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New Phytologist, 2018 DOI: 10.1111/nph.15014

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Main figures: 7 main figures (all in colour)
Supporting information: One file containing Supplementary figures and tables, Tables S3, S4, S5, S6 are in separate Excel files.

Summary

- Several studies have investigated soil microbial biodiversity, but comprehension of the mechanisms underlying plant responses to soil microbiota remains in its infancy. We focused on tomato (*Solanum lycopersicum*), testing the hypothesis that plants grown on native soils display different responses to soil microbiotas.
- Using transcriptomics, proteomics, and biochemistry, we describe the responses of two tomato genotypes (susceptible or resistant to *Fusarium oxysporum* f.sp *lycopersici*) grown on an artificial growth substrate and two native soils (conducive and suppressive to *Fusarium*).
- Native soils affected tomato responses by modulating pathways involved in responses to oxidative stress, phenols biosynthesis, lignin deposition, and innate immunity, particularly in the suppressive soil. In tomato plants grown on steam-disinfected soils, total phenols and lignin significantly decreased. The inoculation of a mycorrhizal fungus partly rescued this response locally and systemically. Plants inoculated with the fungal pathogen showed reduced disease symptoms in the resistant genotype in both soils, but the susceptible genotype was partially protected from the pathogen only when grown on the suppressive soil.
- The "state of alert" detected in tomatoes reveals novel mechanisms operating in plants in native soils and the soil microbiota appears to be one of the drivers of these plant responses.

Key words

Arbuscular mycorrhizal fungi, Defence responses, Lignin biosynthesis, Microbiota, Suppressive and conducive soils, Susceptible and resistant genotypes, Tomato.

Introduction

Crops, like their wild relatives, face many stresses, depending on the soil where they grow, the available nutrients, and other environmental conditions (Mundt, 2002). When grown as genetically homogeneous monocultures, crops are usually more susceptible to severe disease outbreaks than those grown in mixed cultivation. To decrease crop losses to disease, breeders have developed resistant varieties that have morphological and chemical barriers or activate defence responses to pathogens (Agrios, 2005).

Although much research has focused on the effects of plant genotype, the microbiota has recently emerged as an important factor in disease resistance. Plants, like animals, have their own microbiota, which can have a powerful effect on their health. Indeed, many physiological functions require the presence of these mostly benign microbes and the establishment of specific plant–microbe relationships (Ash & Mueller, 2016). In the plant microbiota, bacteria and fungi with beneficial functions, such as root symbionts and growth-promoting rhizobacteria, coexist with endophytes, saprotrophic microbes, and pathogens. Several studies on the plant microbiota have focused on identifying the extraordinary diversity of microbes present on both roots and epigeous organs (Bulgarelli *et al.*, 2012; Bai *et al.*, 2015; Coleman-Derr *et al.*, 2016), while others have examined the influence of the plant's genotype on the composition of the microbiota (Lundberg *et al.*, 2012; Zgadzaj *et al.*, 2016). However, few plant studies have sought to understand how plants build up their microbiota (Lebeis *et al.*, 2015) or whether there is a relationship between microbiota, plant genetics, and nutrient availability (Horton *et al.*, 2014; Hacquard *et al.*, 2017; Castrillo *et al.*, 2017).

A complex interaction of biotic and abiotic factors, such as soil structure, nutrient and water availability, microbiota (including pathogens and symbionts), and plant genotype, affects plant productivity. To begin to untangle these complex interactions, we focused on tomato (*Solanum lycopersicum*), a relevant crop model plant, and tried to understand how plants respond when grown on native soils, as part of a larger project also covering microbial diversity (Poli *et al.*, 2016). Our hypotheses were: 1) microbiotas contained in different soils may trigger different plant responses and 2) different plant genotypes may respond differently to different soils and/or microbiotas. With the identification of the factors that govern plant responses, such hypotheses could allow us to better describe plant responses in conditions closer to those found in the field, rather than in

the lab (Poorter *et al.*, 2016).

To test these hypotheses, we investigated the molecular responses of two tomato genotypes, one susceptible to the soil-borne pathogen *Fusarium oxysporum* f. sp *lycopersici* (FOL) and one resistant. We grew these two cultivars in two soils of different geographical origins, history, biological properties (suppressive or conducive to FOL) and cultivable fungal communities, but comparable textures and nutrient profiles (Poli *et al.*, 2016). As a control, we used a steam-disinfected growth substrate routinely used in tomato greenhouses. Transcript profiling by next-generation sequencing analyses showed that native soil components elicit an alert status in the plant by enhancing the induction of genes involved in defence responses, as compared with plants grown in a disinfected substrate. The disease-suppressive soil was indeed more effective in priming resistance supporting the hypothesis that microbiotas contained in different soils may trigger different plant responses. By contrast, the second hypothesis (plant genotypes may respond differently to different soils and/or microbiotas) was not confirmed, since significant transcriptomics differences were not found betwee the two plant genotypes. Inoculation of an arbuscular mycorrhizal fungus (AMF) in the steam-disinfected soils induced similar responses, suggesting that the soil microbiota was one of the first drivers of the defence responses. Only under pathogen pressure did the plant genotype play a relevant role. These findings, indicate that biotic factors, more than abiotic, elicit specific responses in tomato grown in native soils.

Materials and Methods

Plant, soils, and experimental design

Two commercially relevant tomato genotypes, ‘Cuore di Bue’ and ‘Battito’ were selected. They are genetically related, being both ‘Oxheart’-type Heirloom varieties (Gioia *et al.*, 2010). Seeds were purchased from Ingegnoli (Milano, Italy). ‘Battito’ and ‘Cuore di Bue’ are resistant and susceptible, respectively, to FOL races 1 and 2, as stated by the producer and verified in a previous study (Poli *et al.*, 2016). To remove the seed microbiota (Shade *et al.*, 2017), in all experiments seeds were disinfected as detailed in Chialva *et al.* (2016), at least removing the seed ectosphere. Soils used were the same as those used by Poli *et al.* (2016). Albenga (AL) and Rosta (RO) soils were selected from two Italian regions on the basis of their comparable textures and nutrient profiles,

but different histories (agricultural *versus* meadow soil) and biological properties. Poli *et al.* (2016) have shown in fact that plants grown on AL displayed a moderate ability to suppress FOL growth whereas plants grown on RO allowed more FOL growth, leading to the conclusion that AL can be considered a suppressive soil and RO a conducive soil. In addition, Poli *et al.* (2016) characterized cultivable fungal communities of both soil revealing that the suppressive AL soil showed a higher load in *Fusarium* spp., *Phoma* spp., *Pyrenochaetopsis decipiens*, *Sarocladium strictum*, and *Trichoderma* spp., whilst the RO conducive one was richer in *Trichoderma* spp., *Penicillium* spp., *S. strictum*, and *Fusarium* spp.

