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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 molecular interactions between symbiotic nitrogen-fixing rhizobia and soil fungi (*Trichoderma* spp.), hence shed light on synergistic roles rhizospheric fungi could have in the biology of symbiotic nitrogen fixation bacteria. 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 ×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 generalized rhizobia genotype x fungal genotype interaction, including synergistic, neutral and antagonistic effects on alfalfa symbiotic phenotypes. Differential expression of a high number of genes was shown in rhizobia strains with up to 25% of total genes differentially expressed upon treatment of cultures with fungal spent media. Percentages over total genes and type of genes differentially expressed changed according to both fungal species and rhizobial strain. To support the hypothesis of a relevant rhizobia genotype x fungal genotype interaction, a nested Likelihood Ratio Test indicated that the model considering the fungus-rhizobium interaction explained 23.4% of differentially expressed genes. Our results provide insights into molecular 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.

1. 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 (Berendsen et al., 2012). These interactions are essential for ecosystem functioning and have profound implications for plant health and productivity (Bulgarelli et al., 2013; Finkel et al., 2017). Among the most studied microorganisms associated with plant roots are rhizobia, a group of nitrogen-fixing bacteria that can establish mutualistic symbiotic associations with leguminous plants. These bacteria thrive in soil and could also reside within specialized structures called nodules on plant roots and convert atmospheric nitrogen into ammonia, which can be utilized by the host plant. Another key component of the plant-associated microbiome are fungi (Naranjo-Ortiz and Gabaldón, 2019). Rhizospheric fungi contribute to

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nutrient cycling by decomposing organic matter, releasing nutrients, and enhancing nutrient uptake by plants (Sokol et al., 2022). They can also act as biocontrol agents, suppressing plant pathogens and promoting plant growth. At the molecular level, interaction between *S. meliloti* and fungi has been studied for a tripartite system including rhizobia, arbuscular mycorrhizal fungi, and the host plant *M. truncatula*, reporting extensive effects of microbial mutualists on plant gene expression (Afkhami et al., 2021).

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 (Harman et al., 2004). 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 (Vinale et al., 2008). *Trichoderma* spp. can also promote plant growth by enhancing nutrient availability, stimulating root development, and inducing systemic resistance (Woo et al., 2023). Additionally, they contribute to the decomposition of organic matter, thereby influencing nutrient cycling and soil structure. (Woo et al., 2023; Ramírez-Valdespino et al., 2019).

Reports on co-inoculation of rhizobia and *Trichoderma* spp. demonstrate that in many cases there is a promotion of nitrogen-fixing symbiosis traits (e.g. biomass, grain yield, nodule number) and even some amelioration of antagonistic actions of *Trichoderma* (Bécquer et al., 2013 and Barbosa et al., 2022 for a recent review). However, while many details are known on rhizobia-plant interaction, relatively few studies have investigated the molecular aspects of the interaction between rhizobia and the other relevant components of the root microbiome, i.e. fungi (Afkhami et al., 2021; Afkhami and Stinchcombe, 2016; Hernandez et al., 2019).

Concerning rhizobia, several studies have shown that within a single species large phenotypic difference exists, with a wide spectrum of symbiotic qualities with host legume plants (see for instance Epstein et al., 2023). *Sinorhizobium meliloti* is a model rhizobial species, forming symbiotic associations with leguminous (*Fabaceae*) genera such as *Medicago*, *Melilotus*, and *Trigonella* (Geddes and Oresnik, 2014; Oldroyd, 2013). For *S. meliloti* a large genetic and symbiotic diversity among strains has been reported, and a key relevance of rhizobial genotype x plant genotype partnership for successful symbiotic plant growth promotion has bene shown (Bellabarba et al., 2021; Epstein et al., 2023; Fagorzi et al., 2021; Riley et al., 2023). Such rhizobial genotype x plant genotype interaction has been molecularly deciphered in terms of genomic diversity (Burghardt et al., 2018), but also of transcriptional variation (Burghardt et al., 2017). Indeed, for *S. meliloti*, we detected the transcriptomic signatures of wide genotype x genotype interaction, including epistatic interaction among the main set of symbiotic genes and the rest of the genome in response to the treatment with plant root exudates from three alfalfa (*Medicago sativa* L.) varieties (Fagorzi et al., 2021; Checcucci et al., 2018).

We may hypothesize that genotype x genotype interactions can also be present at other level of biotic relationships, that is between rhizobia and soil fungi (e.g. *Trichoderma*), which could possibly affect overall quality of symbiosis, allowing to interpret the variability of previous results on co-inoculation (Barbosa et al., 2022). While for *Trichoderma* spp. and the symbiosis between *Sinorhizobium* and *Medicago* few reports are present, no molecular (i.e. genetic) details nor clues about the presence of a genotype x genotype interaction have been reported yet. Deciphering 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 potential mutualistic and antagonistic effects down to the molecular level (Burghardt et al., 2017; Fagorzi et al., 2021, 2023; Heath et al., 2012; Smee et al., 2021; Vaccaro et al., 2022).

To test the hypothesis on the presence of genotype x genotype interaction between *S. meliloti* and species of *Trichoderma* and shed light on the molecular determinants of such interactions we then designed experiments using a combination between four *Trichoderma* species and four *S. meliloti* strains. Here, we report the results of such experiments aimed at determining the presence of a potential molecular dialogue between *Trichoderma* and the rhizobium *S. meliloti* which can rule out in a 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 23.4% of total genes) and are also reflected in host plant growth-promoting phenotypes. These results provide novel insights on how rhizospheric fungi are able to influence the legume-rhizobia interactions, especially on genotype-by-genotype molecular dialogue and gene expression.

