bulk soil represents a non-homogenous habitat, even for microbes in close proximity.

### 3.2. Annotation results and abundant CAZy families of class glycosyltransferases

Annotation of the assembled data from the rhizospheric microbiomes of *M. oleifera* resulted in the identification of over 53,000 non-redundant gene queries encoding CAZymes belonging to six CAZy classes, namely GT (glycosyltransferases), GH (glycoside hydrolase), PA (polysaccharide lyase), CBM (carbohydrate-binding module), CE (carbohydrate esterase), and AA (auxiliary activities) (Table S1). Among these, more than 16,000 gene queries encoded CAZymes (Carbohydrate-Active enZymes) from 62 CAZy families within GT class (Table S2). Notably, soil bacteria, followed by eukaryotic taxa (mycobiome or fungiome), harbored a higher number of gene queries encoding CAZymes across CAZy classes or within the GT class (Fig. 2 and Tables S3 and S4). The top GT families, with gene query counts exceeding 125 across soil types and microbial kingdoms, included GT4, GT2, GT51, GT1, GT9, GT35, GT20, GT28, GT26, GT5, GT30, GT39, GT81, GT19, GT47, and GT48 (Fig. 3a and Table S5). Among these, ten GT families exhibited the highest gene abundance (≥1500) (Fig. 3a and 4a and Table S6). Comparison among microbial kingdoms, including Archaea, Bacteria, and Eukaryota, indicated that seven GT families within the Eukaryota kingdom had more than 100 gene queries (Fig. 3b and Table S4). These seven GT families are GT4, GT2, GT1, GT20, GT5, GT47, and GT48 (Fig. 3b).

The results regarding the top ten GT families with a gene abundance of ≥1500 are shown in Fig. 4a and Table S6. Among the seven selected GT families based on the aforementioned criterion, GT5 and GT47 exhibited lower abundance levels (<1500) (Table S6), while the GT1 and GT48 families demonstrated higher abundance in bulk soil microbiome compared to that in rhizospheric soil microbiome (Fig. 4a). Consequently, these four families were not studied further. Additionally, a filtering step based on relative abundance was applied to select GT families for further analysis, focusing on those showing ≥60 % relative abundance across rhizospheric soil microbiomes (Fig. 4b). This filtering criteria led to the exclusion of the GT20 family, resulting in the further analysis of CAZymes from only the GT4 and GT2 families. The number of CAZymes analyzed in depth for the GT4 and GT2 families was 15 and 10, respectively (Fig. 5a and Table S7). It is worth mentioning that one

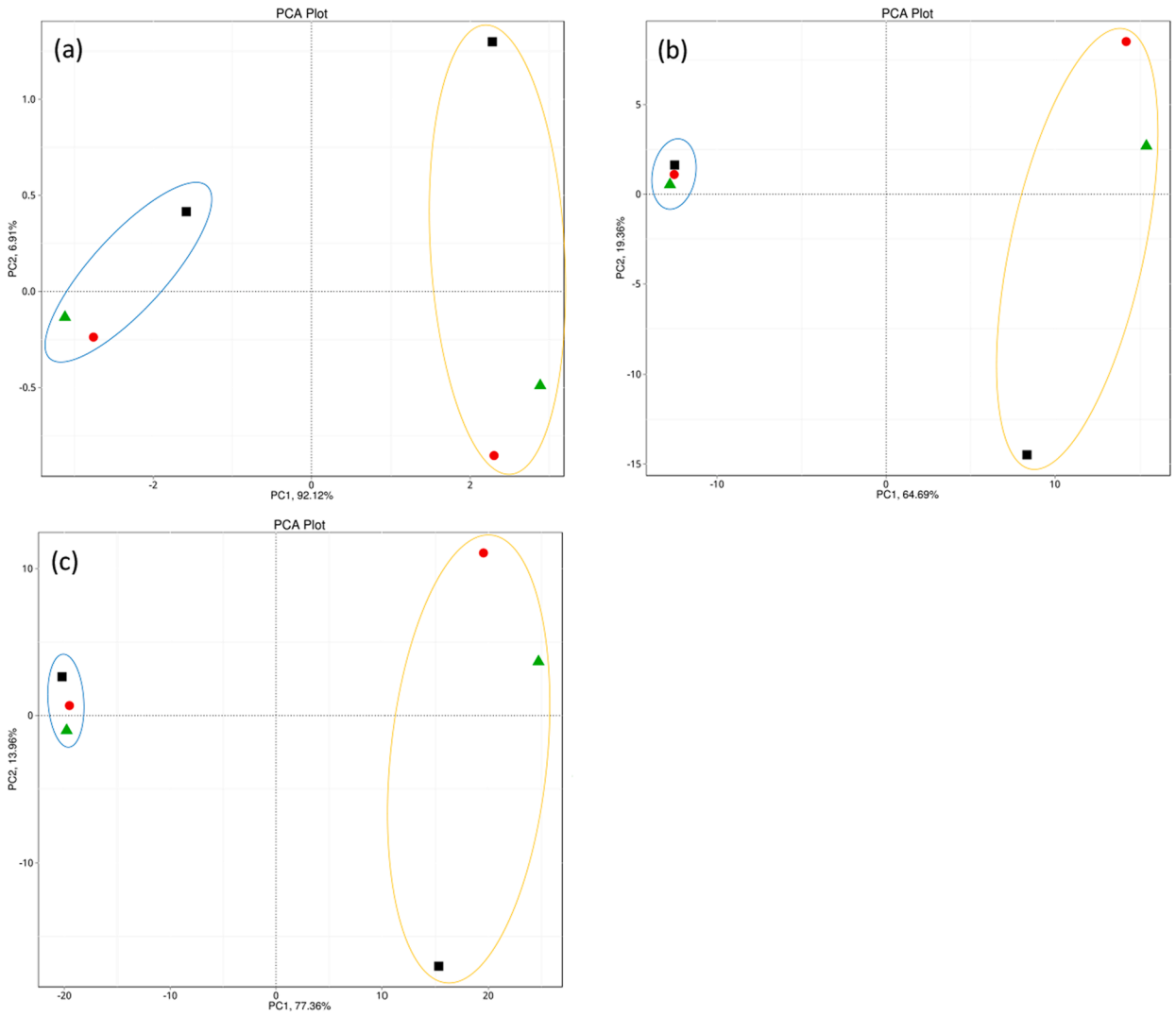


**Fig. 1.** Plots of principal component analysis (PCA) referring to the number/type of genes at CAZy class (a), Family (b) and CAZyme (c) levels in microbiomes collected from bulk (S) and rhizosphere (R) soils of *Moringa oleifera.* Black square, red circle, and green triangle refer to the three replicates of each soil type. Blue circle = samples of rhizosphere soil. Orange circle = samples of bulk soil.

CAZyme with EC of 2.4.1.56 (Fig. S4), from the GT4 family also a member of the GT9 family. This CAZyme exhibits a higher number of gene queries (4622) compared to the other CAZymes (3754) within the same family. In contrast, the GT2 family had 3410 gene queries for its CAZymes (Table S7). The gene relative abundance of CAZymes from the GT4 and GT2 families in the rhizospheric soil was approximately 60 %, while in the bulk soil of *M. oleifera*, it was around 40 % (Fig. 5b, 5c, and Table S8), corroborating the results observed at the family level (Fig. 4b).

