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Negative plant-soil feedback in *Arabidopsis thaliana*: Disentangling the effects of soil chemistry, microbiome, and extracellular self-DNA

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ABSTRACT

Nutrient deficiency, natural enemies and litter autotoxicity have been proposed as possible mechanisms to explain species-specific negative plant-soil feedback (PSF). Another potential contributor to negative PSF is the plant released extracellular self-DNA during litter decay. In this study, we sought to comprehensively investigate these hypotheses by using *Arabidopsis thaliana* (L.) Heynh as a model plant in a feedback experiment. The experiment comprised a conditioning phase and a response phase in which the conditioned soils underwent four treatments: (i) addition of activated carbon, (ii) washing with tap water, (iii) sterilization by autoclaving, and (iv) control without any treatment. We evaluated soil chemical properties, microbiota by shotgun sequencing and the amount of *A. thaliana* extracellular DNA in the differently treated soils. Our results showed that washing and sterilization treatments mitigated the negative PSF effect. While shifts in soil chemical properties were not pronounced, significant changes in soil microbiota were observed, especially after sterilization. Notably, plant biomass was inversely associated with the content of plant self-DNA in the soil. Our results suggest that the negative PSF observed in the conditioned soil was associated to increased amounts of soilborne pathogens and plant self-DNA. However, fungal pathogens were not limited to negative conditions, butalso found in soils enhancing *A.thaliana* growth. In-depth multivariate analysis highlights that the hypothesis of negative PSF driven solely by pathogens lacks consistency. Instead, we propose a multifactorial explanation for the negative PSF buildup, in which the accumulation of self-DNA weakens the plant's root system, making it more susceptible to pathogens.

1. Introduction

Plant-soil feedback (PSF) describes the relative growth of a plant in its own conspecific soil compared to a heterospecific soil conditioned by a different plant species (Bever et al., 1997). As plants grow, they change the biotic and abiotic properties of the soil, which in turn affects the growth and survival of subsequent plants (Van der Putten et al., 2013). PSF can be positive when plant growth is promoted, as in the cases mediated by beneficial microbes and symbiotic mycorrhizal fungi (Klironomos, 2002; Bever, 2003), or negative when affected by depletion of resources, the increase of natural enemies such as pathogenic bacteria, parasitic fungi and nematodes (Huang et al., 2013) and by the excretion of either allelopathic or autotoxic compounds (Zhou et al., 2018; Bennett et al., 2019). Overall, PSFs affect species coexistence and increase diversity when conspecifics are disadvantaged in their own soil compared to heterospecifics (Mazzoleni et al., 2010; Crawford et al., 2019).

Three main hypotheses have been proposed to explain the negative

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PSF: (i) nutrients deficiency in the soil or an imbalance in their availability; (ii) accumulation of soilborne pathogens and parasites in the soil or changes in the composition and diversity of the soil microbiome; (iii) release of phytotoxic compounds, either allelopathic or autotoxic, during the decomposition of plant residues.

First, evidence from both agroecosystems and natural plant communities clearly shows that nutrient deficiency can be ruled out as a primary causal factor for negative PSF (Cesarano et al., 2017). Indeed, the massive use of mineral fertilizers in monocultures fails to recover crop productivity (Lekberg et al., 2018), and the decline in soil quality and fertility due to salinization and acidification is also exacerbated.

Secondly, soilborne pathogens in the soil and their association with symptomatic plants were considered the main cause of negative PSF (Van der Putten et al., 1988). This view is supported by the old observation that soil sterilization can restore plant productivity in soils subjected to monoculture (Savory, 1966). Indeed, the short-term beneficial effects of soil sterilization are well documented and effective for nematodes, oomycetes, fungi, and bacteria (Li et al., 2019). However, the wide range of pathogens reported weakens this hypothesis as it fails to explain the species specificity nor the long-term persistence (even years) of negative PSF under field conditions in both natural and agricultural ecosystems (Chung et al., 2019). In recent years, the basic assumption that negative PSF could be associated with one or a few specific pathogens has increasingly evolved into a more complex hypothesis of a broader imbalance in the soil microbiome that creates inhospitable conditions for conspecifics. Indeed, studies based on high-throughput sequencing of bacterial and fungal rRNA gene markers have shown that soils harboring conspecifics over several generations develop specific microbiomes (Mendes et al., 2013, Idbella et al., 2022). However, the mechanism and/or reasons why such microbiome specificity should cause a negative PSF remain unclear.

Third, the hypothesis that toxic chemical compounds released either by root exudates or by decomposed plant debris could be the cause of the negative PSF dates back to the early 1900 s (Schreiner and Shorey, 1909). Since then, hundreds of cases of plant autotoxicity have been reported (Singh et al., 1999), but most cases are limited to laboratory bioassays. However, the short lifetime (weeks or months at most) of low molecular weight allelopathic compounds such as short-chain organic acids, tannins and phenols in soil (Armstrong & Armstrong, 2001; Blum, 1998) is clearly inconsistent with the long-lasting persistence (years) of negative PSFs.

In this context, the recent discovery of a species-specific inhibitory effect of extracellular self-DNA (Mazzoleni et al., 2015) released during the decomposition of plant litter provided a new solid basis to reconcile the autotoxicity hypothesis (Mazzoleni et al., 2007) with the occurrence of negative PSFs. Indeed, self-DNA is a species-specific fingerprint and its long persistence in soil is a well-established fact (Pietramellara et al., 2006). Moreover, the self-DNA hypothesis is indirectly supported by the effectiveness of soil flooding against negative PSFs in agroecosystems (Cesarano et al., 2017), which is likely related to the leaching of water-soluble autotoxic factors (Nie et al., 2009), which may well include DNA given its water solubility (Poté et al., 2007). However, to date, there are no studies quantifying extracellular self-DNA in soils exposed to monocultures and linking its accumulation to the negative effects on plant performance.

To date, no study has investigated the role of microbes, pathogens, nutrient depletion and autotoxic factors simultaneously. Recently, a clear and exhaustive review of the state of the science on plant-soil feedback (De Long at al, 2023) emphasized the importance of considering plant-soil through more holistic experimental approaches, including soil biota and plant-litter-soil feedback clarifying their priority effects.

Here, using *Arabidopsis thaliana* (L.) Heynh as a model plant in the context of PSF, we designed an adapted soil history approach using the whole soil rather than just an inoculum after a routine conditioning phase to evaluate plant performance in the response phase. For the first time, *A. thaliana* chloroplast rbcL DNA primers were used to quantify self-DNA in soils exposed to different treatments. Moreover, three selective treatments were applied to the conditioned soils of *A. thaliana* to investigate different possible causal mechanisms of negative PSF: 1) addition of activated carbon to remove (small-sized) allelopathic chemicals; 2) washing with water to remove water-soluble putative autotoxic compounds; 3) sterilization by autoclaving to remove harmful microbiota and toxic chemical factors while releasing nutrients. The effects of soil treatments were assessed by a complete chemical characterization and shotgun metagenomics analysis, and all data were analyzed by in-depth multivariate methods. The specific aims of this work were to assess:

i. *A. thaliana* develops negative PSF during a conditioning phase in monoculture;
ii. self-DNA accumulates in the soil during monoculture;
iii. soil conditioning and associated treatments affect the soil microbial composition and functioning.

2. Material & Methods

2.1. Soil bioassay: conditioning and response phases

A soil-history experiment was performed in two subsequent phases: conditioning and response, each lasting three months. The selected target species was *A. thaliana*, a model plant for its short life cycle and small genome size. Seeds of *A. thaliana* (L.) Heynh. Col-0 (186AV) used in this experiment were obtained from the "Centre de Ressources Biologiques" at the "Institut Jean Pierre Bourgin", Versailles, France.

The soil for the experiment was collected in September 2018 from the forest of Parco Gussone, within the campus of the Department of Agricultural Sciences, University of Naples Federico II (40°48'40.3"N; 14°20'33.8"E, 80 m a.s.l). The soil was sampled at a depth of 20 cm and immediately taken to the laboratory, manually homogenized, and sieved (< 2 mm) to remove coarse roots and rocks. The soil physico-chemical properties are reported in **Table S1**.

First, in the conditioning phase, twenty surface sterilized seeds of *A. thaliana* were sown in each of fourteen pots (10 cm opening diameter × 10 cm height × 8 cm base diameter), filled with the collected soil. The seeds were surface sterilized in a 3% sodium hypochlorite solution for 1 min and rinsed several times with sterile water before use. All pots were maintained for 90 days under a photoperiod condition of 12 h per day and watered with deionized water three times per week to maintain field capacity. At the end of the conditioning phase, shoots were cut off at the soil surface and soil and biomass samples were collected to assess the *A. thaliana* self-DNA accumulation and the final plant growth, respectively.

