# Toward deciphering the molecular dialogue in the rhizomicrobiota: Transcriptomic profiling of Trichoderma in rhizobia interaction

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# Toward deciphering the molecular dialogue in the rhizomicrobiota: Transcriptomic profiling of *Trichoderma* in rhizobia interaction

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## ABSTRACT

Microbial interactions are of key importance for the emergent properties of microbiota and ecosystems, playing a pivotal role in plant health, growth, and productivity. This study explores the interactions between soil fungi and rhizosphere bacteria, focusing specifically on fungi belonging to the genus *Trichoderma* and the plant symbiotic bacterium *Sinorhizobium meliloti*. Our aim is to provide evidence of the impact of different strains of the same bacterial species on the fungus. By analysing the effects of four *S. meliloti* strains on gene expression of *T. velutinum*, we revealed the presence of several differentially expressed genes (DEGs) (from 139 to 254 genes) indicating a remodelling of its metabolism and growth. Remarkably, the majority of the DEGs (~90 %) could not be assigned to function, indicating the presence of a large genetic "unknown space" potentially involved in fungal-bacterial interactions. Moreover, results indicated that transcriptomic profiles of *T. velutinum* significantly changed with respect to the four *S. meliloti* strains, suggesting the ability of the fungus to perceive the presence of specific bacterial strains. Our study emphasizes that strain specificity of microbial interactions could play crucial role in shaping microbiota functions, and highlights their potential impact on the success of bioinoculants.

## 1. Introduction

Plant roots are complex ecosystems teeming with life. Within this dynamic environment, an intricate dialogue occurs among a diverse community of microorganisms inhabiting this zone, where a multitude of interactions among plants, microbes and the soil take place (Solomon et al., 2023), fostering both intra- and inter-kingdom relationships (Faust and Raes, 2012; Shi et al., 2022). These microbial communities, collectively referred to as the rhizomicrobiota and consisting of fungi, unicellular eukaryotes and prokaryotes, are critical for various processes such as nutrient cycling, plant disease suppression and plant resilience against environmental stresses (Asghar et al., 2024; Neemisha et al., 2022; Solomon et al., 2023). The nature of intra- and inter-kingdom interactions can vary widely, ranging from competition to commensalism to mutualism (Faust and Raes, 2012), thus being of key importance, since they play a pivotal role in determining plant health, growth, and productivity (Trivedi et al., 2020).

Among microbial communities inhabiting the rhizosphere,

*Trichoderma* species and rhizobia are of particular importance due to their well-established roles in promoting plant growth and providing protection against pathogens (Berg, 2009; Risoli et al., 2023). *Trichoderma* is a widespread genus of filamentous fungi belonging to the Hypocreaceae family, that has gained significant attention over the years. They are involved in diverse functions within the soil ecosystem (Contreras-Cornejo et al., 2016). For example, they can colonize plant hosts, whether on the roots or as endophytes and protect them from a range of soil-borne pathogens, acting as biological control agents (Zhang et al., 2016). These fungi are also known for their plant growth-promoting properties, such as the production of specialized secondary metabolites, i.e. phytohormones, solubilization of nutrients (Contreras-Cornejo et al., 2016; Manganiello et al., 2018; Vicente et al., 2022; Vinale and Sivasithamparam, 2020), and the enhancement of plant defence responses (Chen et al., 2023; Malinich et al., 2019; Morán-Diez et al., 2015; Risoli et al., 2023), thus holding a significant interest and role as a biocontrol agent in agricultural practices. Another model component of the plant-associated microbiome are rhizobia,

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nitrogen-fixing bacteria that can form symbiotic relationships with legumes, supporting legumes in poor soils and enhancing sustainable agriculture (Babalola et al., 2021).

Within rhizobia associated with crops of agricultural relevance, *Sinorhizobium meliloti* is considered one of the most relevant model species. *S. meliloti* forms beneficial relationships with legumes such as *Medicago*, *Melilotus*, and *Trigonella* (Geddes and Oresnik, 2014; Oldroyd, 2013). Within the genus *Medicago*, alfalfa (*M. sativa* L.) has a pivotal importance as forage crop, being cultivated on 35 million ha in more than 80 countries (Radović et al., 2009). Research has shown a significant genetic and symbiotic diversity among *S. meliloti* strains isolated from alfalfa and revealed the importance of the specific combination of rhizobial and plant genotypes for successful plant growth (Bellabarba et al., 2021; Epstein et al., 2023; Fagorzi et al., 2021; Riley et al., 2023).

We recently reported evidences of genotype-by-genotype interaction has also between rhizobia and *Trichoderma* (Vaccaro et al., 2024). In particular we found strain-specific combinations of gene expression pattern of rhizobia in relation to four *Trichoderma* species. Interestingly, one of the *Trichoderma* species tested (*T. velutinum*) showed contrasting results with different strains of *S. meliloti*, strongly suggesting the presence of a strain-specific dialogue in the recognition between such species and *S. meliloti* strains.

These results are shedding new light on how rhizospheric fungi can influence the interactions between legumes and rhizobia, and are opening scenarios for interpreting rhizomicrobiota diversity in terms of strain-specific interaction within the microbial community.

Although the synergistic interactions between these two groups (rhizobia and Trichoderma) have been explored in recent years through inoculant formulations (Barbosa et al., 2022; Freitas Chagas Junior et al., 2021; Marra et al., 2019; Negi et al., 2021; Nirmalkar et al., 2017), the molecular dialogue is much less understood. The dissection of the molecular nature and the extent of this cross-talk is ultimately needed for the development of tailored microbial consortia that work together to enhance plant growth and resilience (Arif et al., 2020; Vaccaro et al., 2022).

To start understanding such strain-specific dialogue we evaluated the presence and the extent of a strain-specific molecular recognition of *S. meliloti* strains by transcriptomic analyses of *T. velutinum*, with the ultimate goal of identifying evidence of a molecular cross-talk between these microbes that could indicate a genotype-specific interaction. These proof-of-principle experiments could allow to start interpreting strain-specific interaction within the rhizomicrobiota and provide insights into the factors that determine the success of microbial consortia, guiding the development of more effective microbial combinations for agriculture (O'Callaghan et al., 2022).

