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Seasonal dynamics of kiwifruit microbiome: A case study in a KVDS-affected orchard

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ABSTRACT

Over the past decade, Italian kiwifruit orchards and overall production have faced a significant threat from Kiwifruit Vine Decline Syndrome (KVDS). Despite the insights gained from metagenomics studies into the microbial communities associated with the disease, unanswered questions still remain. In this study, the evolution of bacterial, fungal, and oomycetes communities in soil and root endosphere at three different time points during the vegetative season was investigated for the first time in a KVDS-affected orchard in the Lazio Region. The fungal and oomycetes genera previously associated with the syndrome, including *Fusarium, Ilyonectria, Thelonectria, Phytophthora, Pythium* and *Globisporangium*, were identified in both groups. In contrast, the characterization of bacterial communities revealed the first instance of the presence of the genus *Ralstonia* in soil and root samples. The microbiome composition shifts between KVDS-affected and asymptomatic plants were significant as evidenced by the results, particularly after a temperature increase. This temperature change coincided with the onset of severe disease symptoms and may indicate a key role in the progression of KVDS.

1. Introduction

Actinidia chinensis var. *deliciosa* is a deciduous vine belonging to the family *Actinidiaceae*, renowned for producing the commercially popular kiwifruit. Native to the temperate forests of China, the kiwifruit was introduced to New Zealand in the early 20th century. Today, with an overall harvested production of 435,696 tons per year, Italy is one of the largest global kiwifruit producers, accounting for 15 % of the world's demand (ISTAT, 2024). However, since 2012 the annual production has been strongly reduced due to the spread of a novel and complex disease known as Kiwifruit Vine Decline Syndrome (KVDS) (Tacconi et al., 2015). Italian kiwifruit production underwent a notable decline from 521,534,000 tons in 2020–391,000 tons in 2023, with a corresponding decrease in cultivated area from 26,690 ha to 25,116 ha in 2023 (ISTAT, 2024).

Typical KVDS symptoms arise from root system damage, characterized by browning, cortical layer detachment, and loss of feeder roots (Tacconi et al., 2014). Root cortical layers exhibit hypertrophy, followed by phloem detachment from the central cylinder, leading to cortical breakdown (Donati et al., 2020). KVDS-affected plants exhibit severe canopy symptoms, including leaf wilting, overall plant decline, and a tendency to collapse, particularly during summer months (Savian et al., 2020). As a consequence of root damage, canopy symptoms often manifest later, when the plant's overall health is significantly compromised. Since 2012, initial observations of KVDS in North Italy (Veneto and Friuli-Venezia-Giulia) indicated a potential plant response to a particularly wet season and anomalous temperature trends (Tacconi et al., 2014). Nevertheless, analogous symptoms were subsequently documented in other regions dedicated to the cultivation of kiwifruit, including Piedmont, Emilia-Romagna, Latium, and Calabria. Approximately 34.5 % of Italian kiwifruit orchards have been affected by this syndrome (Ermacora and Saro, 2020).

Several agronomic and microbiological studies have been conducted to investigate the potential causative factors of KVDS. It has become evident that abiotic stressors, including waterlogging (Tacconi et al., 2015; Tosi et al., 2015; Sorrenti et al., 2016), elevated soil temperature, the use of contaminated irrigation water, and heat stress, play a significant role in the dissemination of the syndrome (Reid et al., 1991; Savian et al., 2019; Bardi et al., 2020). Tacconi et al. (2015) initially proposed the involvement of biotic stressors, a hypothesis that has since been corroborated by other authors (Donati et al., 2020; Spigaglia et al., 2020; Prencipe et al., 2020). Several microbiological studies have highlighted the involvement of the plant microbiota in the multifaced KVDS syndrome spread. Many soil-borne pathogens have been

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associated with the syndrome, including oomycetes such as *Phytophthora* spp., *Pythium* spp. and *Phytopythium* spp., along with fungi from the genera *Desarmillaria*, *Ilyonectria*, *Fusarium* and *Cylindrocarpon* (Tacconi et al., 2015; Donati et al., 2020; Savian et al., 2022; Prencipe et al., 2023, Mian et al., 2023). However, the characterization of bacterial communities remains relatively understudied. To date, only two species of *Clostridium* (*C. bifermentas* and *C. subterminale*) have been associated with the syndrome (Spigaglia et al., 2020) while Donati et al., (2020) reported that no pathogenic bacteria have been isolated from roots of KVDS-affected plants.

This study aims to characterize the bacterial, fungal, and oomycetes communities inhabiting the rhizosphere of plants affected by the syndrome, in comparison with asymptomatic samples. Root and soil samples were collected at three different time-points throughout the season: during vegetative renewal in May, during flowering in June, and at fruit set in July. To the best of our knowledge, this is a pivotal comprehensive investigation of microbial composition and biodiversity within the same field conducted over a single year.

2. Material and methods

2.1. Experimental site

Samples were collected from *Actinidia chinensis* var. *deliciosa* "BOERICA®" plants cultivated in an orchard located in Cisterna di Latina (Lazio Region, Center Italy, 41°33'34.0344''N, 12°47'32.7948''E). The orchard is planted on soil with a high clay content and a low percentage of organic matter, which are known to have an adverse effect on the composition of microbial communities, as well as the structural properties and fertility of the soil (Khanghahi et al., 2019; Dincă et al., 2022). More detailed physical and chemical soil analyses are reported in Table S1. Despite the soil's homogeneous composition, the plantation can be divided into two distinct areas: Area A, characterized by a higher prevalence of KVDS-affected plants, and Area B, where the majority of the plants, at the time of sampling and in the following monitoring activities, exhibit no symptoms on the canopy and roots that can be attributed to KVDS and are thus called asymptomatic (Fig S1).

2.2. Soil and root sampling

In 2022, nine asymptomatic and nine KVDS-symptomatic plants were chosen for the study according to a symptomatology scale shown in Fig S2. Sampling was carried out at three different time-points starting at the end of the vegetative renewal in May, at the end of flowering in June, and at the fruit set in July. From each plant, three soil cores (about 300 g) were drilled using a hand auger at a depth of 30–35 cm, homogenized, and pooled to form a composite sample per vine. At the same time, three root samples from three adjacent plants were collected and pooled together to create a bulk. Thus, three bulk of KVDS soil samples and three bulk of asymptomatic soil samples, together with three bulk of KVDS root samples and three bulk of asymptomatic roots were collected at each of the three time points, for a total of 36 samples.

Soil samples were dried at room temperature and sifted through 4 mm and 2 mm sieves, placed into sterile tubes, and stored at −20°C until DNA extraction. Roots were transferred to sterile tubes, washed with 0.8 % NaCl, vortexed for 1 minute, and then washed again with 1 % sodium hypochlorite and 0.01 % Tween 80. After a final rinse with sterile water, the roots were transferred to new sterile tubes and stored at −20°C until DNA extraction.

2.3. DNA extraction from soil

DNA was extracted from soil using an optimized protocol based on the methods of Zhou et al. (1996), Yeates et al. (1998), and Burgmann et al. (2001). Briefly, 5 gr of soil were mixed with 13.5 mL of extraction buffer [100 mM Tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1 % CTAB] and 100 µl of proteinase K (10 mg/mL) in 50 mL tubes and incubated for 40 min at 37 °C by shaking at 225 rpm. A solution of 20 % SDS (1.5 mL) was added, and the tubes were incubated in a water bath at 65 °C for 2 hours and inverted every 20 minutes. After centrifugation, the supernatant was transferred to new sterile tubes. The pellet was further extracted twice with extraction buffer and SDS, incubated at 65°C, and centrifuged. The supernatant was mixed with an equal volume of chloroform/isoamyl alcohol (24:1), and centrifuged at 7500 g for 30 minutes at 4°C. The aqueous phase was recovered, and 0.6 vol of isopropanol was added and incubated for 1 hour at room temperature. After centrifugation at 9000 g for 20 min at 4 °C, the pellet was washed with 70 % cold ethanol, centrifuged, and resuspended in 500 µl of nuclease-free water. DNA concentration was measured with Qubit™ dsDNA BR Assay Kit.

