GENOTYPE-BY-GENOTYPE INTERKINGDOM CROSS-TALK BETWEEN SYMBIOTIC NITROGEN FIXING *SINORHIZOBIUM MELILOTI* STRAINS AND *TRICHODERMA* SPECIES

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Abstract

In the understanding of the molecular interaction between plants and their microbiome, a key point is to identify simplified models of the microbiome including relevant bacterial and fungal partners which could also be effective in plant growth promotion. Here, as proof-of-concept, we aim to identify the possible interactions between symbiotic nitrogen-fixing rhizobia and soil fungi (Trichoderma spp.), hence shed light on synergistic roles rhizospheric fungi could have in the symbiotic nitrogen fixation with host plants. We selected 4 strains of the model rhizobium Sinorhizobium meliloti and 4 Trichoderma species (T. velutinum, T. tomentosum, T. gamsii and T. harzianum). In an experimental scheme of 4 x 4 strains x species combinations, we investigated the rhizobia physiological and transcriptomic responses elicited by fungal spent media, as well as spent media effects on rhizobia-host legume plant (alfalfa, Medicago sativa L.) symbiosis. Fungal spent media had large effects on rhizobia, specific for each fungal species and rhizobial strains combination, indicating a general rhizobia genotype x fungal genotype interaction. Differential expression of a high number of genes was shown in rhizobia strains. Moreover, changes in rhizobia exopolysaccharide and auxin production were identified in response to fungal spent media. Different rhizobium-fungus combinations were also shown to have synergistic effects on alfalfa symbiotic phenotypes. Our results provide a first insight into interactions involving nitrogen-fixing rhizobia and rhizospheric fungi, highlighting the panoply of genes and genotypic interactions (fungus, rhizobium, host plant) which may concur to plant symbiosis.

Introduction

Soil, rhizosphere, and the rhizoplane, including also internal root tissue, represent complex ecosystems where various organisms interact and influence positively, neutrally, or negatively each other's growth, development, and ecological functions. These interactions are essential for ecosystem functioning and have profound implications for plant health and productivity. Microbes in the roots and rhizosphere can directly and indirectly affect plant growth and development by providing several services to both plants and ecosystems, such as facilitating the nutrient acquisition, promoting hormone synthesis, suppressing pathogens, inducing systemic resistance, contributing to the cycling of organic matter, nitrogen fixation, and degradation of pollutants, thus influencing overall soil fertility and ecosystem sustainability. Such microbes can have various degrees of biotic interaction with plants from free-living commensal to plant symbionts, and include different groups, from bacteria to fungi. Within the plant symbionts are rhizobia, a group of nitrogen-fixing bacteria

that can establish mutualistic symbiotic associations with leguminous plants. These bacteria reside within specialized structures called nodules on plant roots and convert atmospheric nitrogen into ammonia, which can be utilized by the host plant. *Sinorhizobium meliloti* , a model rhizobial species, exhibits a broad host range and forms symbiotic associations with leguminous (*Fabaceae*) genera such as *Medicago* ,*Melilotus* , and *Trigonella* . *S. meliloti* possesses peculiar genetic and physiological traits, allowing it to efficiently colonize plant roots, establish nodules, and fix nitrogen . Several studies have shown the large genetic and symbiotic diversity of *S. meliloti* strains, highlighting the importance of rhizobial genotype x plant genotype partnership for successful symbiotic plant growth promotion and nitrogen fixation . Tough the attention has mainly been focused on discovering the genomic determinants of such symbiotic diversity , recently the focus has shifted to transcriptional variation, as closer proxy to phenotypic differences . Here, for *S. meliloti* , we detected the transcriptomic signatures of wide genotype x genotype interaction in response to the treatment with the symbiotic inducer luteolin and with plant root exudates from three alfalfa (*Medicago sativa* L.) varieties. In addition, to decipher the extent of epistatic interaction among the main set of symbiotic genes and the rest of the genome, a hybrid *S. meliloti* strain was constructed by mobilizing the megaplasmid harbouring most of symbiotic genes .This hybrid strain allowed to discover a large number of unique transcriptional signatures related to intragenomic regulatory interactions which can explain part of strain and plant genotype-specific features of symbiosis.

While many details are known on rhizobia-plant interaction, few studies have investigated the interaction between rhizobia and the other relevant components of the root microbiome, i.e. fungi . Fungi play a crucial role in plant-microbe interactions and soil ecosystem functioning. They form intricate associations with plant as free-living organisms in the rhizosphere or, forming interactions with plant roots as endophytes. Rhizospheric fungi contribute to nutrient cycling by decomposing organic matter, releasing nutrients, and enhancing nutrient uptake by plants . They can also act as biocontrol agents, suppressing plant pathogens and promoting plant growth. Furthermore, rhizospheric fungi influence soil structure and stability, affecting water retention and root penetration. Their interactions with plants and soil make them key players in ecological processes and agricultural sustainability.

Concerning nitrogen-fixing symbiosis, arbuscular mycorrhizal fungi have shown multiple mutualistic effects with *S. meliloti* and the host plant *M. truncatula* and extensive effects of microbial mutualists on gene expression. Among the most studied fungi related to plant growth, used also as bioinoculant, *Trichoderma* spp. hold a special attention. They are a group of filamentous fungi, known for their multifunctional roles in the soil ecosystem. They are widely recognized as biocontrol agents against plant pathogens due to their ability to produce a diverse array of antimicrobial compounds and compete for resources. *Trichoderma* spp. can also promote plant growth by enhancing nutrient availability, stimulating root development, and inducing systemic resistance. Additionally, they contribute to the decomposition of organic matter, thereby influencing nutrient cycling and soil structure. The biocontrol and growth-promoting properties of*Trichoderma* spp. make them valuable components of sustainable agriculture and integrated pest management strategies . Unlike arbuscular mycorrhizal fungi, as *R. irregularis* , free-living filamentous fungi as *Trichoderma* can be easily grown in*vitro* and they are also well-established models with available genome sequences, as well as methods for the study of their secretome .

The deciphering of the relationships and molecular signals taking place in the rhizosphere among rhizobia and *Trichoderma* will then offer in perspective numerous potentialities in providing testable experimental models for understanding and exploiting plant-microbiome interaction and dissecting mutualistic and antagonistic effects down to the molecular level.

However, it is becoming more and more clear that mutualistic effects have a strong intraspecific genotype component, i.e. different combinations of partner's genotypes may lead to significant changes in the outcome of the association (e.g. quality of the symbiosis in rhizobium-legume association) . No data have been reported so far on genotype-by-genotype interactions between rhizobia and rhizospheric fungi and how this can affect the quality of the symbiosis with*M. sativa* as host plant .

Here, we report the results of a study aimed at determining the effect different *Trichoderma* species may

have on the symbiotic nitrogen-fixer *S. meliloti* and the presence of genotype-by-genotype interaction between strains of *S. meliloti*and species of *Trichoderma* . By combining microbiological observation, physiological, metabolic, and transcriptomic analyses, we show the existence of rhizobial strain-specific response to*Trichoderma* species, with the presence of either synergistic, neutral, or antagonistic interactions depending on the rhizobium-*Trichoderma* combination. These interactions determine changes in gene expression of a large fraction of the rhizobial genomes (up to 23.45 % of total genes) and are also reflected in host plant growth-promoting phenotypes. These results provide novel insights into the well-known mutualism between rhizobia and host legumes, emphasizing the role of the molecular dialogue taking place in the plant rhizosphere. Moreover, evidence presented here give a proof-of-concept that careful analysis of microbial interactions can be key for successful development of community-mimicking bioinoculants (also referred to as synthetic communities) in agriculture.

Materials and Methods

Strains and growth conditions

Four *S. meliloti* strains were used for this study: 1021 (indicated also as Rm1021) a spontaneous streptomycin-resistant derivative of the isolate SU47 recovered from *Medicago sativa*root nodules ; AK83, isolated from the root nodules of Medicago falcata in the North Aral Sea Region of Kazakhstan by the Russia Institute of Agricultural Microbiology (RIAM, St. Petersburg, Russia) ; BL225C, isolated from *Medicago sativa* plants grown on soil of Lodi, Italy, during previous experiments ; a cis-hybrid strain between 1021 and BL225C . This cis-hybrid strain harbours the symbiotic megaplasmid pSymA from BL225C strain and the chromosome and pSymB chromid of 1021 strain, resulting in a ca. 30% of 1021 genome substituted by BL225C genome. The strains are cultured from isolates derived from glycerol stocks (25%) stored at -80°C.

