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RESEARCH ARTICLE

Early inoculation with arbuscular mycorrhizal fungi shifts metabolic functions of rhizosphere bacteria in field-grown tomato plants

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Abstract

Aim The study explores the functional plasticity of rhizosphere microbiomes in response to microbial inoculants, a topic not yet fully explored in open-field agroecosystems. Specifically, it assesses the lasting impact of nursery-stage inoculation with the arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* and symbiosis-stimulating compounds on the rhizosphere microbiome of *Solanum lycopersicum*.

Methods Tomato seedlings were inoculated with *R. irregularis* in a commercial nursery. During this period, seedlings were irrigated weekly with strigolactone analogues and the flavonoid quercetin, both with and without AMF inoculation. At harvest, five months post-transplanting, rhizosphere samples were collected for mycorrhizal colonization assessment and predictive metagenomic profiling.

Results Nursery AMF inoculation, especially when combined with the strigolactone mimic SL-M2 and quercetin, significantly enhanced mycorrhizal colonization. Although all plants became colonized, early inoculation did not affect bacterial or fungal taxonomic composition at harvest. However, it induced substantial shifts in predicted bacterial metabolic functions, affecting 137 of 154 KEGG modules. These changes, which included key metabolic shifts—such as reduced energy metabolism, increased carbohydrate degradation over lipid or amino acid metabolism, and enriched biosynthesis of stress-responsive metabolites—occurred independently of later colonization or signalling molecule application.

Conclusions Early AMF inoculation primes the rhizosphere bacteriome toward a stress-adapted functional state, likely mediated by altered root exudation during early symbiosis. The application of SL-M2 and quercetin further supports their role as prebiotics in sustaining inoculant efficacy. This study provides mechanistic insight into how nursery-stage microbial interventions can shape rhizosphere functionality, offering promising strategies for microbiome-driven improvements in sustainable agriculture.

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Introduction

Arbuscular mycorrhizal fungi (AMF) are a group of beneficial soil-borne fungi that establish symbiotic relationships with the roots of the majority of terrestrial plants, including important agricultural crops. The symbiotic relationship between AMF and plants has been demonstrated to provide a number of benefits to the host plant, including enhanced nutrient uptake and modulation of plant defence responses (Stratton et al. 2022). This, in turn, provides a primed state that allows for a more effective response to both biotic and abiotic stresses (Pozo et al. 2015). The establishment and functioning of the AM symbiosis depend on a high degree of coordination between the host plant and the AM fungus, which is initiated during the pre-symbiotic phase in the rhizosphere. The molecular communication between the two partners starts with the production of signalling molecules, such as strigolactones and flavonoids, by the host plant (López-Ráez et al. 2017; Lidoy et al. 2023). These compounds are recognized by the AM fungus, stimulating spore germination and hyphal growth, thereby promoting symbiosis establishment (Akiyama et al. 2005; Bonfante and Genre 2010; Lidoy et al. 2023). Accordingly, there is an increasing interest in using some of these compounds as biostimulants in agriculture to promote beneficial symbiosis.

Tomato (*Solanum lycopersicum* L.) plants, being one of the most valuable horticultural crops worldwide, benefit significantly from mycorrhizal associations (Smith and Read 2008). *Rhizophagus irregularis* (formerly known as *Glomus intraradices* or *Rhizophagus intraradices*) is one of the most extensively studied and commercialized AMF species due to its widespread occurrence in nature, its high sporulation and root colonization ability and significant impact on plant growth and health (Deja-Sikora et al. 2023; Kokkoris et al. 2024). The inoculation of tomato plants with *R. irregularis* has been demonstrated to enhance plant performance, including parameters such as plant biomass, nutritional status and stress resistance (Rivero et al. 2018; Minchev et al. 2024). This enhancement is attributed to the increased efficiency of nutrient uptake facilitated by the extensive hyphal network of the mycorrhizal fungi, which extends beyond the root zone and accesses nutrients that are otherwise unavailable to the plant (Pozo et al. 2015; Bidellaoui et al. 2019). While there is a general consensus on the potential benefits of AMF inoculation, the optimal timing and conditions for inoculation remain a topic of ongoing exploration. Some studies have indicated that early inoculation may facilitate the establishment of a robust symbiotic relationship between the fungi and plant roots, which could subsequently enhance nutrient uptake and plant growth (Janoušková et al. 2017). Other investigations indicate that the specific conditions of the soil and the plant species are the primary determinants of the benefits of AMF inoculation (Jia et al. 2023; Hao et al. 2024). However, the effects seem to depend on the plant genotypes under consideration and the biotic and biotic contexts. It was shown that the inoculation of tomato plants at the nursery stage with *R. irregularis* resulted in higher colonization and arbuscule frequency at harvest, as well as in an enhanced plant growth, yield attributes, and N and P levels in shoots and roots (Subramanian et al. 2006; Bona et al. 2017). In contrast, other tomato varieties pre-inoculated at the nursery stage with *Funneliformis mosseae* and *R. irregularis*, and then transplanted into the field exhibited varying degrees of AMF colonisation, but no yield increase compared to the mock-inoculated controls (Njeru et al. 2017).

The potential contribution of the mycorrhiza-associated changes in the soil microbial communities to the impact on plant performance have been proposed. It is acknowledged that interactions between AMF and the rhizosphere microbial community result in the formation of a more specialised microbial community, which in turn supports plant health and growth and soil quality (Nasslahsen et al. 2022; Yang et al. 2024). The existence of distinct microbial community profiles associated with different AMF species provides further support for the hypothesis that selective association processes occur primarily within mycelia. In particular, *R. irregularis* has been demonstrated to recruit a distinctive hyphosphere populated by a unique soil microbiome and to stimulate their functional profiles, thereby enhancing nutrient cycling and improving soil health (Chen et al. 2022). However, the precise mechanisms by which AMF interact with the hyphosphere soil microbiome remain to be elucidated, and evidence suggests that recruitment may be species-dependent. For example, the mycelial bacterial community of *Rhizophagus cerebriforme* was found to contain 60 bacteriome core taxa that were exclusive to it, while *Rhizophagus clarus* and *R. irregularis* demonstrated the presence of 25 and 9 exclusive taxa, respectively (Lahrach et al. 2024).

In this context, the objective of this study was to investigate the impact of *R. irregularis* inoculation on the metabolic functions of the rhizosphere microbiome under open-field production systems. To the best of our knowledge, there is little information regarding the impact of AMF inoculation on rhizomicrobiome functionality, particularly with regard to the potential lasting effects of early inoculation during the nursery stage in field trials. We tested the hypothesis that *R. irregularis* nursery inoculation affects metabolic pathways of the rhizosphere microbiome of tomato plants, and that this effect persists throughout the growth period of the tomato plants in the field. Additionally, the study aimed to assess the role of the co-addition of signalling molecules that promote AM symbiosis establishment, including strigolactones and flavonoids, in potentially modifying the structure and functionality of the rhizomicrobiome community.

Material and methods

Plant and microbial material

A large-scale open field experiment was performed in collaboration with the agricultural cooperative SAT Hortoventas, Ventas de Zafarraya, Granada (Spain). As it is a standard practice in intensive tomato production systems, we used grafted tomato plants. Tomato (*Solanum lycopersicum* L.) seeds of the root-stock variety Kardia (Syngenta, Spain) and the commercial variety Albenga (Unigen Seeds, Spain) were sown in 150-well starting trays with cell dimensions 3.5 × 3.5 × 6.5 cm, containing blond seedling peat moss without fertilizers (Novarbo, Finland): zeolite mixture (3:1). Half of the root-stock seeds were inoculated with 300 spores of the AM fungus *R. irregularis* (MUCL 57021; Ri plants) provided by Koppert Biological Systems (The Netherlands). The other half served as non-mycorrhizal controls (Nm plants). Seedlings were grown in a commercial nursery (San Isidro, Torrox, Spain) for 4 weeks. Then, scions of the tomato variety Albenga were grafted onto the root-stock Kardia, and maintained in the nursery for another 3 weeks. Both Nm and Ri plants were exogenously treated by irrigation with signalling compounds known to promote AM symbiosis. The synthetic strigolactone analogue GR24^{4DO} (Scaffidi et al. 2014) (StrigoLab, Italy) and the strigolactone mimic SL-M2 (patent ES1641.1974-PRIO) were applied at 0.1 and 1 µM, respectively (Supplementary Fig. S1). The flavonol quercetin (Sigma-Aldrich, Germany) was applied at 1 µM (Supplementary Fig. S1) (Lidoy et al. 2023). Untreated control treatments were irrigated with tap water. The treatments were applied once a week. Finally, 7-week-old tomato seedlings were transplanted into the field.