2.4. DNA extraction from roots

Root samples were grinded with mortar and pestle in liquid nitrogen, transferred in 50 mL sterile tubes, and incubated with 5 mL of lysis buffer [CTAB 2 % (w/v), PVP40 2 % (w/v), Tris-HCl (pH 8) 100 mM, EDTA 25 mM, NaCl 2 M] at 60°C for 30 min, inverting every 10 min. From this solution, 1 mL was transferred into 2 mL tubes and mixed with one volume of chloroform/isoamyl alcohol (24:1) and the tubes were centrifuged at 7000 g for 10 min at 4°C. After repeating the previous step, the supernatant was transferred into a new tube, and 1/10 vol of sodium acetate was added. The tubes were incubated at −20°C for 90 min and then centrifuged at 9000 g for 20 minutes at 4°C. The supernatant was discarded, and the pellet was washed with 70 % cold ethanol and centrifuged for 15 min at 4°C. The pellet was air-dried under the hood and then eluted in 50 µL of nuclease-free water. DNA concentration was measured using a Qubit™ dsDNA BR Assay Kit.

2.5. Amplification of 16S and ITS and sequencing

Amplification, library preparation and Illumina NovaSeq sequencing were carried out by Novogene (Cambridge, UK). Briefly, all PCR reactions were carried out with 15 µL of Phusion® High - Fidelity PCR Master Mix (New England Biolabs), 0.2 µM of forward and reverse primers, and 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98℃ for 10 s, annealing at 50℃ for 30 s, and elongation at 72℃ for 30 s and 72℃ for 5 min. For bacteria, primers 799 F (5'-AACMGGATTAGATACCCKG–3') and 1193 R (5'- ACGTCATCCCCACCTTCC-3') were selected for sequencing of the hypervariable V5-V7 bacterial 16S rRNA region. For fungi and oomycetes, primers ITS5–1737F (5'- GGAAGTAAAAGTCGTAACAAGG-3') and ITS2–2043R (5'- GCTGCGTTCTTCATCGATGC-3') of ITS1 fungal Internal Transcribed Spacer (ITS) gene were used. Sequencing libraries were generated, and indexes were added. The library was checked with Qubit™ and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on Illumina platforms, according to effective library concentration and data amount required.

2.6. Bioinformatic analysis

The microbiome characterization was performed following the Quantitative Insight into Microbial Ecology 2 (QIIME2 v.2023.2.0, Bolyen et al., 2019) pipeline, as previously adjusted by Turco et al., (2024a). The raw merged reads (R1 and R2) were demultiplexed, chimeras were removed, and denoised with the Divisive Amplicon Denoising Algorithm (DADA2), to get the feature table with the Amplicon Sequence Variants (ASVs) and the representative sequences in FASTA format.

Fungal taxonomic classification of the ASVs was performed using the classify-consensus-blast algorithm and an *in-house* database built with

the rescript QIIME2 plugin using the *Actinidia chinensis* sequences (taxID 3625), the NCBI Fungi RefSeq ITS project (PRJNA177353), and the oomycetes sequences present in the International Nucleotide Sequence Databases (www.insdc.org). Bacterial taxonomic classification of the obtained ASVs was performed using the *classify-consensus-blast* algorithm towards the SILVA 138 database (Quast et al., 2013). A phylogenetic tree was built on the bacterial representative sequences using MAFFT and FastTree implemented within the QIIME2 pipeline.

After removal of host-related sequences, including chloroplast and mitochondria, uncharacterized and uncultured sequences as well as low-frequency ASVs according to Bokulich et al. (2013), the four main input files (feature table, taxonomy, representative sequences, and phylogenetic tree) were imported within the R environment v.4.2.3 and further processed with the *phyloseq* package v1.42.0 (McMurdie and Holmes, 2013). The rarefaction curves were obtained with the *vegan* R package v. 2.6 (Dixon, 2003), diversity within samples (alpha diversity) was evaluated with Shannon and Simpson indexes, while richness was evaluated with Chao1 and ACE indexes. Differences in alpha diversity among factors were determined using a Kruskal-Wallis test. The dissimilarity between the samples (beta diversity) was evaluated through Non-metric Multidimensional Scaling (NMDS) and Principal Coordinate Analysis (PCoA) plots based on the Bray-Curtis index. Statistical testing of beta diversity was performed with the permutational multivariate analysis of variance (PERMANOVA, 1000 permutations). Relative microbial abundance was plotted for the 30 most abundant genera, with the least abundant collapsed as "< 1 %". Bacterial ASVs functional classification was performed using *picrust2* plugin within QIIME2, the *ggpicrust2* R package (Douglas et al., 2020; Yang et al., 2023) using DESeq2 for the differential analysis, KEGG Orthology (KO) for pathway annotation and a P value set at 0.05. Functional guilds of the identified fungal ASVs were assigned using the *fungaltraits* v.0.0.3 package within the R environment v.4.2.3. This package leverages information from both the FunGuild and FunFun databases for functional guild assignment (Turco et al., 2024a).

3. Results

3.1. Amplicon sequencing and denoising results

A total of 6.03 M reads were obtained from the Illumina sequencing. After applying DADA2 filtering and denoising, 41,400 reads were assigned to Amplicon Sequence Variants (ASVs). The average of ASVs per sample was 2612, with a range from 1790 to 3518 for the 16S sequencing and 845 ASVs for the ITS, with a range from 559 to 1178 per sample (Table S2). After removal of chloroplast, mitochondria, uncultured and unassigned ASVs, 572 bacterial taxa, 218 fungal taxa and 111 oomycetes taxa were imported within R as a *phyloseq* object and further processed.

3.2. Microbial communities' richness and diversity

The bacterial community richness, expressed by Chao1 and ACE indexes, resulted to be comparable between the two groups, asymptomatic and KVDS samples (Fig. 1A), as well as between soil and root samples (Fig S3A). Conversely, during the vegetative stages, the richness was higher during flowering (second stage) and at fruit set (third stage), compared to the vegetative renewal (first stage), which exhibited the lowest value (Fig. 2A). No statistically significant differences were identified. A similar trend was observed for alpha diversity, as measured by Shannon and Simpson indices. While asymptomatic and KVDS plants exhibited similar heterogeneity, asymptomatic plants showed slightly higher variability (Fig. 1A). Roots exhibited higher diversity than soil samples (Fig S3A). Among the three time points, diversity was highest during flowering, followed by fruit set and vegetative renewal (Fig. 2A). Again, no significant differences were identified.

In contrast to the bacterial communities, fungal richness was higher in the KVDS samples, particularly as measured by the Chao1 index (Fig. 1B). Significant differences were observed among sample types, with soil exhibiting the highest Chao1 and ACE values (Fig S3B). Indices related to the vegetative stages demonstrated a decline in richness during fruit set compared to the other stages (Fig. 2B). No statistically significant differences were identified. Asymptomatic samples exhibited a lower within-sample diversity compared to the KVDS ones (Fig. 1B), but no significant differences were identified. Instead, significant differences were observed between soil and root samples regarding the Chao1 and ACE indexes, with soil samples displaying higher richness (Fig S3B).

During the vegetative season, both communities exhibited a decline in diversity, with values decreasing from their peak during vegetative renewal to a lower level during fruit set (Fig. 2B).

3.3. Microbial communities' dissimilarities

The beta diversity between the samples was evaluated through NMDS and PCoA analysis based on Bray-Curtis distance (Fig. 3). Significant differences were observed in the community structures and compositions, particularly concerning the sample type (roots and soil) and phenological stage (vegetative renewal, flowering, and fruit set).