Trichoderma strains (*T. gamsii* MIAE00029, *T.tomentosum* MIAE01053, *T. harzianum* MIAE00047, *T. velutinum* MIAE00033) belong to the collection "Microorganisms of Interest for Agriculture and Environment" (MIAE, UMR Agroécologie AgroSup/INRAE/uB Plant-Microorganism Interactions Department, Dijon, France). Fungi were stored on Potato Dextrose Agar plates at 4°C. *Trichoderma* spp. liquid cultures were grown on minimal medium containing (mg/ml): glucose 20; $(NH_4)SO_4$ 5; KH_2PO_4 15; $MgSO_4$ 0.6; $CaCl_2$ 0.6; $FeSO_4$ * $7H_2O$ 0.005; $MnSO_4$ *H_2O 0.0016; $ZnSO_4$ * $7H_2O$ 0.0014, and $CoCl_2$ 0.002. All liquid cultures of *Trichoderma*were grown in 100 ml medium, at 25°C, 100 rpm agitation. Inoculation was done with conidial suspensions adjusted to deliver a final concentration of 10^7 spores/ml.

Production and GC-MS analysis of Trichoderma spent media

For each *Trichoderma* species, six independent liquid cultures were prepared (to be sampled after 7, 14, and 21 days, each). After 1 week, 2 and 3 weeks, respectively, cultures were pooled into two meta-samples (each composed by three independent cultures), centrifuged to collect the spent media and pellets were dried to measure fungal biomass. The 1 week (1W) spent media were used both for transcriptomic analysis and phenotypic tests (see below). Regarding the molecular composition, the metabolites from 1W spent media were extracted from a volume of 40 mL using 50 mL of chloroform in a separatory funnel. The extracts were then concentrated in a nitrogen stream to 2 mL and esterified according to the manufacturer's instructions with a BSTFA-TMCS derivatising mixture (99:1 v/v), (Sigma Aldrich, Germany).

Separation of organic compounds in the esterified samples was performed by gas chromatography (GC 7890A, Agilent Technologies, USA) coupled with mass spectrometry (MS 5973c, Agilent Technologies, USA). Samples were injected using an automatic dispenser (7683 Series Injector Agilent Technologies, USA) in a volume of 2µL. The standard deviation of the injection according to the manufacturer's data was a maximum of 0.3%. The injected sample was excited into the gaseous state at 280°C and diluted in a 1:5 v/v helium stream (split). The separation of organic compounds was then carried out using an HP-5MS column (30 m, 0.25 mm I.D., 0.25 µm particle size (Agilent Technologies, USA)), using helium as a carrier gas, at a flow rate of 1 mL/min.

Organic compounds were separated using a temperature gradient. The chromatography column was heated

at 100°C for 5 minutes, followed by a temperature ramp up to 280°C at a rate of 6°C/min. Once the temperature reached 280°C, it was maintained for a further 8 minutes.

The mass spectrometer scanned the separated ions in the mass range 40 to 800 Da) at an ionisation of 70eV, a filament temperature of 150°C, and a temperature of 230°C in the ionisation chamber.

Growth assays on Trichoderma spent media

The effect of *Trichoderma* spp. spent media on *S. melilotis*trains was tested by monitoring bacterial growth of inocula on a 96-well microplate at 30°C for 72 hours with a microplate reader (Tecan Infinite 200 PRO, Tecan, Switzerland). Spent media obtained from each fungal species at 1, 2, and 3 weeks were added to inocula grown in TY liquid medium after normalization with the fungal biomass. Relative biomasses were calculated to the minimum dry-weight pellet. In each well a final concentration of 50% of SM was present. For each strain-spent medium combination, 3 independent cultures were set up and the growth inhibition index was calculated after 72h as: 1- OD_{600}trt/OD_{600} ctr, where trt are treated and ctr the control samples.

Auxins production

The auxins production in the presence of 50% v/v *Trichoderma*spp. was quantified using the Salkowski reagent . Bacterial cultures (5 ml) at initial OD_{600} 0.1 were grown at 30°C overnight in TY medium supplemented with 1.25 mM L-tryptophan and 50% v/v fungal spent medium. After 24 h, cell cultures were centrifuged at 10,000 rpm for 10 minutes. The culture supernatants and the Salkowski reagent (distilled water 300 ml, 95-97% $H_2SO_4$180 ml, 0.5 M $FeCl_3$ 9 ml) were mixed (1:4 ratio) and incubated in the dark for 30 min at room temperature. The absorbance was estimated at 530 nm with a spectrophotometer. The auxins concentration in cultures was estimated based on the IAA standard curve. Four biological replicates (independent cultures) were performed. Production inhibition index was calculated as: 1- OD_{530}trt/OD_{530} ctr where OD represents the values of optical density at 530 nm for treated (trt) and control (ctr) samples.

Exopolysaccharides production

EPS was extracted from bacterial cultures grown at 30°C for 48 hours in TY medium supplemented with 50% v/v fungal spent medium, starting from OD_{600} 0.1. Cultures were centrifuged at 10,000 rpm for 20 minutes at 4°C and EPS was precipitated by adding 1 volume of cold absolute ethanol to the supernatant, followed by incubation at 4°C for 24 h. Crude EPS was harvested by centrifugation at 10,000 rpm for 20 min at 4°C. The pellet was washed and resuspended with dH_2O.

Total sugars estimation was evaluated using Dubois method : 200µl of crude EPS was mixed with 200µl of 5% phenol and 1 ml H_2SO_4. The mixture was vortexed and incubated for 30 minutes at room temperature. Absorbance was measured at 490 nm with a spectrophotometer. Reducing sugars estimation was evaluated using the dinitrosalicylic acid (DNSA) method . EPS crude sample and DNSA reagent were mixed in 1:3 ratio. Samples were heated in a thermostatic bath for 15 minutes at 96°C. After cooling, the absorbance was measured at 540 nm. The reducing and total sugars concentration in the cultures was estimated based on a glucose standard curve.

Data are from 3 biological replicates. Production inhibition index was calculated as: 1- OD_{490} treated/OD_{490}control for total sugars and 1- OD_{540}treated/OD_{540} control for reducing sugars, where OD represents the value of optical density at the indicated wavelength.

Biosurfactants production

The emulsification index was determined as described previously after 48 h incubation with 50% v/v fungal spent media. Equal volumes of culture supernatant and toluene were mixed in a test tube and vortexed for 2 min. The emulsification index was calculated after 24 h using the following formula: Emulsification index (%) =(%)= [emulsion column(cm)/total column]*100. Data are from 3 biological replicates.

Root adhesion test

The adhesion of rhizobial cells to root surface (here used as a proxy of biofilm formation on roots) was estimated as described previously . Five days-old seedlings were transferred in 1.5 ml tubes containing Fahraeus supplemented with 50% v/v spent medium and bacterial culture at OD_{600} 0.1. After 48h, roots of similar length were washed in 500 µl of 0.9% NaCl to remove loosely adherent cells by vortexing for 10 s in 500 µl of 0.9% NaCl. Then roots were transferred to 500 µl of fresh 0.9% NaCl and vortexed for 30 s to collect bacterial cells that are strongly adherent to the root surface. The quantity of bacterial cells was evaluated by qPCR on *rpoE1* gene following an already established method . For each condition, three biological replicates were performed, with three seedlings each. Differences were evaluated by one-way ANOVA Tukey pairwise contrast.