2. Materials and methods

2.1. 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 (Meade et al., 1982); 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) (Roumiantseva et al., 2014); BL225C, isolated from *Medicago sativa* plants grown on soil of Lodi, Italy, during previous experiments (Carelli et al., 2000); a *cis-hybrid* strain between 1021 and BL225C (Checcucci et al., 2018). 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. These strains were chosen based on previous reports indicating they have relevant genomic differences and genotype x genotype variability coupled with transcriptomic changes in the symbiosis with alfalfa (*M. sativa*) varieties (Fagorzi et al., 2021). The *cis-hybrid* allows to test the presence of possible strain-specific epistatic interactions between the symbiotic megaplasmid and the rest of the genome in the response to *Trichoderma*. 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) (Anees et al., 2010). The MIAE structure is holding over 15 300 soil-borne microbial strains. Since 2013 MIAE collection has ISO9001:2000 certification. Fungi were stored on Potato Dextrose Agar plates at 4°C. *Trichoderma* spp. liquid cultures were grown on minimal medium (Penttilä et al., 1987) containing (mg/ml): glucose 20; $(NH_4)SO_4$ 5; KH_2PO_4 15; $MgSO_4$ 0.6; $CaCl_2$ 0.6; $FeSO_4$ * 7 H_2O 0.005; $MnSO_4$ *H_2O 0.0016; $ZnSO_4$ * 7 H_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.

2.2. 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. Based on results of growth obtained after treating S. *meliloti* strains with 1

week, 2 and 3 weeks old media, we selected 1 week (1 W) spent media for transcriptomic analysis and phenotypic tests (see below). Regarding the molecular composition, the metabolites from 1 W 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 7890 A, 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 2 μL. 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 min, 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 min.

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

2.3. Bacterial growth assays with Trichoderma spp. spent media

The effect of *Trichoderma* spp. spent media on *S. meliloti* strains 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 spent medium (SM) was present. For each strain-spent medium combination, 3 independent cultures were set up and the growth inhibition index was calculated after 72 h as: 1- OD_{600} trt/OD_{600} ctr, where trt are treated and ctr the control samples.

2.4. Bacterial auxin production after addition of Trichoderma spp. spent media

The auxins production in the presence of 50% v/v *Trichoderma* spp. was quantified using the Salkowski reagent (Salkowski, 1885). 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.

2.5. Bacterial exopolysaccharides production after addition of Trichoderma spp. spent media

Exopolysaccharide (EPS) was extracted from bacterial cultures grown at 30°C for 48 h 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 min at 4°C and EPS was precipitated by adding 1 vol 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 distilled H_2O.

Total sugars estimation was evaluated using DuBois method (DuBois et al., 1956): 200 μl of crude EPS was mixed with 200 μl 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 (Sumner and Graham, 1921). EPS crude sample and DNSA reagent were mixed in 1:3 ratio. Samples were heated in a thermostatic bath for 15 min 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.

2.6. Bacterial biosurfactants production after addition of Trichoderma spp. spent media

The emulsification index (EI) was determined as described previously (Ashry et al., 2022) 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: EI24h (%) = HE / HT x 100, where HE is the height of the emulsified layer (in cm), and HT is the height of the total liquid column (in cm). Data are from 3 biological replicates.

2.7. Bacterial root adhesion test after addition of Trichoderma spp. spent media

The adhesion of rhizobial cells to *M. sativa* root surface (here used as a proxy of biofilm formation on roots) was estimated as described previously (Fagorzi et al., 2021; Checcucci et al., 2018). Briefly, sterilized seed were germinated in water, then five days-old seedlings were transferred in 1.5 ml tubes containing Fahraeus medium supplemented with 50% v/v spent medium and bacterial culture at OD_{600} 0.1. After 48 h, roots of similar length (~ 3 cm) were washed in 500 μl of 0.9% NaCl to remove loosely adherent cells by vortexing for 10 s. 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 (Checcucci et al., 2016). For each condition, three biological replicates were performed, with three seedlings of *M. sativa* each. Differences were evaluated by one-way ANOVA Tukey pairwise contrast.

2.8. Plant symbiosis assay with combinations of S. meliloti strains and Trichoderma species

The plant symbiosis test was aimed to understand if symbiotic quality is dependent on the different consortia composed by fungal x rhizobium combinations. Symbiotic assays were performed as previously described (Fagorzi et al., 2021) in a chamber of culture. *Medicago sativa* cv. Marina seedlings (Continental Semences SpA, Lodi, Italy) were treated with 24 different conditions: only one rhizobial strain (4 treatments), only one *Trichoderma* species (4 treatments) and 4×4 *Trichoderma* sp.-*S. meliloti* combinations (16 treatments). For each treatment 3 biological replicates were performed, with 3–5 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 16 h photoperiod. After 5 weeks of plant cultivation in a chamber of culture at 26 ± 2°C with a 16 h photoperiod, the following parameters were measured: nodule number, root, shoot and stem length, number of leaves and plant dry weight.

2.9. Bacterial 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 5 ml of TY medium supplemented with 50% v/v of *Trichoderma* 1 week (1 W) spent medium and incubated in the dark at 30°C under shaking (130 rpm) for 24 hours. Three biological replicates (independent cultures) were produced for each strain-spent medium combination, including untreated control with fresh TY 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 previously (Fagorzi et al., 2021). 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 kit (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 Corp. (Korea).

