Transcriptional reprogramming of tomato (*Solanum lycopersicum* L.) roots treated with humic acids and filter sterilized compost tea

Riccardo Scotti[1*], Nunzio D'Agostino[2], Catello Pane[1] and Massimo Zaccardelli[1]

Abstract

Background To counteract soil degradation, it is important to convert conventional agricultural practices to environmentally sustainable management practices. To this end, the application of biostimulants could be considered a good strategy. Compost, produced by the composting of biodegradable organic compounds, is a source of natural biostimulants, such as humic acids, which are naturally occurring organic compounds that arise from the decomposition and transformation of organic residues, and compost tea, a compost-derived liquid formulated produced by compost water-phase extraction. This study aimed to determine the molecular responses of the roots of tomato plants (cv. Crovarese) grown under hydroponic conditions and subjected to biostimulation with humic substances (HSs) and filtered sterile compost tea (SCT).

Results The ^{13}C CPMAS NMR of humic acids (HA) and SCT revealed strong O-alkyl-C signals, indicating a high content of polysaccharides.Thermochemolysis identified over 100 molecules, predominantly from lignin, fatty acids, and biopolymers. RNA-Seq analysis of tomato roots treated with HA or SCT revealed differentially expressed genes (DEGs) with distinct patterns of transcriptional reprogramming.

Notably, HA treatment affected carbohydrate metabolism and secondary metabolism, particularly phenylpropanoids and flavonoids, while SCT had a broader impact on hormone and redox metabolism. Both biostimulants induced significant gene expression changes within 24 h, including a reduction in cell wall degradation activity and an increase in the expression of hemicellulose synthesis genes, suggesting that the treatments prompted proactive cell wall development.

Conclusions The results demonstrate that HS and SCT can mitigate stress by activating specific molecular mechanisms and modifying root metabolic pathways, particularly those involved in cell wall synthesis. However, gene regulation in response to these treatments is complex and influenced by various factors. These findings highlight the biostimulatory effects of HS and SCT, suggesting their potential application in crop biofertilization and the development of innovative breeding strategies to maximize the benefits of humic substances for crops. Further research is needed to fully elucidate these mechanisms across various contexts and plant species.

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Introduction

Future agriculture must increase in sustainability and resilience given the need to conserve non-renewable natural resources, preserve ecosystems over time, and produce more food to feed a growing population [1].

The goal of producing "more with less" is encouraging research and development activities on naturally occurring substances that can act as bioeffectors for plants capable of stimulating the uptake of nutrients and their use efficiency and protecting against (a) biotic stresses [2].

These bioeffectors, formally known as plant biostimulants, group any substance or microorganism capable of acting positively on plant physiology and biochemistry and improving yield and/or quality when applied in ultrasmall and small quantities to the phyllosphere or rhizosphere [3].

Biostimulants are available in a variety of formulations and can be broadly classified as microbial or nonmicrobial [4]. Among the latter, organic nonmicrobial biostimulants can be divided into three large groups based on their origin and content: humic substances (HSs), hormone-containing products (HCPs) and amino acid-containing products (ACPs) [5].

Composts are complex organic soil amendments capable of providing numerous benefits in agricultural applications [6–8]. They can be considered starting materials for the extraction of specific biostimulants, such as humic acids (HAs) [5], and/or for the production of compost tea (CT) [3].

Compost-derived HAs are heterogeneous aggregates of small molecules that are soluble in alkaline aqueous solutions [9]. CT, on the other hand, is an aqueous suspension of useful microorganisms, nutrients and organic molecules (e.g., nitrogen, phosphorus, and potassium; water-soluble humic-like substances; hormone-like compounds, etc.) extracted directly from compost and/or produced by microorganisms during the aerated water-extraction process lasting a few hours/days [10].

Both HAs and CTs have been reported to potentially stimulate improvements in the physiological state of plants, promote root proliferation and vegetative growth, and increase crop quality and yield in different agricultural systems [8].

The addition of HAs to soilless substrates has been shown to significantly stimulate the growth of tomato and cucumber plants [11] as well as the proliferation of pepper roots [12]. The application of HAs to soil via fertigation has been shown to increase potato [13] and vetch [14] yields. In a further study, Eyheraguibel et al. [15] observed positive vegetative and physiological effects on maize (increased root elongation, increased root and shoot biomass, and early flowering) following the addition of HAs to the hydroponic nutrient solution. Several studies have shown that HS can directly stimulate pathways related to primary (secondary root development, net photosynthesis, respiration, the switch-on of enzymes involved in mineral nutrition, and nitrogen assimilation) and secondary metabolism, including changes in root exudation profiles and the plant defense system [16–18].

The putative mechanisms responsible for these events are linked to the "auxin-like" activity of the humus and/or to the activation of the H^+-ATPases of the plasma membrane, which, in turn, affects secondary active transport, resulting in improved root architecture and plant nutrition [19]. Pioneering studies based on cDNA-AFLP profiling in *Arabidopsis thaliana* [20] and microarray transcriptome analysis in winter rapeseed [21] revealed the main metabolic pathways affected by HAs. Tahiri et al. [22] reported that humic substances affect the expression of two ABC auxin transporters both in birch and alder and alter the gene expression levels of an alcohol dehydrogenase and a glutamine synthetase.

These findings confirmed that HAs influence plant growth and development through regulatory mechanisms associated with hormone, carbon and nitrogen metabolism and stress responses. Furthermore, based on microarray experiments, Zanin et al. [23] correlated the physiological effect of water-extractable humic substances (WEHS) on nitrate acquisition in maize roots with changes in the transcriptomic profile of the roots. Indeed, WEHSs have been found to promote the induction of the high-affinity nitrate transport system and modulate the expression of genes related to transcriptional regulation and hormone metabolism. Recent literature indicates that CTs, because of the presence of nutritional and hormone-like elements, can also exert beneficial effects on plant physiology, including plant growth and development, flowering, fruiting, and stimulation of secondary metabolism [24].

Pant et al. [25], after treating pak choi roots and shoots with CT from vermicompost, reported a significant increase in the growth and mineral nutrient content of pak choi leaves. In a nursery, CT application by

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spraying significantly improved the growth and development of tomato, pepper, and melon plants [26]. Similarly, the potential nutritional and biocontrol effects of aqueous compost extracts were highlighted by Bernal-Vicente et al. [27], who reported a significant increase in biomass accumulation in melon plants in greenhouse nurseries and effective control of *Fusarium oxysporum.*

The presence of humic-like substances [28] and plant hormones [29] within CTs, as well as the increase in secondary root branching [30] recorded after the treatment, clearly demonstrated the phytohormone-like activity of CTs.

In other studies, it was shown that the increase in yield was coupled with a peak in plant secondary metabolism activity associated with a marked increase in total antioxidants in *Centella asiatica* [31] and improved organoleptic characteristics of muskmelon [32]. To the best of our knowledge, although CTs are widely involved in the suppression of plant diseases, most likely because of the essential role that microbial communities play as biological control agents [33], the molecular mechanisms through which they stimulate plant growth remain poorly investigated and need to be clarified.

RNA-Seq based transcriptome analysis is certainly the best strategy for characterizing the complex molecular mechanisms triggered by treatments with biostimulants, such as HAs and CTs, in plant tissues. In recent years, next-generation sequencing (NGS)-based transcriptomic analysis has been successfully employed to identify the gene regulatory networks controlling drought stress in maize (*Zea mays* L.) roots [33] and to characterize the effect of seed treatment with microalgal extracts on the growth and development of lettuce [34].

In the present study, the changes in the transcriptome of tomato (*Solanum lycopersicum* L.) roots biostimulated with HAs or sterile compost tea (SCT) were investigated. RNA-Seq was performed to analyse the gene expression profiles at three time points, i.e., 24, 48 and 72 h after treatment. Based on the main results derived from this research, we propose a model that captures and describes the most important molecular events that occur because of biostimulation.

Materials and methods
Production of humic acids and sterile compost tea and their chemical characterization
The HAs and CT were produced from compost (pH 8.36, electrical conductivity 6.27 dS m^{-1}) obtained from agricultural residues of *Chicorium endivia* L. mixed with chipped epigeal parts of *Cynara cardunculus* L. with a dry weight ratio of $80 \div 20$ as reported in Scotti et al. [35]. The HAs were lyophilized, while the CT was sterilized on 0.22 μm filters and then lyophilized before use.