Plant symbiosis assay

Symbiotic assays were performed as previously described . *Medicago sativa* cv. Marina seedlings were treated with 24 different conditions: only one rhizobial strain (4 treatments), only one *Trichoderma*species (4 treatments) and 4x4 *Trichoderma -S. meliloti*combinations (16 treatments). For each treatment 3 biological replicates were performed, with 3 to 5 seedlings per pot. Plants were grown in pots containing a 1:3 mixture of sterile vermiculite and sand. After 24 h, they were inoculated with 500 µl of bacterial suspension at OD_{600} 0.1 and/or 500 µl of fresh mycelial suspension (*T. gamsii* 4.5 mg/ml; *T. harzianum* 2 mg/ml; *T. tomentosum* 4.6 mg/ml; *T. velutinum* 5.16 mg/ml) obtained from 5-days-old fungal cultures grown on PDA plates in a growth chamber at 26±2 °C with a 16h photoperiod. After 5 weeks, the following parameters were measured: nodule number, root, shoot and stem length, number of leaves and plant dry weight.

RNA extraction and RNA-Seq analysis

Cultures of *S. meliloti* , grown overnight in TY medium at 30°C at 130 rpm, were diluted to an OD_{600} 0.1 in 5ml of TY medium supplemented with 50% v/v of *Trichoderma* 1 week-spent medium and incubated for 24 hours. Three biological replicates (independent cultures) were produced for each strain-spent medium combination, including untreated control with fresh medium. A total of 60 samples then were prepared (4 strains x 5 conditions x 3 replicas). After incubation, cells were blocked with RNAprotect Bacteria (Qiagen, Venlo, The Netherlands), and total RNA was extracted using RNeasy Mini kits (Qiagen) from 0.5 ml of culture following the manufacturer's instructions, including on column DNase I treatment as reported in . In particular, after elution, a second DNase I (ThermoFisher, Waltham, Massachusetts, USA) treatment was performed, and the absence of contaminant DNA was verified by qPCR on the *nodC* gene of *S. meliloti* . Quality and quantity of total RNA were checked by Agilent 2200 TapeStation (Agilent) with RNA Screen Tape. NEBNext rRNA Depletion- Bacteria (New England Biolabs) was used for ribosomal RNA depletion. The library was prepared with TruSeq Stranded Total RNA Library Prep Gold Kit (Illumina). Sequencing was performed on an Illumina Novaseq6000 apparatus by Macrogen Crop. (Korea)

Bioinformatic and statistical analysis of RNA-Seq data

Trimmed and demultiplexed reads were mapped back to transcripts using Salmon (version 1.1.0) and quantification was imported into R using the tximport package (version 1.10.1) as previously reported . Differential abundance analysis was performed with the DESeq2 version 1.22.2 package . For each *S. meliloti* strain, genes differentially expressed (log2 fold change of $>|2|$; P value <0.01) under at least one condition relative to the control conditions were identified, and all fold change values for these genes were extracted. Core and dispensable genome were computed with Roary following annotation with Prokka and assignment to Clusters of Orthologous Genes (COG) categories, as previously reported . Comparison of expression values of genes in the core genome of strains and nested likelihood ratio tests (LRTs) were performed as indicated in . All genes of *S. meliloti* strains 1021, AK83, and BL225C were functionally annotated using stand- alone version 2 of eggNOG-mapper with default settings.

Results and Discussion

Fungal spent media affect growth and metabolite production in rhizobia

The inhibitory effects of*Trichoderma* spent media against the four rhizobial strains resulted in different

extents of inhibition (**Figure 1**). In general, the overall profiles of rhizobial growth response among the spent media obtained after 1 week (1W), 2 weeks (2W), and 3 weeks (3W) of fungal growth were different. In fact, Mantel tests performed on the matrices representing the pairwise inhibition values did not show significant correlations (W1 vs. W2 R=-0.496, p-value=0.92; W1 vs. W3 R=-0.402, p-value=0.92; W2 vs. W3 R=0.618, p-value=0.12), indicating that the fungal spent media from 1W, 2W and 3W exert a different effect on rhizobia strain growth. The analysis of the variance (ANOVA) on the growth of individual strains in response to the different fungal spent media showed that all three weeks discriminate among strains (**Supplementary Table S1)** . However, 1W spent media had the highest levels of significance, suggesting that here the four fungal species maximize their differential effects in giving rise to rhizobial strains differential growth inhibition.

Regarding the effects on the *S. meliloti* physiology, as growth and plant-growth-promoting activities, such as auxins, EPS (total and reducing sugars), and biosurfactants production, on the overall (**Figure 1**) we observed effects spanning from growth inhibition to EPS production which were different in relation to both fungal spent media and to the rhizobial strain. The effect detected for total sugars (total EPS) was similar to the growth inhibition effect, while reducing sugars clustered with the effect on biosurfactant production. The effect on biofilm grouped apart from the other phenotypes. A two-way ANOVA (**Supplementary Table S2**) indicated that most of the phenotypes were influenced by both strain, fungus and their interaction, the highest F-values in the interaction being IAA production and growth inhibition. Considering single phenotypes separately (**Supplementary Figure S1**), for growth inhibition (**Figure 1, Supplementary Figure S1a**), *S. meliloti* 1021 and *cis* -hybrid strains clustered together, while AK83 and BL225C formed a second cluster. This clustering is not surprising since the*cis* -hybrid and 1021 share about 2/3 of the genome (they differ for the pSymA megaplasmid only) and suggest that the response to spent media is mainly residing on chromosomal loci (and chromid). Concerning fungi, *T. gamsii* showed the strongest inhibiting effect towards the four strains, while *T. tomentosum* and *T. velutinum* had a milder effect. The BL225C strain was generally less inhibited by the four spent media. Regarding auxins production (**Figure 1, Supplementary Figure S1b**), differential patterns of production related to either rhizobial strains or *Trichoderma* species were recorded. In particular, auxin production by 1021 and BL225C *S. meliloti*strains was more inhibited by *T. gamsii, T. harzianum* and*T. velutinum,* while spent media from these fungi had milder effects on the AK83 and cis-hybrid *S. meliloti* strains. *T. tomentosum* had the lowest effect on the four strains. Interestingly, the 1021 and the *cis* -hybrid *S. meliloti* strains clustered separately, suggesting that the mobilization of pSymA in the*cis* -hybrid strain could affect fungal spent media-related auxin production. Regarding biosurfactants (**Figure 1, Supplementary Figure S1c**), no or very weak production was detected in presence of fungal spent media with 1021 and *cis* -hybrid *S. meliloti*strains, while AK83 and BL225C *S. meliloti* strains showed a different pattern of production under spent media treatment. A pattern related to the combination of fungal species and *S. meliloti*strains (fungal x rhizobial interaction) was also clear for the total EPS production (**Figure 1, Supplementary Figure S1d**) and the biofilm formation on root (**Figure 1, Figure S1e**), while reducing sugar production (**Figure 1, Supplementary Figure S1e**) only distinguished *T. tomentosum* spent medium response from those of the other fungi.

Metabolomic analysis of Trichoderma spent media

To evaluate if the overall differences in rhizobial strains response to*Trichoderma* 1W spent media could be related to the presence of species-specific differences in metabolite composition, a gas chromatography-mass spectrometry (GC-MS) analysis of the*Trichoderma* spp. 1W spent media was performed. The GC-MS profiling detected a total of 987 peaks (**Supplementary Dataset S1**), 76 of which could be identified (cut-off >80% of identification probability). These mainly belong to aromatic, cyclic and aliphatic hydrocarbons, carboxylic acids, alcohols, esters, sugars and their derivatives, terpenes and their derivatives.

Spent media were mainly composed by 2H-Pyran-2-one, 6-pentyl- (a representative metabolite common to the *Trichoderma* genus), 1-Monopalmitin, a compound known for having activity against pathogenic organisms , and succinic acid, monoethyl ester. The peak area of these and of the other compounds varied among samples (see**Supplementary Dataset S1, worksheet 2**). Principal component analysis (PCA)

(**Figure 2**) indicated strong differences in *T. gamsii* and *T. harzianum* spent media, while *T. tomentosum* and *T. velutinum* were more similar. The biplot analysis showed that differences in 2H-Pyran-2-one, 6-pentyl- quantities were associated with *T. gamsii* vs. the other fungi. *T. tomentosum* profiles were related to differences in 1-Monopalmitin. Other compounds contributing to the main differences were Hexadecane and Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester. *T. velutinum* spent medium profiles were associated with differences for 1-Monopalmitin, and succinic acid, monoethyl ester. The compound 2,3,4,5,6-Pentahydroxyhexanal was associated with *T. harzianum* spent medium. It is worth noting that the difference between *T. gamsii* and *T. tomentosum/T. velutinum* highlighted in the biplot is in good agreement with the phenotypic differences observed in the overall panel of rhizobial strain (**Figure 1**), suggesting that possibly some of the compounds mentioned above may target rhizobial metabolism, giving rise to contrasting bacterial responses.