Second, the conditioned soil was submitted to four treatments: (i) control based on untreated soil; (ii) soil sterilization by autoclaving at 121 °C for 30 min; (iii) addition of 10% (v:v) activated carbon (Sigma-Aldrich Co.) that was mixed and homogenized; (iv) soil washing by placing the soil in a nylon net (mesh size 50 μm) and placed under running water at volume of 10 liter per hours for 96 h.

For each treatment, 10 replicated plastic wells (3 cm diameter × 2 cm depth), were filled with the treated soils and sown with ten seeds of *A. thaliana*, for a total of 40 wells and 400 plants. The plastic wells were kept in a growing room under controlled light (400-μmol m^{-2} s^{-1}) and irrigated to field capacity with deionized water three times a week. After 90 days from sowing, the response phase was concluded and all plants were harvested, shoots and roots were washed to remove soil residues, dried at 70 °C for 72 h, and dry weight was recorded. For each soil treatment, rhizosphere samples were collected by gently brushing *A. thaliana* roots and immediately stored at − 80 °C.

2.2. Soil chemistry

At the end of the response phase, the soil was analyzed for 13 parameters: total organic carbon (OC), pH, total nitrogen, electrical conductivity (EC), Phosphorus (P), NO_3, NH_4, potassium (K), magnesium (Mg), calcium (Ca), and sodium (Na), total limestone ($^{2-}CO_3$), and chlorides (Cl). EC and pH were determined in soil-water suspensions at a ratio of 1:5 and 1:2.5, respectively, using a conductivity meter and a pH meter (Czekała et al., 2016). Total nitrogen was determined using the Kjeldhal method (Czekała et al., 2016), while P was determined using the molybdovanadate-phosphate method. NO_3 and NH_4 contents were assessed with a DR 3900 Spectrophotometer (Hach, Loveland, CO, USA) using the manufacturer kits LCK 340 (assay range 5–35 mg/l, ISO 7890–1-2–1986) for NO_3 and LCK 303 (assay range 2–47 mg/l, ISO 7150–1) for NH_4, on samples obtained by mixing 1 g of dry pulverized soil with 1 mL of distilled water in a 2 mL Eppendorf tube, shaken for 20 min and subsequently centrifuged for 5 min at 13,000 rpm. OC was determined by weight loss at 550 °C for 8 h (Silva et al., 2014). K, Mg, Ca, and Na were determined by flame atomic absorption spectroscopy (Peters et al., 2003). Total limestone was determined by the weight method against a strong acid. Attack of the limestone results in gas release of CO_2, the volume of which is measured (LANO: NF ISO 10693). Finally, Cl content in the soil was determined by the volumetric method described by Meldrum and Forbes (1928).

2.3. DNA extraction, shotgun sequencing and functional annotation

DNA was extracted from three technical replicates using the CTAB protocol. In details, 5 g of soil was homogenized on a vortex mixer (S8A Stuart) at 2200 rpm for 5 min with 600 µl of CTAB extraction buffer (2% cetyl trimethylammonium bromide, 1% polyvinyl pyrrolidone, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA). The homogenate was then transferred to a 60 °C bath for 30 min. The resulted lysate was centrifuged at 16,000 g for 5 min. The supernatant was mixed with 5 µl of RNase A and incubated at 37 °C for 20 min. Thereafter, the upper layer was transferred and mixed with an equal volume of chloroform-isoamylic alcohol (24:1 v/v) and centrifuged at 16,000 g for 5 min to separate the phases. The supernatant was then precipitated with 0.7 vol of ice-cold isopropanol and incubated at − 20 °C overnight. The DNA pellets obtained after centrifugation at 14,000 g for 30 min at 4 °C were suspended in 50 µl of endonuclease-free water.

DNA libraries were sequenced on Illumina NovaSeq platform, resulting in 2×150bp, paired end reads. The resulting reads were quality-filtered using PRINSEQ 0.20.4 (Schmieder et al., 2011). Reads with bases that had a Phred score < 15 were trimmed and those < 75 bp were discarded. Taxonomy profiles were obtained by Kraken2/Braken pipeline (Wood et al., 2019). Metagenome functional annotations were obtained using MEGAN6 (Huson et al., 2016). To obtain the gene abundance, short reads were mapped to the genes and the number of mapped reads was normalized using the RPKM method (reads per kilo-base per million mapped reads Mortazavi et al., 2008).

2.4. A. thaliana self-DNA quantification

The same approach used by Foscari et al. (2022) was applied in this study to assess the amount of *A. thaliana* in the soil after the different treatments. DNA was extracted and purified directly from fresh soil aliquots (5 g) after the four treatments using a commercial extraction kit (DNeasy® PowerMax® Soil Kit, Qiagen, USA) according to the manufacturer's instructions. Briefly, each aliquot was placed in a 50 mL Falcon tube containing beads (0.7 mm garnet) and a lysis buffer and shaken horizontally (80 rpm) for 30 min. After centrifugation (4 °C, 3 min, 2500 g), the supernatant was collected and subjected to 5 cycles of purification and centrifugation to precipitate additional organic and inorganic non-DNA compounds, including cell debris and proteins. At the end of the purification, all soil DNA samples were suspended in 5 mL of a 10 mM Tris solution. The purified samples were quantified using the Qubit 3.0 fluorimeter and Qubit dsDNA Assay Kit (Life Technology, Carlsbad, California, USA). Sample quality was assessed by NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), while DNA size distribution was assessed by 0.8% agarose gel electrophoresis.

Amplification of the purified DNA was performed in a final volume of 25 µl using 10 µl of DNA extract, 1X concentrated OneTaq Hot Start Quick-Load, 2X Master Mix with standard buffer (New England Biolabs inc.) and 0.5 µM of rbcL forward and reverse primers. These primers can amplify a 553 bp fragment of the rbcL gene and are recommended by the CBol Plant Working Group (2009) for plant metabarcoding. The primers selected were rbcLa_f 5′-ATGTCACCACAAACAGAGACTAAAGC-3′ and rbcLa_rev 5′-GTAAAATCAAGTCCACCRCG-3′ (Fahner et al., 2016). PCR conditions were 94 °C for 30 s, followed by 40 cycles of 94 °C for 30 s, 64 °C for 60 s, 68 °C for 30 s and a final step of 68 °C for 5 min. A subsequent amplification run integrating relevant flow-cell binding domains and unique indices was performed using the NexteraXT Index Kit (Illumina). Amplification products were sequenced on the MiSeq instrument platform (Illumina, San Diego, CA) using a 300 bp paired end and according to the manufacturer's instructions.

Taxonomic classification was performed using a database of 181133 rbcL sequences downloaded from the NCBI Nucleotide Section on September 9, 2020, using the following key words in search: rubisco [all fields] OR ribulose-1,5-biphosphate carboxylase/oxygenase [all fields]) AND plants[filter] AND biomol_genomic [PROP] AND large subunit [All Fields]. In addition, consensus sequences of *Cyamopsis tetragonoloba* and *Vitis vinifera* were manually inserted. The bioinformatics pipeline steps were as follows: Reads were trimmed using *cutadapt* (Martin, 2011) with default parameters to eliminate primer sequences. Low-quality bases were removed from 3′ with *erne-filter* (Del Fabbro et al., 2013) using default parameters and reads < 60 bp were excluded from further analysis. Reads with an error rate > 1% were removed. Chimeric sequences were removed using the *uchime_denovo* algorithm (Edgar et al., 2011) implemented in *usearch*. Reads were clustered to a minimum identity of 97% using the *cluster_fast* algorithm implemented in *usearch* to produce representative sequences. BLAST was matched against the rbcL database without a minimum identity filter, with the lowest unambiguous taxonomic assignment among all possible blast hits. For hits with the same score indicating different lineages, the most frequent part was indicated. Sequences that could not be taxonomically assigned to *Streptophyta* were discarded.