## 2. Materials and methods

### 2.1. Strains and microbiological methods

Four *Trichoderma* species (*T. gamsii* MIAE00029, *T. tomentosum* MIAE01053, *T. harzianum* MIAE00047 and *T. velutinum* MIAE00033), belonging to the collection "Microorganisms of Interest for Agriculture and Environment" (MIAE, UMR Agroécologie AgroSup/INRAE/uB Plant-Microorganism Interactions Department, Dijon, France) (Anees et al., 2010), were cultured and manipulated as previously reported (Vaccaro et al., 2024).

Four genomically different *S. meliloti* strains were used: 1021 (Meade et al., 1982), AK83 (Roumiantseva et al., 2014), BL225C (Carelli et al., 2000), and a cis-hybrid strain obtained mobilizing the symbiotic megaplasmid pSymA from BL225C strain to the genomic background of strain 1021 (Checcucci et al., 2018). The cis-hybrid strain harbours the chromosome and pSymB chromid of 1021 strain and pSymA megaplasmid of strain 1021, resulting in a ca. 30 % of 1021 genome substituted by BL225C genome. Such cis-hybrid strain allows to test the effect of the presence of epistatic interactions between genes on the pSymA megaplasmid and the rest of the genome. The megaplasmid pSymA is the replicon essential for symbiotic nitrogen fixation with leguminous plants, containing nodulation (*nod*) and nitrogen fixation (*nif*, *fix*) genes and the most variable part of the genome among *S. meliloti* strains (Barnett et al., 2001; Galardini et al., 2013). Details on strains genomic relatedness and isolation of 1021, AK83 and BL225C strains were previously reported (Galardini et al., 2013; Vaccaro et al., 2024).

### 2.2. Dual culture assay

The effect of the four *S. meliloti* strains on four *Trichoderma* spp. isolates growth was tested as described in (Jambhulkar et al., 2018) with some modifications. Bacterial liquid cultures were grown on TY medium (5 g/L tryptone, 3 g/L yeast extract, 0.4 g/L $CaCl_2$) for 48 hours at 30°C and 130 rpm. After determination of OD at 600 nm, a loop of liquid culture was streaked on 90 mm diameter TY agar Petri plates. After a 24-hour incubation at 30°C, 5 mm diameter mycelial plugs were placed at the centre of the plate, at a distance of 3 cm from the bacterial streak. Plugs were obtained from the edges of fully colonized plates of *Trichoderma* spp. incubated for 5 days at 25°C. Dual cultures were incubated at 25°C and the radial growth of the fungi was measured at 24, 48 and 72 hours. Growth inhibition, expressed as Inhibition Index, was calculated as I= [1-(A/B)]x100 where B represents the radial growth of *Trichoderma* in control plates (i.e. without bacterial streak) and A is the radial growth of *Trichoderma*, perpendicular to the bacterial streak. For each strain-fungus combination 9 biological replicates (i.e. independent plates) were set up.

### 2.3. Production of rhizobium spent medium

Pre-cultures of *S. meliloti* strains were grown overnight on TY medium at 30°C, 130 rpm, washed twice with *Trichoderma* minimal medium (MM) (Penttilä et al., 1987) and adjusted to $OD_{600}$ 0.3 in 10 ml MM. Cultures were incubated at 30°C, 130 rpm. After 96 hours cultures were centrifuged at 10000 rpm for 10 minutes. Spent media were filtered with 0.2 μm Vacuum Filter/Storage Bottle System (Corning™) and stored at −20°C.

### 2.4. Spore germination

The effect of the four *S. meliloti* spent media on *T.velutinum* spore germination was tested as described in (Buiatti et al., 1987) with some modifications, by using hanging drop glass slides. $10^4$ spores/ml were inoculated in presence of rhizobial spent media, for a final volume of 180 μl. As controls, spores were also inoculated in PDA and MM medium. Glass slide suspensions were sealed in Petri plates, above sterile filter paper imbued with sterile distilled water to maintain humidity, and incubated at 26°C. Spore germination was evaluated microscopically after 24 hours. For each treatment biological triplicates were done.

### 2.5. RNA sequencing and bioinformatic analysis

*T. velutinum* MIAE00033 (2.5x$10^4$ spores/ml) was inoculated into 12-well cell culture plates containing 2 ml of rhizobium spent media. The plates were shaken in a rotary shaker at 100 rpm and 26°C for 96 h. The mycelium was harvested by filtration and RNA stabilized by adding RNA Protect Bacteria Reagent (Qiagen). Samples were immediately frozen and stored at −80°C for RNA extraction. For each condition, three independent replicates were performed.

RNA extraction, mRNA library preparation (poly A enrichment) and sequencing were performed by Novogene (Cambridge, UK) on an

Illumina NovaSeq X Plus apparatus with a pair-end 150 strategy. Reads were mapped and annotated on the transcriptome assembly of *T. velutinum* CBS 230012 (NCBI Bioprojects PRJNA581607 and PRJNA619608), accessed on the JGI genome portal (https://mycocosm. jgi.doe.gov/Trivel1/Trivel1.home.html).

### 2.6. Statistical analysis

All statistical analyses were conducted in R studio 4.2.0 (Team, 2021). Differentially expressed genes ($|\log_2FC=\geq2$, padj$<0.01$) were identified by DESeq2 version 1.36.2 package (Love et al., 2014). Fold change values of *T. velutinum* DEGs were used to run a PCA using the *prcomp* function and visualized with the *autoplot* function of *ggplot2* package(Wickham, 2009). ANOVA and Fisher exact tests were performed using *stats* package. Heatmap was obtained using *pheatmap* package. GO enrichment analysis was performed with *topGO* (Alexa and Rahnenfuhrer, 2024). *ggvenn* was used to visualise intersecting dataset of DEGs on the different *T. velutinum-S. meliloti* spent media combinations.