In the current experiments, a control substrate (Pomix2, Evergreen, Moncalieri, Italy), which contains a mixture of peat and perlite, was used. This substrate was disinfected with fluent-steam at 100°C for 40 min, followed by 24 h at temperature higher than 80°C, before use. After the disinfection protocol, no cultivable microbes were detected (data not show).

Three experiments were conducted. Experiments 1 and 3 were performed in the greenhouse and Experiment 2 was performed under controlled conditions in a growth chamber. Experiments 1 and 3 were set up in pots using the two tomato genotypes, and plant growing conditions were the same as those described in Poli *et al.* (2016). To investigate the plant transcriptomic response to native soils, microcosms were set-up under greenhouse condition (Experiment 1). 'Battito' and 'Cuore di Bue' tomato genotypes were used and plants were sampled after 30 days. Seedlings were grown in the two soils, AL and RO, plus the steam-disinfected growth substrate as a control (CONT) (Poli *et al.*, 2016). The roots from six plants for each substrate were pooled together, freeze-dried and three pools for each substrate used as replicates for RNA-seq analysis.

For Experiment 2, which aimed to validate transcript profilings data by performing molecular analysis and quantifying phenols and lignin, three subsets of plants maintained in a growth chamber were investigated: a) one set of 'Cuore di Bue' grown in the three substrates, as for Experiment 1, b) one set of seedlings maintained in the steam-disinfected native soils, processed as described for the CONT condition in Experiment 1, and c) a set grown as in conditions a and b with the addition of 30% diluted monospecific inoculum of the arbuscular mycorrhizal fungus (AMF)

Funneliformis mosseae (MycAgro Lab. Dijon, France). Seedlings were transferred into 10x10x12 cm plastic pots, maintained under controlled temperature and light conditions [14-h light (24°C)/10-h dark (20°C)] and watered twice a week with tap water. Five replicates per condition were performed and plants sampled after 90 days.

For Experiment 3, which aimed to understand the role of tomato genotypes, the virulent FOL strain MUT350 was added by mixing the soil with a talc powder containing FOL chlamydospores, at the final rate of 3×10^4 chlamydospores mL^{-1} of soil (Srinivasan *et al.*, 2009). Five replicates per condition were considered; plants were growing in a greenhouse and sampled after fruit set (120 days).

In Experiment 2, the presence of inoculated arbuscular mycorrhizal fungi (AMF) was assessed on fresh roots (Trouvelot *et al.*, 1986) as described in Chialva et al. (2016) observing 60 cm of roots per plant.

RNA extraction and plant transcriptome analysis (Illumina RNA-seq)

In order to study the plant transcriptome under the native soils and the control substrate conditions, material obtained in Experiment 1 was subjected to RNA-seq. The roots were washed in distilled water, blotted on filter paper, frozen in liquid nitrogen and freeze-dried overnight. Total RNA was extracted using a modified 'pine-tree' method (Chang *et al.*, 1993) with the addition of 2% PVPP to the extraction buffer (Guether *et al.*, 2009). RNA integrity (RIN) and concentration were double-checked (after extraction and before sequencing) using the 2100 Bioanalyzer system (Agilent Technologies) discarding samples with a RIN value <7. Details on library preparation, sequencing, and bioinformatics are provided in Methods S1.

Proteome profiling

To complement the transcriptomic data, proteome profiling was performed on the same material used for RNA-seq, but limited to the 'Cuore di Bue' genotype. Total proteins were extracted and analysed by LC-MS/MS on Q-Exactive Orbitrap (Bioproximity Inc. USA). Further details are provided in Methods S2.

Functional enrichment and KEGG pathway analysis

GO terms overrepresented in differentially expressed gene (DEG) lists were identified

in R statistical programming (R Core Team, 2017) in RStudio GUI (RStudio Team, 2016) using the GOseq R Bioconductor package v1.15 (Young *et al.*, 2010) (false discovery rate, FDR<0.1). InterPRO and KEGG pathway terms enriched among DEGs, differentially expressed proteins (DEPs), or gene ID subsets were identified using the 'enricher' function in the clusterProfiler R package (Yu *et al.*, 2012) (P<0.05). Mapping between gene ID (SL.2.4) and GO, InterPro, or KEGG entries was retrieved using BiomaRT queries on Ensembl Plants website (http://plants.ensembl.org). Z-score semantic space was calculated according to Walter et al. (2015). Expressed genes in different contrasts were mapped into the relevant KEGG pathway incorporating color-coded expression values using the pathview R package v1.23 (Luo *et al.*, 2013) as detailed in Matić et al. (2016).

RT-qPCR analysis

To validate RNA-seq data, and to test DEGs emerged from RNAseq in Experiment 2 and 3, reverse transcription quantitative PCR (RT-qPCR) assays were performed on a set of genes listed in Table S1 together with the used primers. RNA was isolated using the modified 'pine-tree method' as described above. Material was quality-checked, processed to remove DNA, and retrotranscribed as described in Chialva *et al.* (2016). RT-qPCR amplifications and data analysis were performed as described in Methods S3.