S. meliloti-Trichoderma combinations differentially affect the quality of symbiosis

Since the most notable and ecologically relevant phenotype of rhizobia is their mutualistic interaction with leguminous plants, giving rise to nitrogen-fixing symbiosis, we evaluated if *Trichoderma* may modify the mutualistic interaction (symbiosis quality) between *S. meliloti* and the host legume plant *M. sativa* . Additionally, we aimed to inspect if the modulation of the mutualism possibly operated by *Trichoderma* could display evidence for genotype-by-genotype interaction, as did the previous results on *S. meliloti*phenotypes (i.e. growth, root biofilm formation, auxin, siderophore, EPS production). Results from *in vitro* symbiosis tests are reported in **Figure 3** and **Supplementary Figure S2** . Symbiosis quality was assessed in terms of root, shoot, and stem length, number of leaves, number of root nodules, and plant dry weight. Differences among combinations were found for all these parameters indicating that the level of mutualism is indeed affected by co-inoculation with *Trichoderma* and at different extent based on the *Trichoderma* species and the rhizobial strain. In particular, while all assessed phenotypes were modulated by the strain and/or the condition (*Trichoderma*), plant dry weight and above ground plant length (shoot length and stem length) showed a statistically significant contribution of the interaction *Trichoderma* species x *S. meliloti* strain. (**Supplementary Table S2**). Going into the details of single combinations, (**Figure 3, Supplementary Figure S2**), plant dry weight was significantly higher when co-inoculated with *T. velutinum* -BL225C, *T. harzianum* -BL225C and *T. tomentosum* -BL225C compared to the other combinations. The highest value of plant dry weight was achieved in presence of the co-inoculation *T. velutinum* -BL225C (**Figure 3d**). In terms of root length, the highest results were obtained with *T. velutinum-S. meliloti* and *T. tomentosum-S. meliloti* co-inoculations, although post-hoc Tukey Test showed no differences among the different groups (**Supplementary Figure S2a**). This phenotype partially mirrors the auxins production profile (**Figure 1**), where these two *Trichoderma* strains showed a less inhibiting power over. We may hypothesize that *T. velutinum* and *T. tomentosum* under the tested symbiotic conditions still allow *S. meliloti* to produce auxins as their spent media do under culture, giving rise to an increased root length . Regarding the number of nodules, values were significantly higher with the *Trichoderma* -AK83 combinations and the single *S. meliloti* AK83 strain. Indeed, this over-nodulating phenotype with AK83 has been already observed and interpreted in terms of higher capacity of competitiveness for nodule colonization , including possible cheating related to reduced nitrogen-fixation ability.

Summing up above-mentioned results, it is possible to distinguish combinations which were neutral (neither increasing nor decreasing the quality of symbiosis), positive (increase of the quality, indicating a synergistic interaction), and negative (decrease of the quality of symbiosis, viz. showing antagonistic interaction) with respect to *M. sativa* phenotypes.**Figure 4** reports an evaluation of the modulation of mutualism by *Trichoderma* species with respect to the phenotypes with significant contribution of the interaction *S. meliloti* strain x *Trichoderma* species (**Supplementary Table S2**). Combinations displaying evidence for synergism, neutralism, and antagonism were present, again emphasizing a genotype-by-genotype effect on modulation of mutualism. Striking results were obtained for *T. velutinum* , which was antagonistic or nearly neutral for all strains but BL225C, which increased 2-fold the shoot length in combination with *T. velutinum* . Moreover, in general BL225C strain was the one showing for most of the combinations a synergistic effect of *Trichoderma* co-inoculation.

S. meliloti transcriptome displays evidence for genotype-by-genotype interactions

Given the large number of phenotypes showing *S. meliloti*strain-specific differential response to *Trichoderma* species, we aimed to identify the transcriptome signatures (in terms of number and type of differentially expressed genes, DEGs) of such genotype-by-genotype interactions. The four *S. meliloti* strains were then exposed to 1W spent media for 24h and their transcriptome was evaluated by RNA sequencing.

The number of differentially expressed genes after treatment (here termed as stimulon) identified is shown in **Table 1** . The list of DEGs can be found in **Supplementary Datasets S2 and S3** . Overall, with respect to the four *S. meliloti* strains, the stimulon due to*T. gamsii* spent medium elicited the highest number of genes (average ca. 20.6%), while *T. harzianum* and *T. tomentosum*treatments resulted in the lowest number of significant DEGs (from 6.0 to 10.0% of total genes) with limited differences between *S. meliloti* strains. *T. velutinum* , on the contrary, showed the ability to stimulate a high number of DEGs in 1021 and in the hybrid strain (ca. 23.8-25%), but in BL225C and AK83 the stimulon dropped to ca. 6.6-9.6%, indicating that a large part of the elicited transcriptome is strictly dependent upon the combination between given*S. meliloti* genotypes and fungal genotypes. It is worth noting that the growth phenotypes under *T. velutinum* 1W spent medium resulted in a quite different response among the 4 *S. meliloti*strains (**Figure 1**).

Since *S. meliloti* harbours an open pangenome with a core set of genes shared by all strains and a dispensable set of genes present in a fraction only of strains we can expect that transcriptomic signatures of genotype-by-genotype interactions could be due to (mainly) the core set or the dispensable set. To clarify if and how much of a *S. meliloti* strain specific response is reflected in a transcriptional rewiring of core genes, we performed a principal component analysis (PCA) on the DEGs belonging to the core gene set (shared orthologs among the four *S. meliloti* strains) (**Supplementary Dataset S4**). Results (**Figure 5**) revealed that, for each strain, the transcriptional responses of the core genome to the different fungal spent media give rise to fungal-related grouping of samples, with the first principal component separating *T. tomentosum* / *T. harzianum* elicited transcriptomes from *T. gamsii* , the second principal component differentiating *T. tomentosum* from *T. harzianum* . This pattern resembles the separation based on spent media metabolome composition (**Figure 2**) and partial *S. meliloti* growth (**Figure 1**). Concerning up-regulated DEGs (**Figure 5a**), the transcriptional response in all the strains treated with *T. harzianum* spent medium is very similar. This clustering is even tighter in the down-regulated genes (**Figure 5b**). Similarly, *T. tomentosum* treatment elicited a similar response among the samples, both in up-regulated and down-regulated genes. However, AK83 and BL225C treated with *T. velutinum* spent medium were found in *T. tomentosum* clusters and, likewise, 1021 and the hybrid treated with *T. velutinum* were found clustering among samples exposed to *T. gamsii* spent medium, indicating that transcriptomic signatures of genotype-by-genotype interaction reside in the shared set of genes (core genome) also. **Figure 6** reinforces the evidence that a large fraction of the transcriptome can be involved in the genotype-by-genotype interaction, in particular concerning strain-specific response to each fungal species. Indeed, most of DEGs identified are unique to the response to single fungal species (**Figure 6a-d**) or at least can be shared among two fungal species (**Figure 6 e-h**), while only a very limited fraction of DEGs is common to all fungal species. Interestingly, depending on the strain, the number of unique up-regulated genes belonging to the dispensable genome spanned from 40.4% to 69.9%, while for down-regulated genes, the dispensable range varied from 51% to 85%, suggesting the importance of the accessory genome in the strain- specific response. The lists of unique genes are reported in**Supplementary Dataset S5** .