2.5. Statistical analysis and data visualization

For the microbial data, alpha diversity metrics, i.e., species richness index, was calculated and presented as boxplots using the software PRIMER 7 (Primer-E Ltd, Plymouth; UK). For assessing variation in community composition at the lowest taxonomic levels, heatmaps were used to represent the most abundant taxa in the fungal and bacterial communities and made by the ComplexHeatmap package implemented in R (version 3.3.2). The significance of variation in diversity metrics, plant biomass, and exDNA abundance between treatments was assessed using the ANOVA test, and means were separated pairwise using the post hoc Tukey test to provide further detail on the level of significance between samples. A heatmap was generated using the ComplexHeatmap package in R. The level of significant differences was evaluated with $p < 0.05$. All statistical analyses were performed using STATISTICA 13.3 software.

Furthermore, an examination of the functional groups present within the fungal community was conducted by utilizing FUNGuild (Nguyen et al., 2016) to identify potential fungal functional groups. The core microbiota was determined by constructing Venn diagrams using the VennDiagram package in R (Chen and Boutros, 2011), which consisted of four sets representing the bacterial and fungal communities for each of the four treatments. The complexity of the microbiome and the

relationships among the different microbial community members were investigated by co-occurrence network analyses of the four different soils focusing on the 50 most abundant species for each taxonomic group (bacteria and fungi). Pairwise correlations between species were calculated by the Hmisc package in R. Based on statistical analysis, only strong and significant (Spearman's $r > 0.6$ or $r < -0.6$ and $p < 0.05$) correlations were considered. Network visualization was performed using Gephi software (version 0.9.2, Bastian et al., 2009). Edges represent robust and significant correlation with nodes corresponding to the taxonomic species. A set of integrative metrics was also computed to describe the network topology.

Multivariate analysis by both numerical clustering, using primer7 software, and principal component analysis, using "factoextra" package in R, were performed on the data matrices of different microbial taxa clusters resulted from the heatmaps, plant biomass, *A. thaliana* DNA, and soil microbiome gene abundance (assessed by metagenomic functional annotation).

3. Results

3.1. *A. thaliana* performance and self-DNA accumulation

In the first experiment, a very significant difference was observed in the biomass of *A. thaliana* in the response phase between plants grown on control and conditioned soil (Fig. 1 **A**). This was associated with a relevant accumulation of *A. thaliana* DNA in the conditioned soil (Fig. 1**B**) and with relevant changes in the microbiota composition (Fig. 1 **C**, **D**). In particular, *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Cyanobacteria* exhibited an increase among the bacteria phyla after conditioning, whereas the fungal community showed a significant increase in pathotroph guilds, particularly plant pathogens.

The second experiment examined the impact of various soil treatments on plant biomass following the conditioning phase. The results confirmed the findings from the first experiment, with low biomass production in the untreated conditioned soil used as a reference. The highest biomass was observed after the sterilization treatment, followed by the soil washing treatment, while the addition of activated carbon did not lead to any significant difference when compared to the untreated conditioned soil. These observations were associated with a substantial

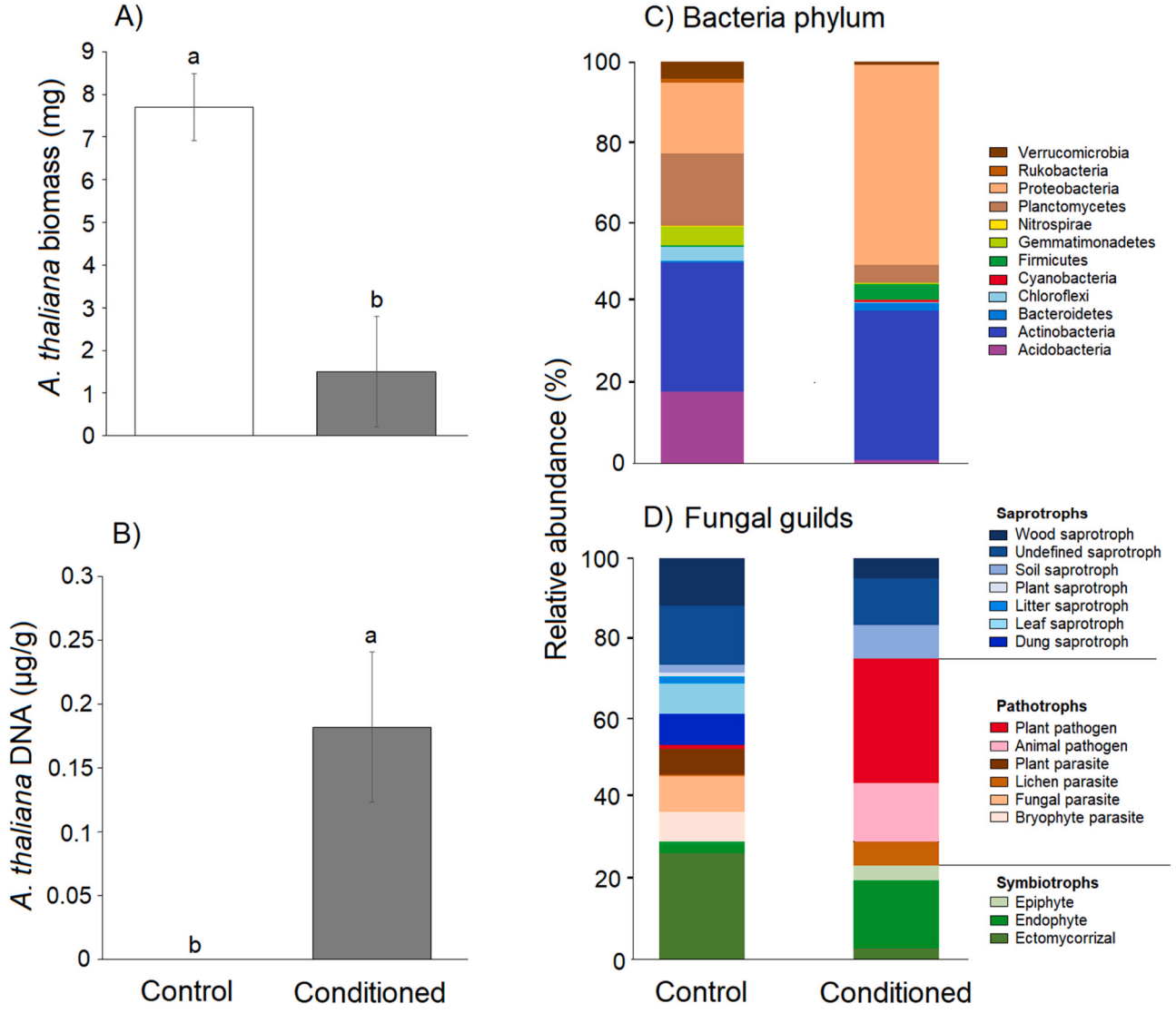

Fig. 1. (A). Total biomass (mg/well) of *Arabidopsis thaliana* in control and conditioned soil. (B). *A. thaliana* self-DNA in the control and conditioned soils. Different letters indicate significant ($P < 0.05$) differences. (C) Stacked bar plots showing the relative abundance of various bacterial phyla between the control and conditioned soils. (D) Relative abundance of fungal functional guilds within the control and conditioned soils.

decrease in *A. thaliana* DNA following both washing and sterilization treatments, whereas the addition of active carbon resulted in a considerable increase in DNA accumulation in the soil (Fig. 2).

3.2. Soil chemistry

The soil chemical parameters showed significant differences between the treated soils (Table 1). Specifically, the sterilized soil had the lowest electrical conductivity and NO_3 content, while the P content was the highest. On the other hand, the soil with activated carbon had the highest organic matter and NO_3 contents, while NH_4 content was the lowest. Total limestone was significantly higher in the control and washed soils, while chloride content was significantly higher in the control and activated carbon soils compared to the other soils. No statistically significant differences were observed between the soils in terms of pH, Mg, K, total N and Na.

Pearson correlations between soil chemical parameters and *A. thaliana* biomass showed significant positive correlation for P content, while significant negative correlations were recorded for Mg, Cl, EC, and *A. thaliana* self-DNA (**Table S2**).

3.3. Microbiome diversity, abundance, and composition in A. thaliana rhizosphere

At the phylum level, *Proteobacteria* was the most abundant in the sterilized soil, followed by the washed soil, the activated carbon soil and in the control (**Fig. S1**). The highest abundance of *Planctomycetes* was found in sterilized soil while the lowest was recorded in activated carbon. *Firmicutes* were more abundant in sterilized soil compared to washed soil. *Bacteroidetes* were more abundant in sterilized soil than in activated carbon soil, while *Actinobacteria* were very abundant in activated carbon soil and decreased in sterilized soil. The fungal community in the treated soils showed significant variation at phylum level (**Fig. S1**). All the soils were dominated by *Ascomycota* with abundances of 93.0%, 91.9, 89.6% and 88.4% in the control, washed, sterilized, and activated carbon soils, respectively. *Basidiomycota,* however, were more abundant in both activated carbon and sterilized soils with 11.3% and 9.9%, respectively, while they were low in control and washed soils with 6.6% and 7.7%, respectively.