Quantification of lignin and total phenols

Since the KEGG pathway analysis and the proteomics profiling identified lignin and phenol metabolism as differentially expressed in tomatoes growing in the three substrates, these compounds were quantified by using plants from the Experiment 2. Lignin was measured in protein-free cell wall material using the acetyl bromide method by Hatfield *et al.* (1996) as described in Moreira Vilar *et al.* (2014). Ten mg of each cleaned cell-wall sample was digested in 0.5 ml of 25% acetyl bromide (v/v in glacial acetic acid) at 70°C for 30 min. Samples were then cooled on ice and 0.9 ml of 2 M NaOH and 0.1 ml of hydroxylamine-HCl was added. Four ml of glacial acetic acid was added to the reaction and after centrifugation (2000xg, 10 min) extracts were diluted 1:4 and A_{280} measured using 10-mm quartz cuvettes. A standard curve was generated using Alkali Lignin (Sigma, 370959) (R^2>0.99) and results expressed as mg g^{-1} cell wall

(CW).

Total phenols (TPs) were extracted and quantified using a modified Folin-Ciocalteu (F-C) assay (Ainsworth & Gillespie, 2007) as described in Zouari et al. (2014). Results were expressed as mg gallic acid equivalents (GAE) g^{-1} of tissue dry weight (DW) using serially diluted gallic acid (Sigma, #G7384) standard solutions from 500 to 7.8 mg/l (R^2>0.99). Spectrophotometric analyses were performed using a Beckman DU 530 UV/VIS spectrophotometer on three to five biological replicates with three technical replicates each.

Statistical analysis

Statistical tests were performed in the R statistical programming environment (R Core Team, 2017) using Rstudio GUI (RStudio Team, 2016). Data normality and homoschedasticity were tested using Shapiro-Wilk (Shapiro & Wilk, 1965) and Levene's test (Levene, 1960) in the 'stats' and 'car' (Fox & Weisberg, 2011) respectively (P<0.05). According to data distributions, ANOVA for normal homoschedastic data or Kruskal-Wallis test for non-normal homoschedastic data (Kruskal & Wallis, 1952) were adopted from the custom R package 'stats' at P<0.05. Pairwise comparisons between treatments were performed when needed, using the appropriate post hoc tests. Tukey's test (Tukey, 1949) in the package 'agricolae' (Mendiburu, 2016) was adopted for ANOVA and Dunn's test (Dunn, 1964) in package 'FSA' (Ogle, 2016) for Kruskall-Wallis, both at P<0.05.

Principal commonent analysis (PCA) on RNA-seq libraries was performed using DESeq2:::plotPCA function in R (Love *et al.*, 2014) and *k-means* clustering analysis in 'stats' R package using 'kmeans' function (R Core Team, 2017) Variance partitioning analyses on transcriptome data sets was performed using the 'varpar' function in the 'vegan' package (Oksanen *et al.*, 2016). Genotype and soil factors were used as explanatory variables. Testable partitions were tested for significance using permutational ANOVA (999 permutation) on the RDA model (P<0.05).

Graphical elaborations were performed using ggplot2 (Wickham, 2009) or 'graphics' package in R environment (R Core Team, 2017).

Data Availability

Raw RNA-seq reads were submitted to the National Center for Biotechnology Information Sequence Read Archive (NCBI-GEO/SRA) under accession SRP126554.

Results

Native soils shape the plant root transcriptome

In Experiment 1, we grew tomato plants on the three substrates, looking for specific effects of AL and RO native soils *versus* the CONT substrate. After 3 months, the plants were healthy on all soils. To examine the plant transcriptome, we sampled tomato roots from the two genotypes and three substrate conditions, extracted total RNA, and produced 18 Illumina RNA-seq libraries, yielding 11–27 million filtered reads (Table S2). The mean mapping rate on the tomato reference genome (Sato *et al.*, 2012) was 93% and we found that 23,759 genes out of 34,675 annotated in tomato were expressed in roots. Counts were further processed with DESeq2 for normalization and identification of differentially expressed genes (DEGs) among conditions. Clustering of normalized counts showed consistency among soil and genotype and all Pearson correlation coefficients for biological replicates were above 0.9 (Figure S1).

To investigate the role of genotype and soil factors, we first performed multivariate analyses on normalized counts. Principal Component Analysis (PCA) with *k*-means clustering (Liu & Si, 2014) split RNA-seq libraries into three clusters (k=3) corresponding to the different soil types (RO, AL, and CONT) (Fig. 1a). By contrast, the analysis produced no clusters for genotype. Variance partitioning of the whole normalized read counts data set (Fig. 1b) confirmed the PCA ordination showing that the soil type significantly explained a large part of transcriptome variability (22% of variance explained, ANOVA, P<0.001), but the genotype, which explained 3% of variance, had no significant influence (ANOVA, P>0.05).

We then focused on the DEGs (fold-change >2, FDR<0.05) among soil conditions (Figure S2). In all the contrasts, the majority of DEG transcripts were upregulated in native soils, compared with CONT (Fig. 1c, Figure S2). We validated the relative expression estimates for 17 DEGs shared among contrasts by using RT-qPCR and found a high correlation with RNA-seq (r^2=0.85, P<0.001; Figure S3), validating our methodology. DEG analysis highlighted a similar number of DEGs when AL and

RO soils were compared with the CONT soil (969 and 847, respectively, Table S3) corresponding to around 4% of the tomato genes expressed in roots (Fig. 1d). However, the two contrasts shared only one-third of the DEGs (289), suggesting that each soil with its microbiota affects sets of tomato genes that only partly overlap. When the AL and RO data sets were directly compared (AL *versus* RO), the number of DEGs was lower (285 genes, corresponding to 1.2% of root transcripts) suggesting that in the two soils, the root transcription profile was largely shared. A similar trend was observed when the comparisons were performed to find enriched Gene Ontology (GO) (Fig. 1e, Table S4) and InterPro (Table S5) terms.