2.10. Bioinformatic analyses of RNA-Seq data and statistical tests

Trimmed and demultiplexed reads were mapped back to transcripts using Salmon (version 1.1.0) (Patro et al., 2017) and quantification was imported into R Studio (ver 4.2.0) using the *tximport* package (version 1.10.1) as previously reported (Fagorzi et al., 2021). Differential abundance analysis was performed with the DESeq2 version 1.22.2 package (Love et al., 2014). For each *S. meliloti* strain, genes differentially expressed (DEGs, 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 genomes were computed with Roary (Page et al., 2015) following annotation with Prokka (Seemann, 2014) and assignment to Clusters of Orthologous Genes (COG) categories, as previously reported (Fagorzi et al., 2021). Comparison of expression values of genes in the core genome of strains and nested likelihood ratio tests (LRTs) were performed as indicated previously (Fagorzi et al., 2021). All genes of *S. meliloti* strains 1021, AK83, and BL225C were functionally annotated using stand alone version 2 of eggNOG-mapper with default settings (Huerta-Cepas et al., 2017).

All statistical analyses were performed in R Studio (ver. 4.2.0) (RStudio Team, 2020). ANOVA and Tukey's tests were performed using respectively *aov* and *TukeyHSD* functions. Mantel's test was performed using *mantel* function from *vegan* package. Permutational analysis of variance (PERMANOVA) (Clarke, 1993) was performed using *adonis2* and *pairwise.adonis2* function from *vegan* package (Oksanen et al., 2017). Heatmaps were produced with package *pheatmap*. Principal Component analysis (PCA) was performed on GC-MS dataset and on DEGs using the *prcomp* function of R visualized with the *ggplot2* package of R (Wickham, 2009). *UpsetR* (Conway et al., 2017) was used to visualise intersecting dataset of DEGs on the different *S. meliloti* strains x fungal spent media combinations.

3. Results and discussion

3.1. Fungal spent media affect growth and metabolite production in rhizobia

The overall profiles of rhizobial growth response among the spent media obtained after 1 week (1 W), 2 weeks (2 W), and 3 weeks (3 W) of fungal growth were different (Supplementary Figure S1). In fact, Mantel tests performed on the matrices representing the pairwise growth index values did not show significant correlations (1 W vs. 2 W R=-0.496, p-value=0.92; 1 W vs. 3 W R=-0.402, p-value=0.92; 2 W vs. 3 W R=0.618, p-value=0.12), indicating that the fungal spent media from 1 W, 2 W and 3 W 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, 1 W spent media had the highest levels of significance in discriminating the effects of fungal spent media on each rhizobial strain, suggesting that here the four fungal species maximize their differential effects in giving rise to rhizobial strains differential growth inhibition. We consequently proceeded with the analysis of rhizobial strains treated with 1 W spent medium only. Indeed, the effects of 1 W *Trichoderma* spent media against the four rhizobial strains resulted in different extents of either inhibition or stimulation (Fig. 1). Regarding the effects of fungal spent media 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 we observed effects spanning from growth inhibition (but in case of BL225C and *T. velutinum* spent medium growth stimulation) to EPS production which were different in relation to both fungal spent media and to the rhizobial strain. Numerical data for each observation can be retrieved from the Supplementary Data**set S1**, while the statistical evaluation of the relevance of rhizobial strain or fungal species on the variability of the observed phenotypes is reported on Supplementary Table S2. The effect detected for total sugars (total EPS) was similar to effect on growth, while reducing sugars clustered with the effect on biosurfactant production. The effect on biofilm grouped apart from the other phenotypes. Considering single phenotypes separately, for growth inhibition (Fig. 1, Supplementary Figure S2a), *S. meliloti* 1021 and *cis*-hybrid strains clustered together, while AK83 and BL225C formed a second cluster. Indeed, a statistically significant relevance of the bacterial strain identity on this phenotype was detected (Supplementary Table S2a). 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 (Fig. 1, Supplementary Figure S2b), 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 significantly 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 (Supplementary Table S2c). 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 (Fig. 1, Supplementary Figure S2c), 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 sugar production (Fig. 1, Supplementary Figure S2d) and the biofilm formation on root (Fig. 1,