Table 1
Chemical analysis of the different treated soils.

Parameters	Untreated	ActiveC	Washed	Sterilized
pH	7.92a	7.79a	7.98a	7.98a
Total limestone (%)	2.25a	1.73b	2.95a	1.91b
Electrical conductivity (mS/cm)	0.59a	0.61a	0.54a	0.39b
Chlorides Cl (g/Kg)	0.51a	0.60a	0.34b	0.30b
Sodium Na (g/Kg)	0.53a	0.54a	0.48a	0.49a
Organic Carbon (%)	7.39b	17.0a	6.23b	6.65b
Total Nitrogen (%)	0.29a	0.27a	0.28a	0.29a
P (mg/Kg)	30.88b	26.57b	31.76b	49.73a
K (g/kg)	2.05a	1.84a	1.77a	2.2a
Mg (g/Kg)	0.87a	0.84a	0.83a	0.81a
NO_3(mg/l)	5.43b	11.17a	5.3b	2.51c
NH_4 (mg/l)	0.89a	0.23b	0.61a	0.78a

Different letters within each parameter indicate significant differences (Tukey test, $p < 0.05$).

The heatmap depicting the relative abundance of bacterial species at the lowest taxonomic level revealed significant variations among treatments (Fig. 3**A, Table S3**). In particular, the sterilized soil exhibited distinct differences compared to other soils, with a specific group of bacterial species belonging to clusters 7 and 8, including *Sphingopyxis macrogoltabida*, *Sphingopyxis lindanitolerans*, *Sphingopyxis alaskensis*, *Planctomyces*, *Georhizobium profundi*, *Sphingopyxis fribergensis*, *Devosia*, and *Microbacterium*, dominating the soil. Conversely, the washed soil was characterized by a high presence of bacteria from clusters 3 and 4, such as *Thermomonas*, *Flavisolibacter*, *Lysobacter soli*, *Microvirga ossetica*, and *Streptomyces lividans*. The soil with activated carbon mainly contained a group of bacteria from clusters 1 and 2, such as *Streptomyces scabiei*, *Sinorhizobium fredii*, *Streptomyces chartreusis*, *Acidovorax*, *Agrobacterium tumefaciens*, and *Bacillus cereus*. On the other hand, the control

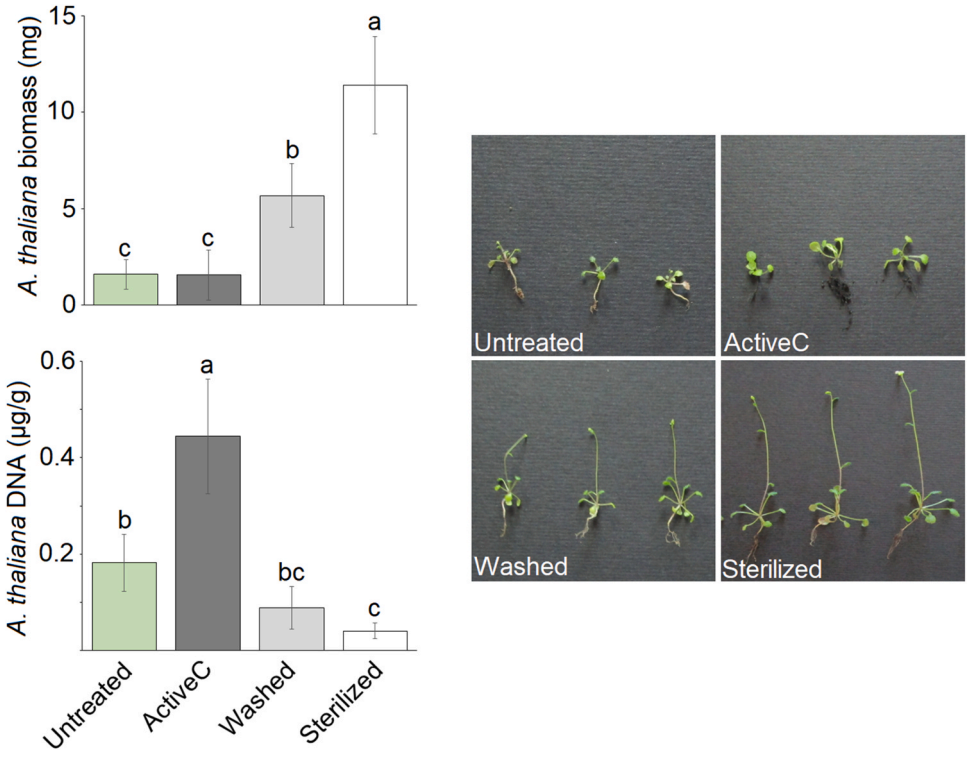

Fig. 2. Total biomass (mg/well) and self-DNA of *Arabidopsis thaliana* in each treatment of the conditioned soil. Different letters indicate significant ($P < 0.05$) differences. Each treatment bar represents the average of 10 replicates.

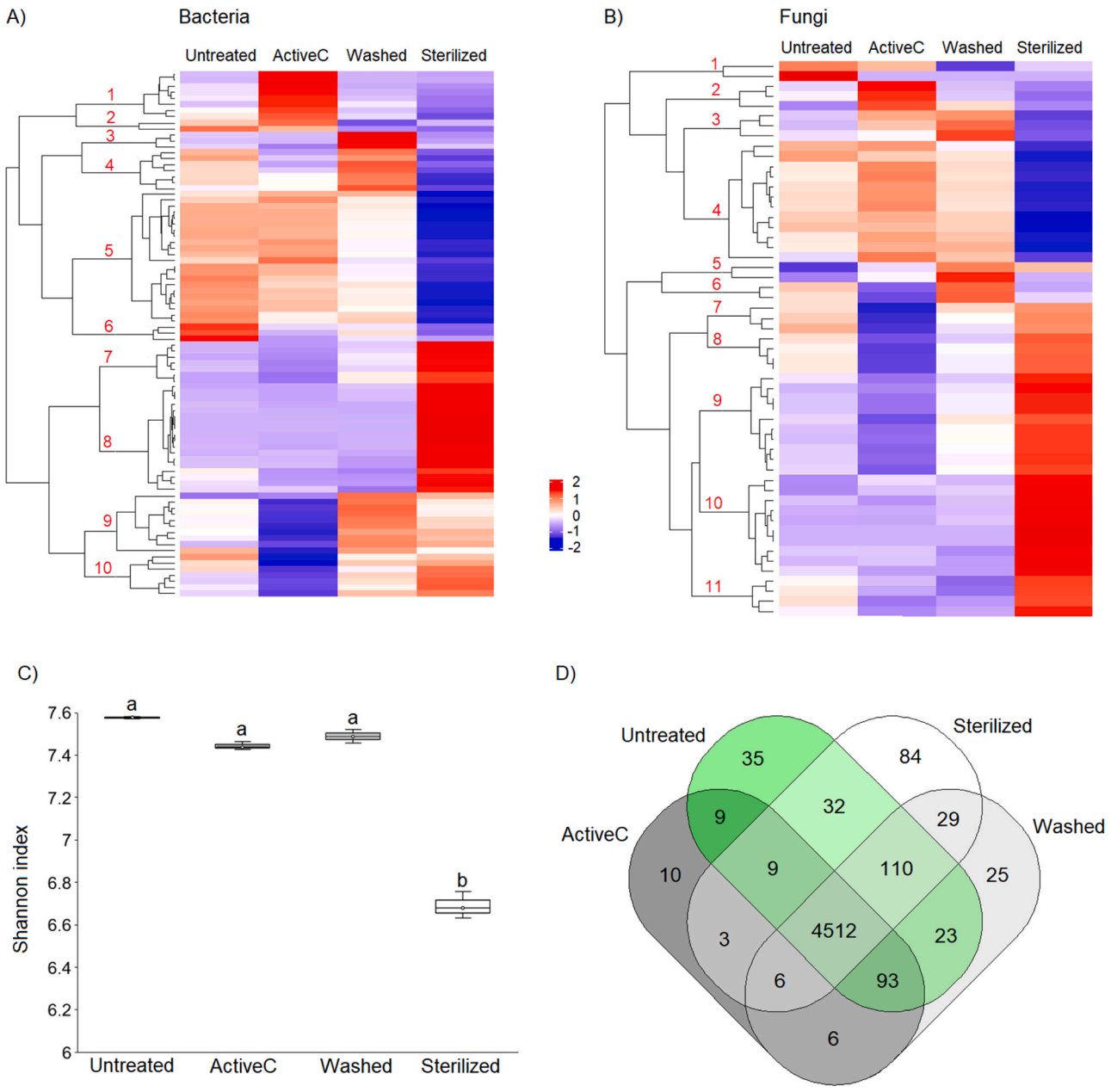

Fig. 3. Heatmaps showing log-transformed relative abundance of the most frequent species in the bacteria (87 species, A) and fungal (55 species, B) communities for each treatment of the conditioned soil and corresponding clustering dendrograms indicating main differentiated groups of correlated species in red numbers. (C) Box plots showing the variation in the Shannon index for microbial communities in each treatment. (D) Venn diagram for microbial community showing the taxa that were shared or not shared by the different studied treatments.