Native soils modulate stress and defence responses in tomato roots

To understand which tomato molecular components were affected by native soils, we focused on the shared set of 289 DEGs that responded in AL (suppressive) and RO (conducive) soils *versus* CONT substrate (FC>2, FDR<0.05) from Experiment 1 (Fig. 1d, Table S6). This set contains mostly upregulated genes enriched in 19 GO and 38 InterPro terms (FDR<0.05) (Fig. 1f). Both enrichment analyses detected the activation of general molecular defences against diverse environmental stresses in plants grown on native soil. The enriched GO terms (Figure S4a) included functions related to the apoplast (GO:0048046) and the response to oxidative stress (GO:0006979). Other enriched terms were related to the cell wall and nutrient transport, such as peroxidase activity (GO:0004601), metal ion binding (GO:0046872), metal ion transmembrane transporter activity (GO:0046873), acid phosphatase activity (GO:0003993), and transmembrane transport (GO:0055085). Similarly, analysis of enriched InterPro categories (Figure S4b) detected 24 categories, most of them related to cell-wall processes, such as laccases (IPR017761), type 1, 2, and 3 multicopper-oxidases (IPR011706, IPR011707, IPR001117), and plant peroxidases (IPR000823). Type III plant peroxidases, which are primarily involved in lignin biosynthesis (Weng & Chapple, 2010), were the most abundant class.

When comparing the GO and InterPro terms enriched in AL *versus* RO soil (Fig.1d), we found only 1 GO term (‘apoplast’, GO:0048046) shared with the terms enriched in AL or RO *versus* CONT (19 enriched GO terms) and none of the previously found InterPro terms. Some interesting GO categories emerged (Figure S5a), such as

those related to general stress responses. When analysing InterPro enriched domains (Figure S5b), functional categories related to central metabolism and plant-pathogen interactions emerged. Among the enriched domains, transcripts encoding peptidase domains (IPR000668, IPR013128) were upregulated in RO, while those encoding chitin-binding (IPR001002) and glutaredoxin (IPR002109) domains were upregulated in AL.

To support the transcriptomic data, we performed a proteome-profiling experiment analysing the same raw root material used for RNAseq. Since the genotype has a negligible role in shaping the plant response to soils (Fig.1b), we considered only the 'Cuore di Bue' genotype (FOL susceptible). The shared features between the proteome and transcriptome (Fig. 2) were either up- or down-regulated. The protein data sets obtained from the three contrasts were enriched in the 'response to oxidative stress' GO term, and in related functions such as metal binding, heme binding, and peroxidase activity (Fig. 2). Proteins belonging to these categories were upregulated in the AL soil when compared to the other two substrates, and slightly upregulated in RO soil when compared with the disinfected substrate, suggesting that plants grown in native conditions (*i.e.* field-collected, non-disinfected substrates) respond to oxidative stress, and that different native soils might trigger responses of different intensities. Among the proteins upregulated in both AL and in RO samples, the peroxidase class was highly represented, including numerous enzymes involved in phenylpropanoid biosynthesis.

Phenylpropanoid metabolism is induced in both native soils

To analyse the Experiment 1 data sets at a deeper level, we mapped RNA-seq and proteome $\log_2$ fold-change values onto KEGG pathways. When comparing gene expression in plants grown in native soils or in CONT, we found consistent regulation of several pathways involved in primary and secondary metabolism. However, when comparing soils with each other, we found only limited differences in the gene regulation in pathways dealing with central metabolism. With respect to primary metabolism, we found upregulation of the citrate cycle (sly00020) in both transcriptomic and proteomic data sets. Moreover, plants grown in AL soil, as compared with RO soil, showed differential regulation of some metabolic pathways in transcriptome data such as glycolysis (sly00010), starch and sucrose metabolism

(slyc00500) and amino acid metabolism (sly00260, sly00280, sly00290, sly00360).

With respect to secondary metabolism, phenylpropanoid biosynthesis (sly00940) was strongly regulated in both data sets in all considered contrasts (Figure S6-8). Several key enzymes were significantly upregulated in roots grown in native soils, as compared with the CONT substrate. The phenylpropanoid pathway originates from phenylalanine and leads to the synthesis of many diverse compounds, from insoluble lignin to soluble compounds (including salicylic acid) involved in defence against UV light, herbivores, or pathogens, as well as in the attraction of pollinators (Almagro *et al.*, 2009; Fraser & Chapple, 2011). In addition to cell wall fortification, lignification is the first constitutive barrier against pathogen attack or abiotic stresses such as salinity (Neves *et al.*, 2010). In the phenylpropanoid pathway, 4 genes coding for beta-glucosidases (EC 3.2.1.21), which are involved in coumarin synthesis, and 14 genes coding for class III plant peroxidases (EC 1.11.1.7) were significantly induced, compared with their expression in plants grown on the CONT substrate. These genes were more induced in AL soil than in RO soil. Class III peroxidases were differentially expressed in both data sets.

To confirm the induction of phenylpropanoid biosynthesis, we conducted independent tests (Experiment 2 a) to quantify lignin and total phenolics in plants grown under controlled conditions in the same three substrates used for the RNA-seq experiment (Fig. 3a, c). We detected a significant increase in the contents of lignin and total phenolics in AL and RO root samples, as compared with CONT, confirming the RNA-seq and proteome profiling. Furthermore, we validated the increased lignin content at a systemic level in leaves, although total phenolics were not changed in leaves (Fig. 3b, d).

The two native soils have different effects on the expression of genes involved in plant–microbe interactions

KEGG pathway analysis highlighted the differential involvement of plant–microbe interaction signalling (sly04626) in tomato roots from native soils *versus* the control conditions (Figure S9–11). In this case, suppressive and conducive soils led to different responses: genes involved in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) were differentially regulated, with an upregulation in the moderately

suppressive AL soil when compared to RO. By contrast, the differential regulation of genes involved in Effector-Triggered Immunity (ETI) was not statistically supported. In particular, we found transcriptional responses to both fungal PAMPs (e.g. regulation of genes encoding cyclic nucleotide-gated channels, which mediate cytosolic calcium signals), and bacterial PAMPs, with the regulation of a *Flagellin Sensing 2* gene (Solyc02g070890.2) belonging to the leucine-rich repeat receptor serine/threonine kinase (LRR-RLK) gene family (EC 2.7.11.1).