For the bacterial communities, there was a clear microbiota distinction among samples collected during the three vegetative stages, both in soil and root samples (Fig. 3, panels A and B). The PERMANOVA supported the NMDS results, indicating a significant association between community similarity and sample type ($R^2 = 0.178$; $P < 0.05$) and phenological stage ($R^2 = 0.258$; $P < 0.05$) (Table S3).

Concerning fungal communities, the phenological stages have also exerted an influence on the structure of these communities (Fig. 3, panels C and D). The data were corroborated by the results of the PERMANOVA, which demonstrated a significant effect of phenological stage ($R^2 = 0.379$; $P < 0.01$) on fungal communities (Table S4).

No significant effects were observed in oomycete communities across different variables, as indicated by NMDS analysis (Fig. 3, panels E and F) and confirmed by PERMANOVA analysis (Table S5).

3.4. Soil and roots bacterial communities

Overall, at the phylum level, the bacterial communities were predominantly composed of Proteobacteria (50 %), followed by Actinobacteriota (35 %), Firmicutes (10 %), and Bacteroidota (5 %). The most abundant genera were *Ralstonia*, *Pseudomonas*, *Streptomyces*, *Methylobacterium*, *Bacillus*, and TRA3–20. Interestingly, microbiome composition changed during the vegetative season in both KVDS and asymptomatic samples (Fig. 4).

At the end of vegetative renewal, *Ralstonia* spp. was the most abundant genus in KVDS root samples (64 %), followed by *Ensifer* spp. (9 %), *Pseudomonas* spp. (2.8 %), and *Bacillus* spp. (2.7 %). It is noteworthy that the relative abundance of *Bacillus* spp. increased significantly during the flowering time-point (10 %). Additionally, bacteria belonging to the *Nitrosomonadaceae* family (7 %), as well as the genera *Pseudomonas* spp. (5.4 %) and *Sphingomonas* spp. (5 %), were present. During the fruit set, the most common genera were *Streptomyces* spp. (25.5 %), *Bacillus* spp. (13.4 %), and *Rhizobium* spp. (6.6 %).

In asymptomatic root samples, *Pseudomonas* spp. (17.4 %), *Bacillus* spp. (8 %), and *Flavobacterium* spp. (5 %) were the most abundant genera found during the first vegetative stage. Furthermore, at the time-points of flowering and fruit set, *Bacillus* spp. was the most common genus, accounting for 13 % and 43 % respectively. Other notable bacteria at the flowering time-point were from the *Nitrosomonadaceae* family (6.5 %), as well as *Sphingomonas* spp. (5.9 %) and *Pseudomonas* spp. (5 %) genera. Fruit set samples exhibit a distinct abundance of *Streptomyces* spp. (14 %) and *Glycomyces* spp. (9 %) genera.

The relative abundance of the soil microbiome exhibited notable differences when compared to that of the root samples. Here, at the end of vegetative renewal, KVDS samples showed a high abundance of

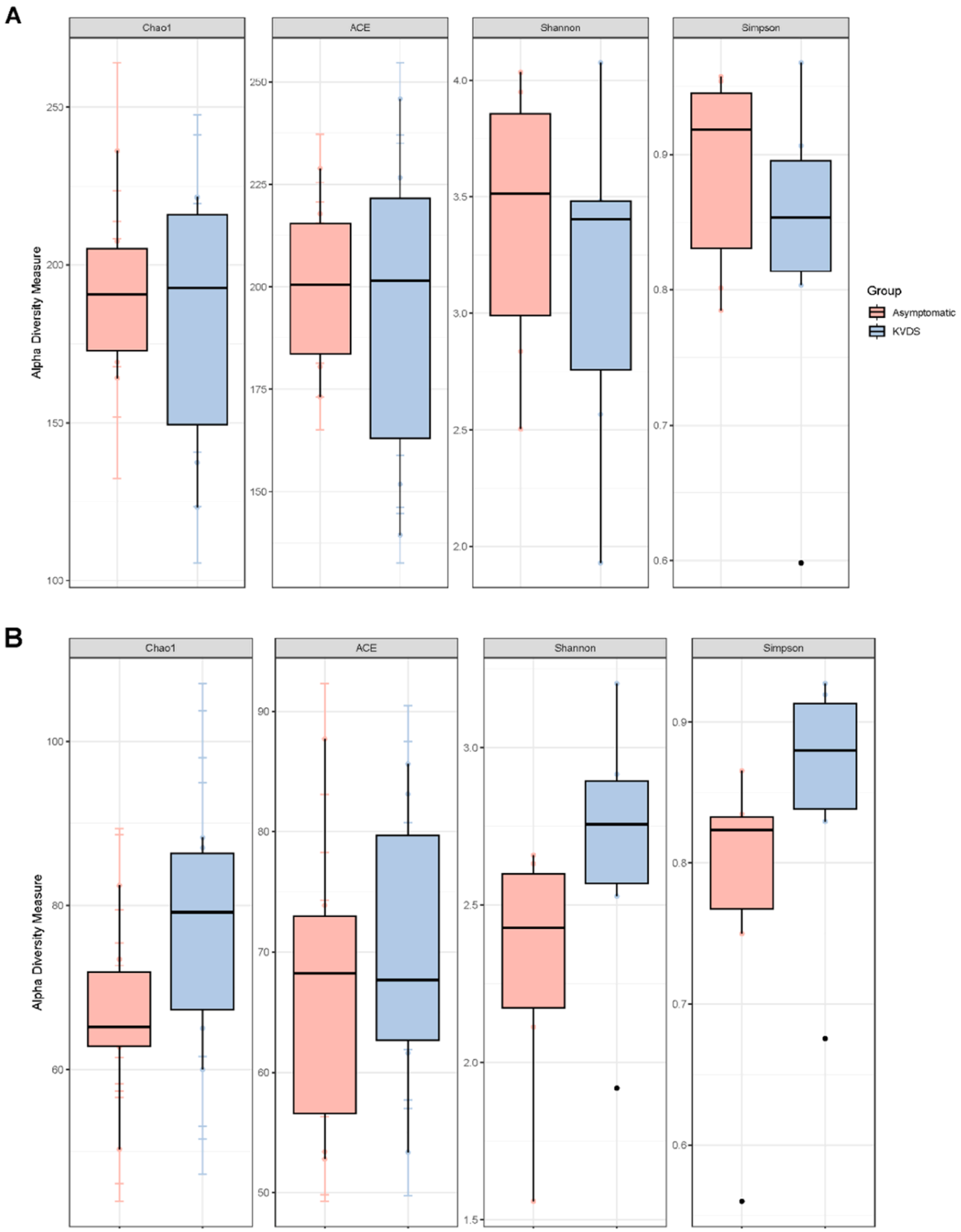

Fig. 1. Alpha-diversity of bacterial (A) and fungal (B) communities in asymptomatic and KVDS samples. Alpha diversity metrics summarize the structure of the microbial community concerning its richness (Chao1 and ACE indexes), and within-sample diversity expressed by Shannon and Simpson indexes. No significant differences ($p < 0.05$) were observed in both communities for all indexes.