### 3.3. Fungiome taxa encoding CAZymes of CAZy families GT4 and GT2

The fungiome taxa with the largest number of gene queries encoding CAZymes from the GT4 and GT2 families, are presented in Fig. 6 and Table S4. These two GT families share seven fungiome taxa, while two additional taxa carry genes encoding CAZymes from the GT2 family (Fig. 6). In our investigation, we have identified seven distinct taxa within the fungiome, each of which has been classified into respective taxonomic families. These taxa include representatives from the taxonomic families Neocallimastigaceae, Glomeraceae (species *Rhizophagus irregularis*), Mortierellaceae (species *Mortierella elongata*), Cunninghamellaceae (species *Absidia glauca*), Lichtheimiaceae (species *Lichtheimia ramosa*), Mucoraceae (species *Mucor ambiguous*), and Phycomycetaceae (*Phycomyces blakesleeanus*) (Fig. 6 and Table S4). Additionally, the remaining two taxa, both belonging to the GT2 group, have been attributed to the Glomeraceae family (species *Rhizophagus* sp. DAOM 213198) and Mucoraceae (species *Mucor circinelloides*) (Fig. 6 and Table S4). It is noteworthy that all of these taxa fall under the phylum Mucoromycota, except for the Neocallimastigaceae family, which is categorized under the phylum Chytridiomycota (Table S4). Furthermore, it is important to highlight that four out of the six remaining families have been assigned to the order Mucorales, with the Glomeraceae family being classified under the order Glomerales and the Mortierellaceae family under the order Mortierellales.

The leading fungal taxa, as indicated by the quantity of gene queries within the two GT families, include *Mucor ambiguous* from the Mucoraceae family (4937 queries), *Mortierella elongata* from the Mortierellaceae family (1691 queries), *Rhizophagus irregularis* from the Glomeraceae family (1325 queries), and *Cunninghamella glauca* from the Cunninghamellaceae family (577 queries) (Fig. 6 and Table S4). The conversion of gene queries into taxonomic units, particularly at the genus level, for the seven common taxonomic families of the two GT families is presented in Table S9. Among these taxonomic units, the most prominent taxon referring to the number of gene queries translated into taxonomic units is the genus *Rhizophagus* from the Glomeraceae family (238 queries), followed by the genera *Mortierella* from the

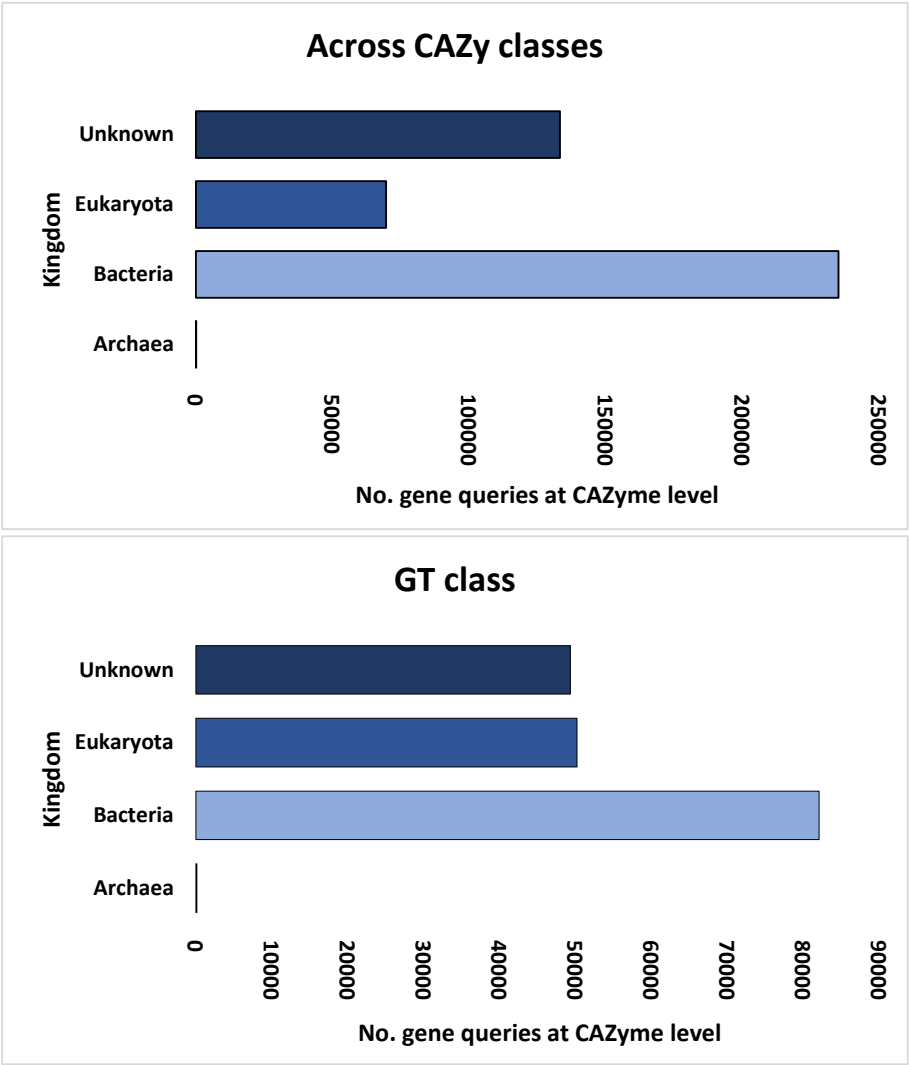


**Fig. 2.** Number of gene queries encoding families of different CAZy classes and that at CAZyme level specifically for glycosyltransferase (GT) class in different kingdoms, e.g., Archaea, Bacteria and Eukaryota, across bulk (S) and rhizosphere (R) soil microbiomes of Moringa oleifera. More information for CAZy classes is available in Tables S3 and S4.

Mortierellaceae family (9 queries), *Lichtheimia* from the Lichtheimiaceae family (7 queries), and *Mucor* from the Mucoraceae family (5 queries) (Fig. 7 and Table S9).

### 3.4. Functional analysis of CAZymes of families GT4 and GT2

A summary of the functional analysis of the CAZymes within the two GT families can be found in Table S10. The findings reveal that out of the 25 GT CAZymes, 22 are involved in KEGG pathways. The remaining three CAZymes consist of one from family GT4 and two from family GT2, and they were not subjected to further investigation. All the GT CAZymes participating in KEGG pathways are associated with a single KEGG category, which is "Metabolism," encompassing five subcategories and nine pathways (Figs. S1–S9). The subcategories under "Metabolism" include "Glycan biosynthesis and metabolism," "Carbohydrate metabolism," "Biosynthesis of other secondary metabolites," "Lipid metabolism," and "Metabolism of terpenoids and polyketides." Subcategory "Glycan biosynthesis and metabolism" contains 12 GT CAZymes distributed across four KEGG pathways. Among these, seven belong to family GT4, while five CAZymes belong to family GT2. The four pathways are "Arabinogalactan biosynthesis – *Mycobacterium*" (involving three CAZymes), "N-Glycan biosynthesis" (involving six CAZymes), "Teichoic acid biosynthesis" (involving two CAZymes), and "Lipopolysaccharide biosynthesis" (involving one CAZyme) (Table S10). The subcategory "Carbohydrate metabolism" incorporates six GT CAZymes found in two KEGG pathways, with three from family GT4 and three from family GT2. These two pathways are "Starch and sucrose metabolism" (involving five CAZymes) and "Amino sugar and nucleotide sugar metabolism" (involving one CAZyme) (Table S10). Within the subcategory "Biosynthesis of other secondary metabolites," two GT4 CAZymes are identified in the KEGG pathway "Neomycin, kanamycin, and gentamicin biosynthesis." The subcategory "Lipid metabolism" includes two GT4 CAZymes participating in the KEGG pathway "Glycerolipid metabolism." Lastly, the subcategory "Metabolism of terpenoids and polyketides" comprises one GT4 CAZyme contributing to the KEGG pathway "Pinene, camphor, and geraniol degradation" (Table S10). It is noteworthy that the GT4 CAZyme, 1,2-diacylglycerol 3-glucosyltransferase, plays a role in two pathways, e.g., "Teichoic acid biosynthesis" and "Glycerolipid metabolism," the two pathways fall under the subcategories "Glycan biosynthesis and metabolism" and "Lipid metabolism," respectively (Table S10).