### 2.7. Data availability

RNA-Seq data are deposited at the European Nucleotide Archive (ENA) under the Project PRJEB75320. Custom scripts used for the analyses can be accessed through the GitHub https://github.com/IacopoPasseri/IacopoPasseri/tree/main.
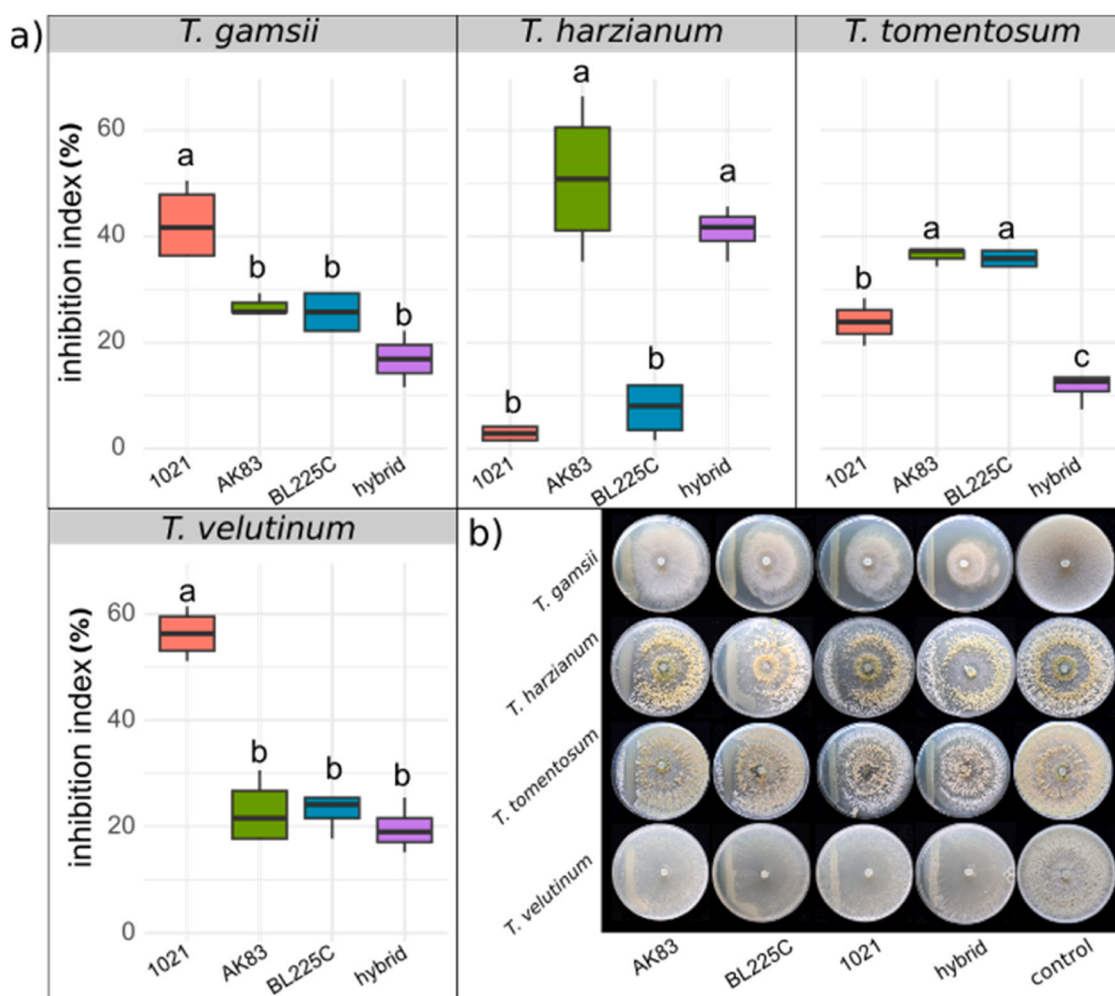
### 3. Results and discussion

#### 3.1. Rhizobia and rhizobia spent media influence Trichoderma phenotypes

The mycelial growth of the four *Trichoderma* species was significantly inhibited (ANOVA) by the four *S. meliloti* strains after 72 hours (Table S1). The degree of growth inhibition depended on the *Trichoderma* species and on the associated *S. meliloti* strain (Fig. 1). *T. harzianum* was in general less inhibited compared to the other three *Trichoderma* species, with *S. meliloti* strain 1021 having the weakest effect on it. Conversely, *T. velutinum* was generally more inhibited than the other *Trichoderma* species. However, the inhibition pattern of each rhizobial strain was significantly different for each *Trichoderma* species (post-hoc Tukey contrasts, $p < 0.05$), indicating a significant strain-related variability. Spore germination was also assessed in *T. velutinum* when tested with spent media from the four *S. meliloti* strains, showed no effect on spore germination (Figure S1).

These results highlighted a rhizobial strain-specific effect on different *Trichoderma* species, suggesting an intimate, genotypic level fungus x rhizobium interaction.



**Fig. 1.** *Trichoderma* mycelial growth is affected by the presence of rhizobia. a) Inhibition index (%) of *Trichoderma* spp. growth in presence of *S. meliloti*. Percentages were determined after 72 h of incubation. Different letters indicate statistically significant results ($p < 0.05$).b) Dual culture Petri plates showing the growth of *Trichoderma* spp. in presence of *S. meliloti*.

### 3.2. Sinorhizobium meliloti spent media modulate T. velutinum MIAE00033 transcriptome

*T. velutinum* showed the highest percentages of inhibition by *S. meliloti* (Fig. 1a) and has previously demonstrated positive effects on the growth of the legume host (*M. sativa*) when combined with *S. meliloti* BL225C (Vaccaro et al., 2024). Therefore, investigated the transcriptome of *T. velutinum* after exposure to spent growth media derived from the four *S. meliloti* strains previously assessed. Results showed that spent media from all four *S. meliloti* strains either increased (upregulated) and decreased (downregulated) the expression ($|log_2FC=\geq 2$) of several genes (Table 1 and Supplementary Dataset S1). Overall, the stimulons due to AK83 and BL225C spent media (i.e. the fraction of both up and downregulated genes) included from 1.28 % to 1.96 % of total fungal genes content. A similar portion of DEGs (1.25–1.78 %) was induced in *S. meliloti* 1021, while the treatment with the cis-hybrid strain spent medium resulted in the lowest number of significant DEGs (1.07–1.23 %). Differences in DEGs among the four treatments were statistically significant (p-value <0.05, Chi-Square test), suggesting distinct biological effects of each *S. meliloti* strains on the *T. velutinum* transcriptome.