Experimental setup

On May 30, 2022, the seedlings were transplanted into the experimental open field (SAT Hortoventas, Ventas de Zafarraya, Granada, Spain 36°57′26″N 4°07′14″O) and maintained during the whole crop cycle from June to October. It was a clayey soil consisting of calcareous fluvisols with 0.12% of N, 3.10% of total C and 0.73% of organic C. The mineral composition of the soil is described in the Supplementary Table S1. The climatological conditions in the area during the experiment are detailed in the Supplementary Table S2. The experimental field set up was a typical open field configuration with plants arranged in a ’castle shape’ (Supplementary Fig. S2), measuring 45 m long and 25 m wide, covering a total area of 1,125 m^2. The inoculation with *R. irregularis* and the co-addition treatments followed a randomized complete block design with four blocks. Each block contained the eight treatments, and each treatment consisted of 12 plants (pseudo-replicates), totalling 384 plants (N = 8 treatments × 4 blocks × 12 pseudo-replicates) (Supplementary Fig. S3). Plants and roots were harvested on October 19, 2022. The rhizosphere soil was obtained from roots individually, placed in 50 ml Falcon tubes and stored at 4ºC until use. The roots were used to quantify mycorrhizal colonization. For the molecular analyses, the twelve pseudo-replicates from each treatment and block were pooled four by four. Three independent replicates per treatment and block (N = 3 × 4 = 12 replicates) were used.

Quantification of colonization rates

Quantification of mycorrhizal colonization was performed by histochemical staining as previously described (García et al. 2020). In brief, roots were harvested, cleared, and digested in a 10% (w/v) KOH solution for 2 days at room temperature. The alkaline solution was then thoroughly washed with tap water and acidified using a 2% (v/v) acetic acid solution. Subsequently, fungal structures were stained with a 5% (v/v) black ink (Lamy, Germany) and 2% acetic acid solution, followed by incubation at room temperature as per Vierheilig et al. (2005). After 24 h, the ink was washed off with tap water, and colonization levels were determined using the gridline intersection method (Giovannetti and Mosse 1980), using a Nikon SMZ1000 stereomicroscope.

Molecular analyses of rhizosphere microbiome

The DNA of the rhizosphere soil was extracted from two replicates of 0.1 g for each homogenised soil sample using the TissueLyzer II method with the aid of a DNeasy Plant Pro Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The two extracted replicates for each rhizosphere soil sample were pooled and concentrated at 35ºC to a final volume of 50 uL using a Savant Speedvac® concentrator (Fisher Scientific, Madrid, Spain). To characterize the microbial communities, the ProV3V4 primers (5' CCTACGGGNBGCASCAG 3' and 5' GACTACNVGGGTATCTAATCC 3') (Lundberg et al. 2013; Takahashi et al. 2014) were used to amplify the V3-V4 hypervariable regions of the bacterial 16S rRNA gene, and the primers ITS4-ITS2_fITS7 (5' TCCTCCGCTTATTGATATGC 3' and 5' GTGARTCATCGAATCTTTG 3') (Ihrmark et al. 2012) were used to amplify the fungal ITS2 region. The samples were sequenced on the Illumina MiSeq platform (2 × 300 nucleotide paired-end protocol) at the genomic facilities of the López-Neyra Institute of Parasitology and Biomedicine (IPBLN-CSIC). Blockers were used to minimize amplification of mitochondria and chloroplasts (Lundberg et al. 2013). Illumina reads were deposited in the Sequence Read Archive (SRA) service of the European Bioinformatics Institute (EBI) database under BioProject ID: PRJNA1192287 (Biosamples accession numbers SAMN45104698- SAMN45104713).

All raw paired-reads from 16S rRNA gene and ITS2 region were analysed using R version 4.2.2 (R Core Team 2022). The DADA2 v1.24.0 pipeline (Callahan et al. 2016) was utilized to process the raw sequences and construct an amplicon sequence variant (ASV) table. ASV taxonomic assignment was performed using the *assignTaxonomy* function (based on naïve Bayesian classifier method) against the SILVA v138.1 (Quast et al. 2013) and UNITE v10.0 (Abarenkov et al. 2024) databases for bacteria and fungi, respectively. An ASV matrix was generated using the Marker Data Profiling module on the MicrobiomeAnalyst web platform (Dhariwal et al. 2017; Chong et al. 2020). ASVs were filtered based on a minimum prevalence in samples of 10% and a minimum variance of 10%. All samples reached a plateau based on the rarefaction curves generated by the MicrobiomeAnalyst tool.

Predictive metagenomic profiles

The Tax4fun v0.3.1 software (Aßhauer et al. 2015), integrated into the MicrobiomeAnalyst Shotgun Data Profiling module (Dhariwal et al. 2017), was employed to predict the functional pathways of soil bacterial communities based on 16S data sets obtained from the SILVAngs web server. This prediction was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) modules- functional units of sets of genes in the KEGG metabolic pathways database that can be linked to specific metabolic capacities and other phenotypic characteristics. These are manually defined sets of genes or reactions that correspond to specific biological functions or pathways (Kanehisa and Goto 2000; Kanehisa et al. 2023).

Statistical analyses

We first assessed the levels of mycorrhizal colonization across the different combinations of nursery stage inoculation with *R. irregularis* (Nm, Ri) × signaling molecules (C, GR24, Q, SL-M2). This assessment was conducted using linear models at two specific times: at the moment of plant transplantation into the field and at the time of fruit harvest.

Next, we employed univariate linear models to test the hypothesis that nursery stage inoculation with *R. irregularis* × nursery stage inoculation with signalling molecules would impact the predicted

KEGG bacterial modules in the rhizosphere samples at the time of fruit harvest. Given the rigorous experimental design—where each NGS sample was a composite sample from the rhizosphere of three plants—and the thorough metagenomic cleaning and filtering processes applied to the samples, no *p*-value correction was performed to avoid inflating type II errors (Moran 2003; Dopheide et al. 2019; Zinger et al. 2019). Alongside with statistical significance, the effect size ($\log_2$ fold change) is shown throughout the paper. Analyses were performed in R 4.2.2 (R Core Team 2022) using the *nlme* package (Pinheiro et al. 2014). We used R packages *ggplot2* (Wickman 2016), *pheatmap* (Kolde 2019) and *EnhancedVolcano* (Bligue et al. 2023) packages for data visualisation.

Results

Strigolactones and quercetin promotes AM symbiosis at the nursery stage

Before transplanting into the field, mycorrhizal colonization levels were assessed in a subset of plants to i) confirm the successful establishment of AM symbiosis and ii) to evaluate the effect of the exogenous application of the different signalling molecules on mycorrhizal colonization. Mycorrhizal symbiosis was successfully established in *R. irregularis* inoculated plants at the nursery stage, although root colonization levels at this stage were low (Fig. 1A). Mycorrhizal colonization was further enhanced approximately twofold by the addition of the synthetic strigolactones analogue GR24^{4DO} and the strigolactone mimic SL-M2, and by the flavonol quercetin (Fig. 1A). Non inoculated control plants did not show any mycorrhizal colonization. Plantlets were then transferred to the field, and final mycorrhizal colonization levels were analysed in roots harvested from the field at the end of experiment. In this case, mycorrhizal colonization was observed in the control, non-inoculated Nm plants (Fig. 1B), confirming the presence of AMF in the field soil. However, higher levels of mycorrhizal colonization were detected in the plants pre-inoculated during the nursery stage, especially after the co-addition of SL-M2 and quercetin (Fig. 1B).