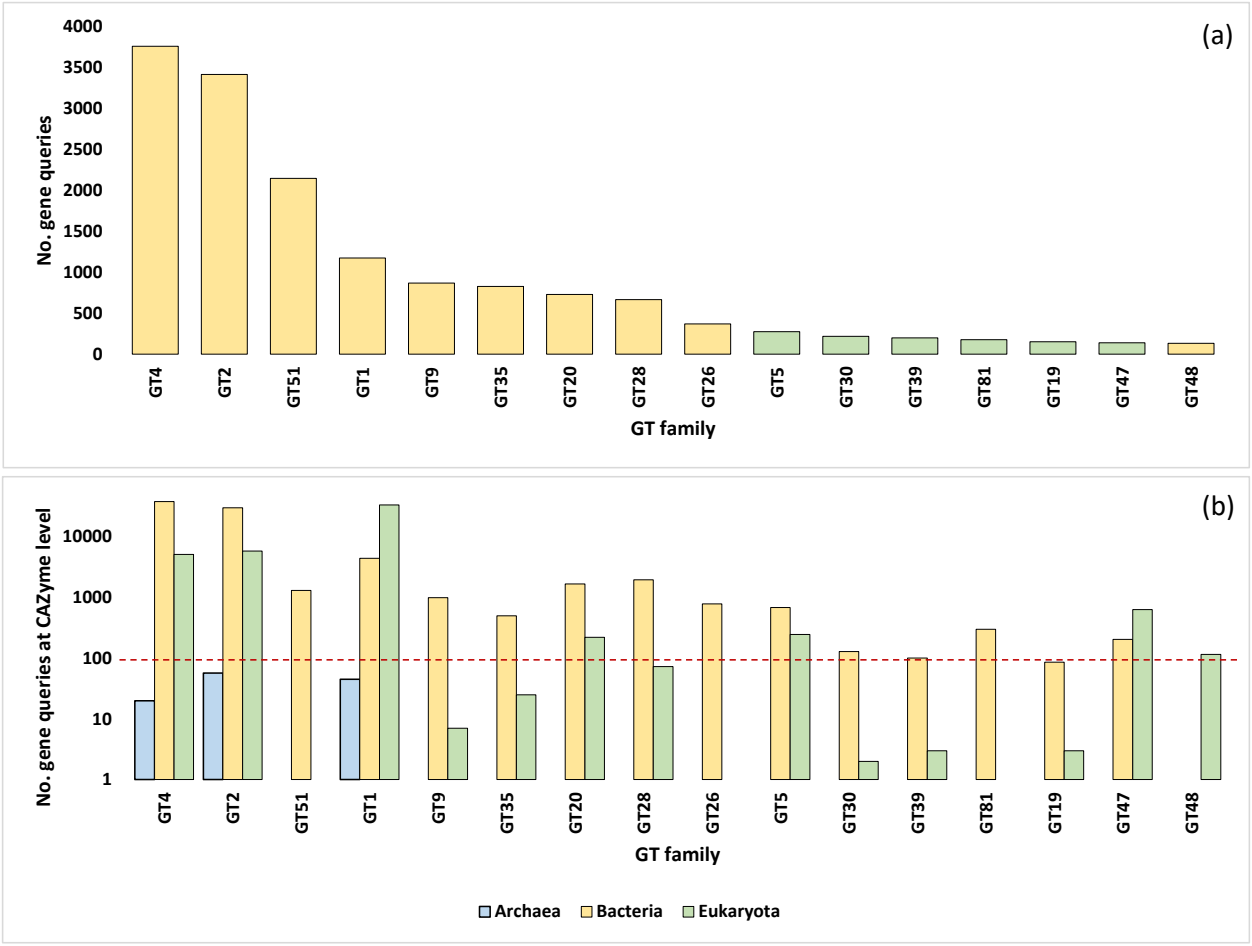


**Fig. 3.** The top glycosyltransferase (GT) families in terms of the number of gene queries (>125) across (a) and among (b) microbial kingdoms, e.g., Archaea, Bacteria and Eukaryota across bulk (S) and rhizosphere (R) soil microbiomes of Moringa oleifera. Orange boxes in (a) refer to the top 10 GT families in terms of abundance of gene queries shown in Table S6. Red dotted line in (b) refers to the required threshold of gene query number (≥100) of GT families in Eukaryota kingdom at CAZyme level for further analysis of respective GT families. More information is available in Tables S4 and S5.

## 4. Discussion

### 4.1. Plant-fungus symbiotic relationship

Up to our knowledge, no prior reports indicated a link between the production of CAZymes by rhizospheric fungiome, and their contribution to the plant-fungus symbiotic relationship. The recent understanding of the plant-fungus symbiotic relationship reveals its role in reprogramming the metabolic pathways of plants, where fungi provide the plant with both metabolites, reducing the need to synthesize these compounds (Kaur and Suseela, 2020). Previous reports have indicated that the fungal taxa in this study, responsible for producing GT CAZymes, are not typically parasitic in nature but rather engage in symbiotic relationships with both plants and rhizobacteria (Benny, 2015; Mora et al., 2018). However, these fungi exhibit various non-mutual actions that benefit the overall health of the plant. These actions, although not strictly categorized as fungus-plant symbiotic relationships, contribute positively to the plant's well-being. This type of action resembles that of the growth-promoting bacteria. Symbiotic fungi within the plant's rhizosphere participate in several advantageous processes, such as the decomposition of plant litter, carbon cycling, soil remediation, the removal of heavy metals, the provision of antibiotic compounds, the synthesis of phytohormones (particularly auxins), and the enhancement of water availability (Hanlon and Coenen, 2011; Yang et al., 2015; Floudas et al., 2020). Furthermore, hyphae extend root system of the plant, facilitating increased nutrient absorption (Floudas et al., 2020). Then, the host plant reciprocates by providing fungi with the required sugars (Rayko et al., 2021).

Mycorrhizae constitute a network comprising fungal mycelium and plant roots (including hairy roots) that collaborate to aid plants in absorbing water and soil nutrients. In our current investigation, mycorrhizal fungi (MF), a part of the Glomeraceae family (e.g., *Rhizophagus irregularis*), which hosts CAZymes from both families GT4 and GT2 (Fig. 6 and Table S4), emerged as the dominant fungal species in terms of gene abundance within the plant rhizosphere (Fig. 7 and Table S9). It is established that exudates of plant roots, e.g., volatile organic compounds and strigolactones, can stimulate fungal spore germination, leading to subsequent fungal colonization and the occurrence of plant-mycorrhizae symbiotic relationship (Rozpądek et al., 2018; Santoyo et al., 2021).

Recent research exploring the relationship between plants and fungi has revealed that the endophytic genus *Mucor* within the Mucoraceae family participates in improving plant growth (Rozpądek et al., 2018). Furthermore, fungi of this genus provide the plant with the tolerant against toxic minerals (Zahoor et al., 2017). Such a symbiotic and mutually beneficial association between the fungal genus *Mucor* and the plant is facilitated by the plant signaling molecule strigolactone (SL), which leads to the colonization of the plant by the fungus and an increased rate of hyphal branching and growth (Rozpądek et al., 2018). As a result of this colonization, the endophytic fungal symbiont supplies the plant with essential nutrients, e.g., potassium, calcium, magnesium, etc., in exchange for the provision of reduced carbon (Baron and