Pathogenesis-related proteins (PRs), which are well-characterized molecular markers for systemic acquired resistance in several herbaceous plants (Zhang *et al.*, 2010) and for early plant responses to AMF (Pozo *et al.*, 2015), were differentially expressed in both the transcriptome and proteome data sets. Among them, two chitinases (Solyc06g053380.2, Solyc11g072760.1), were upregulated in both native soils when compared with the disinfected CONT substrate. Other PRs were more upregulated in the AL soil compared with RO (Figure S9); for example, a class III chitinase (Solyc02g082920.2.1) and a CEVI-1 peroxidase, both belonging to the PR-9 class (Solyc01g006300.2.1) were specifically induced in AL soil. PR-9s are commonly involved in the deposition of phenolics into the plant cell wall during pathogenesis. Both PR-9 proteins were described as markers of tomato resistance to fungal soil-borne pathogens, including *Rhizoctonia solani* (Taheri & Tarighi, 2012).

Looking at other genes potentially involved in the response to biotic stresses, we found that expression of a polygalacturonase inhibitor protein (PGIP) (Solyc09g014590.2) was upregulated in both plants grown on native soils. PGIPs are extracellular leucine-rich repeat glycoproteins that can inhibit the activity of polygalacturonases produced by fungi and bacteria and trigger plant defence reactions (Federici *et al.*, 2006).

Disinfection of the native soils shows that the microbiota elicits phenylpropanoid and defence pathways

Our transcriptomic and proteomic data, as also confirmed by biochemical data, indicated that tomatoes grown in both native soils have increased phenylpropanoid metabolism (Fig. 4, Figure S7-8). These plants also show activation of PTI and defence-related pathways, with stronger activation in the AL soil (Figure S9). To test whether

such events were caused by the biotic or abiotic soil components, we grew 'Cuore di Bue' tomatoes for 90 days on steam-disinfected native soils (Experiment 2b). Under these conditions, the plants showed reduced total phenolics ($P<0.05$) in both root and leaf tissues, as well as less root lignin in AL soil (Fig. 4a, b).

Since disinfection dramatically decreases the microbial presence (Lau & Lennon, 2011; Panke-Buisse *et al.*, 2015), we tested whether this affected other molecular plant response. We took advantage of the RNA-seq results and selected a panel of genes that were related to phenylpropanoid metabolism, oxidative stress, and plant–pathogen interactions as markers of plant responses to native soils. The expression of these genes was investigated using RT-qPCR on plants growing on the two native soils before and after disinfection. We tested transcripts involved in PTI (Cyclic nucleotide-gated ion channel, Solyc01g095770.2, pathogenesis-related protein, Solyc01g106640.2 , Respiratory burst oxidase homolog protein, Solyc01g099620.2), phenylpropanoid biosynthesis (Caffeoyl-CoA O-metyltransfease, Solyc02g093230.2), abiotic (Gibberellin 3-beta-hydroxylase 1, Solyc06g066820.2) and oxidative stress responses (Superoxide dismutase, Solyc11g066390.1). The RNA-seq data were confirmed by RT-qPCR in native soils, because the investigated genes were upregulated in the AL soil, as compared to the conducive RO soil *($P<0.05$)* (Fig. 5). By contrast, after disinfection, the transcripts did not reveal any significant difference. Only superoxide dismutase (SOD) expression remained higher in the disinfected AL than in the RO soil (Fig. 5).

As a further step, we tested whether the induction of total phenolics and lignin observed in native soils can be ascribed to specific microbiota components, such as AMF, as already described in the literature (Rivero *et al.*, 2015). To this end, we chose *Funneliformis mosseae*, the most-represented AMF species in both soils (unpublished results) and one of the best performing partners for tomato (Zouari *et al.*, 2014), and we inoculated it into the disinfected soils (Experiment 2c). At 90 days after inoculation, we found a recovery of total phenolics, similar to the values measured in plants sampled from native soils. No differences emerged in lignin content, with the exception of roots from plants growing in AL, which showed a full recovery after AM inoculation (Fig 4c, d).

Introducing a pathogen: genes involved in PAMP-triggered immunity are activated in the suppressive soil

To test whether putative priming defences induced by native soils and their associated microbiota could counteract FOL disease, we set up a long-term greenhouse experiment (Experiment 3). Tomato plants from both genotypes ('Cuore di Bue' and 'Battito') were grown in AL and RO soils in the presence and the absence of a virulent strain of FOL (MUT 350). Plants were grown for 120 days until fruit-set. 'Battito' showed the expected resistant phenotype (Figure S12), but the response of 'Cuore di Bue' depended on the soil type, since a better performance was detected on the weakly suppressive AL soil (Figure S13).

To test the expression profile of some DEGs identified as being involved in the PTI response (Figure S9), we conducted RT-qPCR on the 'Cuore di Bue' genotype with or without pathogen inoculation (FOL+ and FOL-, Fig. 6). In AL versus RO samples, 6 out of seven tested PTI genes were upregulated ($P<0.05$) in the absence of FOL (Fig. 6a), confirming the RNA-seq results. Two out of seven genes (*CML* and *WRKY22*) were further upregulated in response to FOL inoculation (Fig. 6b). This trend was more evident when comparing FOL+ *vs* FOL- for each individual soil: in the suppressive AL soil, only one transcript, encoding a Plant Respiratory Burst Oxidase Homolog (Solyc01g099620.2), was induced by FOL inoculation (Fig. 6c). Three out of seven genes were upregulated in the permissive RO soil, revealing that the 'Cuore di Bue' genotype reacted more to the pathogen in RO (Fig. 6d) under conducive conditions.

Discussion

Notwithstanding the emerging appreciation of the biological importance of the microbiome concept, plant responses to complex microbial communities have rarely been examined: many reports have carefully investigated the microbial biodiversity associated with plants by using metabarcoding or metagenomics approaches (Bulgarelli *et al.*, 2015), but have generally neglected the effect on plant functions. Our experiments, on two tomato genotypes growing in two native soils with different physiochemical and biological properties (Poli et al., 2016), have revealed some novel plant responses, thus helping us to understand how crops respond to the stimuli that originate from the biotic and abiotic components of soils.