soil was abundant in *Streptomyces fradiae*, *Conexibacter woesei*, *Sorangium cellulosum*, and *Arthrobacter*. As for fungi, the heatmap demonstrated significant variation between the sterilized soil and the other soils (Fig. 3**B, Table S4**). Specifically, the sterilized soil was characterized by the high abundance of fungal groups belonging to clusters 7 to 11, including *Fusarium verticillioides*, *Sugiyamaella lignohabitans*, *Schizosaccharomyces pombe*, *Scheffersomyces stipites*, *Aspergillus oryzae*, and *Zygosaccharomyces rouxii*.

Microbial species diversity assessed by the Shannon index was significantly higher in the conditioned soil, slightly lower in both activated carbon and washed soils and dramatically lower after soil sterilization (Fig. 3**C**). The Venn diagram showed that the core shared microbiota, associated with the most frequent taxa, were represented by 4512 species (Fig. 3**D**). The highest number of unique taxa was found in the sterilized soil (n = 84), followed by the untreated conditioned soil (n = 35), the washed soil (n = 25), while the lowest number was found in the activated carbon soil (n = 10).

The bacterial and fungal communities were numerically clustered and divided into 10 and 11 main groups, respectively (see Fig. 3**A, B**). Further multivariate analysis was conducted to assess the correlation between these clusters and *A. thaliana* biomass and self-DNA in the soil**.** The analysis indicates a clear separation between self-DNA and plant biomass in their association with different microbial taxa, both bacteria and fungi (Figs. 4, 5**)**. The PCA plot shows opposite scores along the first principal component, which represents most of the variability. The dendrograms reveal the most abundant species that characterize each cluster. In the case of bacteria, the numerical clustering and PCA plots indicate that *A. thaliana* self-DNA is associated with beneficial species such as *Streptomyces* sp. and *Sinorhizobium fredii*, and to a lesser extent with a group that includes the pathogen *Agrobacterium tumefaciens*

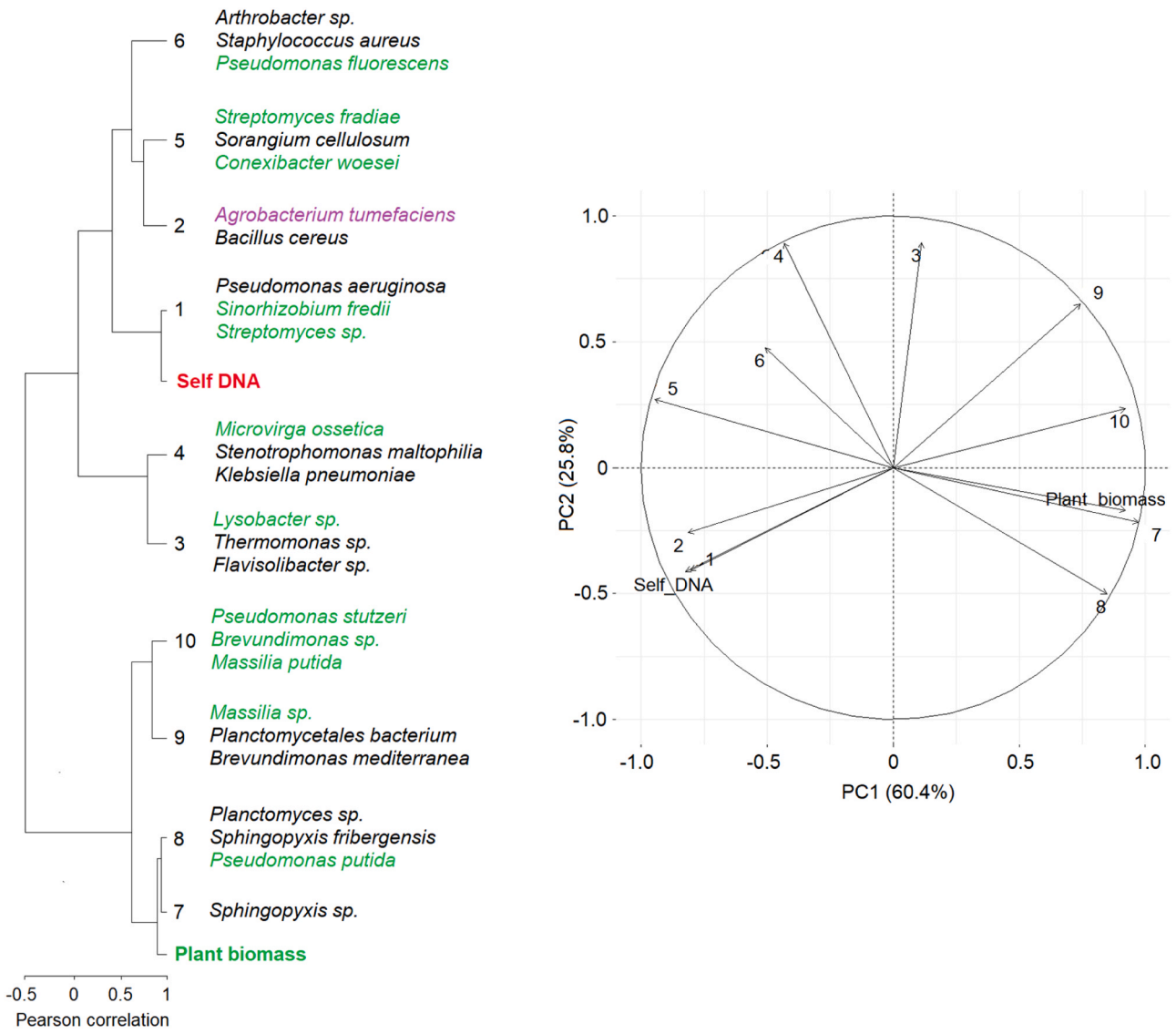

Fig. 4. Dendrogram based on Pearson correlation and Principal component analysis (PCA) with their loading plots of different bacterial clusters with self-DNA and total biomass of *A. thaliana*. Taxa names in the dendrogram refer to the most abundant species within each cluster group.

(clusters 1 and 2). However, other beneficial bacteria are also present in clusters 8, 9, and 10, which are positively correlated with plant biomass (e.g., *Pseudomonas putida*, *P. stutzeri*, *Massilia*, *Brevundimonas*) (Fig. 4**)**. Instead, as for the fungal community, *A. thaliana* self-DNA was grouped with clusters 2 and 4, which contain the pathogens *Fusarium oxysporum*, *Colletotrichum higginsianum*, and *Eremothecium gossypii*. In contrast, plant biomass was associated with different groups, including 11, 9, 10, 7, and 8, which include several beneficial organisms such as *Aspergillus oryzae*, but also three important pathogens, namely *Fusarium verticilloides*, *Botrytis cinerea*, and *Eremothecium sinecaudum* (Fig. 5**)**.

3.4. Co-occurrence network

A co-occurrence network for the untreated conditioned soil and the soils after the three treatments was constructed allowing the calculation of six topological parameters assessing the interactions among microbial species in each of the four networks (Fig. 6**, Table S5**). The microbial networks contained 138 nodes and 3097 edges in the untreated conditioned soil, 141 nodes and 3940 edges in the sterilized soil, 134 nodes and 2910 edges in the washed soil, and 137 nodes with 2923 edges in the soil with activated carbon. The heterogeneity of the network was higher in the untreated conditioned and sterilized soils than in the others, while the centralization of the network was the lowest in the sterilization treatment and in the soil with activated carbon. The clustering coefficient and network density, however, showed no differences between the treated soils. Network modularity measures the strength of subdivision of a network into sub-modules. High modularity reflected dense connections of nodes within modules and reduced connections of nodes of different modules, and it was found highest in the washed soil, followed by the sterilized soil compared to the other conditions.