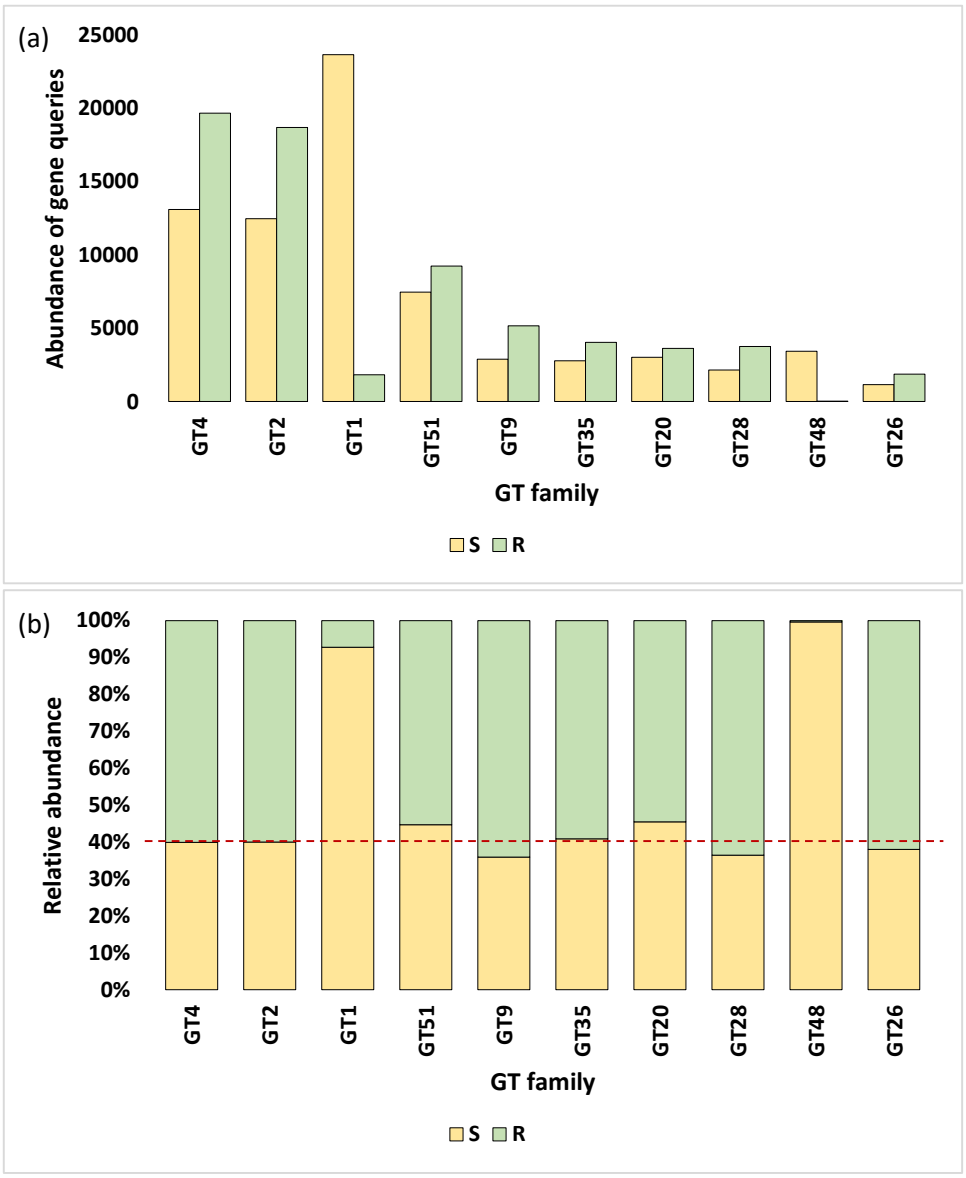


**Fig. 4.** Abundance (a) and relative abundance (b) of gene queries encoding CAZymes of the top 10 CAZy families (gene abundance of ≥1500) of class glycosyltransferase (GT) in bulk (S) and rhizosphere (R) soil microbiomes of Moringa oleifera. Red dotted line in (b) refers to the required threshold of gene query relative abundance of GT families in rhizosphere (≥60 %) soil. More information is available in Table S6.

Rigobelo, 2022).

The soil saprotrophic fungus belonging to the Mortierellaceae family, specifically of the genus *Mortierella*, plays a vital role in ecosystem dynamics. This fungus possesses the capacity to degrade hemicelluloses using the enzyme xylanase, thereby breaking down complex organic matter into simpler sugars that are absorbed by the plant. Such a process is essential for cycling nutrients at the soil level (Webster and Weber, 2007; Boddy and Hiscox, 2016). Notably, members of genus *Mortierella* elicit responses in plants, promoting resistance to various pathogens (Mares-Ponce de León et al., 2018). Additionally, *Mortierella* fungi have a remarkable ability to restructure the rhizospheric bacteriome, fostering interactions that benefit both the plant and its rhizospheric bacteriome and fungiome (Li et al., 2020).

Certain members of the soil rhizospheric genera *Absidia*, belonging to the Cunninghamellaceae family, and *Lichtheimia*, from the Lichtheimiaceae family, participate in the putrefaction of plant litter (Alastruey-Izquierdo et al., 2010; Zhao et al., 2022). It is worth mentioning that the two genera *Absidia* and *Lichtheimia* were previously considered as a single genus. However, their taxonomy was revised in 2007, leading to the separation into two distinct genera (Hoffmann et al., 2007). The saprotrophic genus *Absidia* exhibits the ability to produce chitin, chitosan, chitooligosaccharides, and hydrocortisone, as demonstrated (Kaczmarek et al., 2019; Chen et al., 2020; Zong et al., 2021). These research findings provide evidence for the participation of *Absidia* with plants in a bilateral symbiotic association. This is attributed to the production of the GT2 CAZyme chitin synthase (EC 2.4.1.16), which participates in biosynthesizing chitin, and is essential in mediating the plant-fungus interaction.

### 4.2. Tripartite symbiotic relationships

The leading taxon in terms of the number of genes responsible for encoding CAZymes from the two CAZy families is the mycorrhizal fungal (MF) genus *Rhizophagus* within the taxonomic family Glomeraceae, as presented in Fig. 7 and detailed in Table S9. Previous research has consistently underscored the pivotal role of beneficial microbes in nature, with a primary focus on mycorrhizal fungi (MF) and two microsymbionts: saprophytic root-colonizing rhizosphere bacteria, namely