Within the DEGs, a general enrichment (p < 0.001, Fisher Exact Test) for genes with unknown function, i.e. not annotated in KOG, was observed for all treatments (Table 1). This finding suggests that a large fraction of genes with unknown function in *Trichoderma* genome could play a role in microbial interactions. Given that most microbiological studies so far are performed under pure culture conditions, it is not surprising that these genes often relate to phenotypes rarely studied in classical laboratory conditions, such as co-cultures or biotic relationships. Studies have demonstrated that genes with unknown function in fungi could be involved in a variety of interactions, including symbiosis, parasitism and saprotrophy. For instance, in mycorrhizal fungi, these genes might be essential for the establishment and the functioning of the symbiosis. In the ectomycorrhizal fungus *Tuber melanosporum*, up to the 13 % of the genes expressed in the developmental stages lack known homologs (Tisserant et al., 2011). Similarly, in the transcriptome of the arbuscular mycorrhizal fungus *Glomus intraradices*, only half of the protein coding genes can be functionally assigned (Tisserant et al., 2012). However, in our study, more than 80 % of DEGs have unknown function, suggesting that most genes with unknown functions are involved in bacteria-fungus interaction. In accordance, when the ectomycorrhizal fungus *Laccaria bicolor* is associated with the soil bacteria *Pseudomonas fluorescens*, more than 60 % of the DEGs is represented by hypothetical proteins (Deveau et al., 2007). The co-cultivation experiments of fungi with bacteria showed the induction of fungal silent secondary metabolite gene clusters with the production of several specialized secondary metabolites (Netzker et al., 2015). It is plausible to hypothesize that at least some of the *S. meliloti*-activated *T. velutinum* genes with unknown function may be involved in pathways leading to the production of secondary metabolites, warranting further

investigations on the *T. velutinum*-induced metabolome.

Such scenario of genes with unknown function involved in microbial interactions seem relevant for bacteria as well. For instance, several genes with unknown function have been reported as upregulated during infection of animal hosts by the pathogen *Pseudomonas aeruginosa*, suggesting their potential role in virulence or stress response mechanisms (Valli et al., 2020) and are differentially expressed under mutualistic symbiosis between nitrogen-fixing rhizobia and leguminous plants (Fagorzi et al., 2021; Roux et al., 2014).

Shedding light on the importance of such unknown function genes, provide experimentally testable hypotheses that could deepen our understanding of the complexity of microbial interactions in natural environments, including the rhizomicrobiota.
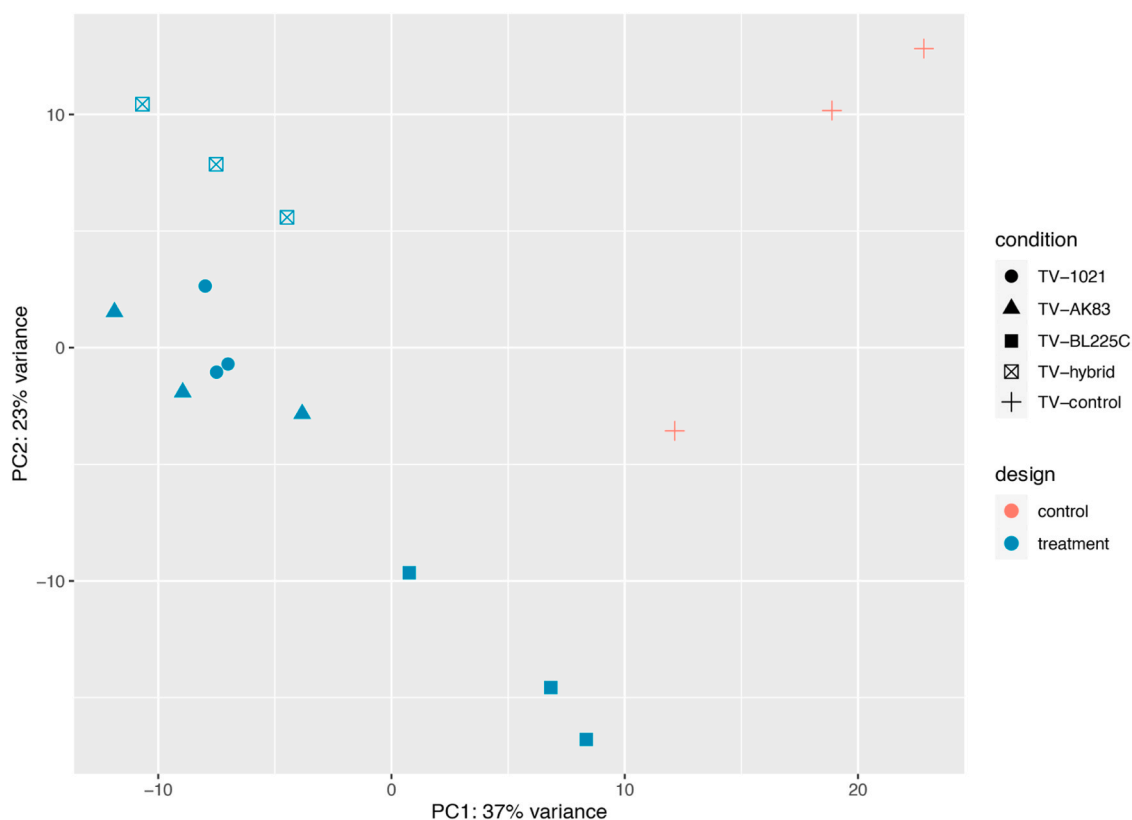
A small number of genes with a functional annotation (KOG) was regulated (77 and 66 up- and downregulated genes, respectively) (Supplementary Dataset S1). A total of 33 and 35 KOG terms for up- and downregulated genes were retrieved, respectively (Table S2). The up- and downregulated gene lists contained exclusive KOG terms apart from one term only (KOG0710, Molecular chaperone - small heat-shock protein Hsp26/Hsp42). The upregulated KOGs included categories related to stress response (e.g., catalase, cytochrome P450 monooxygenase), cytoskeleton and intracellular trafficking remodelling (e.g., Drebrins and related actin binding proteins, Reticulon), membrane transport, and metabolism modification, potentially favoring anaerobic fermentation (e.g., Alcohol dehydrogenase, Fumarate reductase). The downregulated KOGs included genes involved in steroid and terpene biosynthesis pathways (e.g. Tryptophan synthase, Sterol reductase), membrane transporters, a possible reduction of beta-oxidation pathway in the mitochondrion (e.g. Mitochondrial carnitine-acylcarnitine carrier), some ribosomal proteins, alpha-amylase and chitinases. These changes suggest a scenario where rhizobium spent medium might trigger stress response mechanisms with a switch in the main energetic metabolism (from aerobic to fermentation), potentially reducing growth rate, extracellular carbohydrate utilization and variation in biotic interactions due to changes in steroid/terpenoid production. Such results are in accordance with the observation of *T. velutinum* growth inhibition by rhizobia (Fig. 1), but also suggest that the reduction in the expression of sterol reductase and tryptophan synthase genes (related to the biosynthesis of biocontrol molecules and auxin production, respectively)could allow *T. velutinum* to modulate some antifungal activities and plant-growth promoting properties (Castillo et al., 2016; Macías-Rodríguez et al., 2020; Vicente et al., 2022. Based on these results, we cannot exclude that the spent medium, and then the presence of *S. meliloti* in soil, might induce some changes in the ecology and/or the growth of *T. velutinum* (switches between vegetative growth and reproductive behaviour and/or antagonistic effects towards other members of the soil and rhizosphere microbiota).