3.5. Metagenomics soil functional diversity

The heatmap of functional genes belonging to KEGG subsystem 2 (Fig. 7**A**) indicates a significant variation in sterilized soil compared to the other soils with a significantly higher number of genes belonging to various subsystem 1 groups, particularly genes related to membrane transport and cellular processes, but also metabolism and several other processes, whereas with lower levels of genes related to regulation and cell signaling. The active C treatment had the lowest abundance of genes belonging to RNA processing and protein processing subsystems, while showed on the opposite of sterilized soil high number of regulation and

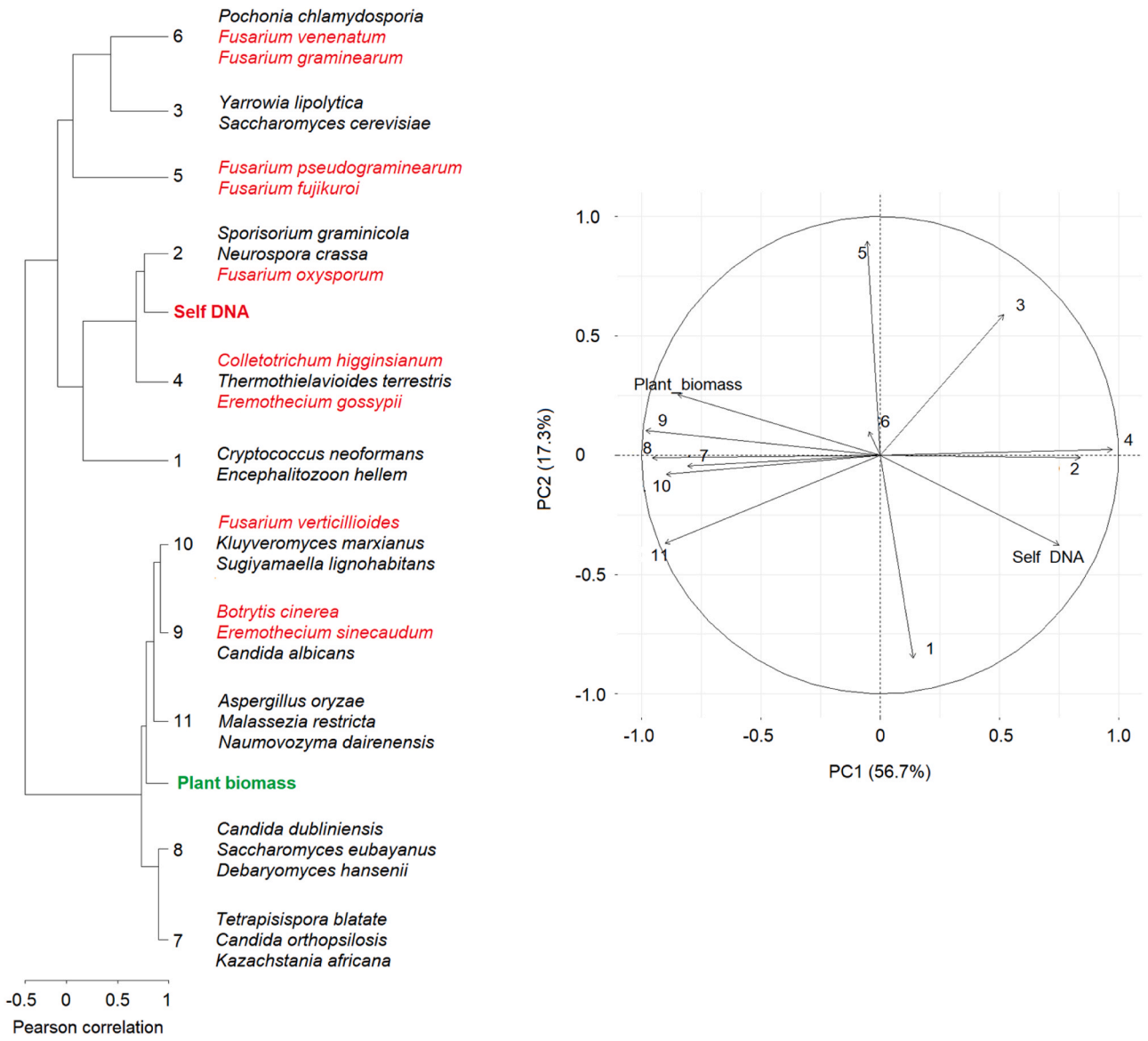

Fig. 5. Dendrogram based on Pearson correlation and Principal component analysis (PCA) with their loading plots of different fungal clusters with self-DNA and total biomass of *A. thaliana*. Taxa names in the dendrogram refer to the most abundant species within each cluster group.

cell signalling genes.

The Venn diagram based on the presence or absence of functional genes shows that the differently treated soils had 591 shared core genes. Only the sterilized soil had six unique expressed genes, while the washed and activated carbon soils did not show any exclusive gene (Fig. 7**B**). Numerical clustering and PCA of these gene expression data, associated with *A. thaliana* biomass and the occurrence of its self-DNA in the soil, show clear discrimination of one cluster containing genes related to regulation cell signalling and self-DNA, in contrast to the majority of other gene categories that group together with plant biomass (Fig. 7**C**, **D**).

4. Discussion

In this work, the response phase was assessed with the entire conditioned soil and not just an inoculum, unlike most feedback studies. This method was preferred in order to study the whole chemical legacies produced by nutrient depletion and decomposition of litter and root exudates, rather than being limited to the microbial legacies alone.

Previous studies reported that soil chemistry is the explanatory factor for variation in the magnitude of PSF effects (Ehrenfeld et al., 2005). In contrast, we found no significant differences between soils in terms of pH, Mg, K, total N and Na content, suggesting that the direction and strength of the negative feedback was not due to soil chemical properties. Some significant differences were observed in the higher electrical conductivity and P content of the sterilized soil, in which the NO_3 content was lower. In addition, we found that although the soil amended with activated carbon elicited the strongest negative feedback effect, it also had the highest organic carbon content. In terms of P content, however, no difference was found between this soil and the washed soil, which on the contrary showed a positive feedback effect. This underlines the fact that the main effects of plant responses cannot be considered only as a matter of resource availability. In agreement with our results, Harrison and Bardgett (2010) also showed an occurrence of PSF effects independently of soil physicochemical conditions in mixed grassland communities.

The beneficial effects of soil sterilization have been known since the 1960 s to reduce the increase in negative PSF (Savory, 1966). Soil sterilization is known to alter biotic and abiotic soil properties and enables nutrient fluxes through the rapid mineralization of dead microbes