These data highlight the relevance of transcriptome variation in strain-specific molecular communication between soil fungi and rhizobia. Such variations also underlie the presence of regulatory interactions in the genome which are differentially affected by the presence of fungi. In this context, the transcriptional response of the hybrid *S. meliloti* strain (e.g. comparing the panels for 1021 and the hybrid strain in **Figure 5**) is different from that of the parental 1021 strain, though they share the same core genome (being different in the symbiotic megaplasmid pSymA only). We may consequently hypothesize the presence of epistatic interactions between genes on pSymA and those harboured by the chromosome and pSymB chromid related to the response to the presence of *Trichoderma* , similar to what has been found for the response to root

exudates . This hypothesis broadens the relevance of pSymA megaplasmid, which could be related not just to the symbiotic interaction with the host plant (see for instance), but also to the molecular dialogue with soil and rhizospheric fungi.

To inspect the type of genes functions affected by fungal spent media, we collapsed DEGs into Clusters of Orthologous Genes (COG) categories. Principal Component Analysis on this dataset (**Figure 7**) showed for the up-regulated genes, a relevant contribution of COG category K (transcription). This observation reinforces the hypothesis about the differential modulation of epistatic interaction in *S. meliloti*genomes triggered by fungal spent media, which may involve various regulons. Among down-regulated genes, an important contribution to variance was found for COG categories G (carbohydrate metabolism and transport) and J (translation), suggesting that fungal spent media may also have differential nutritional effects over *S. meliloti*metabolism. However, we should point out that the highest contribution to transcriptional variance was for genes not found in COG or with unknown function (COG category S), indicating that probably much of the genes relevant for the modulation of interaction taking place in the rhizosphere microbiota has still to be disclosed.

However, concerning specific functional genes with known relevance in plant-rhizobium interaction and which can explain part of the synergistic phenotypes observed (**Figure 3**), among the most highly expressed genes in *S. meliloti* 1021 and in the*cis* -hybrid strain after exposure to *T . gamsii* ,*T . harzianum* and *T . velutinum* , were components of the flagellar apparatus (*flgA* , *C* , *D* ,*F* , *G* , *H* , *I* , *L* and *fliE* ,*G* , *I* , *K* , *L* , *M* , *N*), (**Supplementary Dataset S2**). It is worth noticing that the orthologs of these genes were not induced in BL225C or AK83. To reinforce the presence of epistasis and novel roles for pSymA megaplasmid, while for the *cis* -hybrid strain the same spent media strongly reduced root adhesion (**Figure 1**), in line with the expectation of an active flagellar apparatus,, the same was not true for 1021, which formed an abundant biofilm on the roots when treated with*T. velutinum* spent medium. However, regarding the general differences among *S. meliloti* strains, we must consider that the transcriptome was evaluated after 24h incubation, while the root biofilm after 48h and in the presence of the plant root system. Indeed, the motility-to-biofilm transition often includes an early phase with active flagellar apparatus, followed by an inhibition of flagellar gene transcription . We may speculate that the kinetics of this transition can be differentially modulated by *Trichoderma* species among the tested *S. meliloti* strains, however direct observations of biofilm formation under co-inoculation are needed to sustain such hypothesis.

No evidence was found for changes in the expression of galactoglucan (EPS-II) and succinoglucan (EPS-I) biosynthesis genes, but *exoD*gene was overexpressed in all the strains. This gene has been demonstrated to be needed for the invasion and development of alfalfa nodules .

Regarding genes involved in nodulation and nitrogen fixation, unexpectedly *nodA* expression was found induced in BL225C and 1021 in the presence of *T. gamsii* and *T. velutinum* , respectively, while *nodD3* expression was induced in 1021 in the presence of *T. tomentosum* and *T. velutinum* . The gene was also induced in the hybrid strain when treated with *T. velutinum*and in AK83 in presence of *T. gamsii* . NodD3 does not require specific plant compounds to activate *nod* gene expression , but its expression is activated by a LysR family transcriptional regulator,*syrM* . This latter gene was indeed found overexpressed in the same conditions. This gene also activates *syrA* , which when overexpressed, causes an increase in EPS-I production. Strikingly,*nifN* and *nifH* expression was induced in AK83 in the presence of *T. velutinum* spent medium, questioning the possibility of activation of part of the nitrogenase complex in nonsymbiotic conditions. Concerning other genes which could be relevant for rhizobium phenotypes connected with symbiosis, among the up-regulated genes under spent media treatment those encoding for proteins involved in conjugal transfer (*tra* genes) were retrieved, suggesting that presence of the fungus may promote the mobilization of the pSymA megaplasmid. However, the *rctA* gene, the repressor of the conjugation machinery was found differentially expressed in 1021 and Bl225C strains under *T. gamsii* spent medium treatment (Log2 Fold change ˜ 2), suggesting repression of the megaplasmid conjugation. Interestingly, the same gene was not differentially expressed in the *cis* -hybrid strain, indicating the presence of epistatic interaction among genes on the megaplasmid and those residing on the chromosome and the pSymB chromid.

o quantitatively estimates the number of DEGs whose expression changes among strains and treatments are explained by the rhizobial strain they belong to, by the fungal species and by the interaction between strain and species, a nested LRT model was constructed. Overall results are reported in **Table 2** and the list of genes in**Supplementary Dataset S6.** A total of 327 genes were shared as DEGs among stimulons, which allowed to train a model for a total of 981 strain, fungus, and strain x fungus combinations. Within these genes, a large fraction (ca. 2/3 of total DEGs) showed a significant effect of strain, fungus, or strain x fungus, only 34.1% of their variability in expression not being supported by the models. For 146 genes changes in relative expression levels were modeled with respect to the rhizobial strain, for 270 to *Trichoderma* (giving a total of 416 combinations for both rhizobium and *Trichoderma*), and for 230 genes to rhizobium x fungus interaction. Within this latter list of genes, several are related to redox balance (as *sox gst* , and *nuo* genes), suggesting that part of the genotype-species specific response could be related to a general differential response to stressful conditions.

Conclusions

Interaction between soil fungi and bacteria in the plant root microbiota (rhizomicrobiota) is becoming a central topic in understanding the ecology of the rhizosphere and exploiting the potential of combined manipulation of the fungal and bacterial component in order to improve plant nutrition and growth and resistance to biotic and abiotic stress (for examples see

This interaction can span from almost random microbial assemblies to specific symbiotic associations of fungal hyphae and bacterial cells Concerning *Trichoderma* spp., most of the studies have focused on the antagonistic interaction toward pathogenic bacteria, while positive effect on mutualistic bacteria have been poorly investigated. A recent work on mycorrhizal fungi , showed the alignment of fungal/rhizobial mutualism toward eliciting plant gene expression, but no direct observation has been made on the modulation of rhizobial mutualism.

Evidence presented in this work indicates that *Trichoderma*strains (belonging to four species) have an impact on *S. meliloti*phenotypes, at either physiological and host plant growth promotion levels, and alter gene expression of a large fraction of the genome. Additionally, such effects are different for the different *Trichoderma* species and for the single *S. meliloti*strains, clearly showing a strong component of genotype-by-genotype interaction. In particular, we found that the various combinations of*S. meliloti* strains and *Trichoderma* species elicit the differential expression of a variable fraction of genes, spanning from 6% to 25% of total *S. meliloti* genes. The same fungal species can determine up to 3-fold differences in the extent of the stimulon among strains (e.g. *T. velutinum* from 6.6% to 25.0%), as well as the same *S. meliloti* strain can vary on a similar extent its stimulon among different fungal species treatments (e.g. 1021 from 6.0% to 25.0%). Under a condition simulating the first step of symbiosis, i.e. the recognition of the host plant by the perception of root exudates, the same *S. meliloti* strains displayed stimulons not higher than 19% of total genome and average values around 1%-8% in most strains and conditions . The larger stimulons observed here, with fungal spent media, led to speculate that several functions encoded by the *S. meliloti* genome could involve interaction with members of the soil and root microbiota rather than simply the symbiosis with plant. A metabolic model reconstruction done on *S. meliloti*showed the importance of many genes residing on the chromosome and the pSymB chromid (accounting roughly for 2/3 of the genome) for metabolic adaptation to soil and rhizosphere, while relatively few were relevant for symbiosis .