Transcriptomics and proteomics demonstrated that the overall characteristics of the substrate contribute more than plant genotype to shaping the molecular responses in tomato roots, and that only few genes respond differently in tomato plants grown in the two different native soils. This means that, notwithstanding the significant abiotic and biotic differences of the soils (Poli *et al.*, 2016), tomato roots seem to display a broadly similar expression profile when grown in native soils, as compared with roots grown in the control substrate. Soil is considered the primary force driving plant–microbiota diversity (Jeanbille *et al.*, 2016); our present data reveal that soil is also a key factor that shapes the molecular profile in tomato.

Soil microbiota has a crucial role in the elicitation of phenylpropanoid pathways

Transcriptomics and proteomics data from Experiment 1 led to a second novel result: many of the soil-responsive genes that are similarly modulated in the two native soils compared to an artificial, disinfected substrate, have biological relevance. These genes are mainly involved in the activation of phenylpropanoid metabolism and other defence responses, suggesting that tomato plants activate a pre-alert status, which can be correlated with the biotic and abiotic components of the native soils Similar responses were also detected on Arabidopsis leaves inoculated with non-pathogenic phyllosphere commensals (Vogel *et al.*, 2016).

Phenylpropanoid metabolism is at the intersection of some of the most crucial pathways in plants, from the construction of structural barriers (cell wall and lignification) to the activation of many defence responses (Fraser & Chapple, 2011; Yogendra *et al.*, 2015). Some studies have linked plant–microbiota interactions (or even

selected components of the microbiota, such as AMF) with an increase in the production of lignin and phenolics (Rivero *et al.*, 2015; Beckers *et al.*, 2016). Here, we quantified these compounds and found that tomatoes grown in native soils produce more phenols and lignin in their roots and leaves.

In an attempt to disentangle the effect of the soil physiochemical features from the effect of the microbiota on local and systemic responses, in Experiment 2 we measured total phenolics and lignin content of plants grown in native soils, where a previous disinfection treatment led to an important reduction of endogenous microbes (Lau & Lennon, 2011; Panke-Buisse *et al.*, 2015). Here, we observed a significant decrease in total phenolics, while the decrease in the lignin content was significant only in the AL soil. The inoculation of the AM fungus *Funneliformis mosseae* led to an increase of phenolics back to the original values in the roots. Taken as a whole, the experiments provide evidence that the microbiota, more than the chemico-physical soil features, has a crucial role in the elicitation of phenylpropanoid pathway, and that the AM fungus *F. mosseae* alone largely rescues the activation of this metabolism. Indeed, previous papers have reported that AM fungi activate different steps of the phenylpropanoid pathway (Rivero *et al.*, 2015; Beckers *et al.*, 2016; Bruisson *et al.*, 2016)

The efficiency of the PTI response elicited by soil microbiota depends on soil features and plant genotypes

Plants have developed a complex immune system to protect themselves against pathogen attack (Jones & Dangl, 2006). In addition to pathogen-associated molecular pattern (PAMP)-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI), multiple pathways, including those involving salicylic acid signalling and MAP kinase cascades, form a robust network for plant immunity (Tsuda *et al.*, 2013). To date, however, our understanding of the mechanisms governing plant immunity comes from experiments conducted under controlled conditions, where specific, known microbes are added and their effects on plant immunity are determined as a result of gene–gene interactions (Thomma *et al.*, 2011; Pieterse *et al.*, 2014). Our data, which considered the plant response to the whole microbiota, only revealed the elicitation of PTI, the first and weaker form of defence in response to a microbial pathogen. However, our results did

not show elicitation of other pathways by native soils. The activation of the PTI-related genes likely reflects the plant response to multiple signals from the microbial communities thriving in the diverse substrates. The specific transcriptomic/proteomic picture we obtained might reflect the plant response to long-lasting stimuli produced by a complex soil microbiota. Interestingly, the differential expression of marker genes for plant–microbe interactions, including those for PTI, were detected in plants growing in AL vs RO soil, but disappeared in the plants grown in the two disinfected soils (Experiment 2 b). This strongly suggests that the components of the plant microbiota are directly involved in eliciting priming responses. Taken as a whole, these findings validated our first hypothesis, i.e., that different soils with their diverse microbiotas trigger differential plant responses.

Many recent reports have revealed that plant genotypes affect the establishment of different microbiotas (Bulgarelli *et al.*, 2015; Zgadzaj *et al.*, 2016), but the opposite question (do different plant genotypes respond differently to the same soil microbiota?) remains to be answered. Our experiments did not reveal any change in the transcriptomic profiles of 'Cuore di Bue' and 'Battito' genotypes, which differ in being susceptible and resistant to FOL, respectively. A previous detailed mycoflora analysis revealed that many Fusaria were present in both AL and RO soils, but pathogenic strains were not isolated (Poli *et al.*, 2016), providing a functional explanation for the overlap in the transcriptomic profiles; in the absence of the pathogen, the plant responses are very similar. With Experiment 3, we directly tested the relevance of the two genotypes by introducing the pathogen, and by looking at the phenotype in a long-lasting experiment. The resistant 'Battito' performed better in the presence of the pathogen in all soil conditions, as expected, and confirming previous tests (Poli *et al.*, 2016). Also, the susceptible 'Cuore di Bue' elaborated defences, which allowed it to produce fruits in both the soils. However, measuring the expression of PTI-related genes after 120 days of FOL exposure revealed a surprising result: PTI-related genes were mostly upregulated in "Cuore di Bue" plants grown in the conducive RO soil in the presence of the pathogen. By contrast, in the suppressive AL soil, where the PTI genes were already activated in the absence of the pathogen, important modifications were not detected. The data reveal that the two genotypes respond differently to the pathogen, as expected, but, in addition, the susceptible genotype modulates its defense

responses depending on the soil. The protective effect, elicited by the soil microbiota, is not sufficient, however, to efficiently defeat the pathogen and to guarantee the health of 'Cuore di Bue' plants growing in the conducive RO soil.