The molecular distribution of HAs and organic carbon of the SCT was characterized by ^{13}C Cross Polarization Magic Angle Spinning (CPMAS) and Nuclear Magnetic Resonance (NMR) spectroscopy, as performed in Scotti et al. [36]. For the interpretation of ^{13}C-CPMAS-NMR spectra, the overall chemical shift range was divided into the following main resonance regions: alkyl-C (0–45 ppm); methoxyl-C and N-alkyl-C (46–60 ppm); O-alkyl-C (61–110 ppm); unsubstituted and alkyl-substituted aromatic-C (111–145 ppm); O-substituted aromatic-C (146–160 ppm); carboxyl- and carbonyl-C (161–190 ppm).

Pyrolysis was performed on approximately 100 mg of lyophilized HAs or SCT, followed by the thermochemolysis. The residue was dissolved in 0.2 mL of chloroform and transferred to a glass vial for GC–MS analysis, as described in Monda et al. [37].

Plant material, growth conditions and experimental design
Solanum lycopersicum L. (cv. Crovarese) seeds were provided by La Semiorto Sementi s.r.l. (Lavorate di Sarno, Italy). All seeds were sterilized and placed on water-saturated filter paper in Petri dishes to germinate for three days. Then, seedlings were transplanted on 30-well polystyrene trays floating in an aerated hydroponic nutrient solution, as described by Scotti et al. [38]. The solution was maintained in sterilized dark plastic tanks with acapacity of approximately 7.2 L.

Plants were grown for three weeks in a climatic chamber with 80% relative humidity and a photoperiod of 16/8 h light/dark and a temperature of 25 °C during the day and 21 °C during the night. Tomato plants were treated 22 days after transplanting by adding HAs (50 mg l^{-1}) and SCT (final dilution 1:100) to the hydroponic solution, respectively.

The experimental design was a randomized block, including the three treatments: Control, HA and SCT, each of them distributed in three tanks (replicates). After the treatment, root samples were collected from each replicate by pooling randomly selected roots from 5 plants *per* tank, at 24, 48 and 72 h post-treatment, and immediately frozen in liquid nitrogen and stored at -80 °C until RNA isolation. A total of 270 seedlings (30 seedlings × 3 treatments × 3 tanks) were transplanted and a total of 135 root samples (5 plants × 3 treatments × 3 tanks × 3 times) were collected (for details Supplementary Figure S1).

RNA isolation and sequencing
Total RNA was extracted from 100 mg of root tissue using a RNeasy Mini Kit (Qiagen, Hilden, Germany) coupled with DNase treatment (RNase-Free DNase Set, Qiagen) according to the manufacturer's instructions.

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RNA quality and concentration were assessed with a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), while RNA integrity was checked with an RNA *Bioanalyzer* 2100 *Plant Nano chip* (Agilent Technologies, Santa Clara, CA, USA). The 27 RNA libraries were produced and sequenced in single-end (*read length = 101 bp*) mode by Genomix4Life S.R.L. (Salerno, Italy, http://www.genomix4life.com) on an Illumina HiSeq 2500 platform. The raw sequences were deposited in the European Nucleotide Archive (ENA, http://www.ebi.ac.uk/ena) under the project accession number PRJEB20221.

Bioinformatic analyses

All steps of the analysis, i.e., (i) preprocessing of reads (quality control, filtering of low-quality reads and adapter removal), (ii) alignment of the reads to the reference genome (SL2.50) and (iii) read summarization, were performed exactly as reported in Scotti et al. [38].

Intersample normalization was performed with the trimmed mean of *M*-values (TMM) method implemented within the edgeR package v.3.12.1 [39]. Furthermore, Pearson's correlation coefficient between biological replicates was calculated to evaluate sample homogeneity. Only biological replicates with r^2 values ≥ 0.95 were considered.

Differentially expressed genes (DEGs) were identified using edgeR v.3.12.1 [39], and the gene list was filtered based on *the false discovery rate* (FDR < 0.05) and log^2-*fold* change (FC) (< -2 or > 2). Multiple Experiment Viewer (MeV v.4.9.0) was used for clustering analysis and heatmap generation [40]. The self-organizing tree algorithm (SOTA) coupled with the Euclidean distance measure was used for clustering genes with comparable expression profiles. Gene Ontology (GO) enrichment analysis was performed using topGO v.2.22.0 [41], filtering GO terms for Fisher's exact *p* value > 0.01. The tomato MapMan ontologies were retrieved from the GOMapMan web resource [42] and imported into the MapMan tool v. 3.6.0 [43]. Then, the list of identified DEGs was mapped to bins for data visualization and pathway analysis. All Venn diagrams were produced using the BioVenn web tool [44].

Quantitative real-time PCR

Quantitative real-time PCR (qRT–PCR) was performed to assess significant differences in gene expression profiles, as shown by RNA-Seq, among nine randomly selected genes. The primer pairs used in the qRT–PCR experiments are listed in Supplementary Table S1. The protocol used was the same as that described in Scotti et al. [38]. The relative expression levels of the target genes were calculated using the ΔΔCt method [45] and the elongation factor 1-alpha (Solyc06g005060) as an internal standard.

Results

Chemical characterization of humic acids and sterile compost tea

The ^{13}C CPMAS NMR spectra of HS are characterized by strong signals in the O-alkyl-C range (Fig. 1A). This included a large content of polysaccharides and carbohydrates, which accounted for the 23.7% and 31.4% of the total area in SCT and HA samples, respectively. The different peaks in this chemical shift region (60–110 ppm) were mainly assigned to monomeric units of polysaccharide chains, such as cellulose and hemicellulose. The intense peak at 73 ppm is formed by the overlapping resonances of carbon 2, 3, and 5 in pyranoside structure. The two shoulders at 84–88 ppm derive from the splitting of carbon 4 involved in the glycosidic bond, in two resonances linked either to the crystalline form of cellulose (major chemical shift) and to the amorphous form or to hemicellulose structures (minor chemical shift). The signal at 100 ppm, was assigned to the anomeric carbon 1 of the glucose unit in cellulose chain, while the more deshielded signal at 104/5 ppm was associated with the di-O-alkyl anomeric carbon 1 of the glucose units [30].The region of the NMR spectra around 0–45 ppm includes the broad alkyl-C resonances associated with the presence of aliphatic chains (-CH$_2$- groups) that belong to various lipid compounds, such as fatty acids, vegetable waxes and bio-polyesters. The different resonance peaks at 26 and 30 ppm were related to bulk CH$_2$ groups of amorphous and crystalline aliphatic components, while the intense broader band at 40 ppm, in the SCT spectrum, was mainly attributable to the inclusion of quaternary (C-R) carbons in the assembled rings of sterol derivatives [46].In the range 60–46 ppm, assigned

(See figure on next page.)