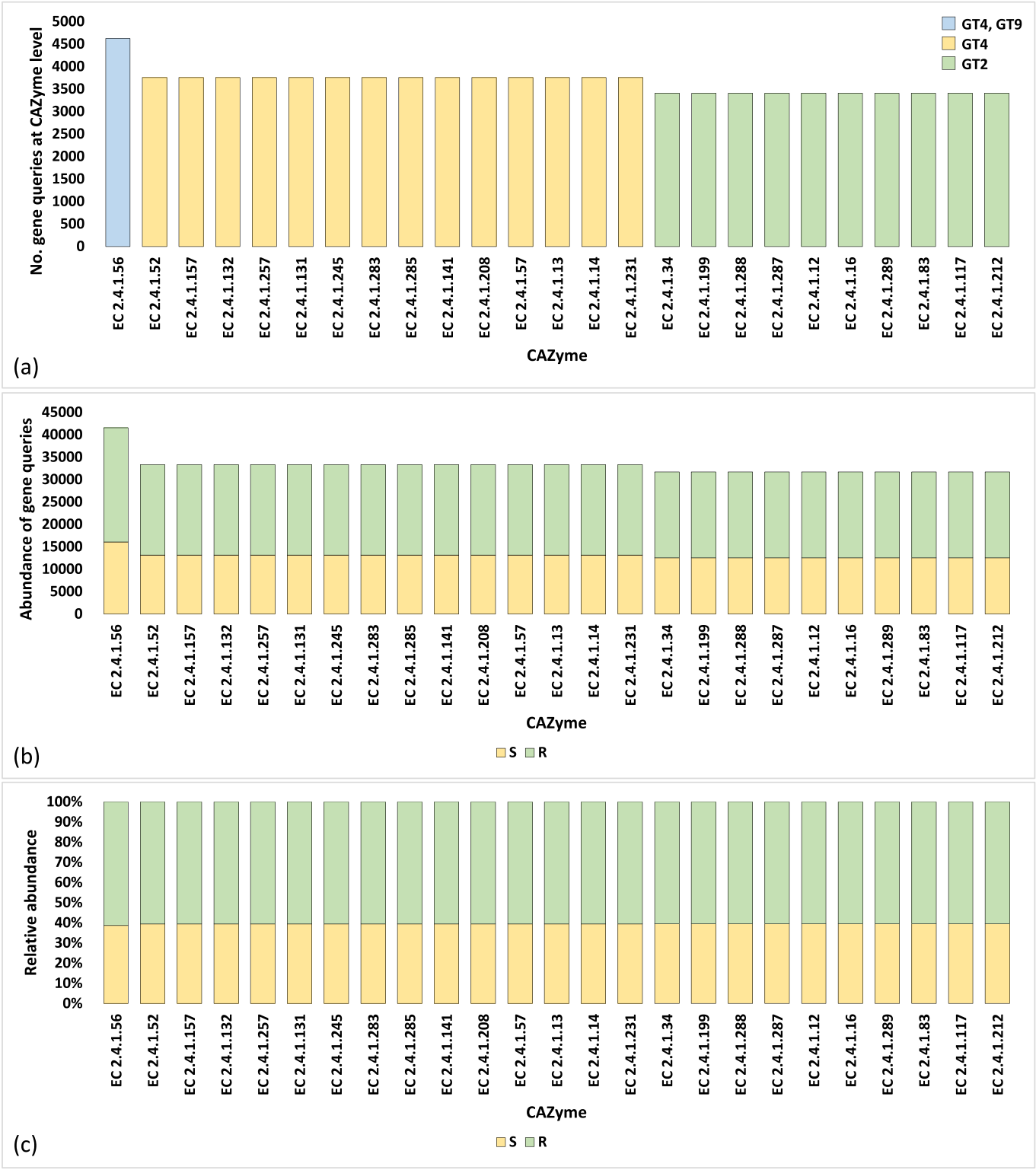


**Fig. 5.** Number at CAZyme level (a), abundance (b) and relative abundance (c) of gene queries encoding CAZymes of glycosyltransferase (GT) families GT4 and GT2 in bulk (S) and rhizosphere (R) soil microbiomes of *Moringa oleifera*. More information is available in Tables S7 and S8.

plant growth-promoting bacteria (PGPB), and $N_2$-fixing bacteria (Meena et al., 2023). The synergistic actions of MF and PGPB on plants contribute to enhancing their growth by providing essential compounds such as phytohormones (including auxins and gibberellins), siderophores, and flavonoids, as recently evidenced (Lidoy et al., 2022; Meena et al., 2023). These microbes also participate in solubilizing micronutrients (e.g., phosphorus, potassium, iron, and zinc), thereby promoting the plant's ability to absorb minerals through its roots. Furthermore, MF and PGPB participate in helping plants tolerate outer stimuli in an additive and synergistic manner (Phour et al., 2020). PGPB also actively participate in nutrient cycling, which aids in the efficient utilization of soil resources (Dobbelaere et al., 2001; Probanza et al., 2002; Pranaw et al., 2023). Meanwhile, $N_2$-fixing bacteria provide a crucial nitrogen (N) source to the biosphere (Guo et al., 2023). The

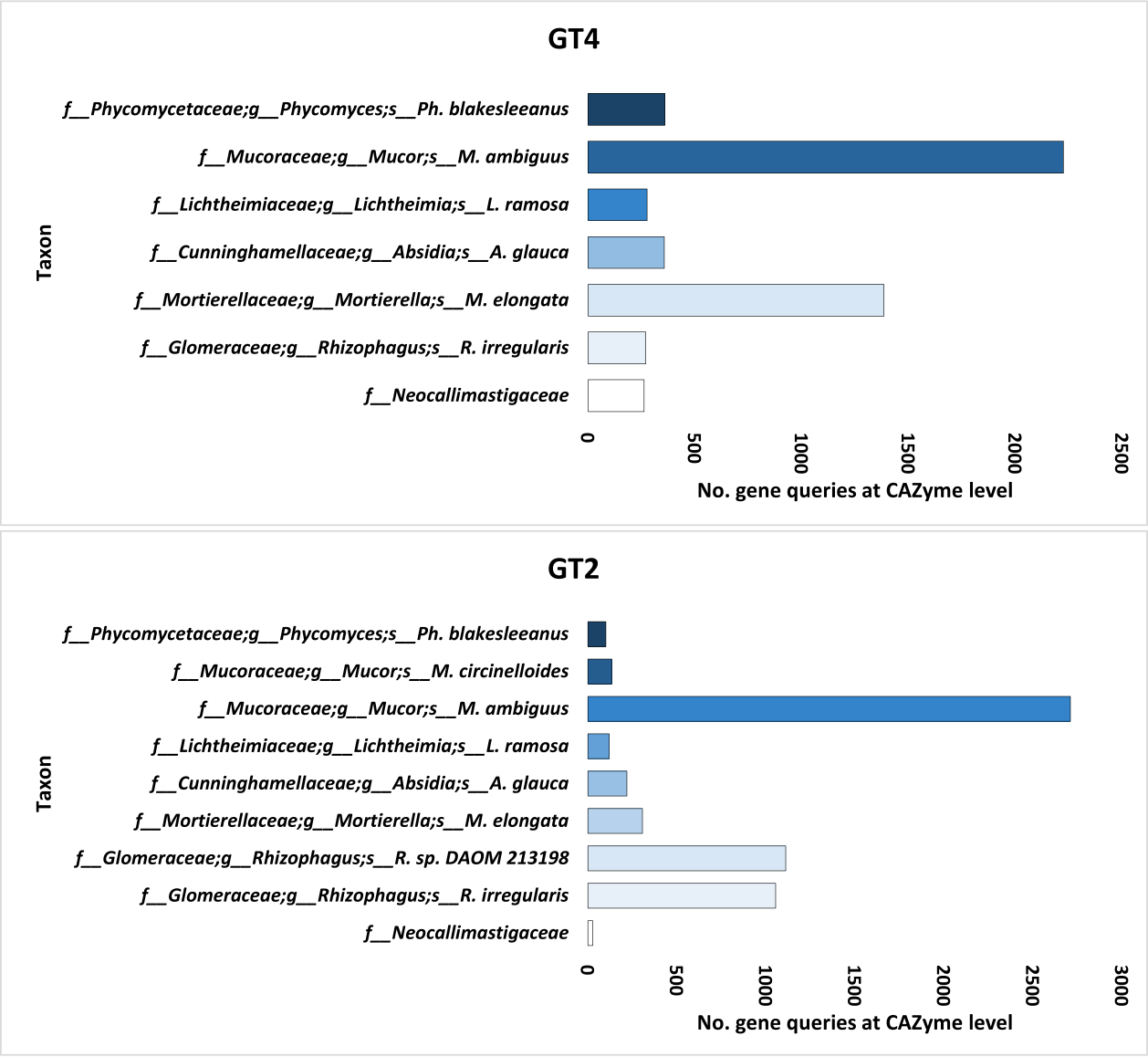


**Fig. 6.** Number of gene queries encoding CAZymes of CAZy families GT4 and GT2 of kingdom Eukaryota at the family taxonomic rank (f) and available downstream ranks, e.g., genus (g) and species (s), across bulk (S) and rhizosphere (R) soil fungiomes of Moringa oleifera. More information is available in Table S4.

mycorrhiza helper bacteria (MHB) are a specialized category of bacteria that facilitate the mycorrhization process by promoting the tripartite interactions involving mycorrhizal fungi and plants (Xing et al., 2018; Zhang et al., 2024). Intriguingly, certain Gram-negative bacteria of the Proteobacterial genus *Pseudomonas* can also enhance overall performance and colonization of MF (Garbaye, 1994). In return for these benefits, MF promotes plant root exudation, which, subsequently, promotes the growth of bacteria (Offre et al., 2007; Hodge and Storer, 2015; Xu et al., 2023).