R. irregularis inoculation at the nursery stage altered bacterial metabolic functions

Despite the establishment of mycorrhiza in both inoculated and non-inoculated plants by the end of the experiment, inoculation with *R. irregularis* at the nursery stage did not alter the taxonomic composition of bacterial or fungal communities after a five-month growth period post-transplantation (Supplementary Fig. S4). Initial findings also indicated that the core taxa of the bacteriome within the rhizosphere bacterial community of tomato plants exhibited limited diversity (Supplementary Fig. S5). However, a shift in the predicted metabolic functions of bacteria in the rhizosphere of tomato plants was observed (Fig. 2A, B). Of the 154 KEGG modules present in the rhizosphere samples, nursery stage inoculation with *R. irregularis* had a significant effect on 137 of them, with some being enriched and others depleted, and with effect sizes ranging from low to moderate values. However, this did not affect the taxonomic diversity of the bacterial and fungal communities, nor the abundance of predicted fungal lifestyles. The exogenous addition of signalling molecules during the nursery stage did not elicit any observable effects on the bacterial communities at the conclusion of the field experiment.

Alterations in predicted energy metabolism

A global reduction in the metabolic activity of rhizobacteria was observed in the nursery stage inoculation treatment with *R. irregularis*, particularly in those related to the synthesis of acetyl-CoA, assimilatory nitrate to ammonia, assimilatory and dissimilatory sulfate reduction to H_2S, and several metabolic pathways related to methane emissions (Fig. 3).

Modulation of predicted carbohydrate and glycan metabolism

Early inoculation with *R. irregularis* resulted in a heterogeneous shift of the carbohydrate metabolism of rhizobacteria (Fig. 4A). Here, a reduction was observed in the Entner-Doudoroff, uronate, ethylmalonate and malonate semialdehyde pathways of rhizosphere bacteria. Conversely, an increase in other metabolic pathways, including galactose degradation, biosynthesis of nucleotide sugars and trehalose pathways was observed.

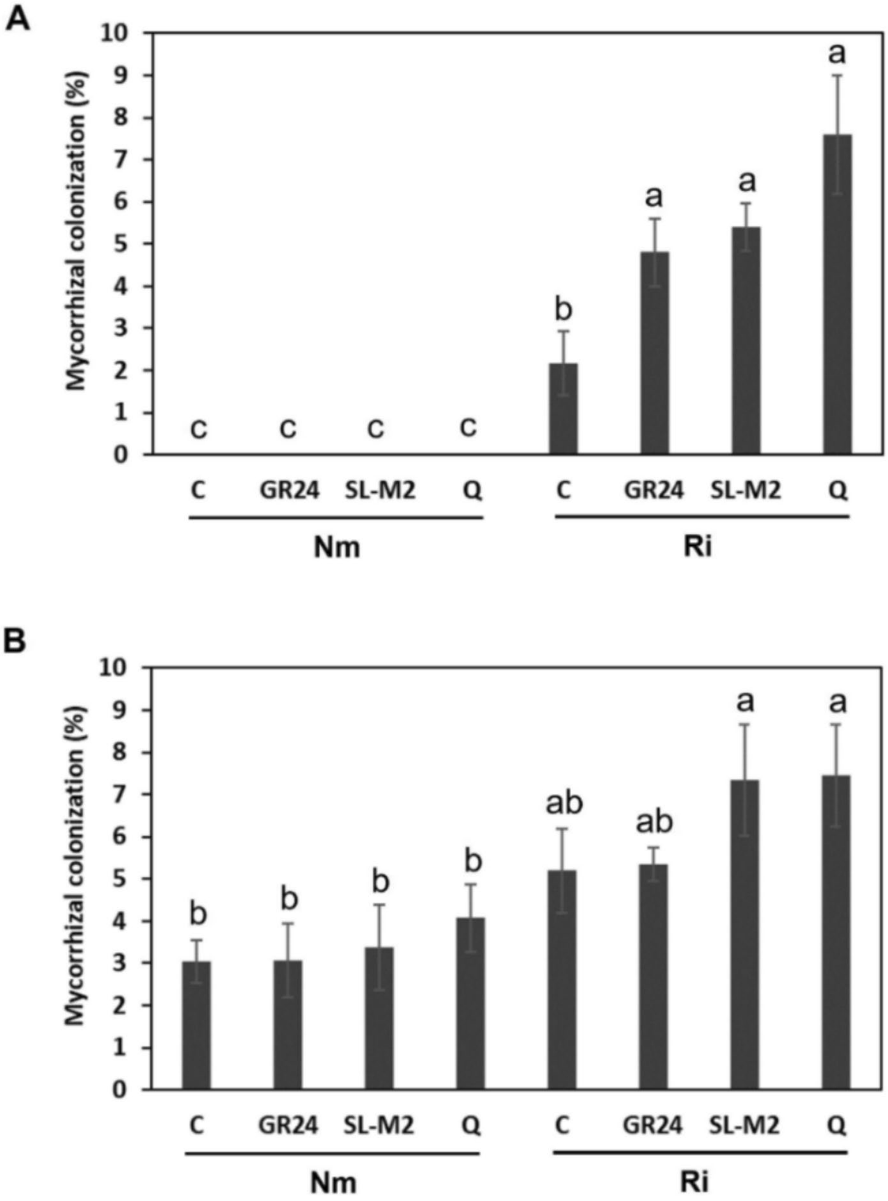


Fig. 1 Levels of mycorrhizal colonisation in the different inoculation combinations at the nursery stage with *R. irregularis* (Nm, Ri) × signalling molecules (C, GR24, Q, SL-M2) at **(A)** the time of transplanting to the field and **(B)** at the time of fruit harvest. Different capital letters indicate significant differences ($P < 0.05$), tested by Tukey's HSD post hoc pairwise comparisons

Furthermore, a slight increase in both the glycolysis and pentose phosphate KEGG modules was identified. Additionally, the degradation of glycans was also enhanced in the metabolism of rhizobacteria by *R. irregularis* inoculation (Fig. 4B).

Changes in predicted amino acids, cofactors and vitamins metabolism

A comparable pattern to that observed in carbohydrate metabolism was identified in the amino acid metabolism (Fig. 5) and in the metabolism of cofactors and vitamins (Fig. 6) of the rhizosphere bacteriome. The inoculation with *R. irregularis* in a nursery setting enhanced the biosynthesis of the amino acids cysteine, GABA, lysine, methionine and proline, while reduced the biosynthesis of acetyl and acetoacyl CoA, lysine, phenylalanine, tyrosine and isoleucine in the rhizosphere of tomato plants after five months of growing in the field (Fig. 5).

With regard to the metabolism of cofactors and vitamins, it is noteworthy that there was an increase in the biosynthesis of biotin and pantothenate, accompanied by a decrease in the biosynthesis of cobalamin, menaquinol and ubiquinol, within the rhizobacteria community metabolism (Fig. 6).

Shifts in n predicted lipid metabolism

The impact of early *R. irregularis* inoculation on the lipid metabolism of the rhizobacteria is shown in Fig. 7. With the exception of an increase in the elongation step of fatty acid biosynthesis, a global reduction in overall lipid metabolism was evidenced, encompassing processes such as acylglycerol degradation, beta-oxidation, acyl-CoA, jasmonic acid and triacylglycerol biosynthesis.

Alterations in predicted nucleotide metabolism

An increase in metabolic pathways associated with the synthesis of adenine nucleotides and the catabolism of purines and pyrimidines, resulting in the production of urea and aminoisobutanoate, respectively, was observed in the nursery stage inoculation treatment with *R. irregularis* (Fig. 8). Conversely, a reduction in purine synthesis was observed, suggesting a promoting effect of AM symbiosis in the urea cycle.