Fig. 1 **A** Solid-state ^{13}C CPMAS-NMR spectrum of humic acids and sterile compost tea. The relative distribution (%) of signal area over the six main chemical shifts (ppm) is shown; alkyl-C (0–45 ppm); methoxyl-C and N-alkyl-C (46–60 ppm); O-alkyl-C (61–110 ppm); unsubstituted and alkyl-substituted aromatic-C (111–145 ppm); O substituted aromatic-C (146–160 ppm); carboxyl- and carbonyl- C (161–190 ppm). **B** GC–MS chromatogram of humic acids and sterile compost tea. Codes on the peaks correspond to the following compounds: Carb = *Carbohydrates*; Lig = *Lignin*; N = *Nitrogenous compounds*; F = *Fatty Acid Methyl Ester (FAME)*; B = *Biopolysters*; Mic = *Microbial*
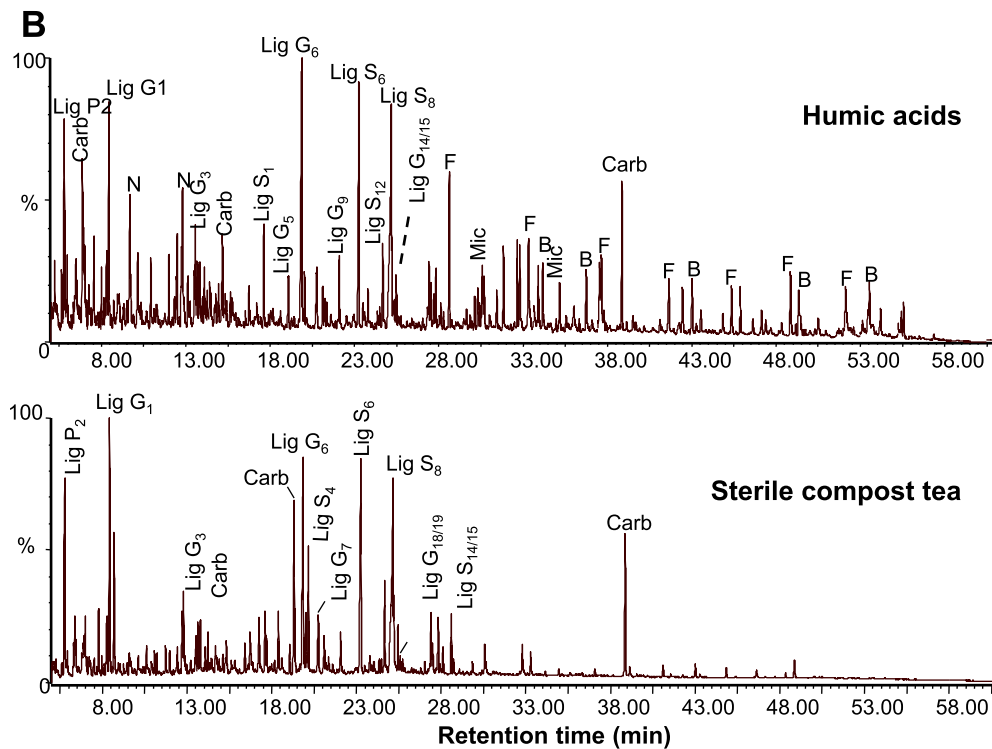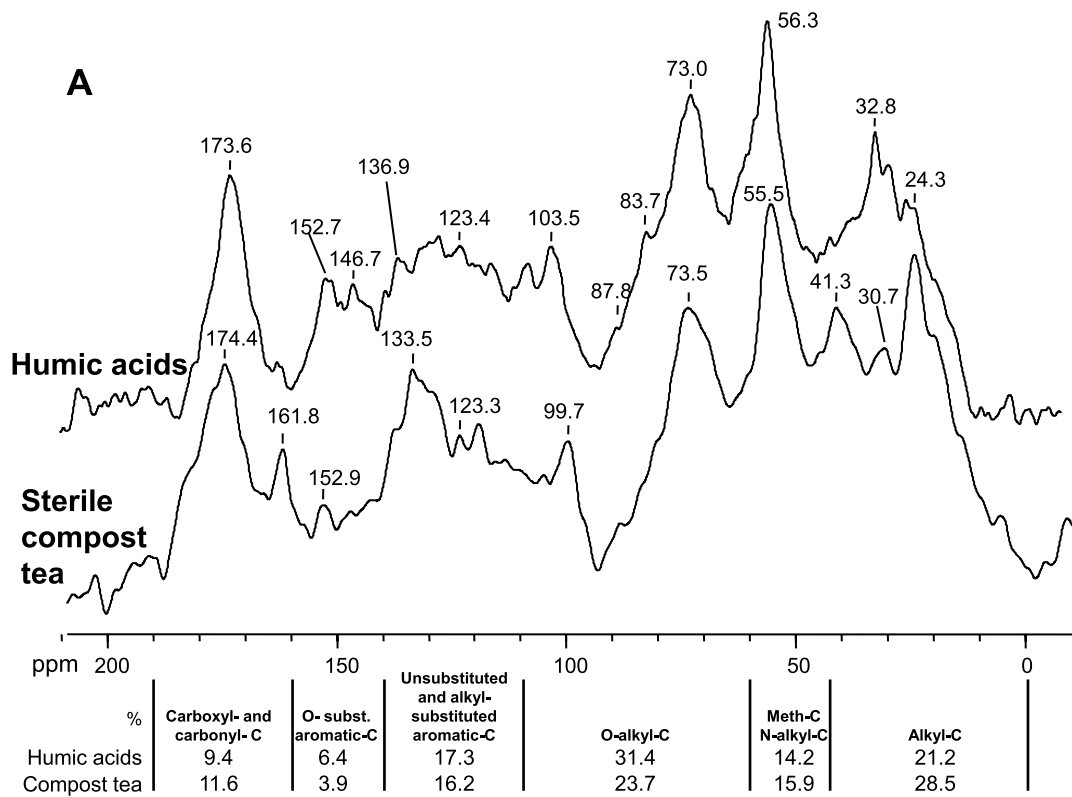
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to protein C, methoxyl C and sterols, the major peak was around 55 ppm and was attributed to O-alkyl groups such as methoxyls in lignin-like structure, suggesting that the aromatic moieties, of the latter, have a high content of lignin C [47].Signals covering the 190–161 ppm range, assigned to carboxylic, ketone and amide C, were dominated by a peak around 174 ppm that was attributed to the COOH carbon of aliphatic acids and amino acid moieties in both biostimulants [48]. In the phenolic C region (160–146 ppm) only a peak was visible at 152 ppm (O substituted aromatic C and phenolic C) due to the oxygen-bound aromatic carbons of the phenolic groups, which constitute different types of lignin [30], as also occur in the aromatic C region (145–111), where the major peaks at 133 and 123 ppm are related to unsubstituted and C-substituted phenyl carbons which belong to lignin monomers of the guaiacyl and syringyl units [49]. Total ion chromatograms (TIC) derived from thermochemolysis of HA and SCT are shown in Fig. 1B, while the compounds identified in the pyrograms are listed in Supplementary Tables S2 and S3. Thermochemolysis applied to HA released more than one hundred different recognizable molecules, identified as methyl ethers and esters of natural compounds. Many of these compounds came from higher plants and microbial by-products and was represented by lignin components, fatty acids, aliphatic biopolymers, hydrocarbons and alcohols (Supplementary Table S2). The distribution of the most representative organic molecules was comparable with previous results obtained by thermochemosys of different substrates [37, 50]. By contrast, the results obtained from the SCT analysis showed a large predominance of lignin components and smaller amounts of carbohydrates and N derivatives (Supplementary Table S3).The current symbols used to distinguish the different structural units have been

associated with the specific monomers of lignin [50]: P, phydroxyphenyl; G, guaiacyl (3-methoxy, 4-hydroxyphenyl); S, syringyl (3,5-dimethoxy, 4-hydroxyphenyl). The pyrogram showed oxidized products of di- and tri-methoxy phenylpropane molecules, with the aldehydic (G4, S4), ketonic (G5, S5) and benzoic-acid (G6, S6) forms as major components. Among the last eluted lignin monomers, 1-(3,4-dimethoxyphenyl)-1 [3]-methoxy-propene (G10/11, G13), 1-(3,4,5-trimethoxyphenyl)-1 [3]-methoxy-propene (S10/11, S13), as *cis* or *trans* isomers, and the 3-(4,5-dimethoxyphenyl)-2-propenoic (G18) and the 3-(3,4,5-trimethoxyphenyl)-2-propenoic (S18) acid forms, have been observed.

RNA-Seq-based gene expression analysis

An NGS-based transcriptomic analysis of tomato roots biostimulated with HAs or SCT was performed. Single-end Illumina sequencing was performed on 27 samples (9 * 3 biological replicates). After filtering out low-quality reads and adapter removal, ~310 million high-quality reads were obtained, with an average of 34.5 million reads *per* sample (Table 1). The percentage of high-quality reads aligned to the tomato reference genome ranged from 86 to 91%.The raw read count matrix underwent *intersample* normalization. Boxplots of the distribution of read counts before and after normalization are shown in Supplementary Figure S2. Pearson's correlation coefficient (r^2) between replicates was not always ≥ 0.95 (Supplementary Figure S3); in the case of SCT-treated samples, one replicate for each time point was discarded, while for HA-treated samples, one replicate for the experimental points at 24 and 48 h was rejected and not considered in downstream analyses.