### *4.3. Contribution of CAZymes of families GT4, and GT2 to soil metabolic processes*

We propose that the metabolites produced by the rhizospheric fungiome of *Moringa oleifera*, resulting from the action of CAZymes from the two GT families, provide new evidence of fungal metabolite contributions to the ecosystem. CAZymes, which belong to the glycosyltransferases (GT) class, play a pivotal role in transferring moieties of sugar from the donor molecule to its acceptor one in order to form glycosidic bonds (Lairson et al., 2008; Andreu et al., 2023). These GT CAZymes function based on a single displacement mechanism, wherein the molecule acting as the acceptor molecule initiates an attack at carbon-1 atom of sugar acting as the donor molecule (Coutinho et al., 2003; Rini et al., 2022). CAZymes within glycosyltransferase family 4 (GT4) feature a two-domain structure that exhibits a Rossman-type fold, while CAZymes of glycosyltransferase family 2 (GT2) are responsible for transferring nucleotide-diphosphate sugars to substrates (Vetting et al., 2008; Rini et al., 2022). A total of 23 CAZymes from these two GT families are actively involved in nine KEGG pathways within the "Metabolism" category, as specified in Table S10. These pathways further demonstrate the significance of GT CAZymes in mediating crucial metabolic processes within the ecosystem.

In our current study, two primary core metabolites, namely UDP-glucose and UDP-GlcNAc, play pivotal roles as the ultimate substrates for CAZymes from the two GT families within the fungiome of *Moringa oleifera* (Figs. 8 and 9). Previous research has affirmed the significance of UDP-glucose in plant cell signaling and its role as a substrate for CAZymes from GT families, contributing to the production of various critical mono-, di-, and polysaccharides (Rademacher, 1988; Janse van Rensburg and Van den Ende, 2017). While, UDP-GlcNac serves as a

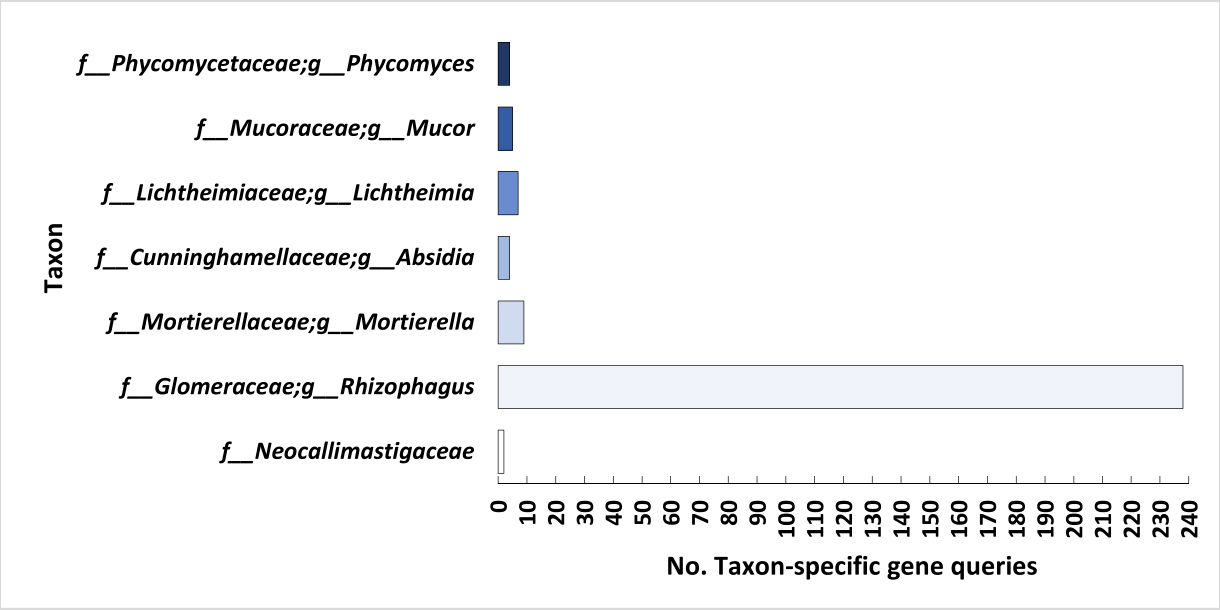


**Fig. 7.** Number of taxon-specific gene queries referring to fungiome family/genus encoding CAZymes of CAZy families GT4 and GT2 across bulk (S) and rhizosphere (R) soil of Moringa oleifera. More information is available in Table S9.

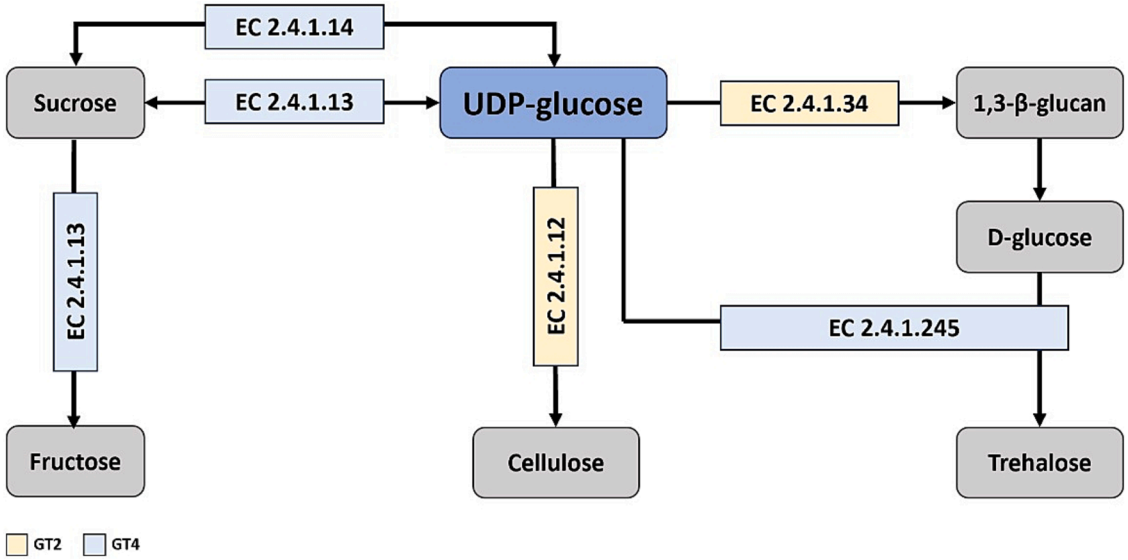


**Fig. 8.** Avenues of the "Starch and sucrose metabolism" KEGG pathway pertain to some CAZymes of the glucosyltransferase (GT) families GT4 and GT2, which are encoded by genes from the rhizospheric fungiome of *Moringa oleifera* and employ UDP-glucose as a substrate. EC 2.4.1.245 = NDP-Glc: alpha-glucose alpha-glucosyltransferase alpha,alpha-trehalose synthase, EC 2.4.1.13 = sucrose synthase, EC 2.4.1.14 = sucrose-phosphate synthase, EC 2.4.1.34 = beta-1,3-glucan synthase, EC 2.4.1.12 = cellulose synthase. CAZyme EC in blue boxes refer to CAZymes of GT4 family, while CAZyme EC in orange boxes refer to CAZymes of GT2 family. Note that each of the two CAZymes with ECs of 2.4.1.13 and 2.4.1.245 participate in two avenues.