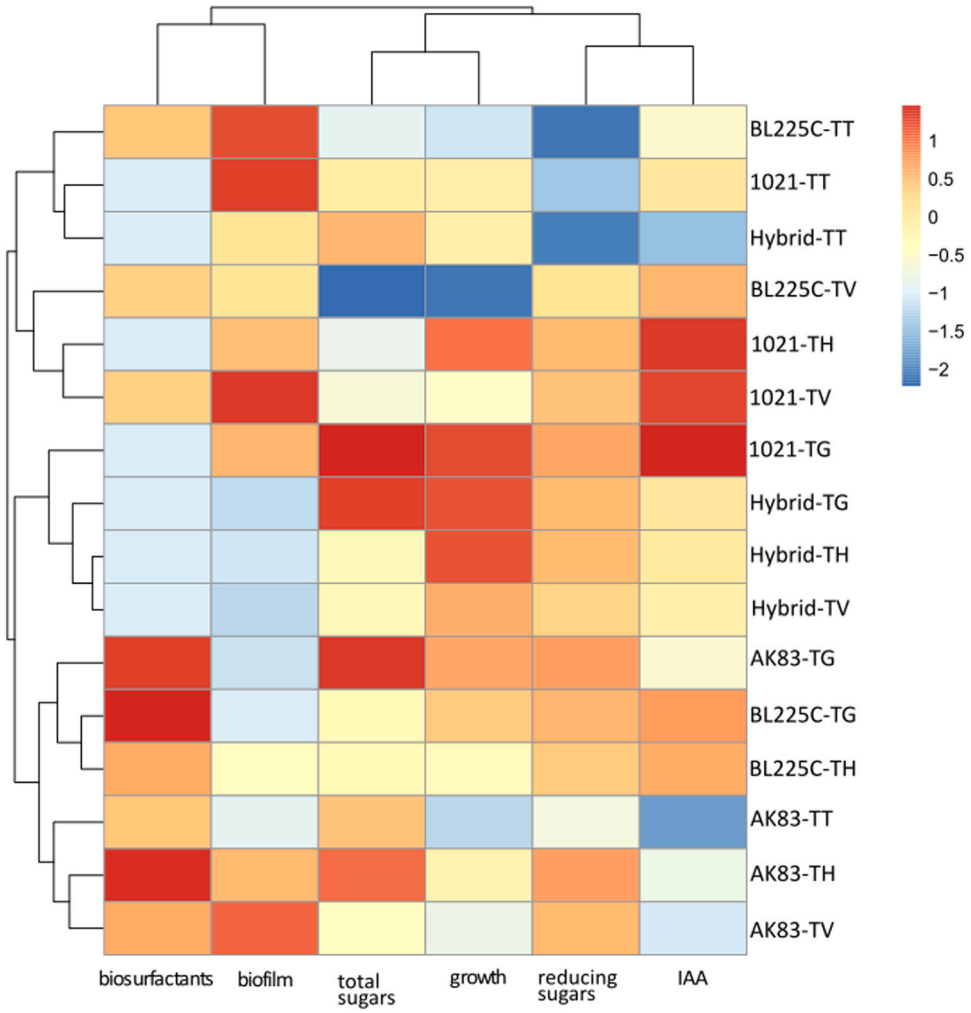

Fig. 1. Genotype-by-genotype interaction in rhizobial phenotypes exposed to *Trichoderma* spent media. Overall variability of observed phenotypes in relation to *S. meliloti* strain and *Trichoderma* species and clustering of strain-fungus combination in relation to the entire set of analysed phenotypes. Row labels reports the combinations between *S. meliloti* strains (1021, AK83, BL225C, Hybrid) and *Trichoderma* species (TG, *T. gamsii*; TH, *T. harzianum*; TV, *T. velutinum*; TT, *T. tomentosum*). Column labels show the phenotypes tested. Positive index values indicate that the ratio trt/ctr is <1, meaning inhibition of the fungus spent media over the bacterial phenotypes. Negative values mean the ratio trt/ctr is >1, then bacteria with fungus spent media display increased phenotypes than controls (absence of inhibition, then stimulation).

Figure S2f), while reducing sugar production (Fig. 1, Supplementary Figure S2e) only distinguished *T. tomentosum* spent medium response from those of the other fungi.

3.2. Metabolomic analysis of Trichoderma spent media

To evaluate if the overall differences in rhizobial strains response to *Trichoderma* 1 W 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. 1 W spent media was performed. The GC-MS profiling detected a total of 987 peaks (Supplementary Dataset S2), 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 2 H-Pyran-2-one, 6-pentyl- (a representative metabolite common to the *Trichoderma* genus), 1-Monopalmitin, a compound known for having activity against pathogenic organisms (Altieri et al., 2009; Reino et al., 2008), and succinic acid, monoethyl ester. The peak area of these and of the other compounds varied among samples (see Supplementary Dataset S2, worksheet 2). Principal component analysis (PCA) (Fig. 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 2 H-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 (Fig. 1), suggesting that possibly some of the compounds mentioned above may target rhizobial metabolism, giving rise to contrasting bacterial responses. Indeed, monoacylglycerol, like 1-Monopalmitin, are known to affect bacterial metabolism, as reported above (Altieri et al., 2009; Reino et al., 2008), as well as organic acids as succinic acid are among the preferred carbon sources of *S. meliloti* (Geddes and Oresnik, 2014). We may consequently hypothesize that the phenotypic differences observed in *S. meliloti* treated with *Trichoderma* spent media could be partially due to differences in the presence of such metabolites in spent media and differential