### 3.3. T. velutinum transcriptome profiles are specific for single Sinorhizobium meliloti strains
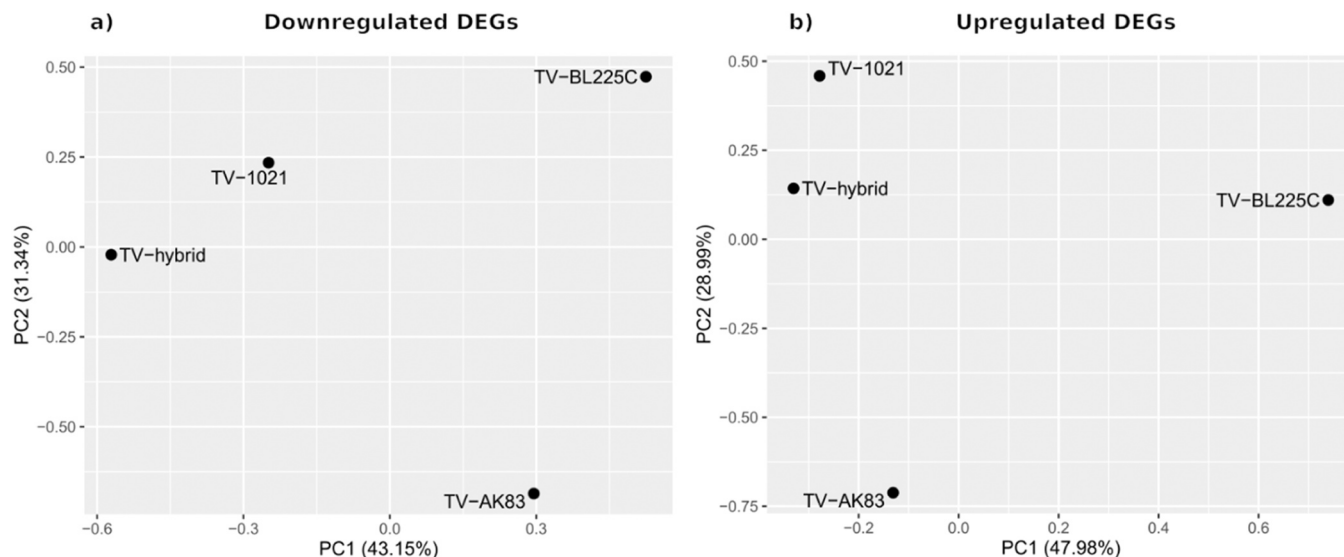
Considering that the four *S. meliloti* strains differently affected *T. velutinum* growth, we hypothesized that such rhizobium strain-related phenotypic variation could be mirrored by the type of genes differentially expressed and by their level of differential expression (i.e. type and $log_2FC$ of DEGs) with respect to control. As illustrated in Fig. 2, the principal component analysis (PCA) of the transcriptome data revealed that the three biological replicates of each condition are clustered, suggesting the data's suitability for further analysis. A clustering of genes and rhizobial strains based on expression values ($|log_2FC=\geq 2$) indicated that expression profiles of *T. velutinum* are differentially affected by the rhizobial strain used (Fig. 3). When considering the DEGs with padj< 0.01, a pattern of expression could be identified depending on the rhizobial spent medium (Figure S2). Overall, a higher number of DEGs is represented by up-regulated genes (Table 1). Transcriptomes induced by the *S. meliloti* 1021 and cis-hybrid strains were the most

**Table 1**

Rhizobia spent media induce large transcriptomic changes in *T. velutinum*. Number of differentially expressed genes (DEGs, $|log_2FC=\geq 2$, padj<0.01) after treatment of *T. velutinum* MIAE00033 cultures with *S. meliloti* spent media. Percentage with respect to total genes in *T. velutinum* CBS 230012 is reported in parentheses. Moreover, the number and percentage over up- and downregulated DEGs (in parentheses) with $|log_2FC=\geq 2$, p < 0.01 of unknown function genes is reported.

| S. meliloti strains | Upregulated | | Downregulated | |
|---|---|---|---|---|
| | Total (%) | Unknown (%) | Total (%) | Unknown (%) |
| 1021 | 231 (1.78) | 207 (89.6) | 162 (1.25) | 146 (90.12) |
| hybrid | 139 (1.07) | 126 (90.64) | 160 (1.23) | 144 (90) |
| BL225C | 241 (1.86) | 225 (93.36) | 166 (1.28) | 149 (89.7) |
| AK83 | 254 (1.96) | 230 (90.55) | 181 (1.39) | 164 (90.6) |

**Fig. 2.** Rhizobium strain-specific response on the global transcriptome of *T. velutinum*. Principal Component Analysis (PCA) of transcriptome variation across treatments with spent media from the four *S. meliloti* strains. The clustering is based on the similarity of their global gene expression patterns, after variance-stabilizing transformation (VST) on the raw count data from *dds* object. The first two components explain, respectively, the 37 % and 23 % of the total variance.