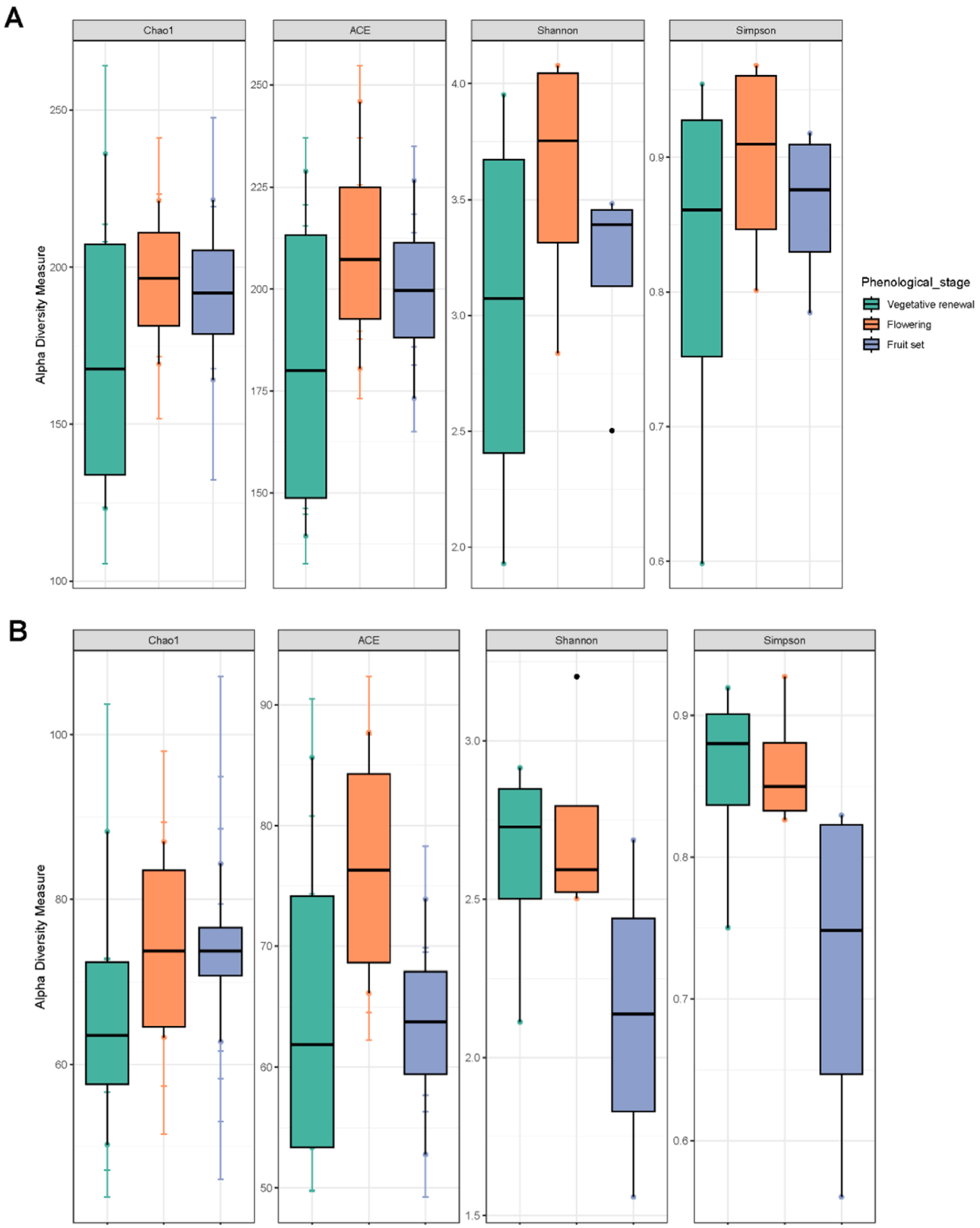

Fig. 2. Alpha-diversity of bacterial (A) and fungal (B) communities during the vegetative season: vegetative renewal (green), flowering (orange), and fruit set (purple). No significant differences ($p < 0.05$) were observed in both communities for all indexes.

Methylobacterium spp. (30 %) and *Pseudomonas* spp. (30 %), followed by *Ralstonia* spp. (7.5 %), and *Flavobacterium* spp. (4.6 %). In asymptomatic samples, *Pseudomonas* spp. was the most abundant genus (21 %), followed by *Flavobacterium* spp. (14.4 %), *Burkholderia* sp. (6.2 %), *Ralstonia* spp. (5.7 %), and *Bacillus* spp. (4 %). During flowering in the asymptomatic samples, there was a significant increase in the relative abundance of *Ralstonia* spp. (41 %), compared to KVDS samples (1.3 %). Asymptomatic samples also exhibited a high relative abundance of

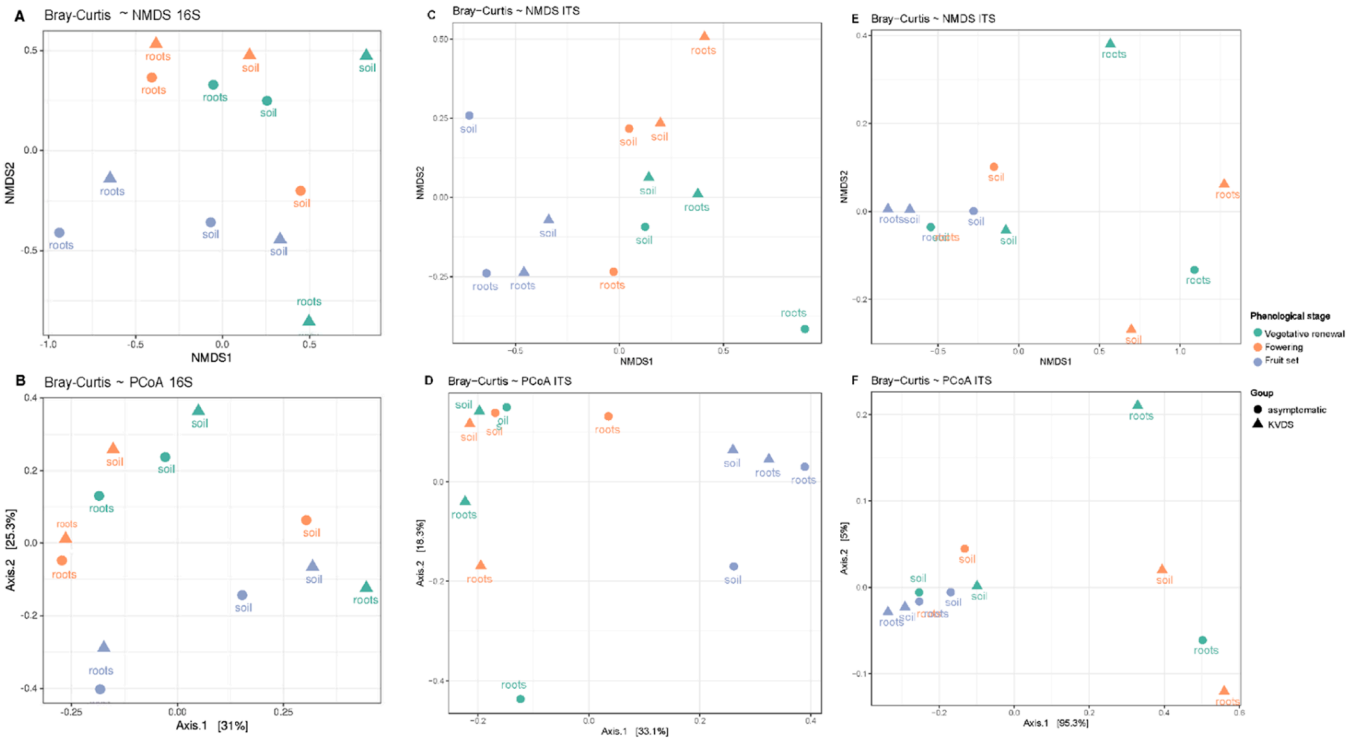

Fig. 3. Non-metric Multidimensional Scaling (NMDS) and Principal Coordinate Analysis (PCoA) plots based on Bray-Curtis similarity of ASV-based bacterial (A-B), fungal (C-D) and oomycete (E-F) communities structure in asymptomatic (circle) and KVDS (triangle) samples in different phenological stages (green = vegetative renewal, orange = flowering, blue = fruit set).