Within these large stimulons, ca. 23% of shared DEGs display a differential expression which is significantly modelled by rhizobium x fungus interaction, again supporting the hypothesis that a relatively important fraction of the *S. meliloti* genome and of its variation could be associated to the interaction with fungi in the rhizomicrobiota. Concerning the functional groups of the overall DEGs contributing to differences among stimulons, relevant contributions of COG categories K (transcription) and S (unknown function) were found. From one side this result highlights that at least some of the genes with still unknown functions may be related to functions normally not assessed in laboratory conditions, and involved in social interaction in the natural environment, from another side it emphasizes the role of transcriptional regulators (e.g. two-component systems) and gene x gene (i.e. epistatic) interactions in social interactions. The relevance of

epistatic interaction is further confirmed by comparing the results from the *cis* -hybrid strain to the two parental strains 1021 and BL225C. Unexpectedly, these results indicate that the swapping of the symbiotic megaplasmid pSymA affects global stimulons in conditions (fungal spent media) apparently unrelated to symbiosis with plant. Indeed, stimulons of the *cis* -hybrid strain do not overlap with those of the two parental strains. Epistatic interactions between genes residing on the megaplasmid pSymA and the rest of genome were already shown, though very few in terms of number of genes involved, by genome reduction experiments and Tn-seq analyses . On the same*cis* -hybrid strain we showed that stimulons from root exudates are, as expected, displaying nonadditive (epistatic) effects . The finding that the transcriptome of the same strain reports similar evidence strongly suggests the presence on pSymA of genes related to social interaction in the rhizomicrobiota.

Moreover, the reported data on the symbiotic quality of the *S. meliloti* - *Trichoderma* combinations disclose the possibility that the rhizomicrobiota may modulate rhizobium-plant mutualistic interactions (synergistically or antagonistically, depending on the genotypic combinations, again emphasizing a genotype x genotype effect). Indeed, also results obtained in culture with spent media indicated effects, including the genotype-species specific response of plant-growth promoting related phenotypes of *S. meliloti* , which could modulate symbiotic quality, as the production of indole-3-acetic acid (IAA). IAA production in *S. meliloti* has been in fact demonstrated to positively modulate symbiotic quality . Moreover, within some stimulons (e.g. the *cis* -hybrid strain treated with *T. velutinum*) we found the presence of some *nod* genes, which are required to produce the lipochito-oligosaccharide molecule (Nod Factor), the first molecular signal directed toward the host plant by rhizobia . Modulation, e.g. synergistic effect, by fungi on rhizobium symbiotic quality has been observed for mycorrhizal fungi but until now not for non-mycorrhizal fungi as *Trichoderma* and for the effect of fungi on rhizobium physiology and genome-wide transcriptional patterns. Data obtained on symbiotic tests with *S. meliloti* - *Trichoderma*combinations demonstrated that alfalfa growth performances are indeed affected by *S. meliloti* - *Trichoderma* mix. However, a plant variety dependency over the effect of the different combinations was observed, which points out to the importance of the tripartite interaction between host plant genotype and the genotypes of the rhizomicrobiota, here oversimplified with with *S. meliloti* -*Trichoderma* combinations.

We may conclude hypothesizing that rhizobium fitness in symbiosis, and consequently rhizobial genome evolution and genomic diversity, could be modulated by rhizospheric microbes, which may have favoured or antagonized the overall differentiation pathway leading to nitrogen-fixing root nodules. Under this hypothesis we propose novel models for studying rhizobium-plant interaction, which should include other components of the rhizomicrobiota, in the present proof-of-concept exemplified by *Trichoderma* . Such novel models should clarify the role of genes, many of them still with unknown function, in the tripartite social interaction between rhizobium, host plant, and microbiota, leading to understanding the multifaceted selective pressures over rhizobium in soil, rhizosphere and the benefits of symbiosis.

In perspective, such models would allow to translate laboratory evidence into the application of novel bioinoculant consortia due to high relevance for agriculture of the nitrogen-fixation operated by rhizobia and the biocontrol by *Trichoderma* , considering that for both formulations and regulation on their use already exist , as well application to alfalfa cultivation .

Acknowledgments

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References

Data accessibility

RNA-Seq data are deposited at the European Nucleotide Archive (ENA) under the Project PRJEB63390. Scripts used for the analyses are deposited in GitHub (https://github.com/IacopoPasseri/IacopoPasseri/tree/main).

Author contributions

FV and AM conceived the idea. FV performed microbiological experiments, transcriptomic and statistical analyses. IP performed bioinformatic analysis of RNA-Seq data. NA, NJ, MM helped in setting microbiological experiments. PB guided the experiments with fungal strains. PEC provided fungal strains and guided interpretation of fungal-related data. KDA, LK, RS, KP provided support, guidance and data interpretation for plant experiments, metabolomic analysis and assays of rhizobial phenotypes. AM and FV interpreted the whole set of data and drafted the manuscript. All authors contributed in the writing of the final version of the manuscript.

Tables and Figures

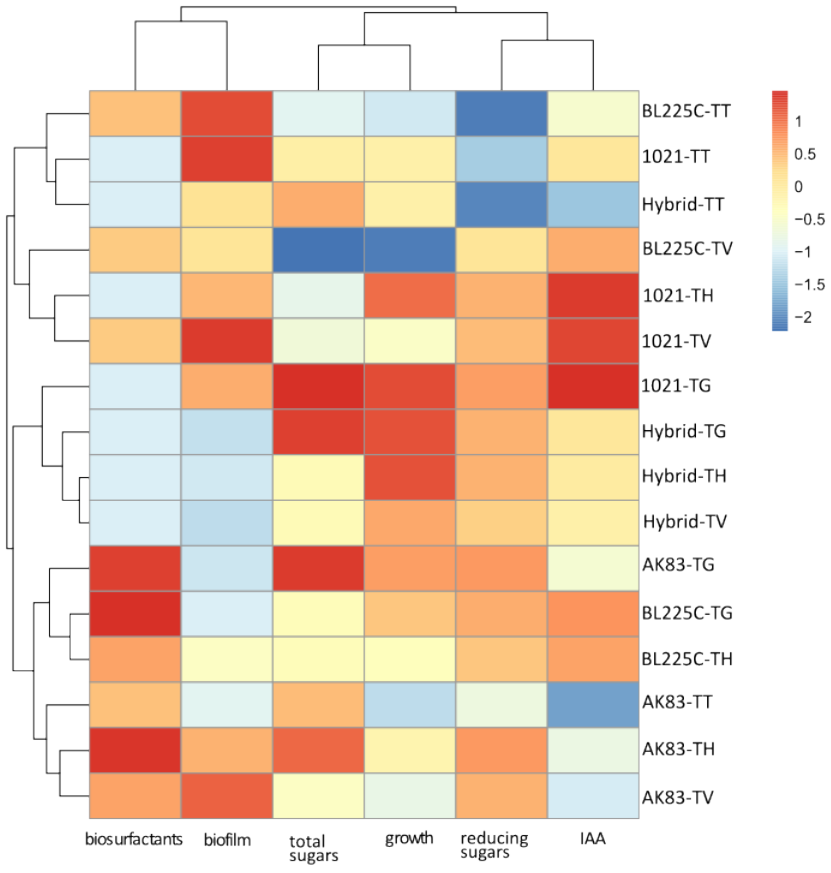

Figure 1 - Genotype-by-genotype interaction in rhizobial phenotypes exposed to *Trichoderma* spent media . Overall variability of observed phenotypes in relation to *S. meliloti*strain and *Trichoderma* species and clustering of strain-fungus combination in relation to the entire set of analysed phenotypes.