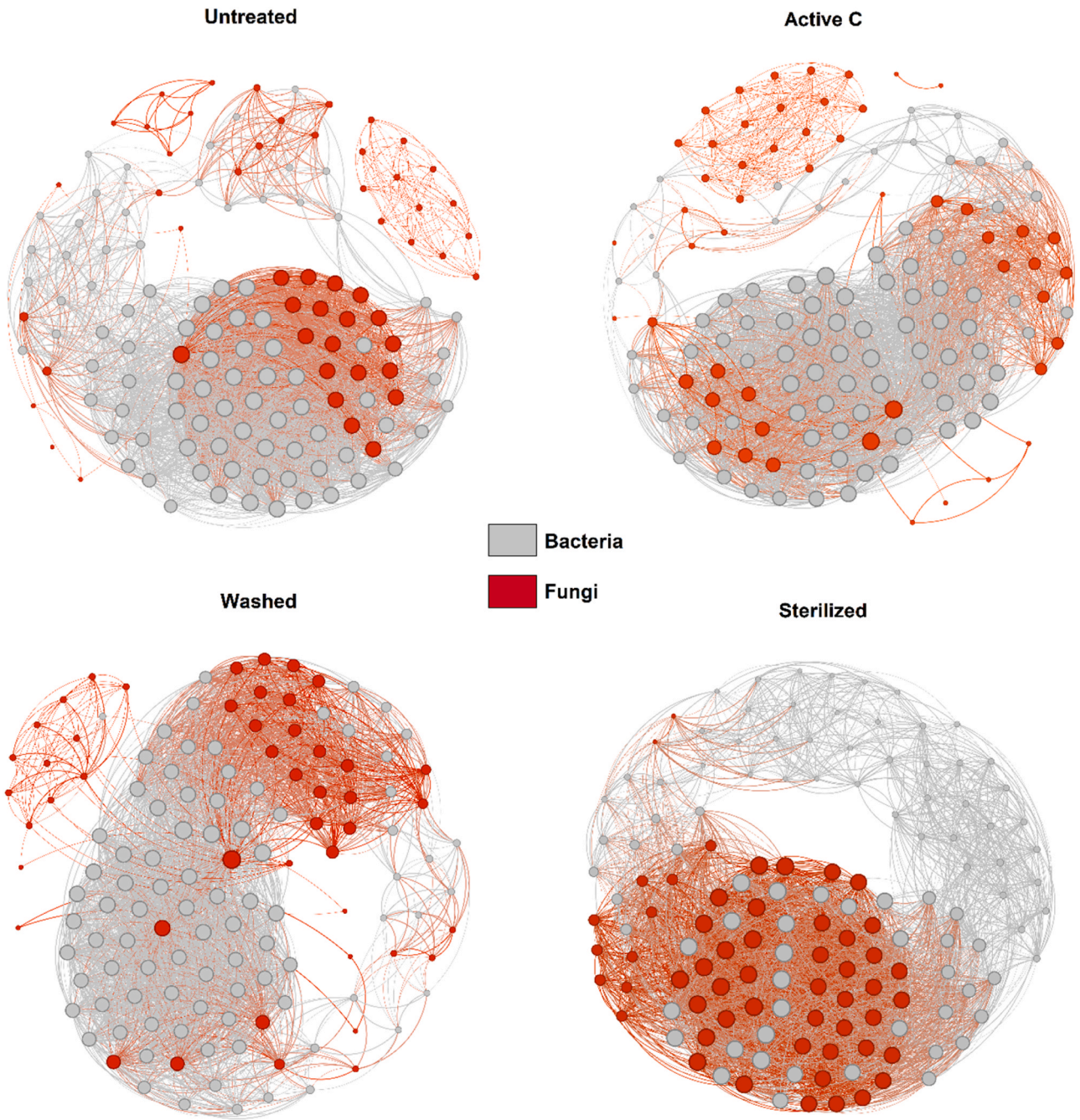

Fig. 6. Correlation based network between bacterial and fungal families within soil microbial communities in four different treatments. The most frequent species for each kingdom (87 species for bacteria and 55 species for fungi) are reported. The nodes are coloured by kingdom level. Edges length inversely represents correlation strength. Connections stand for significant correlation (P-value<0.05).

(Troelstra et al., 2001). Besides, organic phytotoxic compounds can be thermally degraded. Therefore, soil sterilization can both reduce negative PSF and promote plant growth. Indeed, our results showed that the sterilized soil had the highest P content. Changes in soil P concentration have been reported in previous studies as the main factor for changes in microbial community composition (Wei et al., 2020). Accordingly, we found that sterilized soils contained greater amounts of early-succession phyla such as Proteobacteria, Planctomycetes, Firmicutes, and Bacteroidetes, while late-succession phyla Actinobacteria and Acidobacteria were the least abundant. Most bacteria belonging to the Proteobacteria and Firmicutes tend to be fast-growing copiotrophs (Ling et al., 2022) that rapidly consume soil resources, while Actinobacteria are considered oligotrophic in soil (Yan et al., 2021). In addition, our results showed that sterilization by autoclaving not only changed the composition of the microbiota but also reduced its diversity in the soil. We also observed that the highest proportion of positive co-occurrence interactions between microbial communities was found on sterilized soils, probably due to the elimination of previously established competing microbial communities (Troelstra et al., 2001), leaving empty niches for beneficial microorganisms influenced by ongoing root exudation (Broeckling et al., 2008).

Soil washing treatment has never been tested in terms of the feedback effect. In this context, it is worth noting that the negative PSF occurs mainly in terrestrial systems, while it has been rarely observed in aquatic environments (Mazzoleni et al., 2007), which in a way supports our results, as washing under running water could mimic the aquatic

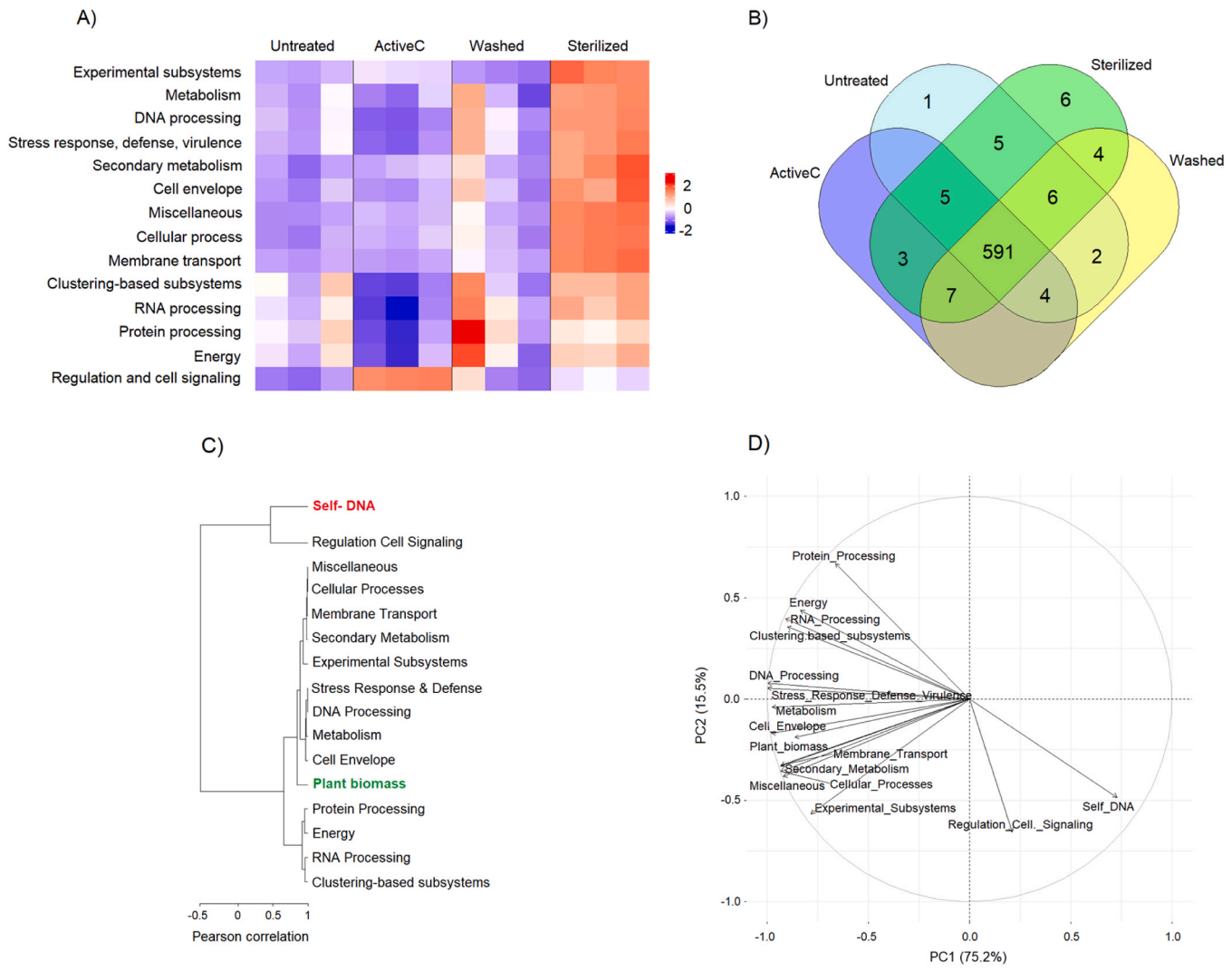

Fig. 7. (A) Heatmap showing log-transformed relative abundance of genes sub-categories (level 1) based on KEGG pathways in different soil treatments. (B) Venn diagram showing the genes that were shared or not shared by the different studied treatments. (C) Dendrogram based on Pearson correlation and (D) Principal component analysis (PCA) of different genes sub-categories (level 1) based on KEGG pathways with self-DNA and total biomass of *A. thaliana*.

environment. We found that washed soil contained fewer Proteobacteria and fewer Firmicutes compared to sterilized soil. These results could explain the difference in observed growth between sterilized and washed soil. Similarly, several previous studies have shown that washing soil drastically affects the development of the microbiome in a new environment (Howard et al., 2017).