The EdgeR software package was used to identify DEGs in the biostimulated tomato roots. With an

Table 1 Overall count of RNASeq reads. Data are average of three biological replicates at 24, 48, and 72 h for control, humic acids and sterile compost tea treated samples. It is reported the number of raw reads from Illumina single-end sequencing, the number of high quality reads resulting from the pre-processing step and the number of reads successfully aligned along the tomato reference genome (SL2.50). In brackets are reported the percentages with respect to high quality reads

Sample	Raw reads	High quality reads	Total number of reads aligned along the reference genome		Number of uniquely aligned reads along the reference genome	
Control 24 h	43,541,173	40,034,246	37,274,513	(93.1%)	36,199,840	(90.4%)
Control 48 h	43,102,047	39,510,522	36,448,295	(92.2%)	35,435,030	(89.7%)
Control 72 h	44,406,670	39,674,193	35,274,273	(88.9%)	34,317,392	(86.5%)
Humic acids 24	35,189,605	28,829,393	26,165,712	(90.8%)	25,456,564	(88.3%)
Humic acids 48	39,724,450	32,462,905	30,064,506	(92.6%)	29,240,315	(90.1%)
Humic acids 72	38,841,109	33,577,290	30,613,085	(91.2%)	29,785,631	(88.7%)
Compost tea 24 h	36,315,573	32,249,435	30,329,777	(94.1%)	29,469,552	(91.4%)
Compost tea 48 h	41,331,284	37,556,829	35,221,259	(93.8%)	34,270,708	(91.3%)
Compost tea 72 h	33,987,636	26,846,736	25,224,197	(93.9%)	24,554,980	(91.5%)

FDR < 0.05 and an FC of ± 2, 175 and 478 nonredundant DEGs were identified in the HA- and SCT-treated samples, respectively (Supplementary Tables S4 and S5). HA treatment induces tomato transcriptional reprogramming mainly at 24 and 72 h. Approximately 89% of the 156 DEGs at 24 h were downregulated (Fig. 2B), while approximately 97% of the 40 DEGs at 72 h were upregulated (Fig. 2B). None of the DEGs were shared between all-time points (Fig. 2C). Conversely, SCT treatment induced tomato transcriptional reprogramming mainly at 24 h (Fig. 2A), as approximately 62% of the 470 DEGs were upregulated (Fig. 2B). Only 2 DEGs

were shared between all-time points, as shown by the proportional Venn diagram in Fig. 2C.

GO enrichment and MapMan annotation of differentially expressed genes

Hierarchical clustering was performed by grouping DEGs after both treatments into clusters based on similarity in gene expression profiles. In the HA-treated samples, five gene clusters were identified (Fig. 3). The 39 DEGs in Cluster I showed strong downregulation at 24 h and a slight increase in expression at 72 h. Clusters II and III included the smallest number of genes (8 and 7, respectively). Genes in both clusters were strongly upregulated
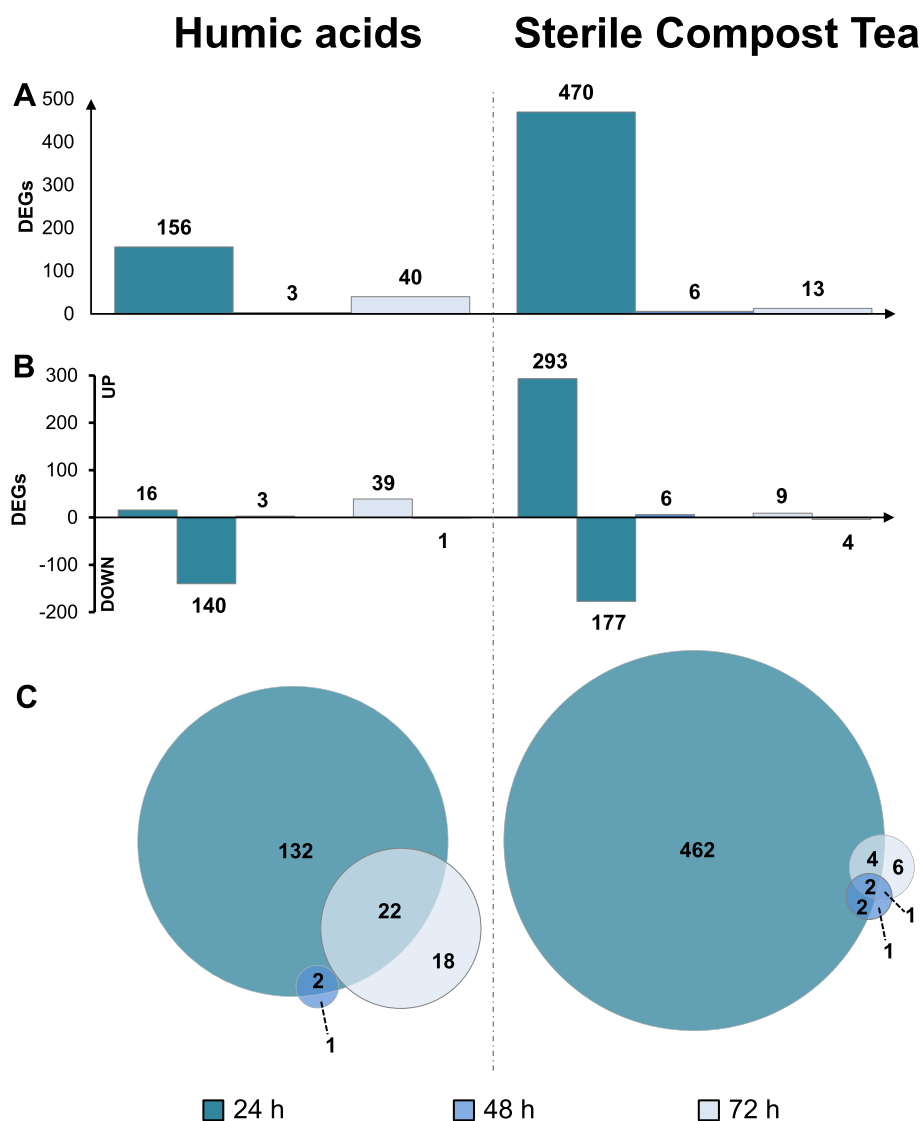
Fig. 2 Overview of transcriptional response of tomato roots following treatment with humic acids (on the right) and sterile compost tea (on the left). Bar charts plotting the number of (**A**) differentially expressed genes (DEGs) and (**B**) of up or downregulated DEGs at 24, 48, and 72 h post-treatment. (**C**) Area-proportional Venn diagrams displaying the overlap of DEGs between samples

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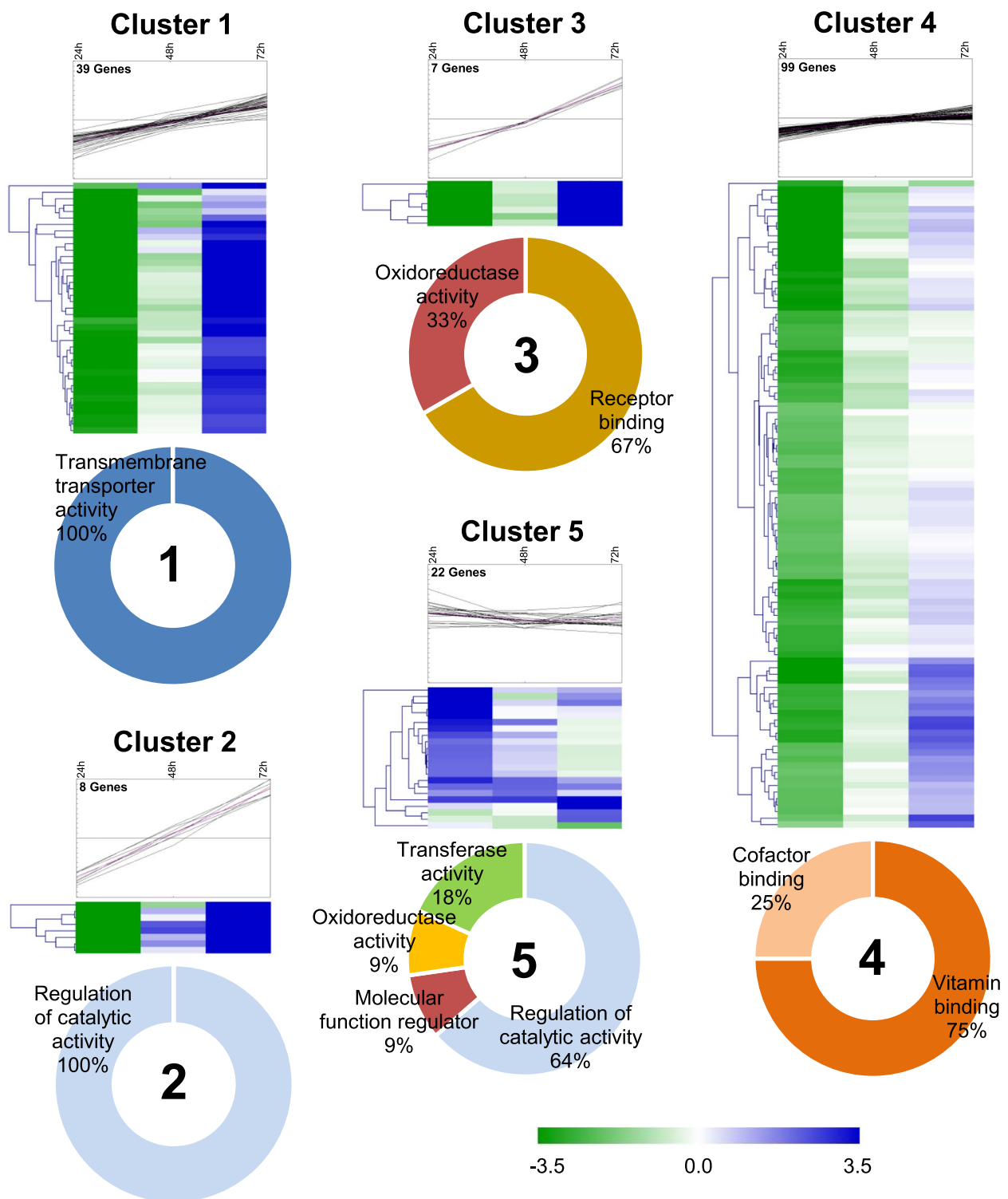
Fig. 3 Changes in tomato root gene expression after treatment with humic acids. One hundred seventy-five (175) genes, that were differentially expressed in at least one of the three time points (24, 48, and 72 h post-treatment), were grouped in 5 SOTA clusters. For each cluster it is reported the corresponding heatmap and the percentage frequency distribution of enriched 3rd level GO terms within the *molecular function* domain