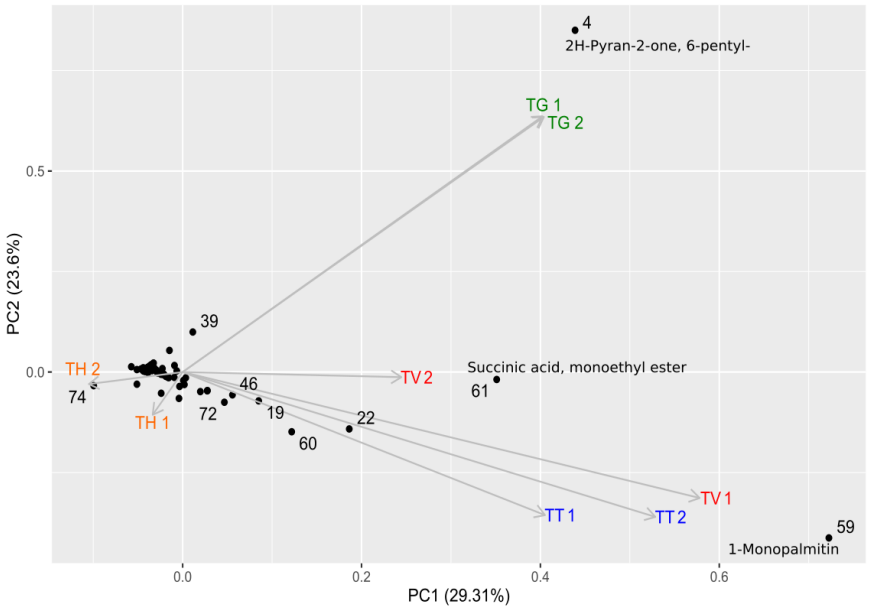

Figure 2 - *Trichoderma* **spent media metabolites differ among species.** PCA biplot from GC-MS analysis of *Trichoderma* spp. 1 week- spent media. Centroids report the code number of the compounds and vectors indicate the loadings of the spent media with respect to fungal species. TG, *T. gamsii* ; TH, *T. harzianum* ; TV, *T. velutinum* ; TT, *T. tomentosum* . Numbers after species codes indicate the biological replica (see **Dataset S1** for raw data and details of the statistical analysis).

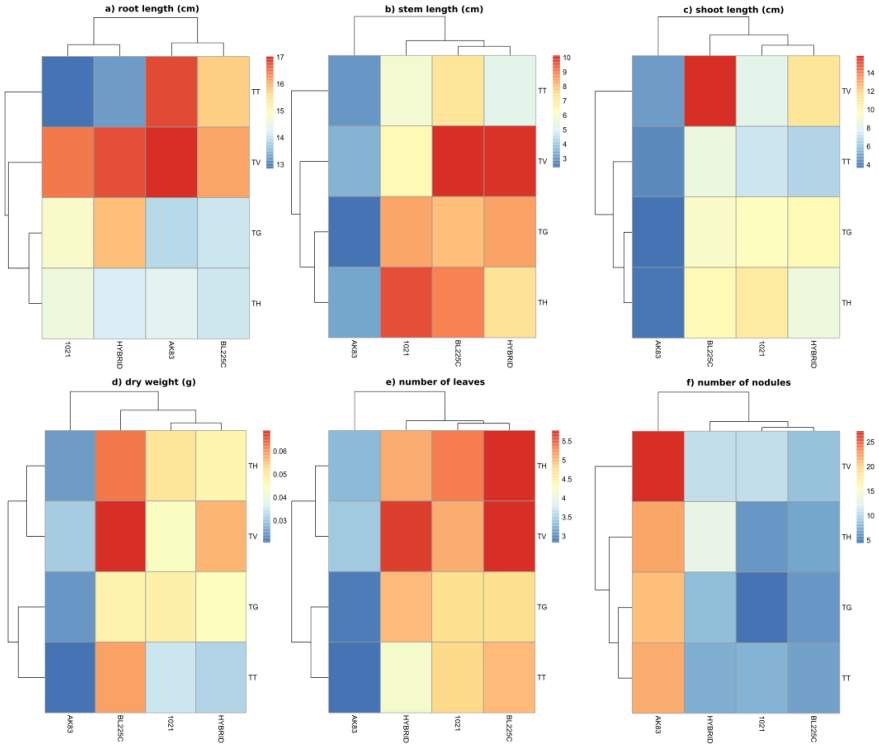

Figure 3 - Genotype-by-genotype effect of *S. meliloti*-*Trichoderma* on the quality of symbiosis . a) root length; b) shoot length; c) aerial part length; d) dry weight; e) number of leaves; f) number of nodules.

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`image4.emf` available at https://authorea.com/users/756381/articles/727343-genotype-by-genotype-interkingdom-cross-talk-between-symbiotic-nitrogen-fixing-sinorhizobium-meliloti-strains-and-trichoderma-species

Figure 4 - Modulation of rhizobial mutualism by *Trichoderma* species. The ratio between values of symbiosis quality obtained from the *S. meliloti* / *Trichoderma*combinations and the *S. meliloti* alone are reported (trt/ctr). Values above 1 indicate that *Trichoderma* exert a positive effect non symbiosis quality (synergism), below 1 indicate a negative effect of*Trichoderma* on symbiosis quality. TG, *T. gamsii* ; TH, *T. harzianum* ; TV, *T. velutinum* ; TT, *T. tomentosum* .

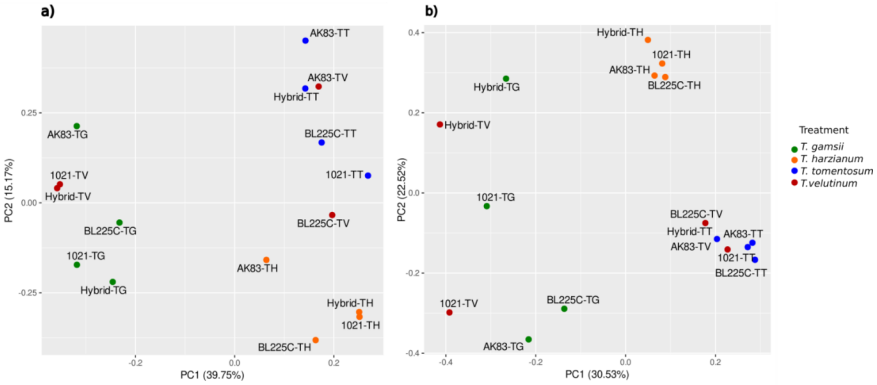

Figure 5 – Fungal species are the main drivers of*S. meliloti* core genome transcriptional response . Principal Component Analysis (PCA) of the expression profiles from stimulons of strains grown in the presence of the four fungal spent media. The clustering is based on a) up-regulated and b) down-regulated DEGs (2-fold change in expression and padj < 0.01) belonging to the core sets. The first two components explain respectively the 55.45% and 53.05% of the total variance.