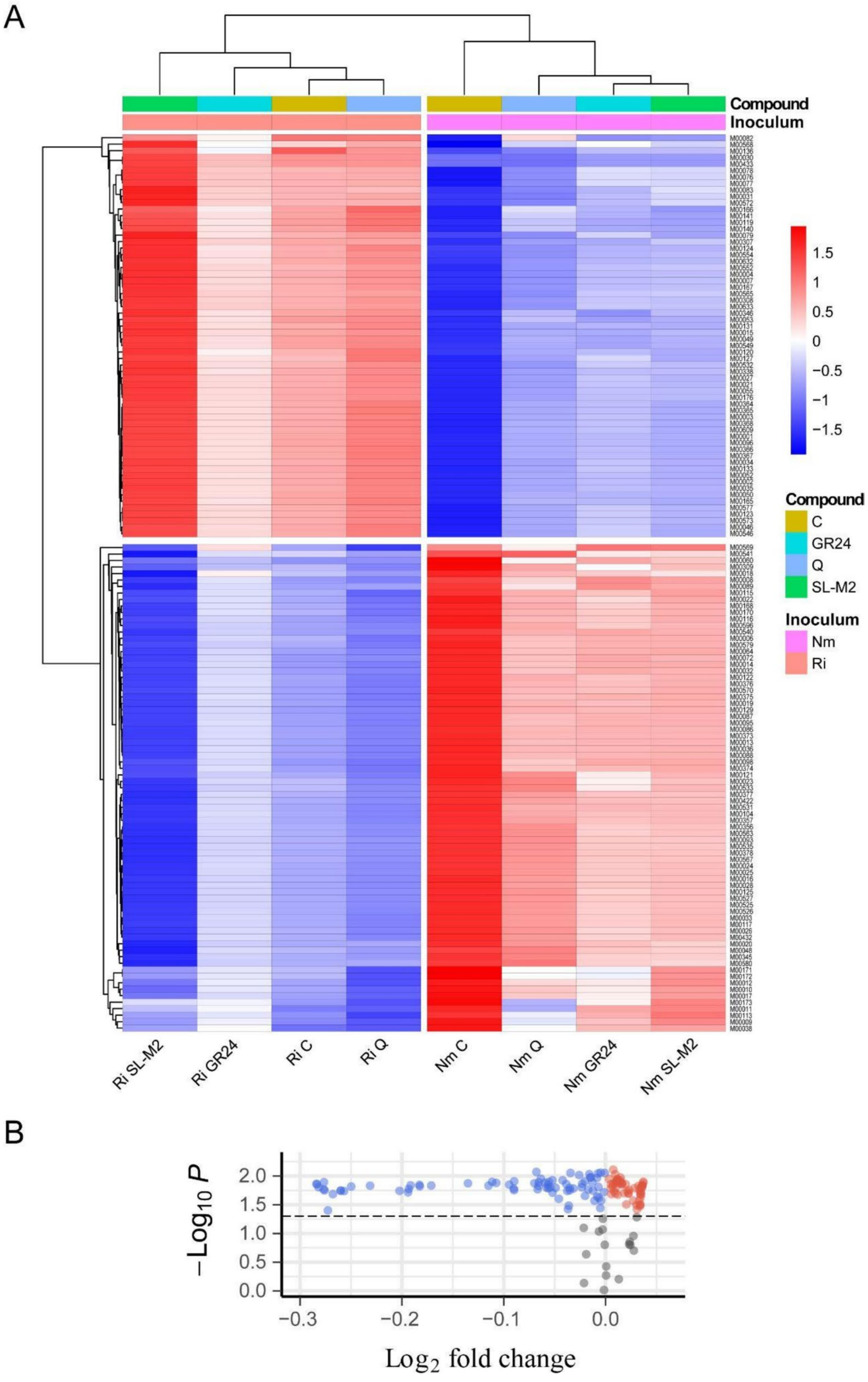
Modulation of predicted terpenoids, polyketides and xenobiotics pathways

The early inoculation of *R. irregularis* led to an enhancement of KEGG modules associated with the biosynthesis of terpenoids and polyketides in the rhizobacteria of cultivated tomato (Fig. 9A). This was particularly evident for the biosynthesis of C10-C20 isoprenoids and the deviation of C5 isoprenoid biosynthesis from the mevalonate pathway to a non-mevalonate pathway. Additionally, a considerable number of KEGG modules associated with xenobiotic degradation potential pathways in rhizobacteria were found to be diminished, with the majority of these modules reliant on coenzyme A (Fig. 9B).

Discussion

Mycorrhizal inoculation has been proposed to have an impact on the rhizosphere communities, but there is a gap in the research examining the lasting impact of inoculation at the nursery stage on the rhizosphere community, particularly in terms of functional diversity. The present study constitutes an assessment of the impact of early inoculation with AMF on the structure and functionality of the rhizosphere microbiome of tomato plants at the harvesting period, after five months of growth in the field. We show that early inoculation with the AMF *R. irregularis* during the nursery stage efficiently colonized tomato seedlings. Moreover, the exogenous addition of signaling molecules further promoted symbiosis establishment, showing their potential as prebiotic biostimulants. Thus, only inoculated plants were mycorrhized at the time of transplanting to the field. However, at the end of the experiment, mycorrhizal colonization was also observed in non-inoculated plants after growing in the field, albeit with slightly lower levels compared to inoculated plants. This is likely due to the presence of AMF in the agricultural soil, either native isolates or remnants from previous campaigns in the agricultural soil.

Prior research has demonstrated that AMF inoculation can influence the soil-borne fungal community, increasing the diversity and abundance of beneficial fungi while suppressing pathogenic species (Cao et al. 2024). The initial findings of this study indicate that the bacteriome core taxa within the rhizosphere bacterial community of tomato plants exhibited a limited range of diversity. Similarly, the findings by Lahrach et al. (2024) suggest that the number of core bacteriome taxa in the mycelial bacterial community of *R. irregularis* is very limited. The consistency in feature-level taxa across both inoculated and non-inoculated plants, with comparable relative abundance, along with beta-diversity analysis, indicates that *R. irregularis* inoculation in the nursery stage had no impact on the taxonomic diversity of the bacterial community in the rhizosphere of tomato plants after a five-month growth period in the field. Nevertheless, the nursery inoculation resulted in alterations to the potential metabolic functions of the rhizosphere bacteria. The exogenous addition of signalling molecules during this stage had no effect on the bacterial communities at the end of the field experiment, likely due to their short-lived nature (Akiyama et al. 2010; Wang et al. 2016). It was shown that the introduction of AMF into the soil can modify bacterial functionality without altering the composition of the bacterial community (Sangwan and Prasanna 2022; Lahrach


A
Compound
Inoculum
1.5
1
0.5
0
−0.5
−1
−1.5
Compound
C
GR24
Q
SL-M2
Inoculum
Nm
Ri
Ri SL-M2
Ri GR24
Ri C
Ri Q
Nm C
Nm Q
Nm GR24
Nm SL-M2
B
−Log10 P
2.0
1.5
1.0
0.5
0.0
−0.3
−0.2
−0.1
0.0
Log2 fold change

◂**Fig. 2** (**A**) Heatmap classifying mean rhizosphere bacterial KEGG module values per nursery stage inoculation with *R. irregularis* (Nm, Ri) × signalling molecules (C, GR24, Q, SL-M2). The colour gradient indicates the magnitude and direction of the standardized coefficients, with shades of red representing enriched modules and shades of blue representing depleted modules. Coefficients close to zero are depicted in white. Hierarchical clustering (average method) was applied to both bacterial KEGG modules and treatment combinations to highlight the degree of similarity within the data. The dendrograms along the axes show the clustering structure. (**B**) Volcano plot illustrating the average $\log_2$ fold change of rhizosphere bacterial KEGG modules in the nursery inoculation treatment with *R. irregularis* (Ri) compared to the control (Nm). Each point on the graph represents a specific KEGG module. The y-axis shows the $-\log_{10}$ *p*-value, indicating the statistical significance ($P < 0.05$) of the observed changes, and the x-axis shows the $\log_2$ fold change, indicating the magnitude of the change in abundance between conditions. Grey dots indicate bacterial KEGG modules for which there is no significant effect, red dots indicate those enriched by nursery stage inoculation with *R. irregularis*, and blue dots indicate those depleted by nursery stage inoculation with *R. irregularis*