All together, these data suggest that in native soils and in the presence of heterogeneous microbial communities living in the plant ectosphere and endosphere, tomato plants modulate some of their metabolic pathways, among which phenylpropanoid metabolism and PTI pathway (Fig. 7). Tomato plants respond to both native soils by activating a first level of defences based on cell wall fortification. However, the PTI pathway is regulated differently accordingly to the disease-suppressiveness of the two soils. In the suppressive soil, these PTI-like responses are induced to higher levels, providing a protective shield when a pathogen such as FOL is added to the system. This result is in agreement with the concept of 'general suppression' suggested by Raaijmakers & Mazzola (2016). Our results agree with those of Vogel *et al.* (2016), who described a comparable defence response elicited by phyllosphere commensals on *Arabidopsis thaliana*. This confirms that the rules governing the responses to the microbiota are similar in different plant compartments.

Next-generation sequencing techniques and the resulting data sets have provided us with many pieces of a complex puzzle, *i.e.* identification of tomato metabolic processes related to plant immunity, production of compounds related to cell-wall fortification and lignin. In conclusion, even if the puzzle is far from complete, we have started to reveal the multi-level mechanisms that operate in plants living in realistic conditions that are closer to those experienced by field-grown plants, rather than by plants grown in artificial growth substrates. These mechanisms bring into play multiple factors (soils, microbiotas, genotypes) that affect plant health, overcoming the reductionist approach of one-to-one interactions.

Acknowledgments

The authors thank Andrea Berruti (CNR-IPSP, Torino) for helping in multivariate statistical analysis; the HuGeF and DBIOS Genomics Platform for sequencing assistance (Francesco Neri, Danny Incarnato and Salvatore Oliviero); Ginevra Nota and Paolo Perret for lab assistance, Maria Teresa della Beffa for maintaining plant cultures, and the whole Mycoplant consortium. The authors thank Dr. Jennifer Mach (Plant

592 Editors Cooperative) for the language editing, the Editor, Dr. Francis Martin, and the
593 three anonymous referees for their help in improving the manuscript.
594

595 ## Author contributions

596 MChial., SP, AS and P.Bonfante designed research; MChial performed research with
597 the contribution of SD and MC for proteomics, AS for molecular analysis, PBag and SG
598 for bioinformatics. MN performed AMF analysis and manuscript editing; DS set up the
599 greenhouse experiments; MChial and P.Bonfante analyzed the data and wrote the paper.
600

601 ## References

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Figure legends

Fig 1. Analysis of the root transcriptome of tomato (*Solanum lycopersicum*) plants grown in native and artificial soils. (**a**) PCA plot with *k*-means clustering of RNA-seq libraries showing the two principal components (PC1 and PC2), which separated the samples by soil type. *K*-means clusters (k=3) are represented by ellipses and group sequencing libraries by soils. In the legend, the first letter indicates genotype ('Cuore di Bue' or 'Battito') and the following letters represent the substrate (RO, AL, or CONT). (**b**) Donut plot showing the amount of transcriptome variability explained from soil (22%) and genotype (3%) factors. Data were tested using permutational ANOVA (999 permutations, *P<0.001; ns = not significant). Collinearity between genotype and soils explained none of the variance. (**c**) Heatmap of DEGs (differentially expressed genes) across the three soil contrasts analysed with DESeq2 (FC>1, P<0.05) shows that native soils mostly upregulate transcription when compared to the control transcriptome from plants grown in disinfected soil. (**d**) Venn diagrams showing the number of shared DEGs categories (FDR<0.05) across the three main contrasts. (**e**) The 20 most-enriched GO categories shared between AL *versus* CONT (red bars) and RO *versus* CONT (blue bars).
AL='Albenga' suppressive soil; RO='Rosta' conducive soil; CONT=neutral control soil.

Fig. 2 Overlap of GO-categories enriched in both proteome and transcriptome experiments in tomato (*Solanum lycopersicum*) 'Cuore di Bue' genotype. The y-axis in the bubble plot represents the -$\log_2$ of adjusted P-value of proteome enrichment; the

x-axis shows the z-score computed on DEPs contained in each GO category (z-score > 0 prevalence of upregulation, z-score < 0 prevalence of down-regulation). Categories from each of the three GO domains are indicated by colours (BP='biological process', CC='cellular component', MF='molecular function'). Categories with $\log_2$ adj. *P*-value > 25 or z-score < -1 are labelled. The size of each bubble represents the number of differentially expressed proteins for each term. AL='Albenga' suppressive soil; RO='Rosta' conducive soil; CONT=neutral control soil.

Fig. 3 Total phenols and lignin concentrations in tomato (*Solanum lycopersicum*) plants grown in native and artificial substrates. (**a, b**) Total phenols (TPs) in roots and leaves, respectively. (**c, d**) Lignin content in root and leaves, respectively. Letters indicate significant differences among treatments (ANOVA, Tukey's post-hoc test, $P<0.05$). TPs are expressed as mg of gallic acid equivalents (GAE) per grams of dry weight (DW) material. Lignin amount is expressed as mg per grams of cell-wall (CW) material. $N=5$. AL='Albenga' suppressive soil; RO='Rosta' conducive soil; CONT=neutral control soil. Boxplots display the median (horizontal line), the quartiles (boxes) and the 1.5-times interquartile range (whiskers).

Fig. 4 Levels of total phenols and lignin in tomato (*Solanum lycopersicum*) 'Cuore di Bue' genotype grown in disinfected native soils and disinfected soils inoculated with *F. mosseae*. (**a** and **b**) Total phenols (TPs) in roots and leaves, respectively. (**c** and **d**) Lignin content in root and leaves, respectively. Letters indicate significant differences among treatments (ANOVA, Tukey's post-hoc test, $P<0.05$). TPs are expressed as mg of gallic acid equivalents (GAE) per grams of dry weight (DW) material. Lignin amount is expressed as mg per grams of cell-wall (CW) material. $N=5$. AL='Albenga' suppressive soil; RO='Rosta' conducive soil; CONT=neutral control soil. Boxplots display the median (horizontal line), the quartiles (boxes) and the 1.5-times interquartile range (whiskers).

Fig. 5 RT-qPCR of native-soil induced gene expression in tomato (*Solanum lycopersicum*) plants under native and steam-disinfected treatments in suppressive *versus* conducive soils. Dotted red lines indicate the threshold at fold change=1. Asterisks indicate statistically supported differences (Kruskall-Wallis test at $P<0.05$).