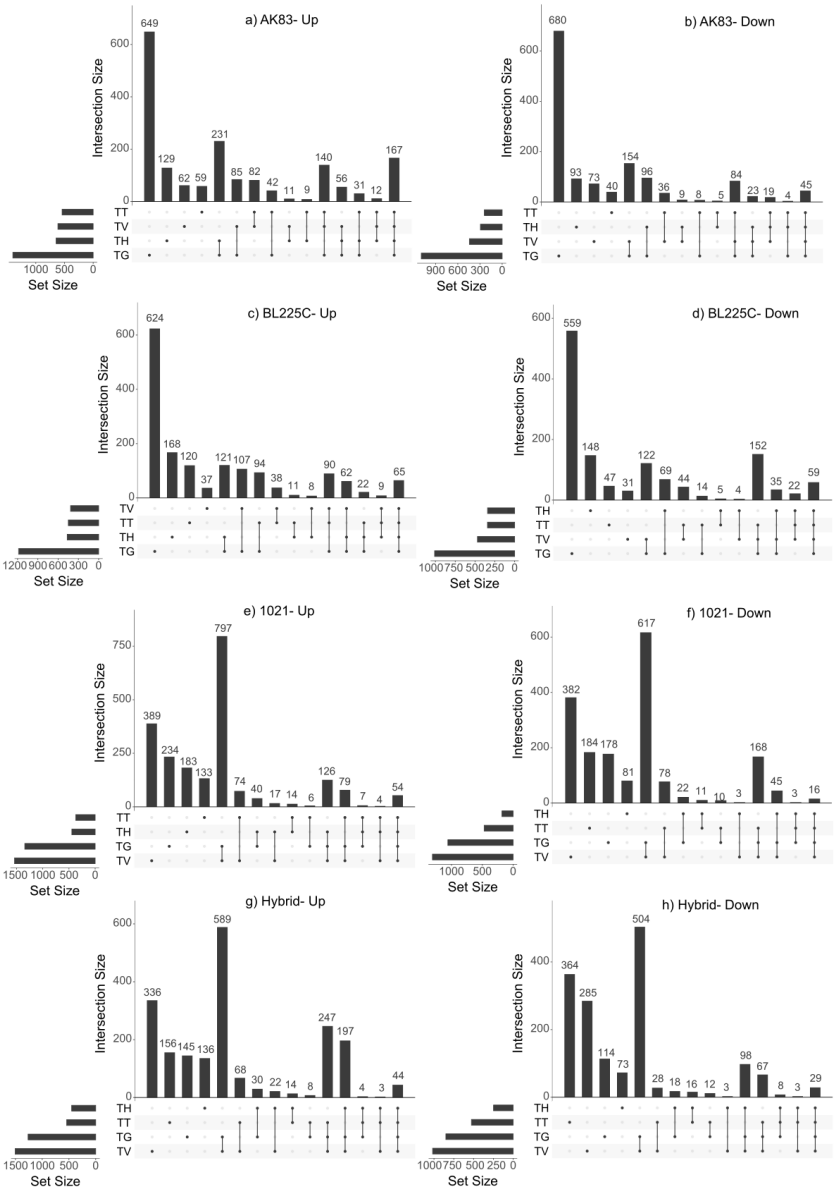

Figure 6 – Most of the DEGs are unique to fungal species . Intersection between up- and down-regulated genes under all conditions. The numbers of up-regulated (a, c, e, g) and down-regulated (b, d, f, h) genes under each condition for each strain are reported. (a and b) AK83; (c and d) BL225; (e and f) Rm1021; (g and h) hybrid strain. Each row of the matrix corresponds to a *Trichoderma* treatment, with the number of up-regulated/down-regulated genes reported to the left as a bar plot. Each column corresponds to one intersection: cells are either empty, indicating that up- or down-regulated genes under the specified conditions are not part of the intersection, or filled, indicating that the genes present under the specified conditions are participating at the intersection. Bars on the top show the size of the intersection reported on the bottom. TG, *T. gamsii* ; TH, *T. harzianum* ; TV, *T. velutinum* ; TT, *T. tomentosum* .

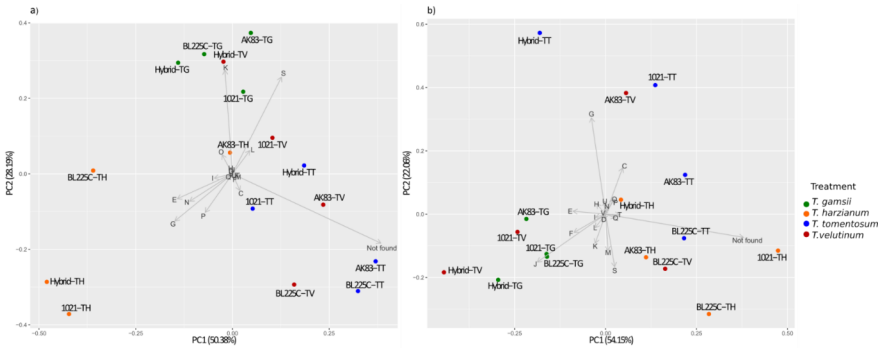

Figure 7 - *S. meliloti* **functional gene categories differentially elicited by fungal spent media.** PCA biplots of COG categories percentages among a) up-regulated and b) down-regulated DEGs. Centroids report the name of all *S. meliloti* -*Trichoderma* conditions and vectors indicate the loadings of the COG categories. Definitions for observed COG categories are: [J] Translation, ribosomal structure, and biogenesis; [K] Transcription;[L] Replication, recombination and repair; [M] Cell wall/membrane/envelope biogenesis; [O] Posttranslational modification, protein turnover, chaperones; [C] Energy production and conversion; [G] Carbohydrate transport and metabolism; [E] Amino acid transport and metabolism; [F] Nucleotide transport and metabolism; [H] Coenzyme transport and metabolism; [I] Lipid transport and metabolism; [P] Inorganic ion transport and metabolism; [S] Function unknown.

Table 1 - The extent of stimulons in *S. meliloti*strains treated with *Trichoderma* sp. spent media. The number of significant DEGs with respect to the black control (>|2|-fold change in expression and an adjusted P value of <0.01) and the percentage with respect to the total number of genes in each *S. meliloti* genome are reported**.**

	1021	**BL225C**	**AK83**	**Hybrid**
T. gamsii	1343 (21.7)	1186 (18.8)	1401 (21.8)	1264 (20.0)
T. tomentosum	371 (6.0)	451 (7.1)	542 (8.4)	545 (8.6)
T. velutinum	1546 (25.0)	416 (6.6)	615 (9.6)	1506 (23.8)
T. harzianum	445 (7.2)	466 (7.4)	646 (10.0)	450 (7.1)

Table 2 – Many shared DEGs account for genotype-by-genotype interaction. Number of combinations and percentage over of 981 total (327 genes), which resulted significant in a nested LRT model. Significance was based on an FDR-corrected p-value < 0.05.

	S. meliloti	Trichoderma	*S. meliloti* **and** ***Trichoderma***	*S. meliloti* **x** ***Trichoderma***	**None**
Number of DEGs	146 (14.9%)	270 (27.5%)	416 (42.4%)	230 (23.4%)	335 (3

Supplementary Information

Supplementary Dataset S1. GC-MS profiles of fungal spent media and peak attribution. Worksheet 1 reports for each sample the retention time (min), the peak area (%), the name of the possible compound and probability of attribution. Tg, Th, Tv, Tt, indicates *T.gamsii* , *T. harzianum* , *T. velutinum*and *T. tomentosum* , respectively. Worksheet 2 matrices with the 76 compounds identified with >80% probability and grouped on the basis of chemical class. The matrices report percentage and dimensionless values for the peak area. File type .xlsx

Supplementary Dataset S2. List of total upregulated Differentially Expressed Genes (DEGs).

Each datasheet contains the list of DEGs, with fold change of >2; padj <0.01 for each strain and fungal spent media treatment. For each gene information on functional annotation and pangenome is present. Fungal codes are as indicated above. File type .xlsx

Supplementary Dataset S3. List of total downregulated Differentially Expressed Genes (DEGs). Each datasheet contains the list of DEGs, with fold change of < -2 and padj<0.01, retrieved for each strain and fungal spent media treatment. For each gene information on functional annotation and pangenome is present. Fungal codes are as indicated above. File type .xlsx

Supplementary Dataset S4. Statistically significant DEGs present in the core genome of the strains. The list of DEGs with fold change of >|2|; P value <0.01, from genes present in the core genome of the strains is reported. File type .xlsx

Supplementary Dataset S5. List of unique DEGs. For each strain, each datasheet contains the list of unique upregulated and downregulated DEGs retrieved from the intersection of the 4 sets (corresponding to Trichoderma species). For each gene, information on fold change, padj, functional annotation and pangenome details is present. File types .xlsx

Supplementary Dataset S6. List of core DEGs resulted from the nested model of the likelihood ratio test (nLRT). For each gene fold change and padj are reported. The effect column indicates the term of the model (strain, fungus, or strain:fungus) being tested. In column sign, significance after BH correction for false discovery rate is reported with "*" for padj<0.05 ; "**" for padj<0.01

Supplementary Figure S1. Effects of *Trichoderma* spent media on S. meliloti growth and plant-growth promotion phenotypes . a) growth inhibition b) Auxin (IAA) inhibition of production; c) biosurfactants production; d) total EPS production; e) reducing EPS production, f) root biofilm formation

Supplementary Figure S2 - Effect of *S. meliloti*-*Trichoderma* on symbiotic phenotypes . a) root length; b) stem length; c) shoot length; d) dry weight; e) number of leaves; f) number of nodules. Different letters indicate significant differences at $P < 0.05$

Supplementary Table S1. *S. meliloti* response to spent fungal growth media . Results from one-way ANOVA evaluating the effect on rhizobial growth of different fungi on each *S. meliloti*strains for 1W, 2W and 3W spent media are reported.

Supplementary Table S2 . **Effect of coinoculation of*S. meliloti* strains with *Trichoderma* (condition) on symbiosis quality** . Results from ANOVA are shown for the different phenotypes recorded. Asterisks indicate statistically significant differences (*, p<0.05; **, p<0.01; ***, p<0.001).