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at 72 h, while genes in cluster II showed increased expression only at 72 h. The expression of genes in cluster III was downregulated at 24 h and then progressively increased at 72 h. Cluster IV included the greatest number of DEGs (99 genes), which were slightly downregulated at 24 h. Cluster V grouped 22 genes that were, in general, upregulated at 24 h. (Fig. 3, Supplementary Table S4).

In the case of the SCT-treated samples, hierarchical clustering produced four different clusters (Fig. 4). Clusters I and II included the largest number of genes (177 and 200, respectively). In the case of cluster I, all DEGs were downregulated at 24 h, unlike the DEGs in cluster II, which were all slightly upregulated at 24 h. Clusters III and IV included the lowest number of genes (47 and 54, respectively). Genes in both clusters were strongly upregulated at 24 h, but while genes in cluster III also showed a slight increase in expression at 72 h, the expression of genes in cluster IV progressively decreased up to 72 h. GO enrichment analysis of the *molecular function* domain was performed on each individual gene cluster as previously defined by SOTA, and then, gene ontologies were collapsed into 3rd level GO terms, whose percentage frequency distribution is shown in Figs. 3 and 4.

Based on Fisher's exact *p* value > 0.01, 23 and 39 GO terms were associated with DEGs for the HA and SCT treatments, respectively (Supplementary Tables S6 and S7). For changes in tomato root gene expression after HA treatment, "transmembrane transporter activity" was the only GO term associated with DEGs in cluster I. The GO functional category that characterized, to a large extent, the DEGs in clusters II and V was "regulation of catalytic activity", while the main GO functional category associated with DEGs in clusters II and IV was "binding activity" (Fig. 3).

Regarding changes in tomato root gene expression after SCT treatment, "transporter activity" was the most represented GO term within cluster I. The GO functional category that characterized, to a large extent, DEGs in clusters II and IV was "transferase activity", while DEGs in cluster III were mainly associated with the GO term "enzyme regulator activity" (Fig. 4). Supplementary Figure S4 provides an overview of the DEG distribution at 24, 48 and 72 h based on MapMan ontology [43] for tomato roots biostimulated with HA and SCT. Additionally, each DEG was associated with the MapMan BIN code and name, allowing the reader to easily navigate through the data (Supplementary Tables S4 and S5).

A metabolic overview map showing DEGs affected by HA biostimulation was generated only at the experimental time point of 24 h (Fig. 5A), as no substantial changes in gene expression were detected at the remaining time points. To highlight the effects of

biostimulation on transcriptional reprogramming, pathway sketches of cell wall precursors, secondary metabolism and hormone metabolism were depicted (Fig. 5A). The metabolism overview map provides a clear indication of the transcriptional changes that take place during biostimulation of the tomato plant (Fig. 5A) and shows a series of downregulated genes involved in the major (starch and sucrose; BIN 2) and minor pathways of carbohydrate metabolism (BIN 3). Glycolysis and fermentation processes were negatively affected by HA, as several genes belonging to the phosphofructokinase, phosphoenolpyruvate and pyruvate families (BIN 4 and 5) were downregulated.

Some of these genes are also involved in the biosynthesis of cell wall precursors and thus affect the organization of the cell wall structure. The alteration of cell wall metabolism is evident from the general decrease in the expression levels of genes related to cell wall degradation (subBIN 10.6.2) and the upregulation of genes responsible for the synthesis of hemicellulose and cell wall precursors (subBIN 10.3).

HA treatment also resulted in the reprogramming of tomato root secondary metabolism (BIN 16), particularly the metabolism of phenylpropanoids and flavonoids (subBIN 16.2 and 16.8, respectively). Specifically, genes belonging to the UDP-glucosyltransferase family were upregulated, while genes encoding cytochrome P450 proteins and members of the phenylalanine ammonialyase family were downregulated.

Biostimulation with HA also affected a small portion of genes assigned to hormone metabolism (BIN 17). The biostimulated plants exhibited changes in the expression of genes involved in the ethylene (BIN 17.5) and jasmonate (BIN 17.7) synthesis pathways. Notably, genes belonging to the aminocyclopropane-1-carboxylate oxidase (ACO) or synthase (ACC) family or involved in ethylene signalling, such as ethylene-responsive transcription factors (ERFs), were downregulated. The application of HA to tomato roots also repressed the metabolism of auxin (BIN 17.2), abscisic acid (BIN 17.1) and brassinosteroid (BIN 17.3).

HA also affects protein metabolism; indeed, genes involved in posttranslational modification (BIN 29.4) were downregulated. In contrast, genes encoding proteinase inhibitors (BIN 29.5) were upregulated. Overall, for the "RNA transcription regulation" BIN (27.3), biostimulation with HA repressed the transcriptional activity of genes encoding transcription factors belonging to the ERF, MYB and WRKY families or containing zingfinger, LOB or U-box domains.