et al. 2024). Nevertheless, to date, no research has investigated the effect of nursery inoculation on the bacterial rhizosphere community. Our findings indicate that bacterial communities are highly dynamic and demonstrate the capacity to adapt to the introduction of AMF, with these adaptations persisting throughout the growing and development period. The observed shift towards more specialised metabolic functions within the bacterial community could be attributed to a number of factors, including functional redundancy, metabolic plasticity, microbial interactions and resource allocation. It is important to note that a significant proportion of bacterial species are capable of performing analogous functions (Singavarapu et al. 2023). Additionally, bacteria can modify their metabolic pathways in response to environmental changes, such as variations in nutrient availability or the presence of root exudates from mycorrhizal plants (Parter et al. 2007; Wani et al. 2022). Consequently, even if the community composition remains unaltered, the functional roles of the bacterial community may shift in order to adapt to the novel environmental conditions created by the AM symbiosis. Mycorrhizal plants can also impact bacterial activity through the release of signalling molecules, which can enhance or suppress specific functions while maintaining the overall composition of the bacterial community (Sangwan and Prasanna 2022). Moreover, mycorrhizae have the capacity to reallocate soil resources (Huey et al. 2020). In response, bacteria may alter their functional roles in order to exploit new niches or resources provided by the mycorrhiza, resulting in changes in functionality without a change in community composition. These findings support the assertion that bacterial communities are dynamic and that they have the capacity to adapt their functional capacity in response to root colonization by AMF. Nevertheless, there is currently a gap in the research examining the impact of nursery inoculation on the bacterial rhizosphere community, particularly in terms of functional diversity. In the present study, predictive metagenomics was employed to explore the potential functional capabilities of rhizosphere bacterial communities. While this approach offers valuable insights, it is essential to recognize its inherent limitations. These include a strong dependence on the completeness and accuracy of reference genome databases —which may not fully represent the vast diversity of environmental microbiomes, particularly in complex ecosystems like soils—, as well as a lack of strain-level resolution and the possibility of introducing biases in functional inference (Sun et al. 2020). Nonetheless, predictive tools such as Tax4Fun have demonstrated robust performance across diverse environments, showing strong correlations with whole-genome metagenomic profiles in terms of functional prediction accuracy (Djemiel et al. 2022; Lema et al. 2023). Their utility is especially pronounced in agricultural and ecological studies, where large sample sizes and field conditions often limit the feasibility of deeper sequencing approaches.

In our case, predictive metagenomics provided meaningful insights into the restructuring of rhizosphere metabolic functions following early AMF inoculation. It can be reasonably inferred that the reduction in the predicted energy-intensive processes observed in the rhizobacterial community from inoculated plants can be attributed to differential resource allocation. The presence of AMF and symbiosis establishment likely facilitate more efficient nutrient uptake by the plant, prompting bacteria to reduce their energy metabolism activities and focus on metabolic processes that support the new ecological balance. This shift may be due to changes in substrate availability and/or to the emergence of alternative metabolic pathways that are more advantageous in the mycorrhizal environment (Chen et al. 2022; Luo et al. 2024). In our experimental setup, the reduction

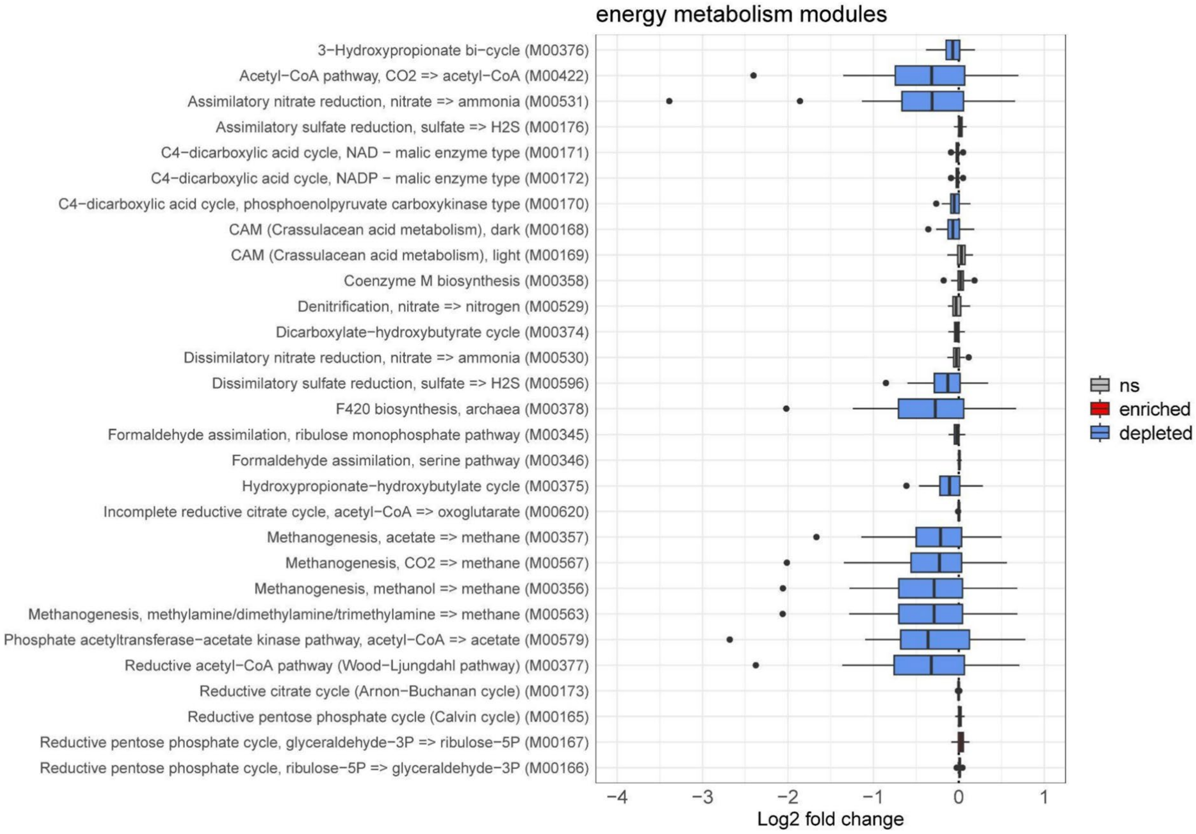


Fig. 3 Effect of nursery inoculation with *R. irregularis* on predicted KEGG pathway molecular functions (KEEG modules) associated with Energy Metabolism in rhizosphere bacteria. The $\log_2$ fold change of nursery inoculation with *R. irregularis* (Ri) with respect to the mean of the treatment without mycorrhizal inoculation (Nm) is shown. Red indicates significant enrichment, blue indicates significant depletion and grey indicates no significant effect

of predicted pathways associated with the synthesis of acetyl-CoA, assimilatory nitrate to ammonia, assimilatory and dissimilatory sulfate reduction to H_2S, and methane emissions in the presence of mycorrhizal plants may be indicative of several ecological and metabolic shifts. The acetyl-CoA pathway is fundamental to the survival and growth of a multitude of bacterial species, particularly in environments where organic carbon sources are scarce (Bernal et al. 2016). In this metabolic pathway, the coenzyme A facilitates the conversion of CO_2 and H_2 into acetyl-CoA, which can then be employed for the synthesis of cellular components or for energy production. Indeed, a reduction in the inferred ethylmalonyl-CoA pathway, which plays a central role in the carbon metabolism of numerous soil bacteria and converts acetyl-CoA into precursor metabolites for cell carbon biosynthesis, was observed. The decrease in nitrate and sulphate reduction pathways and methane emissions supports the hypothesis that bacteria are undergoing a shift away from certain metabolic pathways involved in carbon metabolism.

Therefore, a decrease in this activity may result in a diminished carbon metabolism, encompassing the Entner-Doudoroff, pentose phosphate, uronate, ethylmalonyl and malonate semialdehyde pathways. The Entner-Doudoroff pathway provides an alternative route to glycolysis, while the pentose phosphate pathway operates in parallel to glycolysis. The observed slight increase in glycolysis and galactose predicted catabolism may be linked to these pathways, indicating that bacteria may have shifted to them as a means of catabolizing glucose. In this context of decreased carbon metabolism, the uronate and malonate semialdehyde pathways, which facilitate the breakdown of complex organic compounds, were also reduced.