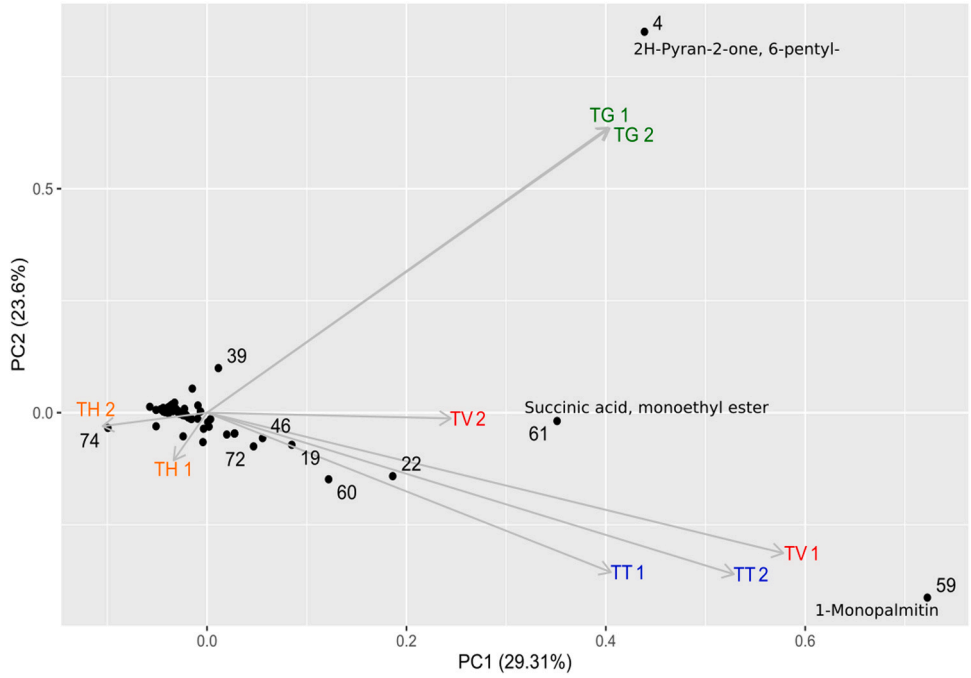

Fig. 2. *Trichoderma* spent media metabolites differ among species. PCA biplot from GC-MS analysis of *Trichoderma* spp. 1 week- spent media. Centroids report the code number of the compounds and vectors indicate the loadings of the spent media with respect to fungal species. TG, *T. gamsii*; TH, *T. 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).

sensitivity for the same metabolites among *S. meliloti* strains.

3.3. *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* (as inoculant) 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* inoculation could display evidence for genotype-by-genotype interaction, as did the previous spent medium results on *S. meliloti* phenotypes (i.e. growth, root biofilm formation, auxin, siderophore, EPS production). Results from *in vitro* symbiosis tests are reported in Supplementary Table S3 and Supplementary Figure S3. 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. We tested the hypotheses of effects (variability) due to: i) the bacterial strain (all fungal species combined), ii) the fungal species (all bacteria strains combined), and iii) the bacteria x fungi combinations. Results indicated 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 also a statistically significant contribution of the interaction *Trichoderma* species x *S. meliloti* strain (Supplementary Table S4), supporting the hypothesis that genotype x genotype interaction between *S. meliloti* and *Trichoderma* can also affect the rhizobium host plant symbiotic-related phenotypes. Going into the details of single combinations, (Supplementary Table S3, Supplementary Figure S3), 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. Post-hoc test Tukey test showed that all treatments had the same effect on this trait but *T. gamsii* (Supplementary Table S3). 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 Table S3). This phenotype partially mirrors the auxins production profile (Fig. 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 (Camerini et al., 2008; Ferguson and Mathesius, 2014). 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 (Galardini et al., 2011) and interpreted in terms of higher capacity of competitiveness for nodule colonization (Bellabarba et al., 2021), including possible cheating related to reduced nitrogen-fixation ability (Checcucci et al., 2016).

Summing up above-mentioned results, it is possible to distinguish, in a sociomicrobiological framework *sensu* Hamilton (Hamilton, 1964), 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, such as showing antagonistic interaction), with respect to *M. sativa* phenotypes. Fig. 3 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 S3). Combinations displaying evidence for synergism, neutralism, and antagonism in the quality of symbiosis 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. However, we should consider that this test was done under controlled conditions and that no physiological measures of the