A metabolic overview map was also generated for DEGs at 24 h after SCT treatment (Fig. 5B), along with information on the cell wall precursor pathway,
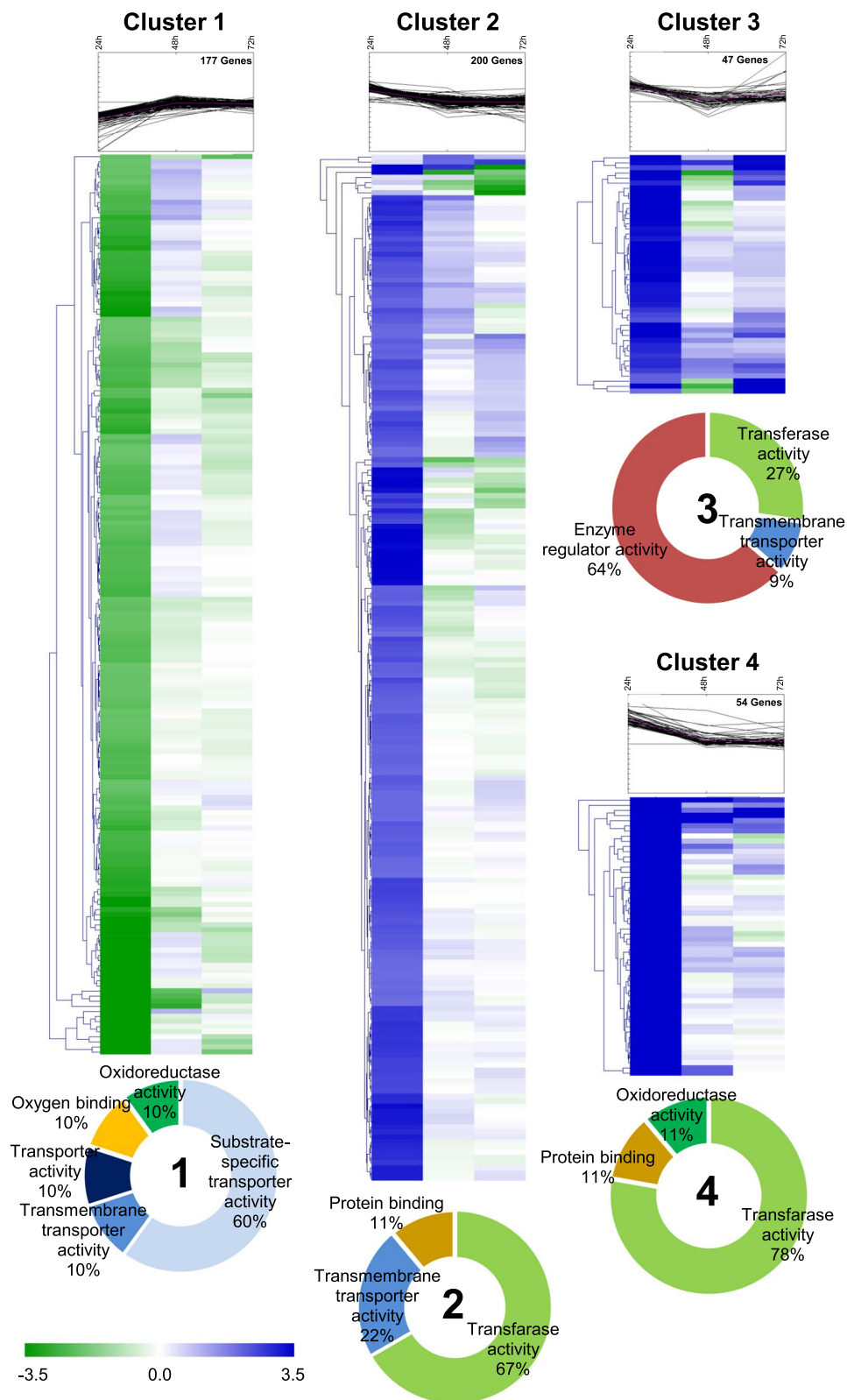
Fig. 4 Changes in tomato root gene expression after treatment with sterile compost tea. Four hundred seventy-eight (478) genes, that were differentially expressed in at least one of the three time points (24, 48, and 72 h post-treatment), were grouped in 4 SOTA clusters. For each cluster it is reported the corresponding heatmap and the percentage frequency distribution of enriched 3rd level GO terms within the *molecular function* domain

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Tomato root metabolism overview
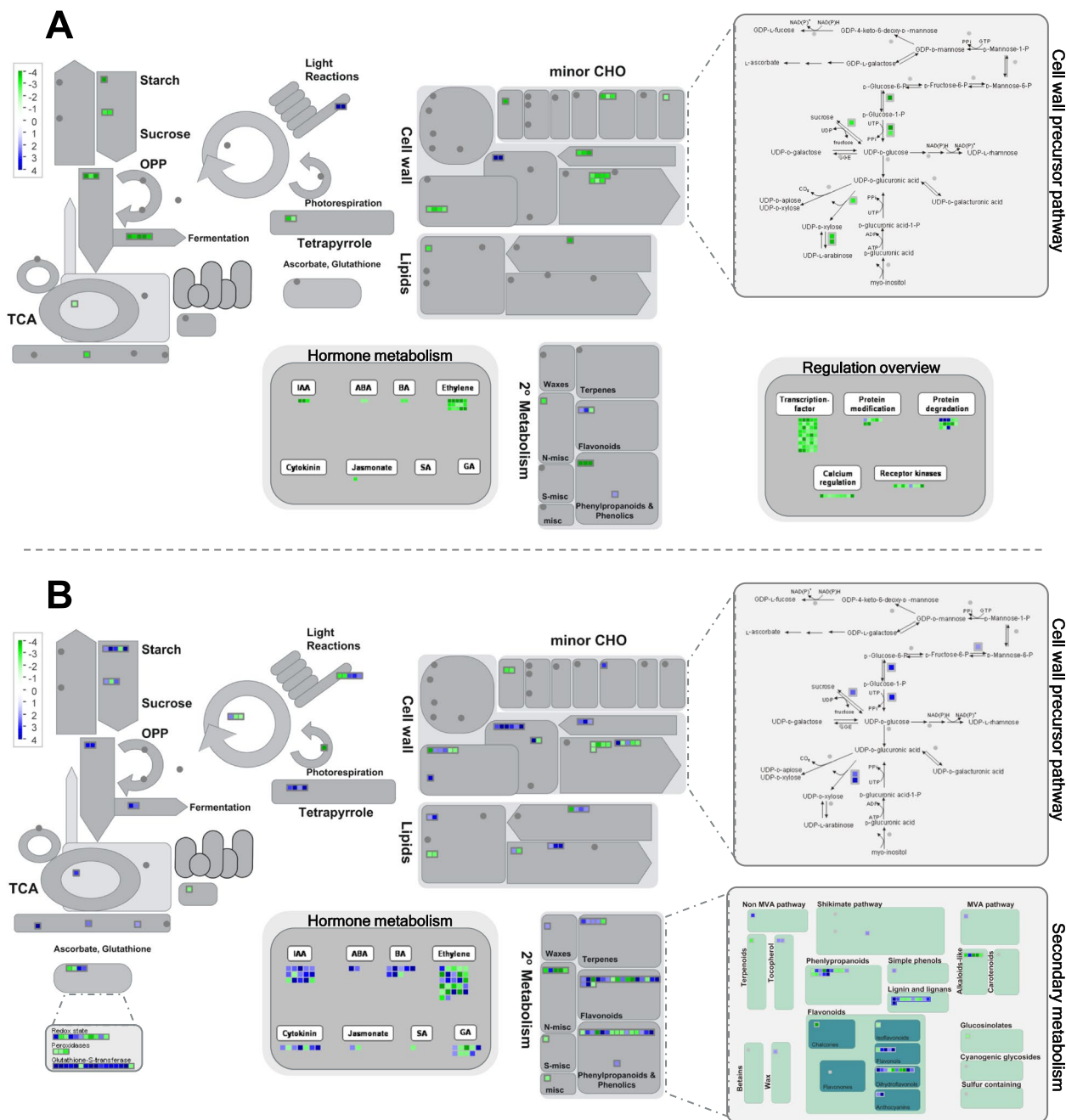
Fig. 5 MapMan metabolism overview showing differences in gene expression levels following treatment with humic acids (**A**) and sterile compost tea (**B**) at 24 h post-treatment. Blue and green boxes correspond to up and downregulated genes, respectively

secondary metabolism, and ascorbate and glutathione metabolism.

SCT also affected the major pathway of carbohydrate metabolism (starch and sucrose; BIN 2) given the upregulation of genes encoding cytochrome P450 proteins. Cell wall metabolism (BIN 10) involved a general

downregulation of genes responsible for cell wall degradation (subBIN 10.6.2 and 10.6.3) and an upregulation of genes involved in the synthesis of hemicellulose and cell wall precursors (subBIN 10.3 and 10.1).

A focus on the biosynthesis pathway of cell wall precursors highlighted the upregulation of key genes,

such as those encoding proteins of the phosphofructokinase family or mannose-6-phosphate isomerase 2.

SCT results in more extensive reprogramming of secondary metabolism (BIN 16) than HA, specifically regarding phenylpropanoid and flavonoid metabolism (subBIN 16.2 and 16.8, respectively). Indeed, several genes in the lignin biosynthesis pathway (subBIN 16.2.1) were affected by biostimulation; among these genes, cinnamyl alcohol dehydrogenase (CAD) and 4-coumarate:CoA ligase (4CL) showed increased gene expression; conversely, phenylalanine ammonia-lyase (PAL), caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT) and ferulate 5-hydroxylase (F5H) were downregulated.

SCT treatment affected several DEGs within the flavonol and dihydroflavonol metabolic pathways (subBIN 16.8.4 and 16.8.3). Specifically, genes belonging to the UDP-glucosyltransferase family were upregulated, whereas genes encoding proteins of the cytochrome P450 and the 1-aminocyclopropane-1-carboxylic acid (ACC) families were downregulated.

In contrast to biostimulation with HA, SCT influenced the expression of a greater portion of genes involved in the hormone metabolism pathway (BIN 17). This resulted in extensive alteration of ethylene (BIN 17.5) and jasmonate (BIN 17.7) metabolism, including several genes belonging to the ACC and ERF families. The application of SCT to tomato roots also affected auxin metabolism; specifically, genes encoding auxin efflux carriers were upregulated, whereas genes encoding auxin-induced/responsive proteins were downregulated. Furthermore, brassinosteroid and abscisic acid metabolism in BINs were affected by SCT treatment.