**Fig. 3.** Rhizobium strain-specific impact on differentially expressed genes (DEGs) of *T. velutinum*. a) and b) Principal Component Analysis (PCA) of transcriptome variation under treatment with spent media from the four *S. meliloti* strains. The clustering is based on down-regulated DEGs and up-regulated (2-fold change in expression and padj < 0.01). The first two components explain respectively the 76.97 % and 74.49 % of the total variance.

similar (as expected, due to the genomic similarity between these strains with ca. 60 % of the genome identical).

The relevance of such rhizobium strain specificity on *T. velutinum* transcriptome is highlighted by the results of the principal component analysis (Fig. 3). Here, the transcriptomes of treatments with the four strains are strongly separated (the first two principal components explaining more than 70 % of total variance). *S. meliloti* 1021 is close to

the cis-hybrid strain, which are separated from BL225C on the first principal component and from AK83 on the second principal component, for both up- and downregulated genes. This pattern, though not in line with growth inhibition results (Fig. 1), where *S. meliloti* 1021 and cis-hybrid do not have similar effects, nicely mirrors previous metabolomic analyses on the spent media from *S. meliloti* 1021, cis-hybrid and BL225C (Checcucci et al., 2018). These data strongly suggest that the

strain-specificity of the DEGs pattern in *T. velutinum* can be directly related to the genomic similarity of the *S. meliloti* strains and hence to their extracellular metabolites. Nonetheless, the fact that *S. meliloti* 1021 and the cis-hybrid strain spent media do not overlap, reinforces previous evidence of epistatic interaction between the genomic element involved in plant symbiosis (the pSymA megaplasmid) and the rest of the genome (chromosome and pSymB chromid). Genomic rearrangement in such rhizobial genome could not only give rise to marked differences in symbiotic phenotypes (as already shown, Checcucci et al., 2018), but also to different interactions with other members of the soil microbiota such as *Trichoderma* spp. In the previous study, where we analysed the transcriptome of the same *S. meliloti* strains using spent media from *Trichoderma* spp. (Vaccaro et al., 2024), we observed similar evidence, the transcriptome of *S. meliloti* 1021 and cis-hybrid strains being different under treatment with the spent medium from the same *Trichoderma*.

Collectively, this evidence supports the hypothesis that *S. meliloti* and *Trichoderma* spp. can recognize each other's presence at the single genotypic level, possibly giving rise to cross-talk and coordinated changes in their physiology.

This strain-specific recognition, evidenced by the high specificity of the *T. velutinum* transcriptome to the *S. meliloti* strains, is further confirmed by the analysis of shared and unique DEGs. Indeed, most of the DEGs are unique to each rhizobium strain (Fig. 4). Interestingly, also the spent medium from the cis-hybrid strain induced specific genes (12 up regulated, 74 down regulated), suggesting that the sole change of the symbiotic megaplasmid in *S. meliloti* can strongly affect *S. meliloti*-fungal interaction, even though they are in principle not related to the establishment of rhizobia symbiosis with host plant. Concerning the shared genes, 45 upregulated DEGs were retrieved, including ABC-2 type transporter, 17 beta-hydroxysteroid dehydrogenase type 3, cytochrome P450, catalase and a predicted methyltransferase, suggesting that *T. velutinum* is responding to rhizobial spent media by enhancing its capabilities for detoxification, stress response, and potentially altering its metabolic pathways. The 27 downregulated genes, including ferric reductase, hydroxymethylglutaryl-CoA synthase and mitochondrial carnitine-acylcarnitine carrier protein, suggest a metabolic adjustment of *T. velutinum* in response to rhizobial spent media reprogramming of fungal metabolism, likely driven by the specific nutrient and chemical composition of the rhizobial environment.

To better investigate the functions possibly linked to strain-specific patterns of DEGs, an enrichment analysis of Gene Ontology (GO) terms was performed on both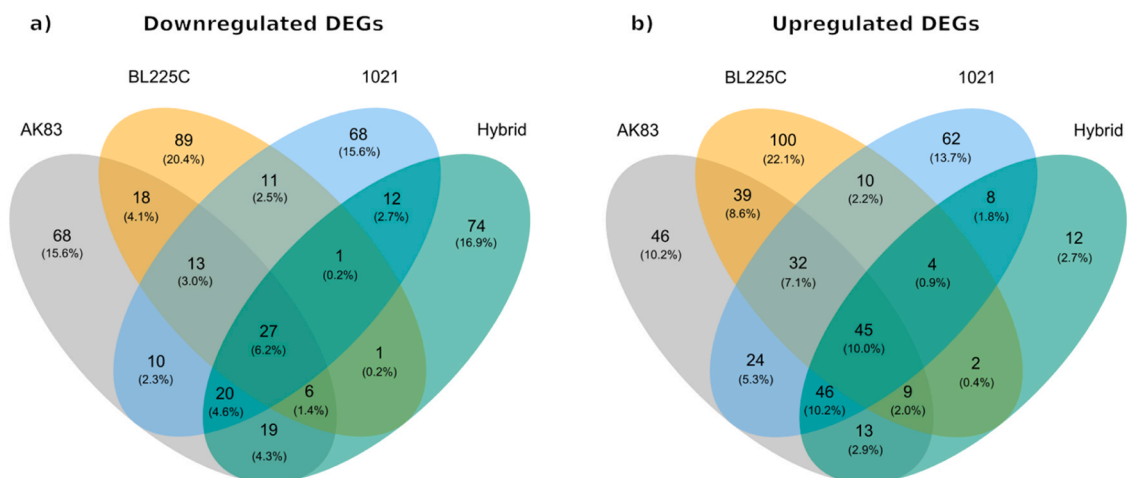 upregulated and downregulated DEGs (Fig. 5, Supplementary Dataset S2). Among the enriched terms that shared similar significance patterns across the conditions, two groups could be identified: one related to anabolic processes and stress responses to reactive oxygen species (ROS), and the other related to cellular homeostasis and responses to environmental stresses, with a prevalence of catabolic processes.