Data are mean ± SD, *n*=3. CCoAOMT=Caffeoyl-CoA O-methyltransferase (Solyc02g093230.2); G3B=Gibberellin 3-beta-hydroxylase 1 (Solyc06g066820.2); SOD=superoxide dismutase (Solyc11g066390.1); Rboh=Respiratory burst oxidase homolog protein (Solyc01g099620.2); PR=Pathogenesis-related protein (Solyc01g106640.2); CNG=Cyclic nucleotide-gated ion channel (Solyc01g095770.2).

Fig. 6 RT-qPCR of PTI-related gene expression in FOL-inoculated (FOL+) and non-inoculated (FOL-) tomato (*Solanum lycopersicum*) plants in both soils. (**a, b**) Relative expression of genes in AL *versus* RO under FOL- and FOL+ treatments; (**c, d**) Relative expression of genes in FOL+ versus FOL in AL and RO native soils. Dotted red lines indicate the threshold at fold change=1. Asterisks indicate statistically supported differences (Kruskall-Wallis test at $P<0.05$). Data are mean ± SD, *n*=3; FOL=*Fusarium oxysporum* f.sp. *lycopersici*. AL='Albenga' suppressive soil; RO='Rosta' conducive soil.

Fig. 7 Proposed model of tomato (*Solanum lycopersicum*) plant response to soil microbiota. The scheme illustrates the main pathways which are differentially regulated between the suppressive vs. conducive soil on the basis of transcriptomic and proteomics analysis. The highlighted pathways were validated with chemical quantification of total phenols and lignin, as well as RT-qPCR of genes involved on tomato defence on both native and disinfected soils. In this model, irrespectively of their genotype, tomato would perceive microbial MAMPS (as flagellin, flg22, and chitin) through specific receptors (FLS2 and CERK1, respectively) whose transcripts were upregulated. The signalling cascade would activate a PTI-like mechanism upregulating Cyclic nucleotide-gated channels (CNGCs) which increase the amount of cytosolic calcium, inducing in turn the expression of calmodulin (CaM)/CaM-like (CML) proteins and calcium-dependent protein kinases (CDPKs). Transcript profiling revealed other downstream events: an *Rboh* (Respiratory burst oxidase homolog) gene leads to a ROS burst, and to the activation of transcription factors as WRKY 22 and 33. We suggest that the latter elicits plant defences through the presence of pathogenesis-related proteins (PRs) and of the phenylpropanoid pathway. It would start with the up-regulation of the phenylalanine ammonia lyase (PAL) enzyme, followed by the up-

689 regulation of the caffeoyl-CoA O-methyltransferase (CCoAOMT) gene and of class III
690 peroxidases (PRX (III)) which are directly involved in lignin and total phenolics (TPs)
691 biosynthesis. All these pathways may confer an increased resistance against *Fusarium*
692 *oxysporum* f.sp. *lycopersici* not only in the resistant genotype, but also in the susceptible
693 one (long-term greenhouse Experiment 3).
694 Rectangular splitted boxes represent the expression ratio (log_2fold-change) in
695 suppressive versus conducive soils contrast in 'Cuore di Bue' genotype for both
696 transcripts (left box) and proteins (right box). Red colour represents upregulation, blue
697 downregulation.

Supporting Information

Figure S1 Correlation heatmap of RNA-seq libraries using Euclidean distances between samples calculated on RLD (regularized log transformation) normalized reads counts.

Figure S2 MA plots obtained from DESeq2 Differential Expression analysis using all considered contrasts with both separated or pooled genotype.

Figure S3 Correlation plot of DEGs between RT-qPCR and RNA-seq analysis.

Figure S4 GO and InterPro domain enrichment analysis on the DEG set shared in native *versus* control substrate contrast.

Figure S5 GO and InterPro enrichment analysis of DEGs in AL versus RO soil contrast.

Figure S6 Phenylpropanoid biosynthesis KEGG pathway (sly00940) in AL *versus* RO and in 'Cuore di Bue' genotype

Figure S7 Phenylpropanoid biosynthesis KEGG pathway (sly00940) in AL *versus* CONTROL and in 'Cuore di Bue' genotype

Figure S8 Phenylpropanoid biosynthesis KEGG pathway (sly00940) in RO *versus* CONTROL and in 'Cuore di Bue' genotype. Regulated transcripts and proteins are displayed in left and right box, respectively. Up-regulated IDs are in red, down-regulated in green.

Figure S9 Plant–pathogen interaction KEGG pathway (sly04626) in AL *versus* RO and in 'Cuore di Bue' genotype.

Figure S10 Plant–pathogen interaction KEGG pathway (sly04626) in AL *versus* CONTROL and in 'Cuore di Bue' genotype.

Figure S11 Plant–pathogen interaction KEGG pathway (sly04626) in RO *versus* CONTROL and in 'Cuore di Bue' genotype. Transcripts and proteins regulation is displayed in left and right box respectively. Up-regulated IDs are in red, down-regulated in green.

Figure S12 *S. lycopersicum* cv 'Battito' plants grown in native soils with and without FOL inoculation.

Figure S13 *S. lycopersicum* cv 'Cuore di Bue' plants grown in native soils with and without FOL inoculation. (a, b) plants growing in RO, and AL (c, d) native soils inoculated (b, d) or not (a, c) with FOL pathogen.

Table S1. RT-qPCR primers used in this study.

730 **Table S2.** Sequencing and genome mapping statistics.

731 **Table S3.** Differentially expressed transcripts in the three soil contrasts (FDR<0.05,
732 FC>2).

733 **Table S4.** GO enrichment analysis (*P*<0.1) on DEGs from Table S3 by soil contrasts.

734 **Table S5.** InterPro domains enrichment analysis (*P*<0.1) on DEGs from Table S3 by
735 soil contrasts.

736 **Table S6.** Shared DEGs between AL versus CONT and RO *versus* CONT soil contrasts
737 from Table S3.

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739 **Methods S1.** Illumina RNA sequencing and bioinformatic analysis.

740 **Methods S2.** Proteome Profiling

741 **Methods S3.** Real-Time PCR protocol used in the study.