Finally, redox metabolism (BIN 26) mediated by glutathione S-transferase enzymes (BIN 26.9) is strongly induced by biostimulation with SCT.

Overlap of DEGs between humic acid and sterile compost tea biostimulation

The similar chemical composition and common origin of HAs and SCT led us to compare the lists of DEGs independently identified after treatment with HAs or SCT (Supplementary Figure S5). The two biostimulants promoted a similar response at the root level and induced a strong alteration in gene expression within 24 h, mainly with repressive activity in the case of HAs (Supplementary Table S8). At this time point, 3 genes were upregulated in both treatment groups, while 21 genes were downregulated. Both treatments caused a general upregulation of genes after 48 h (3 common genes) and especially after 72 h (4 common genes).

Gene expression validation using qRT–PCR

To validate the RNA-Seq-based gene expression profiles, qRT–PCR was performed on nine randomly selected genes (Supplementary Figure S6). Each qRT–PCR experiment was carried out in triplicate and repeated three times. The expression profiles obtained by qRT–PCR were consistent with those detected by RNA–seq for both experiments at all three time points (Pearson's correlation coefficient, $r^2 > 0.85$), with the exception of the Solyc02g085660 ($r^2 = 0.29$) and Solyc07g063640 ($r^2 = 0.72$) genes for the HA and SCT experiments, respectively (Supplementary Figure S6).

Discussion

Chemical characterization of humic acids and sterile compost tea

The ^{13}C-CPMAS-NMR spectra of SCT and HA indicate, in general, an overall similar molecular distribution of organic C while, if we look more in detail, there are specific signals that show clear differences in the molecular composition between the two matrices. Both spectra were characterized by similar strong signals in the O-alkyl-C interval (Fig. 1A), indicating a large content of polysaccharides and carbohydrates, mainly cellulose and hemicellulose [30]. The sharp intense peak at 56 ppm, compared to the low abundance of the O-aromatic lignin components in the range from 148 to 155 ppm, also suggested the large contribution of the peptidic moieties to the global resonance in the 46 to 60 ppm range. Lignin molecules indicate the presence of both fresh decomposing plant residues and microbially processed organic materials [7].

Not considering the multiple possible origins of the C16 and C18 acids, the significant release of heavier molecules (>C20), and the predominance of even carbon atoms indicated that plant waxes are a prevalent source of straight-chain aliphatic acids. These compounds are derived from the breakdown of long-chain esters, although a possible origin from the terminal oxidation of other components, such as linear hydrocarbons and aliphatic alcohols, cannot be excluded [51]. The prevailing role of plant intake in lipid composition was also suggested by the detection of C24, C26 and C28 aliphatic alcohols (Table S2), which are common components of the wax layer of nonlignified tissues [51]. Offline pyrolysis of HAs also produced a notable yield of the methylated form of ω-hydroxy alkanoic acids and of alkan-dioic acids (Table S2). These molecules, namely, cutin and suberine, are the main constituents of the external protective barriers of fresh and lignified plant tissues. The relatively less abundant lipid compounds were high-molecular-weight tetra- and pentacyclic triterpenes. The sterol and

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triterpenol moieties have been tentatively identified as methyl ethers and esters of both methyl/ethyl cholesten-3-ol structures and ursane, lupeane and oleanane derivatives that are characteristic lipid components of aerial and root plant tissues [51].

The relatively lower amount of carbohydrate derivatives found among the pyrolysis products may be related to the lower efficiency of off-line pyrolysis techniques for detecting carbohydrate units of polysaccharides in complex matrices [51]. The thermal behaviour and pyrolytic rearrangement of poly-hydroxy compounds, combined with the basic reaction conditions of the TMAH reagent solution, are believed to negatively interfere with the release of carbohydrates from oligosaccharides and polysaccharides.

Molecular response of tomato plants to humic substances

RNA-Seq has already been applied to explore the transcriptional responses of tomato roots while interacting with different soil microorganisms [38, 52, 53]. However, information on host downstream signalling pathways following root biostimulation by abiotic components is still scarce. In this manuscript, we reported the results of the gene expression profiling of tomato roots biostimulated by HS and SCT. The major changes at the tomato root transcriptome level are discussed in detail below.

The introduction of HS, incorporated directly or via CT into the nutrient solution, resulted in a significant downregulation of numerous genes associated with key pathways, such as glycolysis and fermentation processes. This includes the downregulation of genes belonging to the phosphofructokinase, phosphoenolpyruvate, and pyruvate families.Although previous studies suggested that humic acid could enhance glycolysis and primary metabolic processes in general [20, 54], our findings suggest a contrary response where the observed downregulation could signify an adaptive reaction by plants to perceived stress.This stress could be caused by the use of HS. The observed downregulation may represent a strategic reallocation of resources and energy aimed at strengthening the plant's ability to cope with changes introduced in its environment, even if these alterations ultimately prove beneficial [55].On the other hand, other affected genes are also involved in the biosynthesis of cell wall precursors and thus affect the organization of the cell wall structure. The alteration of cell wall metabolism is evident from the general decrease in the expression levels of genes related to cell wall degradation and the upregulation of genes responsible for the synthesis of hemicellulose and cell wall precursors.The proactive conversion of root cell metabolism towards the development of cell wall structures observed after exposure to HS suggests that the treatment has biostimulatory effects, which is also

widely appreciated in the current literature [56, 57]. On the other hand, it is conceivable that the stasis observed at the level of cell wall synthesis could stem from inadequate adaptation to the conditions of floating cultivation. The continuous immersion of roots in the nutrient solution may not align well with the genetic make-up of the tomato cultivar used, known for its drought-tolerant characteristics and inclination towards low water regimes [58]. Indeed, it has recently been demonstrated that the cultivation environment has a significant effect on root architecture [59] and therefore on the physiological processes involved.From a more in-depth analysis of the genes downregulated at 24 h, it seems that most of them are involved in the reaction to abiotic stress. This may indicate that the plant molecular response was in a sort of stress-priming state [60], probably due to the less adaptability of the cultivar to the experimental system [61], and the addition of HS or SCT to the solution relieved the plant system, downregulating all the genes affected by the stress conditions [56, 62].As matter of fact, for example, changes in the expression of genes that are part of the ethylene and jasmonate synthesis pathway could be linked to the subtle influence of the water solution in which the roots remained immersed. Despite maintaining a conductivity level (1.95 dS m^{-1}) typically suitable for hydroponic tomato cultivation, the unique traits of the cultivar might render it less compatible with this specific hydroponic environment.Moreover, the application of jasmonic acid in combination with HAs was found to be more effective in minimizing the stress response of forage sorghum plants exposed to salinity [63]. Notably, genes belonging to the aminocyclopropane-1-carboxylate oxidase (ACO) or synthase (ACC) family or involved in ethylene signalling, such as ethylene-responsive transcription factors (ERFs), were downregulated.

Several regulatory genes linking pathways involved in ethylene signalling are activated in the mitigation of salt stress, as well as in adaptation to osmotic imbalances [64, 65]. On the other hand, SCT resulted in broader reprogramming of secondary metabolism than HS, particularly regarding the metabolism of phenylpropanoids and flavonoids. The latter play an essential role in defense mechanisms and environmental adaptation [66, 67]. Nevertheless, the potential direct effect of these phytohormone-like treatments on the plant growth should also be considered.

As matter of fact, two randomly selected genes for the RNA-Seq results validation by qPCR, involved in the auxin mediated plant responses (Solyc01g068410, coding for an Auxin Efflux Carrier; and Solyc06g084070 coding for Auxin responsive protein), showed an upregulation over time following root exposure to HA and SCT. Phenylpropanoids are a class of secondary metabolites

involved in diverse plant processes, among which the biosynthesis of lignin is noteworthy.

Alterations in gene expression along this pathway confirm the role of these compounds in facilitating the transformation of root cell metabolism to construct cell wall structures, thereby emphasizing their biostimulatory effects. In particular, the upregulation of cinnamyl alcohol dehydrogenase (CAD) and 4-coumarate:CoA ligase (4CL) suggests that SCT promotes lignin production [68], providing structural support to plants and improving their resistance to pathogens and environmental stress.