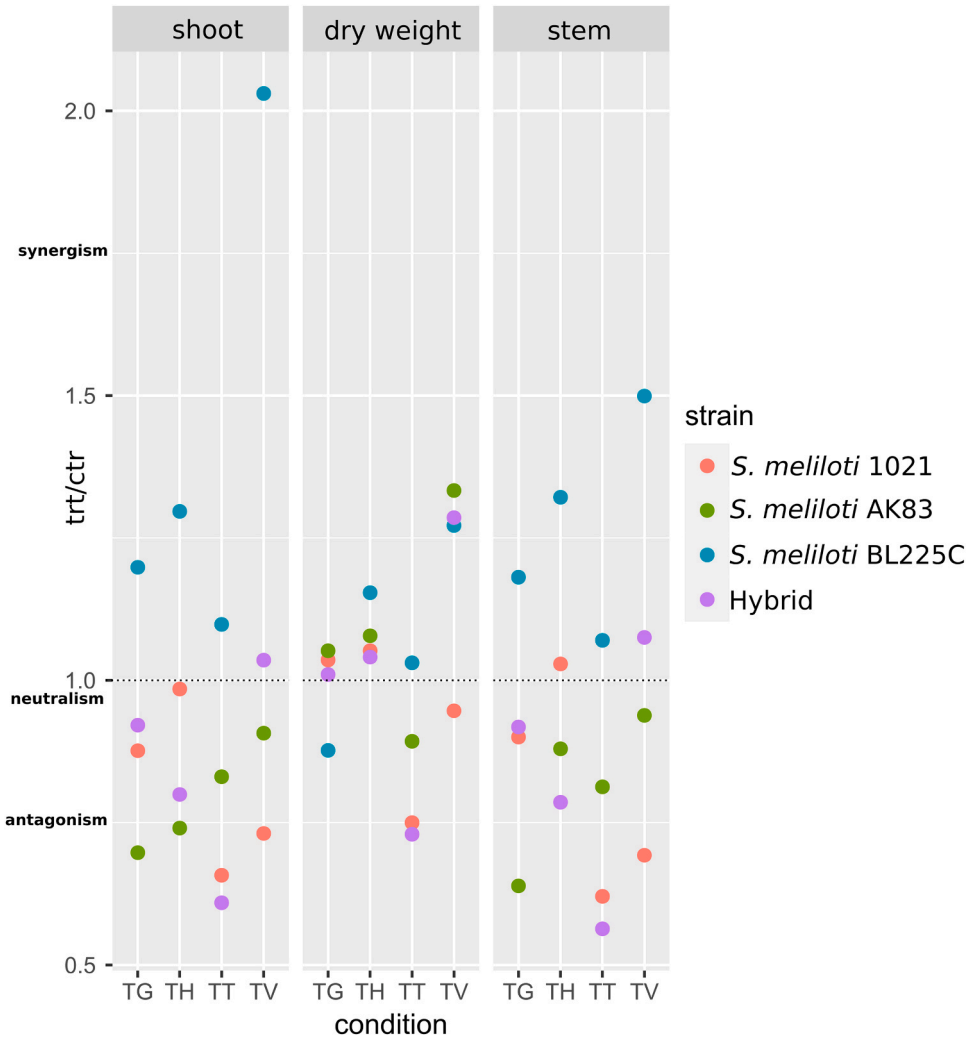

Fig. 3. Modulation of rhizobial mutualism by *Trichoderma* species. The ratio between values of symbiosis quality obtained from the *S. meliloti/Trichoderma* combinations and the *S. meliloti* alone are reported (trt/ctr). Values above 1 indicate that *Trichoderma* exert a positive effect non symbiosis 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*.

plants were performed (e.g. fixed nitrogen). Consequently, we should be very cautious about the interpretation of such sociomicrobiological results in terms of predictors of actual synergism, neutralism, or antagonism toward alfalfa biomass productivity of co-inoculation in the field.

3.4. 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 1 W spent media for 24 h 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 S3 and S4. 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

Table 1
The extent of stimulons in *S. meliloti* strains treated with *Trichoderma* spp. 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%)

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* 1 W spent medium resulted in a quite different response among the 4 *S. meliloti* strains (Fig. 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 (Galardini et al., 2013) 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 and S5). Results (Fig. 4) 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, supported by a PERMANOVA (Supplementary Table S5 and S6), resembles the separation based on spent media metabolome composition (Fig. 2) and partial *S. meliloti* growth (Fig. 1). Concerning up-regulated DEGs (Fig. 4**a**), 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 (Fig. 4**b**). 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. Fig. 5 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 (Fig. 5**a-d**) or at least can be shared among two fungal species (Fig. 5 **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 S6.

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 Fig. 4) 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 (Fagorzi et al., 2021). 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 (diCenzo et al., 2014)), 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 (Fig. 6) 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 (that have no matches to any known orthologous groups in the COG database) 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 (Fig. 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 S3). It is worth noticing that the orthologs of these genes were not induced in BL225C or AK83. To reinforce the

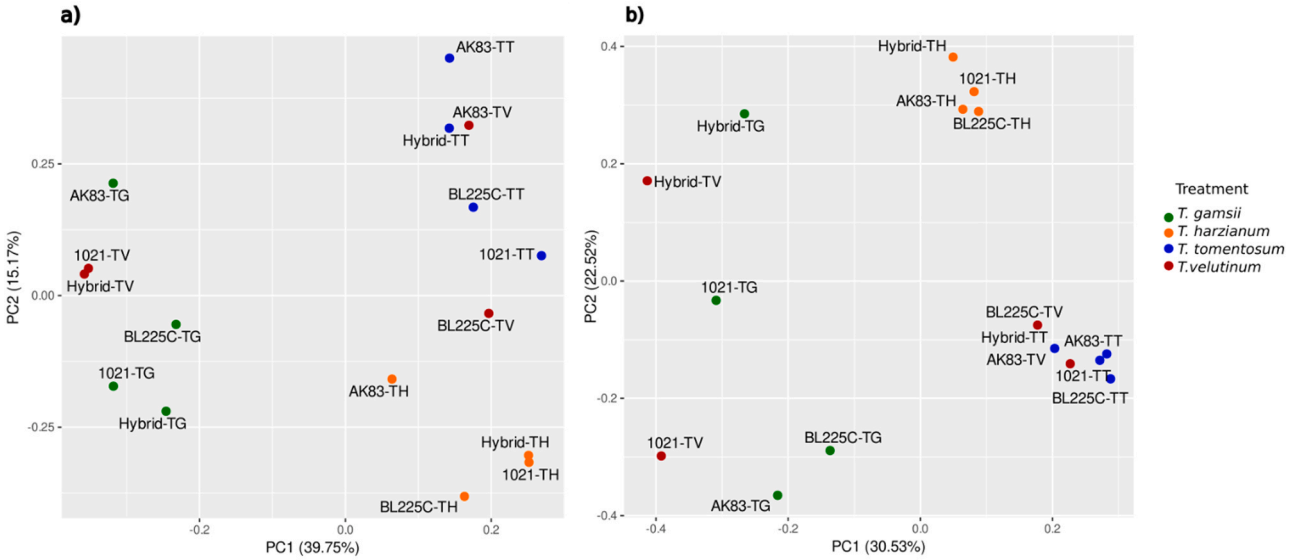

Fig. 4. Fungal species are the main drivers of *S. meliloti* core genome transcriptional response. Principal Component Analysis (PCA) of the expression profiles from stimulons of strains grown in the presence of the four fungal spent media. The clustering is based on a) up-regulated and b) down-regulated 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.