precursor for glycoconjugates and participates in cell wall synthesis through a series of reactions. In addition, it plays a crucial role in glycosylation reactions in eukaryotes, including fungi and plants, which leads to the biosynthesis of *O*-linked GlcNAc (Rodríguez-Díaz et al., 2012; Vigetti et al., 2012; Gauttam et al., 2021). The UDP-glucose and UDP-GlcNAc are actively involved in the production of an array of important glycan and glycan-linked compounds. UDP-glucose contributes to the formation of cellulose and 1,3-β-glucan, as shown in Fig. S5. Meanwhile, UDP-GlcNAc participates in the biosynthesis of eukaryotic cell wall chitin, lipopolysaccharide (LPS), mycolyl-arabinogalactan-peptidoglycan complex (mAGP), and C-polysaccharide teichoic acid of cell wall (WTA), as evidenced in Figs. S1, S3, and S6. Additionally, UDP-glucose is implicated in the biosynthesis of the monosaccharide fructose and the disaccharides sucrose and trehalose, as depicted in Fig. S5. Alternatively, UDP-GlcNAc participates in the production of N-glycan precursor Dol-P-P-GlcNAc (Fig. S2) and two aminoglycosides, namely paromamine and neomycin B (Figs. 9 and S7). Two CAZymes with ECs of 2.4.1.283 and 2.4.1.285 (Fig. 9), participate in the production of these two aminoglycosides. Paromamine, derived from apramycin, and neomycin B are both antibiotics with bactericidal activity against Gram-negative aerobic and some anaerobic bacteria, as they bind to the 30S ribosome subunit, inhibiting its assembly with the 50S subunit (Mehta and Champney, 2003). Notably, this appears to be the sole harmful action of the GT4 CAZymes from fungiome detected in relation to

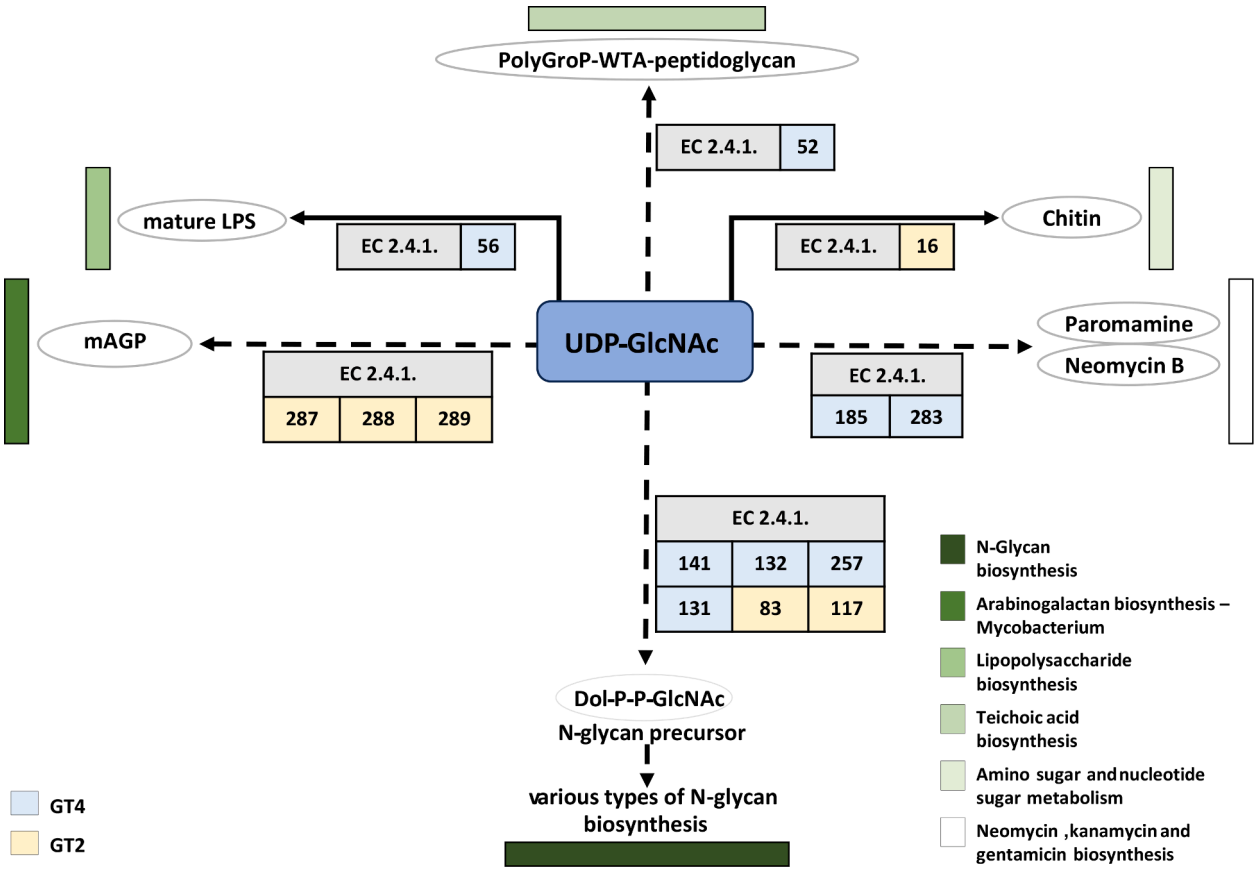


**Fig. 9.** Avenues of six different KEGG pathways pertain to some CAZymes of the glucosyltransferase (GT) families GT4 and GT2, which are encoded by genes from the rhizospheric fungiome of *Moringa oleifera* and employ UDP-GlcNAc as a substrate. EC 2.4.1.288 = UDP-Galf: galactofuranosyl-galactofuranosyl-rhamnosyl-N-acetylglucosaminyl-PP-decaprenol beta-1,5/1,6-galactofuranosyltransferase, EC 2.4.1.287 = UDP-Galf: rhamnopyranosyl-N-acetylglucosaminyl-PP-decaprenol beta-1,4/1,5-galactofuranosyltransferase, EC 2.4.1.289 = dTDP-L-Rha: N-acetylglucosaminyl-PP-decaprenol alpha-1,3-L-rhamnosyltransferase, EC 2.4.1.132 = GDP-Man: Man1GlcNAc2-PP-dolichol alpha-1,3-mannosyltransferase, EC 2.4.1.257 = GDP-Man: Man2GlcNAc2-PP-dolichol alpha-1,6-mannosyltransferase, EC 2.4.1.131 = GDP-Man: Man3GlcNAc2-PP-dolichol_Man4GlcNAc2-PP-dolichol alpha-1,2-mannosyltransferase, EC 2.4.1.141 = digalactosyldiacylglycerol synthase, EC 2.4.1.83 = dolichyl-phosphate beta-D-mannosyltransferase, EC 2.4.1.117 = dolichyl-phosphate beta-glucosyltransferase, EC 2.4.1.52 = alpha-glucosyltransferase, EC 2.4.1.56 = lipopolysaccharide N-acetylglucosaminyltransferase, EC 2.4.1.16 = chitin synthase, EC 2.4.1.283 = UDP-GlcNAc: 2-deoxystreptamine alpha-N-acetylglucosaminyltransferase, EC 2.4.1.285 = UDP-GlcNAc: ribostamycin alpha-N-acetylglucosaminyltransferase.

rhizobacteria.

Furthermore, the GT4 CAZymes with ECs of 2.4.1.208 and EC 2.4.1.337 (Fig. S8), which are enriched in the rhizospheric fungiome of *Moringa oleifera*, contribute to the biosynthesis of lipocarbohydrate lipoteichoic acid (LTA) using 1,2-Diacyl-*sn*-glycerol as the substrate (Fig. S8). It is important to note that the GT4 CAZyme with EC of 2.4.1.57 was removed from the KEGG database in 2017 and was therefore not analyzed further in this study.