Pseudomonas spp. (13 %), and *Bacillus* spp. (5 %). The most abundant genus found in KVDS samples was *Pseudomonas* (37.5 %), followed by *Bacillus* spp. (4.7 %), *Sphingomonas* spp. (3.4 %), TRA3–20 (3.2 %), and *Flavobacterium* spp. (2.6 %).

Ralstonia spp. was the most abundant genus in KVDS samples at the last time point – fruit set - with a percentage of 36.3 %, followed by *Streptomyces* spp. and *Pseudomonas* spp. (5.2 % and 4 % respectively). Furthermore, the microbiome consists of *Sphingomonas* spp. (3.7 %), and *Bacillus* spp. (2.7 %). Asymptomatic samples exhibited a higher number of abundant genera compared to KVDS samples. The *Ralstonia* genus was the most abundant even in asymptomatic samples, accounting for 20.3 %. Additionally, *Bacillus* spp. (10.7 %), *Micromonospora* (9 %), *Streptomyces* spp. (8.7 %), *Pseudomonas* spp. (8.1 %), and *Sphingomonas* spp. (2.7 %) were also abundant.

The distribution of bacterial members within the different groups (asymptomatic and KVDS) and sample types (roots and soil) was deeply investigated and represented by a Venn diagram (Fig S5). A core of 279 ASVs (48.8 % of the total) was reported between soil and root samples in both groups. The root core microbiota consisted of 33 ASVs (5.8 %), predominantly represented by *Nitrosomonas*, *Solimonas*, *Clostridium*, *Exiguobacterium*, *Luteimonas*, and *Terrisporobacter*. The KVDS root sample showed a total of 12 unique ASVs (*Actinomyces, Bacteroides, Polyangium, Coprococcus, Xanthobacter, Ciceribacter, Eubacterium, Luedemannella, Agathobacter, Lachnospiraceae*, and two genera belonging to the *Sphingobacteriales* and *Lachonospirlaes* orders). *Halobacillus, Anaerococcus*, and *Caenimonas* were the most representative of the 9 unique ASVs of asymptomatic root samples. *Haemophilus, Mogibacterium, Psuchoroglaciecola, Pirellula, Kytococcus, Acetitomaculum, Aurantimonas, Thermoactinomyces,* and *Fusobacterium* were the most representative genera of soil core microbiota (60; 10.5 %). *Friedmanniella, Rhodobacter, Martelella*, and AD3 were found in KVDS soil, but not in the asymptomatic ones.

3.5. Functional analysis of bacterial communities

In silico functional analysis of the most significantly abundant bacterial communities was performed with *picrust2* through a KEGG annotation (Fig S7). In total, 30 metabolic functions were identified and correlated with ASVs relative abundance values. The results showed that the highest relative abundance of ASVs related to KVDS samples belonged to a family of transcription factors (DtxR) associated with the management of manganese transport, followed by enzymes involved in cysteine and methionine metabolism, specifically (2 R)-3-sulfolactate dehydrogenase (NADP+), and methane metabolism, particularly the subunit alpha and beta of malate-CoA ligase. Two families of transporters, the MFS and the ABC families, were identified as the most abundant. These families contain proteins related to the transport of quinolones, galactorates, and drugs.

3.6. Soil and roots fungal and oomycete communities

The soil and roots fungal communities were predominantly composed of Ascomycota (69.2 %), Basidiomycota (23.8 %), and Mucoromycota (5.5 %). The most prevalent fungal genera in KVDS samples were identified as *Fusarium, Starmerella, Ilyonectria/Thelonectria*, *Neurospora,* and *Mortierella* (Fig. 5). Notable changes in the fungal community profile occurred during the vegetative season, with the soil compartment exhibiting a higher prevalence of different genera than the root endosphere (Fig. 5).

During the vegetative renewal, the most abundant genera in KVDS root samples were *Fusarium* spp. (19.1 %), *Linnemannia* spp. (17.2 %), *Ilyonectria* spp. (11 %), and *Starmerella* spp. (9 %), followed by *Epicoccum* spp. (8.5 %), *Dactylonectria* spp. (6.7 %), *Plectosphaerella* spp. (5 %) and *Alternaria* spp. (4.2 %). *Starmerella* was the most prevalent genus in the flowering stage, accounting for 35.3 % of the total, followed by *Mortierella* spp. (12.3 %), *Fusarium* spp. (10.2 %) and *Alternaria* spp. (9 %). A remarkable increase of *Fusarium* abundance up to 56.7 % was

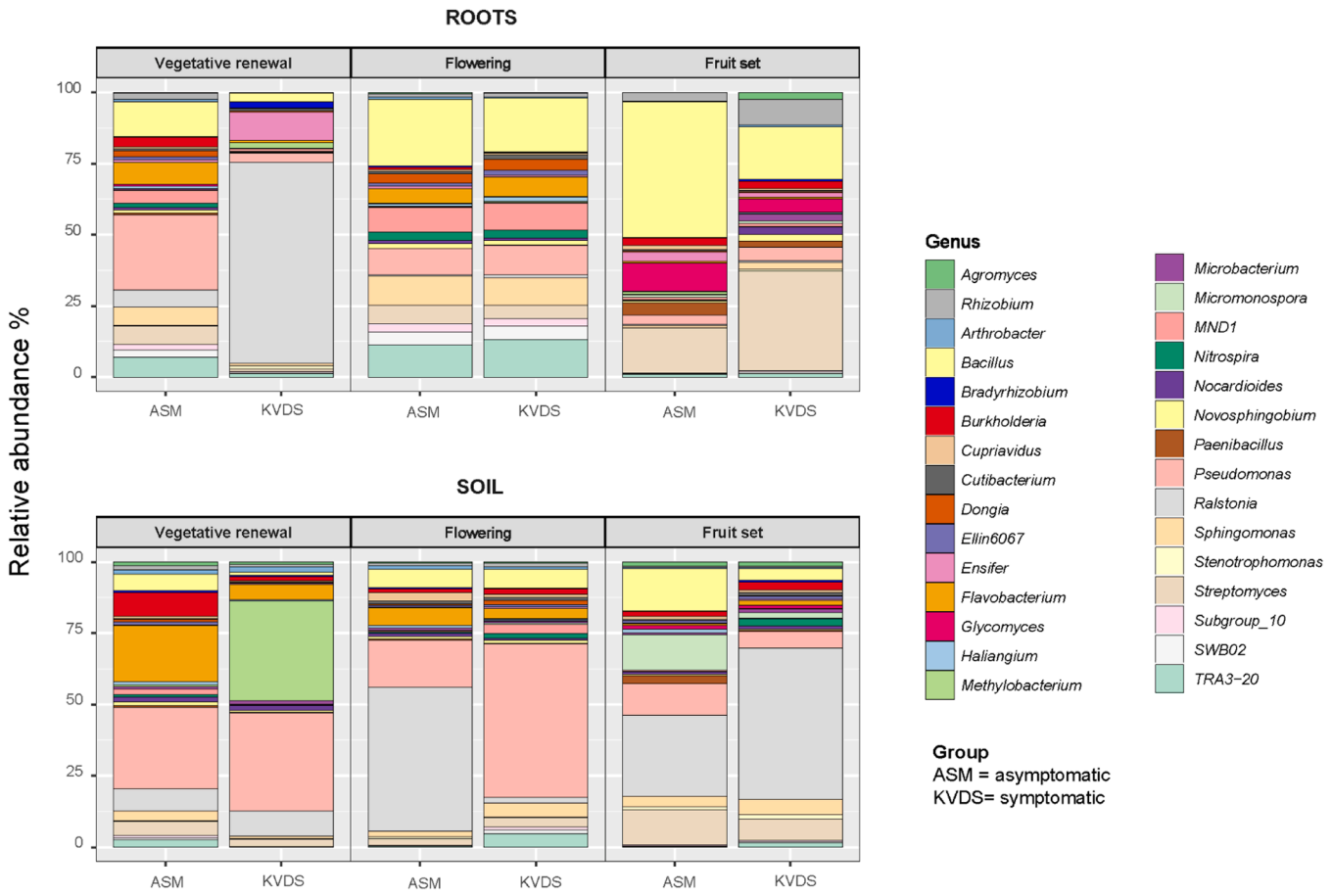

Fig. 4. Taxonomy plots showing the relative abundance of the top 30 genera of bacterial communities in roots and soil of asymptomatic and KVDS samples during the vegetative season.

observed during the fruit set, followed by *Neurospora* (15.4 %) and *Ceratobasidum* (6.6 %) (Fig. 4).