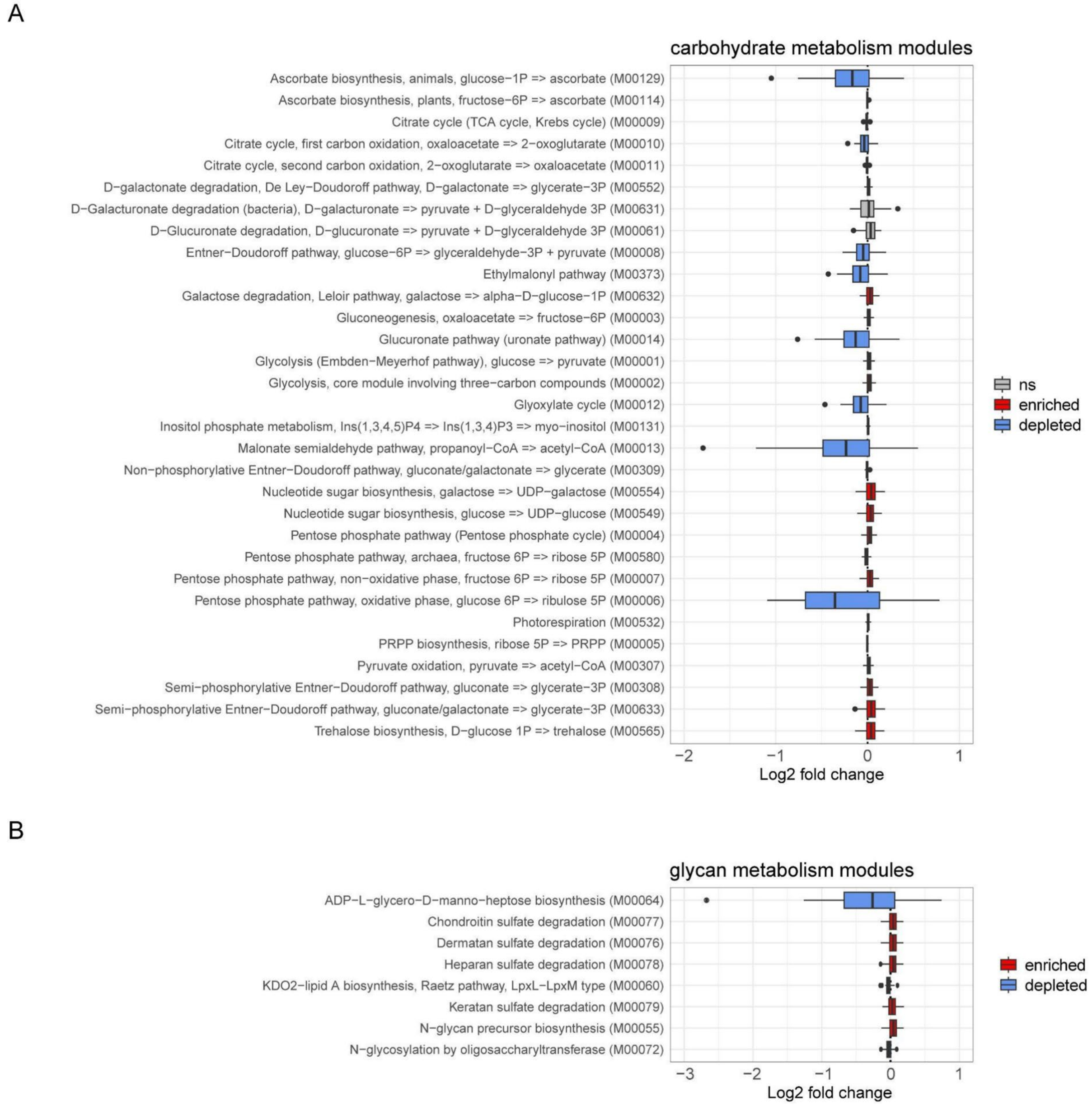


Fig. 4 Effect of nursery inoculation with *R. irregularis* on predicted KEGG pathway molecular functions (KEEG modules) associated with (**A**) Carbohydrate Metabolism and (**B**) Glycan Metabolism in rhizosphere bacteria. The $\log_2$ fold change of nursery inoculation with *R. irregularis* (Ri) with respect to the mean of the treatment without mycorrhizal inoculation (Nm) is shown. Red indicates significant enrichment, blue indicates significant depletion and grey indicates no significant effect

Nonetheless, the increase in predicted KEGG modules associated with glycan metabolism indicates that the rhizosphere bacteria are adjusting to utilise long chains of monosaccharide molecules with greater efficiency. This may be attributed to the AM symbiosis increasing the accessibility of complex carbohydrates in the soil (Saia et al. 2019), which are then metabolised by bacteria for energy and growth.

The observed decline in the predicted lipid metabolism is consistent with the shift in the bacterial community's energy metabolic pathways. This may be a response to the influence of the AMF or the AM

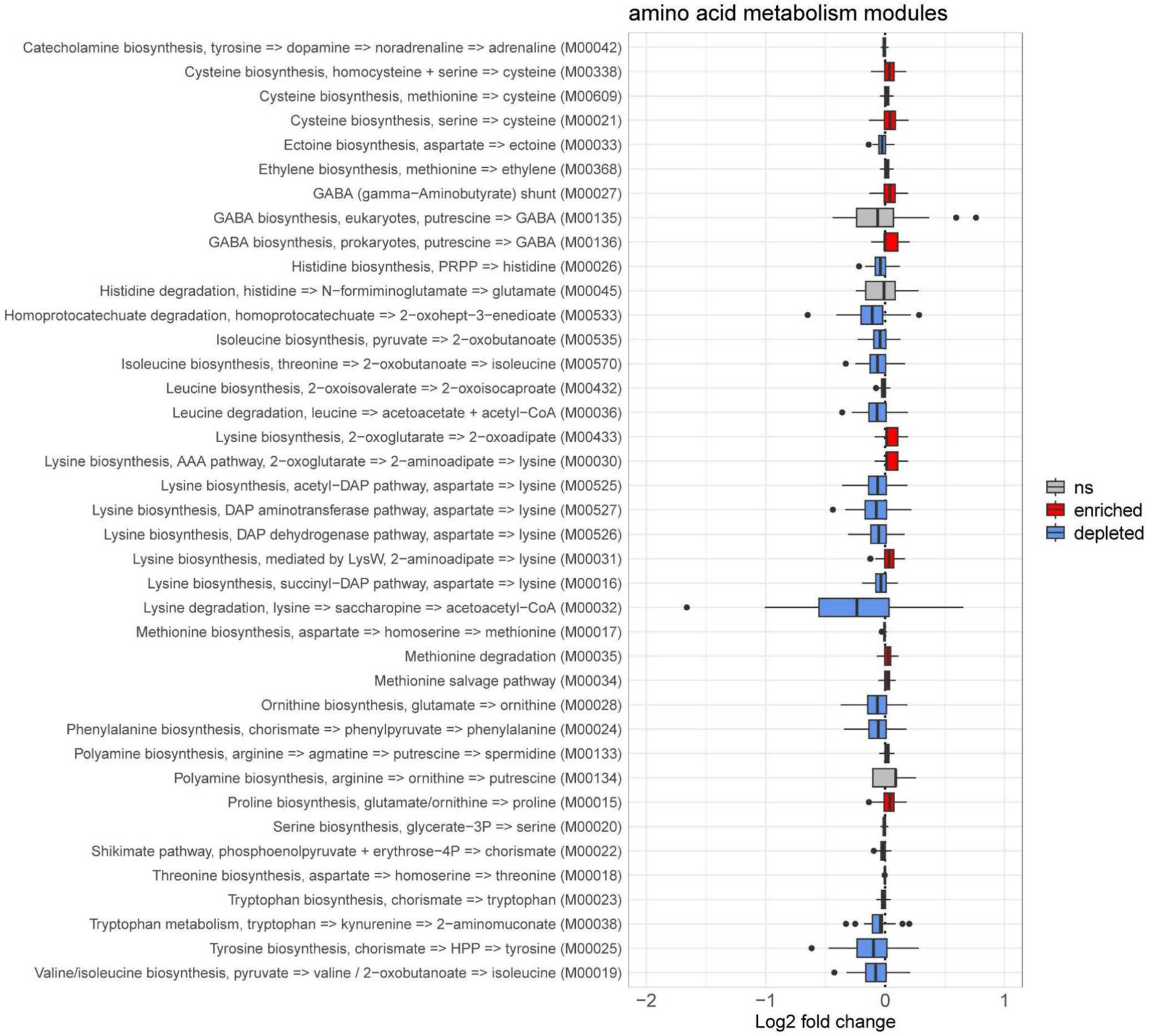


Fig. 5 Effect of nursery inoculation with *R. irregularis* on predicted KEGG pathway molecular functions (KEEG modules) associated with Amino Acid Metabolism in rhizosphere bacteria. The $\log_2$ fold change of nursery inoculation with *R. irregularis* (Ri) with respect to the mean of the treatment without mycorrhizal inoculation (Nm) is shown. Red indicates significant enrichment, blue indicates significant depletion and grey indicates no significant effect