Our results show that the activated carbon soil had similar levels of bacteria as the conditioned soil, with a high proportion of Actinobacteria. In contrast, previous studies have reported that the content of Actinobacteria in soil is positively correlated with the addition of activated carbon (Jaiswal et al., 2017; Wang et al., 2020). Indeed, activated carbon has shown the potential ability to suppress plant diseases (Elmer and Pignatello, 2011) and increase plant productivity (Kolton et al., 2016). However, our results show that the addition of activated carbon did not contribute to plant recovery. In contrast to our observations, Wang et al. (2020) showed that the application of activated carbon mitigated the negative PSF in a Sanqi (*Panax notoginseng*) production system, suggesting that this effect is dependent on more complex interactions depending on the plant-soil system.

In general, evidence that soil-borne pathogens are consistently isolated from symptomatic plants has been taken as apparent support for the pathogenicity hypothesis of negative PSF. However, our results clearly showed that pathogens were also found in soils where growth was boosted. For example, *Colletotrichum higginsianum*, a known plant pathogen causing anthracnose disease in *A. thaliana* (Yan et al., 2018), was unexpectedly found most abundant in all treated soils. Moreover, other relevant soilborne pathogens were detected in differently treated soils, e.g., *Fusarium oxysporum* and *Cercospora beticola* in the ActiveC treatment, *F. venenatum* and *F. fujikuroi* in the washed soil, while *Botrytis cinerea* was most abundant in the sterilized soil. Thus, we could not establish a clear relationship between pathogen incidence and plant performance, as we observed significant biomass production in the presence of pathogens. The relevant point to be underlined here is that the presence of a known pathogen species is not associated with its effective virulence under the specific conditions.

Recent studies have suggested that the net effect of PSF must be related to the balance between beneficial and harmful microbes (van der Putten et al., 2016; Hannula et al., 2017). Notably, the type of mycorrhizal association with a plant species has been reported to explain the variation in negative PSF, with trees with arbuscular mycorrhizal suffering stronger negative effects than trees with ectomycorrhizal associations (Bennett et al., 2017). In this work, we used a model plant that does not establish symbiotic relationships with mycorrhizal fungi. However, we found several beneficial bacteria such as *Georhizobium profundi*, the denitrifying *Pseudomonas stutzeri*, *Streptomyces fradiae* and *Microvirga ossetica* in the different soils, with no evidence of a net

outcome for *A. thaliana* growth, which depends on antagonistic and synergistic interactions within the soil microbiome, with the bacteria being dominant in the soil compared to the fungi.

The discovery of the inhibitory effect of extracellular self-DNA in plants by Mazzoleni et al. (2015) opened new perspectives for understanding the autotoxicity of litter and negative PSFs. These early results and subsequent work on the model plant *A. thaliana* (Chiusano et al., 2021; Lanzotti et al., 2022) provided significant evidence that fragmented extracellular self-DNA has species-specific inhibitory effects on plant growth. Here, we quantified for the first time the amount of accumulated extracellular self-DNA of *A. thaliana* directly from the soil after a conditioning phase. Such an accumulation of DNA molecules may be caused by the degradation of *A. thaliana* residues (Nielsen et al., 2007). Correlation analysis between plant biomass and soil variables showed a significant negative association with self-DNA, suggesting a functional relationship with the species-specific negative PSF. We observed that sterilized and washed soils had the highest growth and lowest concentration of self-DNA, while conditioned soils and activated carbon treated soils had the lowest growth and highest concentration of *A. thaliana* self-DNA. The persistence of extracellular DNA in the soil environment depends on various chemical, physical and biological conditions and processes. DNA persists in soil by adsorption to soil minerals, humic substances and organo-mineral complexes (Levy-Booth et al., 2007). Once DNA is bound to these particles, it is physically protected from degradation by microbial DNases and nucleases, enabling its long-term persistence over years (Agnelli et al., 2007; Nielsen et al., 2007). Such persistence ability combined with a specific inhibitory function is highly consistent with the observed long-lasting negative PSFs in soils in both natural and agricultural ecosystems (Miller, 1996; Hawkes et al., 2013). Sterilization of soils has been reported to reduce negative PSFs (Packer and Clay, 2000; Klironomos, 2002; Kardol et al., 2007). Sterilization methods such as autoclaving are also known to affect DNA molecules (Gefrides et al., 2010) by reducing their overall amount by fragmentation, degradation and denaturation (Maity et al., 2009; López-Andreo et al., 2012).

In flowing waters, i.e., streams, exDNA is washed out (Bonanomi et al., 2022) and can be transported over long distances to a considerable extent (Jerde et al., 2016). Moreover, a previous study has shown that environmental DNA concentration decreases by 16% per hour under tap water (Maruyama et al., 2014). Furthermore, extracellular DNA in aquatic environments can sometimes also be degraded rapidly, i.e., within minutes, by hydrolysis, oxidation and microbial activity (Lindahl, 1993; Torti et al., 2015). Conversely, recent studies have shown that the presence of activated carbon protects extracellular DNA from degradation by DNase I (Fang et al., 2021). Indeed, there is evidence that activated carbon has a high adsorption affinity for DNA, which increases its persistence in soil (Wang et al., 2014; Fang et al., 2021; Calderón-Franco et al., 2021). These known effects on freely circulating environmental DNA are very consistent with our observations that DNA in soil is either removed or stabilized by washing or active carbon treatments, respectively.

We believe that the weakening of the plant exposed to its extracellular self-DNA increases its susceptibility to pathogens attack. Chiusano et al. (2021) showed that exposure of *A. thaliana* to its self-DNA limits root cell permeability, impairs chloroplast function and increases the accumulation of reactive oxygen species, leading to a general inhibition of metabolism and cell cycle arrest. Recently, Lanzotti et al. (2022) demonstrated in the same plant model that the self-DNA inhibition is reflected by a significant change of metabolomic profile corresponding to a strong reduction of cellular RNA turnover. In particular, the accumulation of various RNA components such as nucleobases, ribonucleosides, dinucleotide and trinucleotide oligomers, including their cyclic and methylated forms, in the cells of the inhibited plants shows a specific response to self-DNA exposure, which could represent a predisposing condition for increased pathogen attack.

Bonanomi et al. (2022) have shown for the first time under field conditions that self-DNA, but not heterologous one, exerts acute toxic effects on the roots of *Alnus glutinosa* L. in field conditions. In this study, we succeeded in quantifying extracellular self-DNA directly from soil, following the approach shown by Foscari et al. (2022). This made it possible to significantly attribute the observed negative PSF to the concentration of plant self-DNA in the soil. On the one hand, these results, combined with the previous evidence that self-DNA is only toxic to conspecific and, on the other hand, the lack of consistent evidence that the occurrence of specific pathogens can only be linked to negative PSF conditions, suggest that the inhibitory effects of self-DNA should be considered as the triggering and driving mechanism explaining the species-specific negative PSF, a phenomenon in which the observed changes in the microbiome should be considered as consequences rather than causal factors.

5. Conclusion

This research sheds light on the multifaceted mechanisms underlying PSF. It emphasizes the importance of considering a wide range of factors, including soil chemistry, microbial communities and extracellular self-DNA, to understand the dynamics of plant-soil interactions. These findings contribute to our evolving understanding of the complex web of relationships in terrestrial ecosystems and emphasize the need for further investigations into the role of self-DNA and its impacts on plant growth and community dynamics.

CRediT authorship contribution statement

Zotti Maurizio: Writing – review & editing, Visualization, Software, Data curation. **Foscari Alessandro:** Writing – review & editing, Methodology, Investigation, Data curation. **Fechtali Taoufiq:** Writing – review & editing, Visualization, Validation, Conceptualization. **Abd-ElGawad Ahmed M.:** Writing – review & editing, Visualization, Validation, Investigation. **Mazzoleni Stefano:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization. **Incerti Guido:** Writing – review & editing, Visualization, Investigation, Data curation. **Idbella Mohamed:** Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **De Filippis Francesca:** Writing – review & editing, Validation, Software, Data curation. **Bonanomi Giuliano:** Writing – original draft, Methodology, Investigation, Conceptualization.

Data availability

I have shared the links and the accession number of the deposited data in the manuscript.

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Declarations of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2024.127634.

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