In asymptomatic roots, during the first vegetative stage, the most abundant fungal genus was *Metarhizium* spp., accounting for 46.1 % followed by *Fusarium* spp. (12.5 %), *Boeremia* spp. (10.6 %), and *Dactylonectria* spp. (5.1 %). During the flowering stage, the most abundant genus was *Fusarium* spp. (39.4 %), followed by *Linnemannia* spp. (18.5 %), *Trichoderma* spp. (10.1 %), *Metarhizium* spp. (6.7 %), and *Plectosphaerella* spp. (4.7 %). As for the KVDS root samples, the population of *Fusarium* spp. showed a notable increase up to 67 % in the asymptomatic ones during the fruit set. Moreover, *Coniochaeta* spp. (9.6 %) and *Cephalotrichum* spp. (7 %) were present at this time point.

Both asymptomatic and KVDS soil samples exhibited a comparable composition in the fungal genera, even though the percentages differed between groups and during the vegetative season. *Fusarium* spp. was present at all time points, with a percentage of 45.5 % in KVDS samples during the fruit set and 20.2 % in asymptomatic samples during flowering. At this time-point the presence of *Linnemannia* spp. was detected and accounted for 37.7 % in asymptomatic samples. *Neurospora* was the most abundant in asymptomatic samples during the fruit set (39 %), followed by *Coniochaeta* (11 %), which was also present in KVDS samples (11.8 %).

When looking at the distribution of fungal community members across the various groups (asymptomatic and KVDS) and sample types (roots and soil), a core of 112 ASVs (representing 51.6 % of the total) was identified (Fig S6). The soil core microbiota was composed of 16 ASVs (7.4 % of the total), representing the following fungal genera: *Debaryomyces, Elsinoë, Symmetrospora, Ramicandelaber, Termitomyces, Absidia, Sporocadus, Atractiella, Darksidea, Remersonia, Gongronella, Stagonospora, Entomocorticium, Lepiota, Chlamydocillium, and Botryosphaeria*. Two unique ASVs (*Lobariella* and *Montagnula*) were identified in asymptomatic soil samples, while no unique ASVs were observed in the KVDS samples. A total of 4 ASVs (*Pyricularia, Stachybotrys, Clavulina, and Clonostachys*) were identified in the root microbiota, and no unique ASVs were observed in the KVDS root samples. Conversely, only 3 unique ASVs (*Synnemellisia, Pseudoarthrographis, and Scedosporium*) were detected in the asymptomatic root.

In contrast, the oomycete community was represented by three main genera: *Globisporangium, Phytophthora* and *Pythium*. During the flowering stage, in KVDS samples, the most abundant genus was *Globisporangium*, showing higher abundance in roots (93.7 %) than soil (72.6 %). Overall, the abundance of *Phytophthora* was higher in soil samples than in root samples at all time points, in both groups (asymptomatic and KVDS). The highest prevalence was observed in KVDS samples during the fruit set phase, with a percentage of 96.6 % in roots and 91.1 % in soil, respectively. The genus *Pythium* was present in root samples (ASM, 1.7 %; KVDS, 21.4 %) during the vegetative renewal phase, as well as in soil samples (ASM, 2.5 %; KVDS, 4 %) during the flowering phase. (Fig. 6)

3.7. *In silico analysis of the fungal functional groups*

The functional potential of the identified fungal taxa was assessed using FunGuild, revealing a predominance of plant pathogens belonging to the Ascomycota phylum (Fig S8). The largest group of fungal genera, including *Aspergillus*, *Alternaria*, *Epicoccum*, *Fusarium*, *Ilyonectria*, *Cladosporium*, and *Phaeosphaeria*, have been identified as plant pathogens. Some of these fungal taxa have also been classified as endophytes

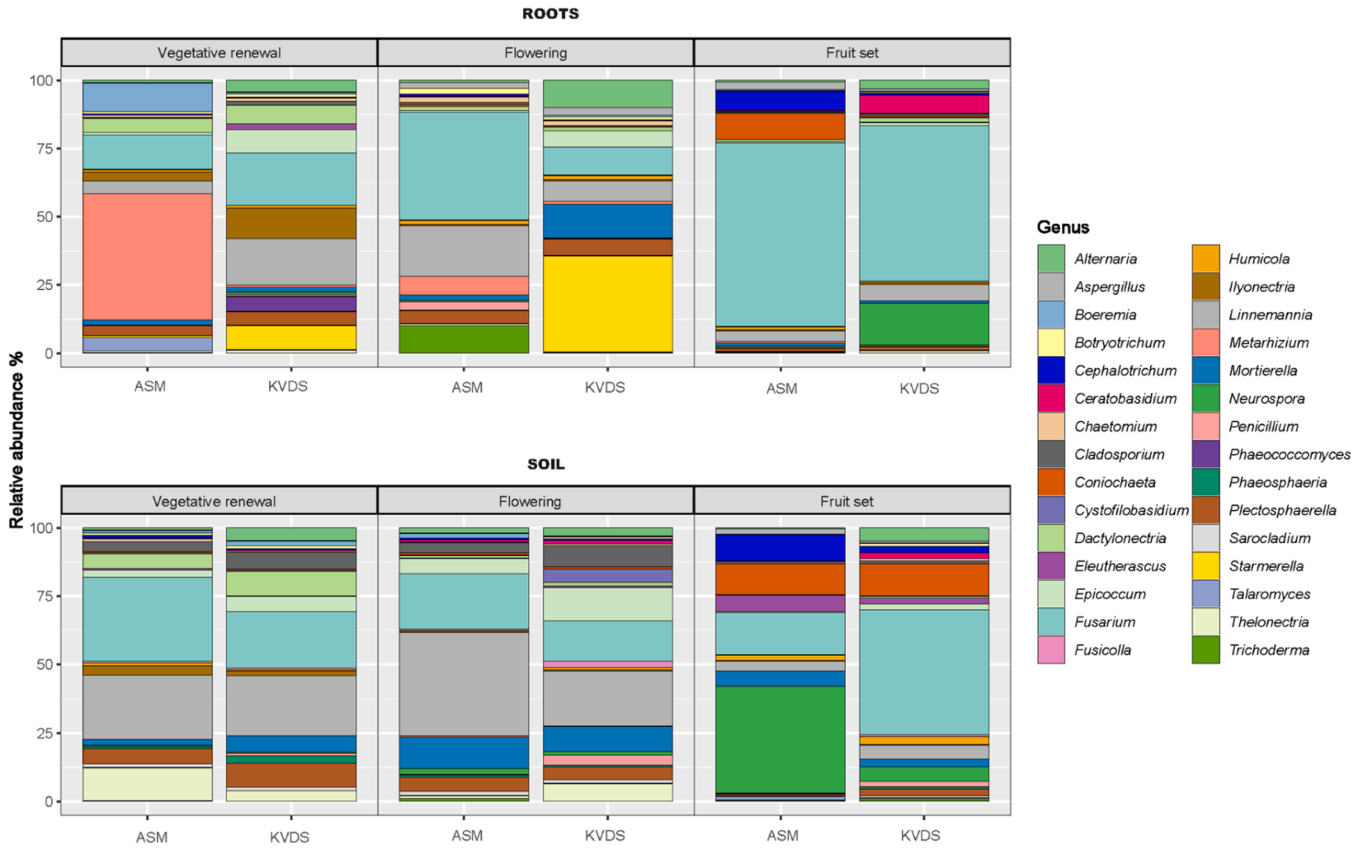

Fig. 5. Taxonomy plots showing the relative abundance of top 30 genera of fungal communities in roots and soil of asymptomatic and KVDS samples during the vegetative season.