In contrast, the downregulation of genes encoding enzymes such as phenylalanine ammonia-lyase (PAL), caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT), and ferulate 5-hydroxylase (F5H) in response to SCT treatment could be due to feedback inhibition mechanisms [69]. Enzyme activity within metabolic pathways may be subject to feedback inhibition.

SCT contains lignin and other lignin-like molecules, as evidenced by ^{13}C-CPMAS-NMR spectra, which are produced downstream of the phenylpropanoid pathway and may act as inhibitors, signalling that plants reduce enzyme production early in the pathway, such as PAL, COMT, and F5H [69].

It is important to note that the exact mechanisms involved in the regulation of these genes in response to SCT can be complex and vary depending on the specific composition of the CT, plant species and environmental conditions. Further research and experimentation are needed to pinpoint the precise reasons for these changes in gene expression in a particular context.

Finally, the addition of HS and SCT to the aqueous nutrient solution activated the vegetative state of the plant by simulating the buffering effect of organic matter in the soil. Hydroponic systems can sometimes expose plants to stressors such as rapid nutrient fluctuations or imbalances. Humic substances present in HA and SCT can help mitigate these stresses by stabilizing nutrient availability and pH, creating a more favourable environment for plant growth [70].

Conclusions

The objective of this study was to shed light on the early transcriptional response in tomato plants when biostimulated with humic substances, aiming to expand our understanding of the involved molecular pathways and to identify potential target genes for future crop breeding programs.

In summary, the experimental systems chosen for this study may have posed some challenges due to potential stressors that have not been recorded in this experimental study. However, humic substances present in both HS and SCT may have been involved in the mitigation activating some specific molecular mechanisms, unfortunately the gene regulation in response to these treatments are complex and may vary based on various factors, warranting further investigation in specific contexts and plant species.

Additionally, it is worth considering that the general alterations of the root metabolic pathways and in the cell wall synthesis may be indicative of a biostimulation caused by HS and SCT.

This study, which focused on the biostimulation of humic acid on tomato plants, contributes to a better understanding of the belowground events occurring between plants and biostimulants and provides the optimal conditions for this type of experiment, highlighting the complexity of gene regulation in response to biostimulants and emphasizing the need for further investigations in various contexts and crops.

After further investigations that may also involve shoot response, our results may help to develop new strategies for crop biostimulation as well as to design new breeding strategies for the selection of crop varieties with improved ability to benefit from humic substances.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05602-7.

> Supplementary Material 1. Experimental design and sample collection scheme used for the RNA-Seq experiment.
>
> Supplementary Material 2. Box plots of the distribution of read counts before and after the "trimmed mean of M-values" normalization in control and treated (humic acid and filter sterilized compost tea) samples.
>
> Supplementary Material 3. Scatter plots and Pearson's correlation coefficient (r2) showing the relationships among expression estimates in three biological replicates of control (A), humic acid-treated (B) and sterile compost tea-treated (C) samples at 24, 48 and 72 hours posttreatment.
>
> Supplementary Material 4. Distribution of DEGs in MapMan functional categories (bins) at different time points (24, 48 and 72 h) after treatment with humic acids (above) and sterile compost tea (below). The blue and green boxes correspond to up- and downregulated genes, respectively. Numbers refer to bin designations as defined in MapMan: 1) Photosystem; 2) Major carbohydrate metabolism; 3) Minor carbohydrate metabolism; 4) Glycolysis; 5) Fermentation; 6) Gluconeogenesis/glyoxylate cycle; 7) OPP cycle; 8) TCA/organic transformation; 9) Mitochondrial electron transport/ATP synthesis; 10) Cell wall; 11) Lipid metabolism; 12) N-metabolism; 13) Amino acid metabolism; 14) S-assimilation; 15) Metal handling; 16) Secondary metabolism; 17) Hormone metabolism; 18) Co-factor and vitamine metabolism; 19) Tetrapyrrole synthesis; 20) Stress; 21) Redox; 22) Polyamine metabolism; 23) Nucleotide metabolism; 24) Biodegradation of xenobiotics; 25) C1-metabolism; 26) Miscellaneous enzyme families; 27) RNA; 28) DNA; 29) Protein; 30) Signalling; 31) Cell; 32) Micro RNA, natural antisense; 33) Development; 34) Transport; 35) Not assigned.
>
> Supplementary Material 5. Comparison overview of the transcriptional response of tomato roots following treatment with humic acid (HA) and sterile compost tea (SCT). Bar chart plotting the number of unique and common DEGs that were up- or downregulated at the same time points between the two treatments. All the data were collected at 24, 48, and 72 hours posttreatment.

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Supplementary Material 6. Comparison between RNA-seq (bar charts) and RT–qPCR (dotted lines) expression data of 9 randomly selected DEGs following treatment with humic acids (above) and sterile compost tea (below). The data are the means of three biological replicates at 24, 48, and 72 hours posttreatment. Bars represent the standard deviation between biological replicates. All plots have the same scale on the x- and y-axes for easy comparison. Pearson's correlation coefficient (r2) shows the relationships among expression estimates by RNA-Seq and RT–qPCR.

Supplementary Material 7. Table S1. Identified products released by off-line thermochemolysis of humic acids. Code: Biop = biopoliester; N = nitrogen derivates; Carb = carbohydrates; Cy = cyclopropane; CH3O = methoxy; DIME = dimethyl ester; FAME = fatty acid methyl ester; Lig = lignin; ME = methyl ester; Mic = microbial. Table S2. Identified products released by off-line thermochemolysis of sterile compost tea. Code: Biop = biopoliester; N = nitrogen derivates; Carb = carbohydrates; Cy = cyclopropane; CH3O = methoxy; DIME = dimethyl ester; FAME = fatty acid methyl ester; Lig = lignin; ME = methyl ester; Mic = microbial. Table S3. Complete list of genes differentially expressed at least one of the three time points (namely, 24, 48, and 72 hours) following treatment with humic acids. For each gene, the expression level estimate (log^2-fold change) and the statistical significance value (FDR) calculated by edgeR are reported. The expression and statistical significance values that met the threshold criteria (-2 $\leq$ Log^2-fold change $\geq$ 2 and FDR <0.05) are highlighted in grey. The iTAG 2.40 functional annotation, SOTA cluster membership and MapMan ontologies separated by semicolons are reported for each gene. Table S4. Complete list of genes differentially expressed at least one of the three time points (24, 48, and 72 hours) following treatment with sterile compost tea. For each gene, the expression level estimate (log^2-fold change) and the statistical significance value (FDR) calculated by edgeR are reported. The expression and statistical significance values that met the threshold criteria (-2 $\leq$ Log^2-fold change $\geq$ 2 and FDR < 0.05) are highlighted in gray. The iTAG 2.40 functional annotation, SOTA cluster membership and MapMan ontologies separated by semicolons are reported for each gene. Table S5. For each SOTA cluster, a list of enriched GO terms (Fisher's exact *p* value > 0.01) in the molecular function domain associated with DEGs identified as a result of humic acid treatment is shown. Table S6. For each SOTA cluster, a list of enriched GO terms (Fisher's exact p value > 0.01) in the molecular function domain associated with DEGs identified as a result of sterile compost tea treatment is shown. Table S7. Complete list of DEGs common to samples treated with humic acids and sterile compost tea. For each gene, the expression level estimate (log^2-fold change) and the statistical significance value (FDR) calculated by edgeR are reported. The expression and statistical significance values that met the threshold criteria (-2 $\leq$ Log^2-fold change $\geq$ 2 and FDR < 0.05) are highlighted in gray. The iTAG 2.40 functional annotation is reported for each gene. Table S8. List of primer pairs used in qRT–PCR experiments. All primers used for both experiments (control vs humic acid treatment and control vs sterile compost tea treatment) were used; however, primer pairs labelled with * and ■ were used to amplify DEGs identified in tomato root samples treated with humic acid and sterile compost tea, respectively.

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Authors' contributions
RS, NDA and MZ designed the experiment. RS and NDA wrote the manuscript. RS and CP set up the experiment, processed the samples for RNA isolation, performed the RNA-Seq analysis, carried out the qRT–PCR experiments and were involved in the data interpretation. RS analysed and analysed the ^{13}C-CPMAS NMR spectroscopy, thermochemolysis and GC–MS spectra. NDA developed the workflow for RNA-Seq data analysis, coordinated and supervised bioinformatic work and contributed to data interpretation. MZ conceived the study and critically revised the manuscript.

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Declarations

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Competing interests
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