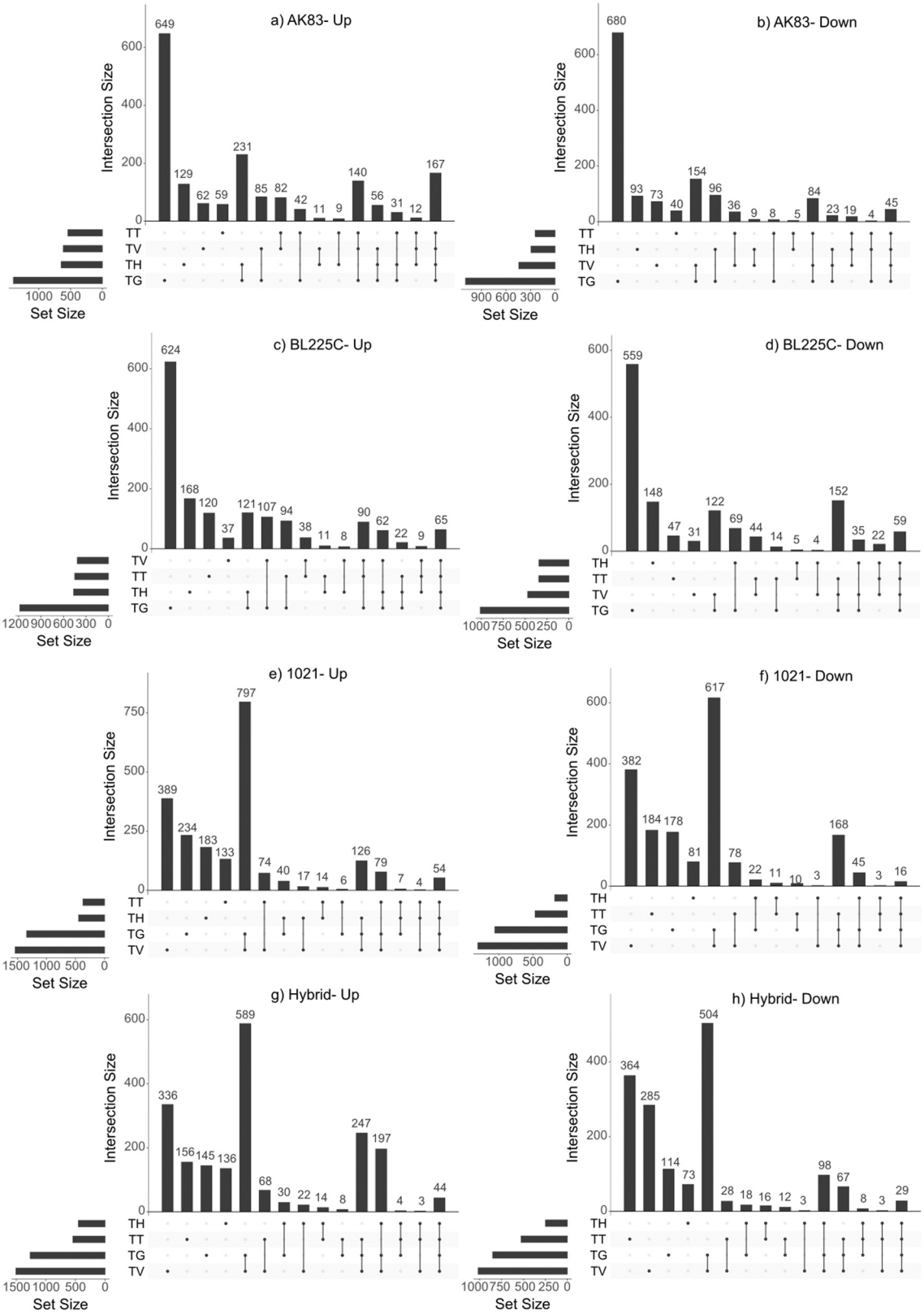

Fig. 5. Most of the DEGs are unique to fungal species. Intersection between up- and down-regulated genes under all conditions. The numbers of up-regulated (a, c, e, g) and down-regulated (b, d, f, h) genes under each condition for each strain are reported. (a and b) AK83; (c and d) BL225; (e and f) Rm1021; (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*.