Regarding the common upregulated genes, all four treatments activated oxidative stress responses, particularly those related to hydrogen peroxide and ROS management, indicating that oxidative stress is a central feature of their effects. Among the common downregulated DEGs, the spent media from *S. meliloti* 1021, AK83, and BL225C primarily impacted amino acid metabolism and biosynthesis, particularly involving the glutamine family of amino acids, such as glutamate and arginine. This consistent GO enrichment pattern suggests that these treatments reduce cellular biosynthetic and metabolic activities, potentially by affecting shared pathways related to amino acid metabolism. Each rhizobial strain also induced unique *Trichoderma* pathway activations.
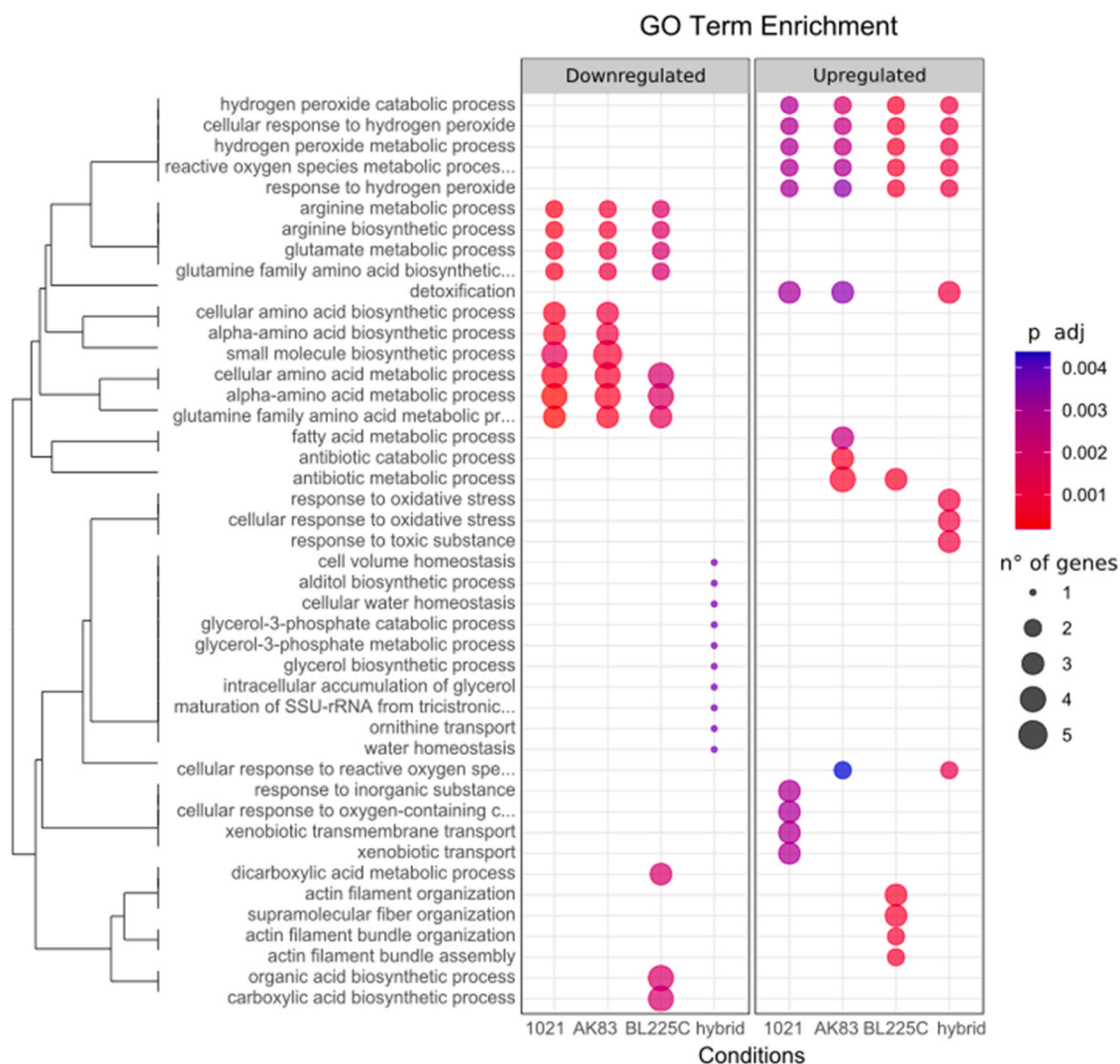
For *S. meliloti* 1021, a unique enrichment in GO terms related to xenobiotic processes (e.g., xenobiotic transport and xenobiotic transmembrane transport) and detoxification was observed, along with terms related to inorganic substances and oxygen-containing compounds. This possibly indicates that this treatment involves exposure to substances that require detoxification and xenobiotic handling, which were not observed in the other treatments. The *S. meliloti* AK83 spent medium was uniquely enriched for fatty acid metabolic processes, potentially pointing to differences in energy demands or stress adaptation. The *S. meliloti* BL225C spent medium uniquely downregulated genes involved in dicarboxylic acid metabolic processes and the biosynthesis of organic and carboxylic acids, suggesting a broader impact on cellular metabolism.

Regarding the upregulated DEGs, the *S. meliloti* BL225C spent medium was responsible for the unique enrichment of terms related to actin filament and cytoskeleton organization, indicating potential structural changes and responses to specific chemical stressors. The *S. meliloti* cis-hybrid strain spent medium displayed a distinct GO enrichment profile related to water homeostasis, glycerol metabolism, and cell volume regulation. This suggests that the hybrid strain secretome could influence cellular processes involved in osmoregulation and maintaining cellular hydration, rather than metabolic pathways. Moreover, the strain showed unique GO enrichment for responses to toxic substances, oxidative stress, and cellular responses to oxidative stress.

These results, for both up- and downregulated GO terms, further



**Fig. 4.** Spent media from different *S. meliloti* strains elicit unique transcriptomes in *T. velutinum*. Venn diagrams illustrating the distribution of DEGs across conditions. Diagram a) represents upregulated DEGs, while diagram b) depicts downregulated DEGs. Overlapping regions in each diagram indicate DEGs shared between conditions, and distinct sections highlight unique DEGs for each condition. Percentages shown in the diagrams reflect the proportion of DEGs relative to the total number of DEGs in the entire dataset.