Glycans represent polysaccharides comprised of monosaccharides linked together through glycosidic linkages, a fundamental feature of their structure (McNaught and Wilkinson, 1997). These polysaccharides are categorized as glycoconjugates, capable of forming conjugates with proteins and lipids, leading to the formation of glycoproteins and glycolipids, respectively (Dwek, 1996). Within the rhizospheric fungiome of *Moringa oleifera*, six GT CAZymes are actively involved in the production of the N-glycan precursor, a pivotal component of glycan biosynthesis (Fig. S2). These CAZymes have ECs of 2.4.1.132, 2.4.1.257, 2.4.1.131, 2.4.1.141, 2.4.1.83 and 2.4.1.117 (Fig. 9). Other notable examples of glycans include cellulose and chitin, e.g., two most prevalent carbohydrates existing on the Earth (Klemm et al., 2005; Chen et al., 2010). These essential compounds are shaped and maintained by specific GT CAZymes. Cellulose, a fiber-forming glycan, is biosynthesized by the enzyme with EC of 2.4.1.12 (Fig. S5). The monomeric unit of cellulose consists of β-1,4-linked D-glucose subunits. It is worth noting that cellulose is a fundamental building block that bolsters the overall strength of plant primary cell walls. The biosynthesis of chitin, another glycan, is facilitated by chitin synthase (EC 2.4.1.16), illustrated in Fig. S6. The chitin's monomeric unit is GlcNac. Chitin serves is an entity of the primary cell wall in fungi that provides strength and integrity (Klemm et al., 2005). Moreover, in bacteria, cellulose plays a pivotal role in biofilm formation, acting as a protective shield for bacteria like *Komagataeibacter xylinum*, guarding them from the surrounding environment (Klemm et al., 2005), while chitin can also provoke and attenuate fungal immune responses and pathogenesis (Lenardon et al., 2010). Alternatively, in the cell members of the Mortierellaceae family, cellulose and chitin are biodegraded to facilitate growth in unfavorable soil conditions (Telagathoti et al., 2022). β-1,3-glucan represents another important glycan that contributes to the fungal cell wall's elasticity and tensile strength (Lesage and Bussey, 2006). This spring-like structure is synthesized by the CAZyme with EC of 2.4.1.34 (Figs. 8 and S5). These examples underscore the critical roles that glycans play in diverse biological processes, ranging from structural integrity to pathogenic interactions.

Previous research has established that in the rhizospheric soil of *Moringa oleifera*, trehalose, synthesized by the enzyme with EC of 2.4.1.245 (Fig. 8), serves as a protective agent against unfavorable environmental stimuli (Suárez et al., 2009). Additionally, rhizospheric soil sucrose, produced by the enzyme with EC of 2.4.1.13 (Fig. 8), has a

major role in promoting plant–microbe symbiosis (Tian et al., 2021). This intriguing function highlights the complex interactions within the rhizospheric ecosystem. Moreover, fructose in the rhizospheric soil has been associated with mineralization of soil organic carbon (SOC), a process that facilitates the absorption of minerals by plant roots. This phenomenon, which contributes to nutrient availability, has been explored in research conducted earlier, emphasizing the multifaceted contributions of these compounds to plant health and ecosystem dynamics and health (Hamer and Marschner, 2005).

Glycoconjugates and lipoglycans encompass a variety of compounds, with one example being lipopolysaccharide (LPS). In the rhizosphere soil fungiome, LPS is synthesized by the CAZyme with EC of 2.4.1.56 (Figs. 9 and S4). LPS consists of a hydrophobic lipid component known as lipid A, which acts as an anchor for the LPS molecule within the outer cell membrane (Li et al., 2017). This lipoglycan participates in membrane bilayer structure of Gram-negative bacteria. It contributes to the cell structural integrity, serving as a mechanism by which bacteria adhere to other surfaces (Li et al., 2017). Furthermore, LPS functions as an efficient permeability barrier within the cell membrane, safeguarding against harmful molecules (Farhana and Khan, 2022). These properties emphasize the pivotal role of LPS in cell structure, integrity, and interactions with the environment.

Regarding fungal enriched GT CAZymes in the "Teichoic acids biosynthesis" pathway, there are specific enzymes biosynthesizing teichoic acids in cell wall, such as the polyglycerol phosphate wall teichoic acid peptidoglycan (PolyGroP-WTA-peptidoglycan) found in Gram-positive bacteria. In this pathway, alpha-glucosyltransferase (EC 2.4.1.52) participates in the production of this glycopolymer. The glycopolymer teichoic acid (TA) is a phosphate-rich molecule exclusively existing in Gram-positive bacteria (Swoboda et al., 2010). This glycopolymer provides essential structural and functional properties within these bacteria. The two GT CAZymes of pathway "Glycerolipid metabolism", with ECs of 2.4.1.337 and 2.4.1.208 (Fig. S8), participate in the production of lipoteichoic acid (LTA). Gram-positive bacteria have no outer membrane, but they compensate for this with a thick structure of peptidoglycan layers (Swoboda et al., 2010). These layers form a matrix that not only stabilizes the cell membrane but also offers a platform for the attachment of other molecules (Neuhaus and Baddiley, 2003). The two types of TAs contribute to the negative charge and structural integrity of the cell wall (Neuhaus and Baddiley, 2003). WTA participates in maintaining the tensile strength, elasticity, and porosity of the cell wall that contributes to the mechanical and physical integrity of the cell envelope (Doyle and Marquis, 1994). LTA, in turn, serves to uphold the integrity of the envelope (Neuhaus and Baddiley, 2003; Schlag et al., 2010). Absence of LTA in bacteria was observed to exhibit temperature sensitivity, growth inhibition, and reduced ion homeostasis across the cell membrane (Schirner et al., 2009). Such information underscores the participation of teichoic acids in maintaining the entity and functional properties in cell walls.

In the rhizospheric fungiome of *Moringa oleifera*, three specific GT CAZymes participate in the generation of the core structure of mAGP within the "Arabinogalactan biosynthesis - *Mycobacterium*" pathway, as illustrated in Fig. S1. These CAZymes play a critical role in this complex. These CAZymes have ECs of 2.4.1.288, 2.4.1.287 and 2.4.1.289 (Fig. 9). The mAGP is an integral part of the cell wall's glycolipid structure found in certain Gram-positive bacteria (ex., *Mycobacterium*). This complex is crucial for maintaining the viability and structural integrity of cells (Alderwick et al., 2015). Within the mAGP, the presence of arabinan is essential, as it serves as an anchor for mycolic acid, contributing to the overall structural integrity of the cell envelope (Vilchèze, 2020). It is recently noted that conformational changes taking place at the arabinogalactan portion of the cell envelope occur due to the shift in the surrounding environmental conditions (Shen et al., 2020).

In contrast to fungi and plants, which feature a phospholipid bilayer membrane, Gram-negative bacteria are known to have an asymmetric bilayer membrane with the LPS presents on the outer side, while phospholipids on the cytoplasmic side (Farhana and Khan, 2022). Alternatively, Gram-positive bacteria have a strong structure made up of thick layers, however they do not have exterior membranes of peptidoglycan anchored to WTA and LTA (Swoboda et al., 2010). The mycolyl-arabinogalactan-peptidoglycan complex (mAGP) is a highly specific compound synthesized primarily by *Mycobacterium* species, a type of bacteria. While cellulose can be synthesized by plants and certain Gram-negative bacteria (ex., Acetobacter xylinum) (Jonas and Farah, 1998), there is no prior research confirming that fungi are capable of synthesizing cellulose. In fact, prior evidence suggests that fungi, particularly *Basidiomycetous* fungi, are known for their ability to degrade cellulose rather than produce it (Baldrian and Valaskova, 2008). Similarly, there is no prior research supporting the idea that fungi can synthesize compounds like LPS, WTA, LTA, mAGP, or cellulose. Therefore, there is no sufficient justification to claim that fungi can biosynthesize these bacterial-specific membrane or cell wall compounds for their benefit. Instead, the current understanding suggests that fungi might provide these compounds to assist bacteria in their division and response to changing environmental stimuli. Research is still required to substantiate the nature of this potential symbiotic relationship between fungi and bacteria.

## 5. Conclusion

We conclude that the CAZymes belonging to the two predominant glycosyltransferase (GT) families encoded by genes in the fungiome of *Moringa oleifera* play significant roles in cell membrane and cell wall processes. The presence of GT4 and GT2 CAZymes suggests a symbiotic relationship between fungi and bacteria, and between plants and bacteria. This study also refers the potential existence of tripartite symbiotic relationships involving plants, their rhizospheric bacteriome, and fungiome. This information may be valuable for the application of biotechnological tools and metabolic processes to restructure the rhizospheric soil fungiome, with the aim of enhancing plant growth and its ability to withstand adverse environmental conditions.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2024.103956.

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