(*Epicoccum, Ilyonectria, Cladosporium, Neurospora, Trichoderma*) or wood saprophytes (*Aspergillus, Alternaria, Fusarium*, and *Chaetomium*). *Mortierella* and *Gilbertella* were the two genera of the phylum Zygomycota classified as a saprotroph and plant pathogen, respectively. The Basidiomycota phylum comprises two genera, *Ceratobasidium* and *Cotinarius*, which are classified as ectomycorrhizal.

4. Discussion

The increasing focus on microbiological studies in plant pathology emphasizes the crucial role of plant microbiota in sustaining plant health and fitness. In land plants, roots are the primary site for interactions with microbes in the soil, which represents the largest reservoir of microbial diversity known (Reinhold-Hurek et al., 2015). The so-called endorhizosphere is a complex and dynamic environmental niche, recruited and established through several factors that contribute to its structure and composition, such as pH, nutrient availability, and organic matter, chemical molecules (de Luna et al., 2005; Priya et al., 2021; Pantigoso et al., 2022). This recruitment is also influenced by the plant genotype and directly involved in conferring resistance/tolerance to plant diseases (Berendsen et al., 2012; Du et al., 2024). Perturbation of microbial homeostasis leads to dysbiosis and may have a negative impact on plant fitness (Du et al., 2024). Recent insights into the spread and occurrence of KVDS in Italy suggest that microbial dysbiosis may be a significant driver of the disease. However, it is clear that other factors also contribute to the dissemination and progression of the syndrome. Previous research has characterized the microbial communities associated with KVDS, identifying distinct niches, particularly involving fungi and oomycetes, present across all affected regions (Savian et al., 2021; 2022; Mian et al., 2023; Prencipe et al., 2023; Guaschino et al., 2024; Mosca et al., 2024). The role of bacterial communities in the etiology of the syndrome remains unclear, and to date, they have not been definitively linked to the disease in the same way as fungi and oomycetes.

In this study, the bacterial, fungal, and oomycete communities were characterized in plants exhibiting KVDS symptoms, in comparison to asymptomatic plants, across three different stages of the vegetative season. Our findings suggest that the root microbiome undergoes greater variation throughout the vegetative season than the soil microbiome. This is likely due to the influence of the plant on the rhizosphere environment, which varies according to the physiological stage and health of the plant. It is established that plants play a role in reshaping the microbial communities that inhabit the soil (Santoyo, 2022), and root activities modify microbial activity and the physicochemical properties of the surrounding soil or rhizosphere (Shi et al., 2015).

Overall, *Ralstonia*, *Pseudomonas*, and *Bacillus* were the most prevalent bacterial genera in the core root microbiota. Understanding the dynamics of these bacterial populations throughout the vegetative season is crucial, especially during stages when plants are more vulnerable to syndrome-induced damage.

The *Ralstonia* genus, particularly abundant in KVDS-affected root samples during the vegetative renewal, includes gram-negative bacteria belonging to the *Burkholderiaceae* family, which currently consists of six species: *R. insidiosa, R. mannitolilytica, R. pickettii, R. pseudosolanacearum, R. solanacearum*, and *R. syzygii*. These bacteria typically colonize different environments. *R. pseudosolanacearum, R. solanacearum*, and *R. syzygii* have been identified as plant pathogens (Safni et al., 2014), as forming *Ralstonia solanacearum* Species Complex, or RSSC. Over 310 species of plants belonging to 42 plant families are infected by *Ralstonia* (Genin, 2010; Genin and Denny, 2011; Paudel et al., 2020). *Ralstonia* typically colonizes the root xylem tissues and infects the roots of both susceptible and resistant plants through small wounds following a rapid migration to the stem tissues (Wang et al., 2023). Exopolysaccharide, a substance produced by *Ralstonia*, causes blockage in the xylem that leads

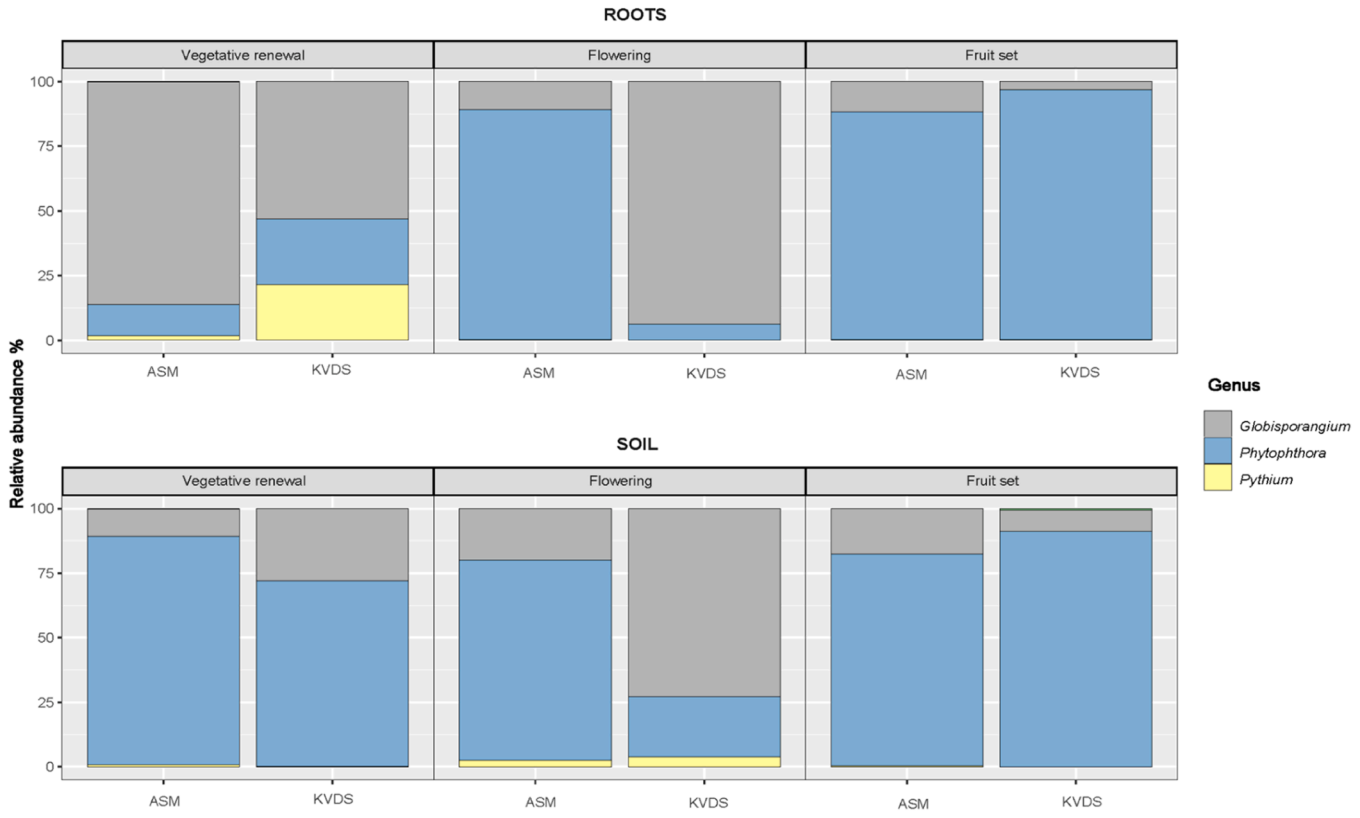

Fig. 6. Taxonomy plots showing the relative abundance of oomycetes communities in roots and soil of asymptomatic and KVDS samples during the vegetative season.

to the appearance of wilting in plants (Ingel et al., 2021). Bacteria move from plant roots to soil when plants wilt (Wang et al., 2023). To date, no correlation has been established between this genus and kiwifruit diseases. While the potential link between *Ralstonia* and KVDS is intriguing, further investigation is needed to clarify this association, especially considering the limitations in species-level identification using 16S sequencing (Johnson et al., 2019).