**Fig. 5.** GO enrichment pathway analysis of *T. velutinum* differentially expressed genes (DEGs, 2-fold change in expression and padj < 0.01) induced by rhizobia spent media. For each panel, the horizontal axis represents the conditions and the vertical axis the biological processes names enriched by differential genes. The size of the bubble indicates the number of differential genes contained. The colour of the bubble also corresponds to the degree of significance. Blue corresponds to smaller the p-value, red is for higher p-values. The hierarchical clustering is based on grouping GO terms by their similarity in significance across conditions, using a distance metric and average linkage, indicating which biological processes or molecular functions may be co-regulated or similarly impacted by experimental treatments.

emphasize previous evidence that genes on the *S. meliloti* pSymA megaplasmid impact not only symbiosis with the host plant, but also interactions among members of the rhizomicrobiota.

## 4. Conclusions

The ability of soil and rhizosphere microbiota components to interact and communicate with each other is a key aspect of our understanding of the microbial world and has large impacts over the functionality and ecosystem services provided by such biotic communities. For example, fungal-bacterial interactions play a crucial role in supporting plant productivity and maintaining the structure and function of soil ecosystems (Trivedi et al., 2020). Therefore, investigating and leveraging these complex interactions is pivotal for mechanistic understanding of the microbiota and provides the possibility to predict the success of newly developed microbial inoculants in agriculture (Poppeliers et al., 2023).

In this study, we revealed the presence of intricate strain-specific interactions between the fungus *T. velutinum* and the plant symbiotic bacterium *S. meliloti*. The rhizobia strains exhibited varying degrees of inhibitory effects on the mycelial growth of four *Trichoderma* species, mirroring previous findings on *S. meliloti* growth under the presence of

*Trichoderma* spp. (Vaccaro et al., 2024). When one of the *Trichoderma* species was analysed for its transcriptomic response to rhizobia presence (simulated by using rhizobia spent media), we observed the presence of many highly ($|\log_2 FC = \geq 2$) differentially expressed genes (DEGs). Most of the DEGs lacked functional annotation (unknown function genes), suggesting that the biotic interactions we are reproducing under this experimental condition may involve novel genes not typically studied (and consequently functionally annotated) under the classical pure culture settings. However, for functionally annotated genes (KOG functional analysis), we found a wide spectrum of functions from metabolic processes (e.g. steroid and terpene biosynthesis, membrane transport, carbohydrate utilization) to stress responses and growth (e.g. cytoskeleton remodelling), which could possibly make sense over the biotic interaction between rhizobia and *Trichoderma*. Actually, when *S. meliloti* strains were treated with *Trichoderma* spp. spent media (Vaccaro et al., 2024), plant-microbe interaction phenotypes of rhizobium were affected differentially including increased indoleacetic acid (auxin) production. Here we found that *S. meliloti* spent media reduce the expression of the tryptophan synthase gene, which could lead to speculate about a cross-talk between *S. meliloti* strains and *Trichoderma* spp. to synergistically modulate the overall auxin production in the

rhizosphere. Clearly, this hypothesis needs experimental testing under, possibly, soil conditions. However, an indication of synergism toward plant growth between *T. velutinum* and *S. meliloti* was previously reported (Vaccaro et al., 2024), as well as between *P. fluorescens* and *L. bicolor* (Deveau et al., 2007), and *Candida albicans-Pseudomonas aeruginosa* (De Sordi and Mühlschlegel, 2009).

Surprisingly, each *S. meliloti* strain spent medium was able to induce unique and significant changes in the gene expression patterns (and Gene Ontology functional enrichment) of *T. velutinum.*, This points up that, despite belonging to the same species, the genetic variation among strains from the same rhizobial species can profoundly and differentially influence their fungal partners and possibly their ecological dynamics. Indeed, we detected wide range of variability even in presence of highly genomically similar *S. meliloti* strains. We could hypothesize that such variability creates the basis for a network of strains (fungal and microbial) with balancing selection effects in the rhizomicrobiota, giving rise to the well-known presence of a high microbial diversity in the plant rhizosphere, even for members of the very same species (Kumar et al., 2015).

Moreover, the present results highlight the importance of considering microbial specificity when developing bioinoculants, as a one-size-fits-all approach may overlook crucial interactions that influence microbial efficacy and plant health (Yadav and Yadav, 2024). It is worth noting that the implications of this research extend beyond understanding rhizobium-fungal interactions: it also questions the existing methods of developing bioinoculants, raising new issues. The variability observed in the elicited transcriptomes from the different fungal-rhizobial combinations, reinforces the evidence for the relevance of genotype-by-genotype interactions on consortia or synthetic communities' performances and need to develop tailor-made bioinoculants. Additionally, given the large fraction of not yet identified genes, future research should focus on elucidating the roles of these unknown functions, in order to dissect their contribution to microbial interactions and ecosystem dynamics (Rappsilber, 2024). Using advanced genomics, metabolomics and co-culture experiments we could possibly clarify their roles in natural environments. Additionally, by revealing how specific microbial strains affect fungal behaviour at molecular level, our work can offer some models and data to help design targeted and effective bioinoculants. Investigating microbe-microbe interactions and how these interactions play out in field conditions will be critical for translating laboratory findings on potential novel microbial bioinoculants into practical agricultural solutions (Canfora et al., 2021; Massa et al., 2022).

In summary, our work suggests that a deeper understanding of the molecular basis of microbial interactions is essential for advancing sustainable agriculture. Exploring this fascinating and complex area of study could reveal secrets of microbial communication, potentially leading to more effective and environmentally friendly farming practices.

## CRediT authorship contribution statement

**Bettini Priscilla P.:** Methodology, Supervision, Writing – review & editing. **Courty Pierre-Emmanuel:** Methodology, Supervision, Writing – review & editing. **Vaccaro Francesca:** Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Fagorzi Camilla:** Conceptualization, Supervision, Visualization, Writing – review & editing. **Mengoni Alessio:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. **Passeri Iacopo:** Formal analysis, Methodology, Visualization. **Sarrocco Sabrina:** Methodology, Supervision, Writing – review & editing.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2025.128180.

## Data availability

Data have been submitted to a public database and the accession number has been mentioned in the manuscript

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