symbiosis on the plant's root exudates. In general, carbohydrates are more abundant in root exudates than lipids when AMF is present. The decline in lipid metabolism that has been observed is consistent with the shift in the bacterial community's energy metabolic pathways. These findings align with previous research showing that AMF presence can modulate root exudate composition, leading to an increased release of carbohydrate-rich compounds, such as sugars and organic acids (Pasquini et al. 2023). This enhanced nutrient accessibility may diminish the need for bacteria to rely on lipid metabolism for energy, consequently resulting in a reduction in pathways such as beta-oxidation and acylglycerol degradation (Koshila Ravi and Muthukumar 2024). Consequently, this is associated with a decline in acyl-CoA biosynthesis, a process that plays a pivotal role in the storage and utilisation of energy. In relation to the reduction in jasmonic acid (JA) production, it is a well-established fact that plants and certain fungi

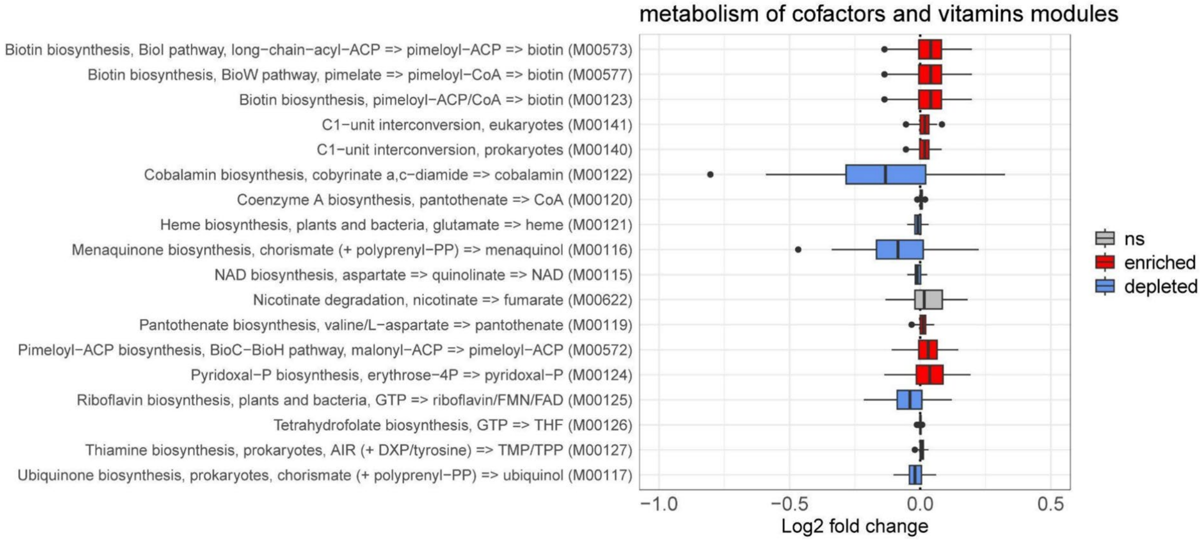


Fig. 6 Effect of nursery inoculation with *R. irregularis* on predicted KEGG pathway molecular functions (KEEG modules) associated with Metabolism of Cofactors and Vitamins in rhizosphere bacteria. The $\log_2$ fold change of nursery inoculation with *R. irregularis* (Ri) with respect to the mean of the treatment without mycorrhizal inoculation (Nm) is shown. Red indicates significant enrichment, blue indicates significant depletion and grey indicates no significant effect

are capable of synthesising this compound (Eng et al. 2021). Moreover, research has demonstrated that specific endobacteria are able to increase the levels of JA in root tissues (Zhu et al. 2021). Nevertheless, the precise function of JA in rhizosphere bacteria remains uncertain, although it is plausible that it may perform a signalling role.

The decrease in some amino acid synthesis inferred pathways provides further evidence of a potential shift in metabolic priorities, which may be attributed to the mycorrhiza providing amino acids directly in the rhizosphere as an energy source (Wang and Feng 2021). However, the observed increase in the synthesis of specific amino acids, including cysteine, GABA, lysine, methionine, and proline, suggests that the bacterial community may be engaged in a more specialised metabolic function. This shift has several implications at the ecological and functional levels. Increases in GABA and proline are frequently associated with stress responses. GABA plays a role in stress tolerance, while proline acts as an osmoprotectant, helping bacteria to cope with osmotic stress (Chun et al. 2018; Sita and Kumar 2020). This may suggest that the presence of AM symbiosis also induces a stress response in rhizosphere bacteria, resulting in the production of protective compounds. Moreover, cysteine and methionine are sulfur-containing amino acids essential for a number of metabolic processes. The increased synthesis of these amino acids could indicate enhanced sulfur metabolism, which may in turn improve the availability of essential nutrients for both plants and microbes (Korenblum et al. 2022; Yang et al. 2024). The rise in some of the biosynthesis pathways of lysine may be related to enhanced symbiotic interactions between plants and bacteria, as lysine is a precursor for the synthesis of various secondary metabolites that can influence plant–microbe interactions, promoting further mutual benefits.

The observed increase in predicted nucleotide metabolism KEGG modules, which are crucial for nucleic acid synthesis, may initially suggest that the rhizosphere bacteria associated with AM plants are experiencing enhanced growth and division. This is accompanied by the use of purine as a nitrogen source, which is likely to be degraded into urea following hydrolysis into ammonia and carbon dioxide by the enzyme urease. Nevertheless, elevated nucleotide metabolism may also signify augmented microbial activity and adaptation to the changing environment brought about by the mycorrhizal inoculation early at the nursery stage. In particular, pyrimidine

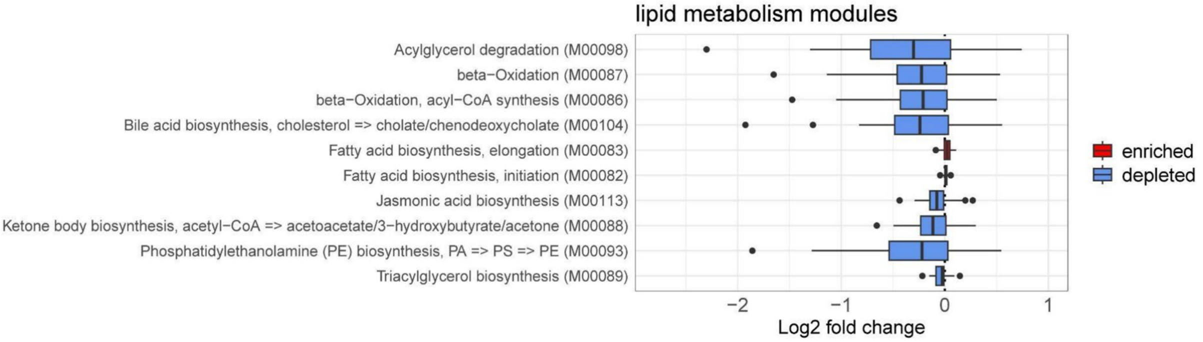


Fig. 7 Effect of nursery inoculation with *R. irregularis* on predicted KEGG pathway molecular functions (KEEG modules) associated with Lipid Metabolism in rhizosphere bacteria. The $\log_2$ fold change of nursery inoculation with *R. irregularis* (Ri) with respect to the mean of the treatment without mycorrhizal inoculation (Nm) is shown. Red indicates significant enrichment, blue indicates significant depletion and grey indicates no significant effect