The *Pseudomonas* genus, detected in both symptomatic and asymptomatic roots, includes species with a longstanding association with *Actinidia*, like *Pseudomonas syringae* pv. *syringae*, causal agent of floral bud necrosis (Balestra and Varvaro, 1997), *Pseudomonas virdiflava*, responsible of kiwifruit bacterial blight (Balestra and Varvaro, 1998) and *Pseudomonas syringae* pv. *actinidiae*, the causative agent of the kiwifruit bacterial canker (Balestra et al., 2009; Mazzaglia et al., 2021; Turco et al., 2024b). On the other hand, plant-associated *Pseudomonas* species may act as plant growth promoters with a beneficial role, like the nitrogen-fixing species *Pseudomonas stutzeri*, or *Pseudomonas fluorescens* with biocontrol activity (Mehmood et al., 2023).

Known for its plant growth-promoting activity, as well as for antibacterial and antifungal compound production (Saxena et al., 2020; Poveda et al., 2021), the *Bacillus* genus, consistently present in both groups, showed a higher prevalence in asymptomatic roots during flowering and fruit set. This occurrence may be attributed to plant physiology and temperature increase. Indeed, several studies have demonstrated the capacity of certain *Bacillus* species to enhance crop development under conditions of heat stress (Abd El-Daim et al., 2014; Mukhtar et al., 2020).

The relationship between soil bacteria and kiwifruit plants exhibiting decline symptoms was investigated by Manici et al. (2022), demonstrating the impact of orchard age on bacterial communities. Older orchards showed a significant decline in beneficial genera such as *Pseudomonas* and *Bacillus* compared to adult orchards. Overall, the reduced and modified bacterial communities in older orchards may contribute to the diminished soil health and plant growth. Our findings indicate a notable increase in dynamic activity within root bacterial communities during the vegetative season. This could be attributed to the plant's ability to modulate and reshape the microbiome in response to fluctuating climate parameters, such as temperature and humidity, as well as its interaction with fungal communities.

On the other hand, fungal communities exhibited a markedly disparate pattern compared to the bacterial ones, particularly those of the soil, which showed greater dynamism and variability throughout the season.

The fungal genus *Fusarium* was the most prevalent and abundant taxon in all samples, especially in KVDS-affected roots. This genus encompasses several pathogens known to cause root rot diseases across a wide range of plants. *Fusarium solani*, for instance, is a pathogen that attacks several plant species, including olive, soybean, pea, bean, and certain cucurbits. Infected plants often exhibit various symptoms, including a generalized decline, wilting, and root necrosis (Kurt et al., 2020). The fungus's ability to penetrate cell walls and destroy vacuoles at the root level causes damage to the plant. Another soil-borne pathogen is *Fusarium oxysporum* which causes vascular damage and root rot in more than 120 economically important plant species. Both *F. solani* and *F. oxysporum* have been previously isolated from *Actinidia* roots exhibiting symptoms of KVDS (Savian et al., 2020; D'Ippolito et al., 2022).

The *Ilyonectria* and *Thelonectria* genera were identified in both the soil and root samples from the KVDS and asymptomatic samples. These genera are typically included in the *Cylindrocarpon*-like family, encompassing various fungi that cause disease and result in plant decline (Lawrence et al., 2019). This family has been observed to affect many species, including apple, cherry, grapevine, kiwifruit, peach, and walnut. *Ilyonectria* species were isolated from roots exhibiting symptoms of KVDS (D'Ippolito et al., 2022) and were identified in the roots' endosphere compartment through a metabarcoding approach (Savian et al.,

2022).

The oomycetes community was predominantly represented by *Phytophthora* and *Globisporangium*. The genus *Phytophthora* comprises a group of oomycete pathogens that are known to cause severe diseases in a wide range of crops, leading to significant agricultural losses on a global scale. Different *Phytophthora* species represent a significant threat to kiwifruit, causing root and crown rot leading to vine decline and plant collapse (Akilli et al., 2011; Kurbetli and Ozan, 2013; Li et al., 2022). Previous studies have demonstrated a correlation between the presence of *Phytophthora* spp. and KVDS (Donati et al., 2020; Prencipe et al., 2020; Savian et al., 2022). Additionally, *Pythium* spp. and *Phytopythium* spp. have been frequently isolated from affected plants. The second most prevalent genus identified was *Globisporangium*, which includes pathogens known to cause root rot in kiwifruit. In particular, the species *Globisporangium intermedium* was identified as the predominant microorganism isolated from symptomatic kiwifruit plants (Türkkan et al., 2022). Mian et al. (2023) reported the presence of *Globisporangium* spp. in *Actinidia* genotypes resistant to KVDS, especially in the root endosphere. The authors noted that the high presence of *Pythium* clade F (*Globisporangium intermedium*) counteracted the presence of *Phytophthora* sp., suggesting that it may compete with the recruitment of the latter species, explaining the onset of KVDS symptoms in susceptible genotypes. The involvement of specific oomycete populations in the emergence of KVDS-related symptoms is becoming increasingly evident. In this study, although to a much lesser extent than in other studies, the characterization of the oomycete community corroborates previous findings in areas where KVDS is present.

Overall, a comparative analysis of the microbiome composition in asymptomatic and KVDS-showing plants revealed a state of dysbiosis that occurred during the vegetative season. Specifically, during the peak periods of KVDS symptom development in summer, alterations in microbiota and microbiome network can influence the health status of plants susceptible to pathogen attacks. The identification of known genera previously associated with KVDS further supports the observations made in other studies. Nevertheless, the relationship between bacterial communities and KVDS-related fungal and oomycete communities in establishing conditions of biocontrol, antagonism, and/or promoting the onset of symptoms remains understudied. A more comprehensive examination of microbiome evolution can enhance our understanding of syndrome spread, especially during critical phases. It became increasingly evident that the interaction between soil microorganisms, abiotic stressors, and plants is a pivotal factor in the emergence and progression of the syndrome.

The relationship between KVDS and dysbiosis remains inconclusive, as recent studies have yielded conflicting results. Guaschino et al. (2024) demonstrate that stochastic processes primarily drive the assembly of rhizosphere bacterial, fungal, and oomycete communities, regardless of plant health status. In contrast, Mosca et al. (2024) observed a marginal shift from deterministic to stochastic processes in the assembly of the bacterial root microbiome. However, this shift was not evident in the fungal and oomycete communities. These findings suggest that KVDS may have a limited impact on the diversity and structure of the plant rhizosphere and root microbiomes. This suggests that microbial dysbiosis may not be the primary factor contributing to KVDS.

Last but not least, it would be interesting to explore the contribution of soil composition to the KVDS syndrome. Some soils possess a natural ability to prevent the establishment of pathogens or to inhibit their pathogenic activities, referred to as soil suppressiveness (Baker and Cook, 1974). On the other hand, soils that promote disease development are known as conducive soils, as the abiotic and biotic factors are favorable for pathogen infections (Gómez Expósito et al., 2017, Schlatter et al., 2017).

All these considerations emphasize the need for further investigations to understand the influence of soil composition and kiwifruit plant microbiome on KVDS establishment and spread.

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CRediT authorship contribution statement

Silvia Turco: Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Antonella Cardacino:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Giorgio Mariano Balestra:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2024.128044.

Data availability

The raw data related to this study are available on the NCBI SRA Database under the BIOPROJECT accession number PRJNA1170226

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