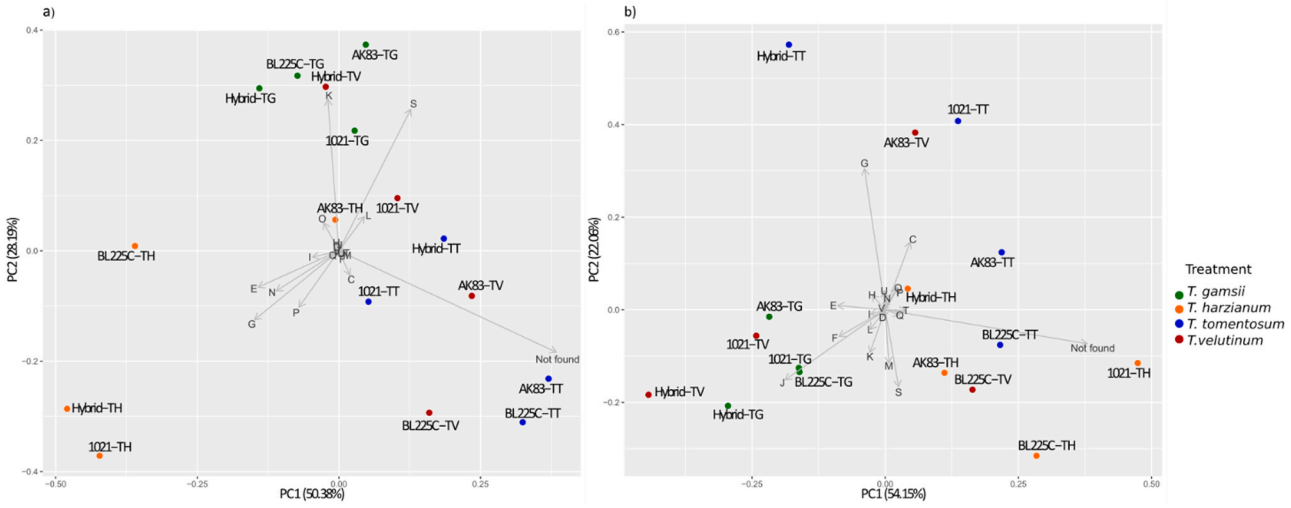

Fig. 6. *S. meliloti* functional gene categories differentially elicited by fungal spent media. PCA biplots of COG categories percentages among a) up-regulated and b) down-regulated DEGs. Centroids report the name of all *S. meliloti-Trichoderma* conditions and vectors indicate the loadings of the COG 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. "Not found" refers to genes or proteins that have not matched to any known orthologous groups within the COG database.

presence of epistasis and novel roles for pSymA megaplasmid, while for the *cis-hybrid* strain the same spent media strongly reduced root adhesion (Fig. 1), in line with the expectation of an active flagellar apparatus (Guttenplan and Kearns, 2013), 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 24 h incubation, while the root biofilm after 48 h 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 (Guttenplan and Kearns, 2013). 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 (Reed and Walker, 1991).

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 (Barnett and Long, 2015), 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 (Nogales et al., 2013) 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.

Finally, to 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 S7. 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 modelled 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.

4. 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 del Barrio-Duque et al., 2020, 2019; Li et al., 2019; Villalobos-Escobedo et al., 2023)

This interaction can span from almost random microbial assemblies to specific symbiotic associations of fungal hyphae and bacterial cells (del Barrio-Duque et al., 2020). Concerning *Trichoderma* spp., several studies have focused on the antagonistic interaction toward pathogenic

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. A gene can be associated with S. meliloti strain (gene differentially expressed only between strains), Trichoderma species spent medium treatment (gene differentially expressed only between different Trichoderma species treatments), S. meliloti and Trichoderma only (gene differentially expressed in relation to S. meliloti strain and Trichoderma but not considering the full model strain x Trichoderma), or the interaction between S. meliloti strain and Trichoderma (S. meliloti x Trichoderma column).

	S. meliloti	*Trichoderma*	*S. meliloti* and *Trichoderma*	*S. meliloti* x *Trichoderma*	None	Total
Number of DEGs	146 (14.9%)	270 (27.5%)	416 (42.4%)	230 (23.4%)	335 (34.1%)	981

bacteria, and some on the positive effect of co-inoculation with mutualistic bacteria (rhizobia) (Barbosa et al., 2022). A recent work on mycorrhizal fungi (Afkhami et al., 2021; Afkhami and Stinchcombe, 2016; Hernandez et al., 2019; Kavadia et al., 2021), showed the alignment of fungal/rhizobial mutualism toward eliciting plant gene expression, but no direct molecular 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 (Fagorzi et al., 2021). 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 (diCenzo et al., 2016).

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 (diCenzo et al., 2018a, b). On the same *cis*-hybrid strain we showed that stimulons from root exudates are, as expected, displaying nonadditive (epistatic) effects (Fagorzi et al., 2021). 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 (Defez et al., 2016; Imperlini et al., 2009). 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 (Ghantasala and Roy Choudhury, 2022). Modulation, e.g. synergistic effect, by fungi on rhizobium symbiotic quality has been observed for mycorrhizal fungi (Afkhami et al., 2021; Afkhami and Stinchcombe, 2016; Hernandez et al., 2019; Kavadia et al., 2021) 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 *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 (Woo et al., 2023), as well application to alfalfa cultivation (Buysens et al., 2016; Shwerif, 2018; Zhang et al., 2020).

CRediT authorship contribution statement

Francesca Vaccaro: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Iacopo Passeri:** Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation. **Nur Ajijah:** Resources, Methodology. **Priscilla Bettini:** Validation, Methodology, Investigation. **Pierre Emmanuel Courty:** Validation - review & editing - original draft, Resources, Supervision. **Klaudia Dębiec-Andrzejewska:** Writing – original draft, Supervision, Resources, Methodology, Funding acquisition. **Namrata Joshi:** Resources, Methodology, Investigation. **Łucja Kowalewska:** Supervision, Resources, Methodology, Investigation, Funding acquisition, Data curation. **Robert Stasiuk:** Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation. **Marcin Musialowski:** Resources, Methodology, Investigation. **Kumar Pranaw:** Writing – review & editing, Writing – original draft, Validation, Supervision, Funding acquisition, Conceptualization. **Alessio Mengoni:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data Availability

RNA-Seq sequence data have been deposited to the European Nucleotide Archive (ENA) under the study PRJEB63390

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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).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2024.127768.

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