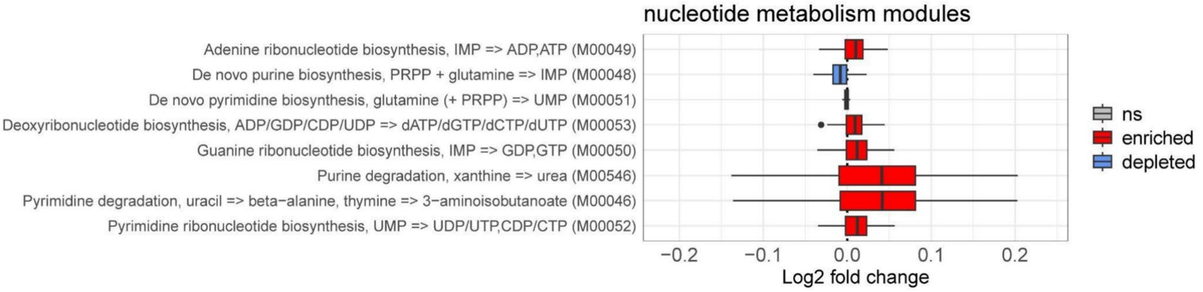


Fig. 8 Effect of nursery inoculation with *R. irregularis* on predicted KEGG pathway molecular functions (KEEG modules) associated with Nucleotide Metabolism in rhizosphere bacteria. The $\log_2$ fold change of nursery inoculation with *R. irregularis* (Ri) with respect to the mean of the treatment without mycorrhizal inoculation (Nm) is shown. Red indicates significant enrichment, blue indicates significant depletion and grey indicates no significant effect

nucleotides, such as uridine monophosphate (UMP) and cytidine monophosphate (CMP), serve as signal molecules involved in a range of cellular processes, including biofilm formation (Garavaglia et al. 2012) and bacterial chemotaxis (Liu and Parales 2008).

The early inoculation with *R. irregularis* at the nursery stage also increased the predicted abundance of bacterial KEGG modules associated with terpenoid biosynthesis, with several potential ecological implications. Terpenoids fulfil a variety of functions, including acting as signalling molecules, antimicrobial agents and stress protectants. These compounds frequently serve as defensive mechanisms, protecting bacteria from predators, pathogens, and environmental stressors. For example, terpenoids have the capacity to influence the behaviour of other microorganisms and plants through the process of signalling (Fordjour et al. 2022; Li et al. 2023). Furthermore, terpenoids and polyketides play a role in the degradation of complex organic molecules, thereby contributing to the cycling of nutrients in the soil (Bi et al. 2024). The capacity to synthesise a plethora of these compounds endows soil bacteria with the ability to adapt to diverse environmental conditions. There is evidence to suggest that the symbiosis with *R. irregularis* may result in increased expression of genes involved in terpenoid biosynthesis, leading to higher production of these compounds in plants (Kapoor et al. 2016). It seems reasonable to suggest that AMF or AM symbiosis induce rhizobacteria to produce a greater amount of these compounds, thereby enhancing plant defence mechanisms and facilitating more effective plant–microbe interactions.

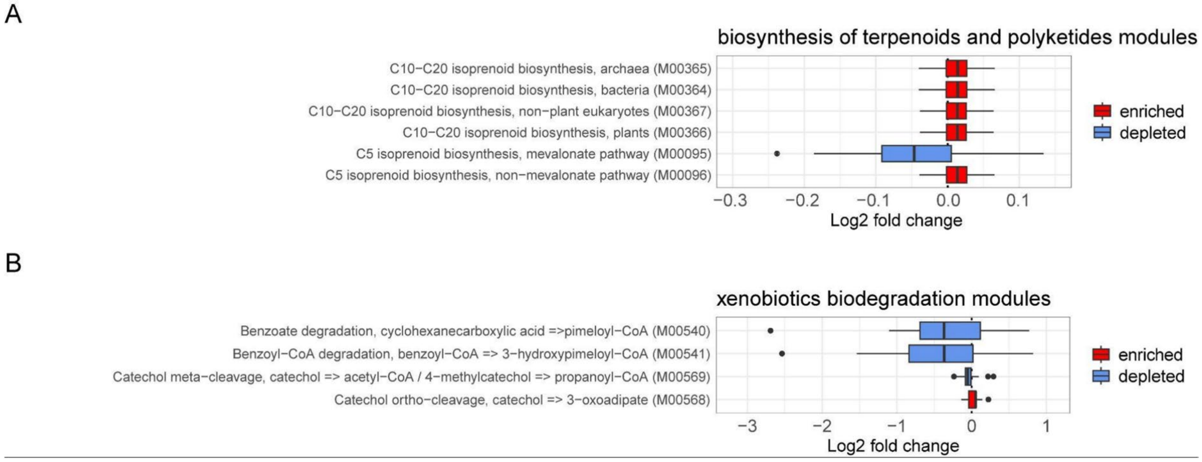


Fig. 9 Effect of nursery inoculation with *R. irregularis* on predicted KEGG pathway molecular functions (KEEG modules) associated with **A)** Biosynthesis of Terpenoids and Polyketides and **B)** Xenobiotics biodegradation in rhizosphere bacteria. The $\log_2$ fold change of nursery inoculation with *R. irregularis* (Ri) with respect to the mean of the treatment without mycorrhizal inoculation (Nm) is shown. Red indicates significant enrichment, blue indicates significant depletion and grey indicates no significant effect

Finally, the capacity for bacteria to adapt to contaminated environments is well established. It is well documented that a number of bacteria possess the capacity to degrade synthetic compounds that are not naturally present in the environment and utilise them as carbon or nitrogen sources (Mishra et al. 2021). A reduction in the number of pathways involved in the breakdown of xenobiotic compounds may indicate that mycorrhizal plants play a role in maintaining soil stability and reducing the necessity for bacteria to degrade harmful compounds. It is noteworthy that these biochemical pathways rely on acetyl-CoA as a substrate, so the observed reduction may be related to the decrease in its biosynthesis.

Conclusions

The exogenous application of symbiosis-promoting signalling compounds, combined with AMF inoculation in tomato plants during the nursery stage, enhanced AMF colonization rates. This suggests that prebiotic compounds (e.g., strigolactones, flavonoids) could optimize the efficacy of microbial inoculants in agricultural systems. Furthermore, early inoculation with *R. irregularis* altered the metabolic potential of the rhizosphere bacteriome, with changes persisting at the end of the five-month growth period following transplanting to the field. Notably, non-inoculated plants eventually developed mycorrhizal colonization at harvest, yet early-inoculated plants exhibited distinct bacterial metabolic shifts, including reduced energy metabolism and increased stress-defense compound synthesis, accompanied by a shift in metabolic focus towards the degradation of carbohydrate compounds, rather than lipids or amino acids (Fig. 10). These differences likely reflect divergent mycorrhizal adaptation trajectories during field establishment, underscoring the critical role of inoculation timing in microbiome engineering. The results of the present study provide a mechanistic basis for leveraging nursery-stage AMF inoculation to prime metabolic shift of rhizosphere microbiomes for sustained functional benefits, thus advancing sustainable crop management strategies.

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Authors' contributions JA.L. and MJ.P. developed the original ideas presented in the manuscript. JA.L., MJ.P., and E.B. designed the research. J.L, L.E., A.R., JM.G., JA.L., and MJ.P. performed the research. B.M., J.L., A.R., JM.G., and L.E. conducted the methodology. B.M. and M.A. performed the bioinformatic analysis. B.M., J.L. and M.A. handled the statistical analysis. E.B., B.M., and M.A. prepared the original draft. JA.L., J.L., and MJ.P. were responsible for writing-review and editing. JA.L., MJ.P., and E.B. managed project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Data availability All raw Illumina sequence data were deposited in the Sequence Read Archive (SRA) service of the European Bioinformatics Institute (EBI) database under BioProject ID: PRJNA1192287 (Biosamples accession numbers SAMN45104698- SAMN45104713).

Declarations

Competing interests The authors declare no competing interests.

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