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Article

PRELIMINARY STUDY ON THE EFFICACY OF OZONATED WATER IN THE CONTROL OF GRAPEVINE DISEASES AND ITS INFLUENCE ON THE PLANT MICROBIAL DIVERSITY

ESTUDO PRELIMINAR SOBRE A EFICÁCIA DA ÁGUA OZONIZADA NO CONTROLO DE DOENÇAS DA VIDEIRA E A SUA INFLUÊNCIA NA DIVERSIDADE MICROBIANA DA PLANTA

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SUMMARY

Environmental contamination and biodiversity loss associated with the use of conventional phytosanitary products in crops has driven the search for sustainable alternatives. Ozone is a powerful disinfectant with proven applications in the agri-food chain. In recent years, water ozonation systems have been developed for agricultural sprayers, yet research on their effectiveness remains limited. This study evaluated the viability of ozonated water (OW) for grapevine disease control and its impact on plant microbiota. Six application strategies were tested in two vineyards of the 'Albariño' cultivar, assessing disease severity and microbial communities in leaf and berries. Disease incidence was generally low, except for downy mildew, which was prevalent. Consequently, the efficacy of OW was only assessed against this disease. While conventional treatments effectively controlled downy mildew, OW treatments failed, resulting in significant yield losses. Microbial analysis allowed to identify 400 fungal and 270 bacterial genera. Overall, plants treated with conventional products exhibited lower microbial abundance, highlighting the detrimental impact of chemical products on biodiversity. In conclusion, although OW is beneficial for the microbial communities compared to conventional products, it is not recommended for controlling downy mildew in regions with high disease pressure.

RESUMO

A contaminação ambiental e a perda de biodiversidade associadas ao uso de produtos fitossanitários convencionais nas culturas têm impulsionado a procura de alternativas sustentáveis. O ozono é um poderoso desinfetante com aplicações comprovadas na cadeia agroalimentar. Nos últimos anos, foram desenvolvidos sistemas de ozonização da água para pulverizadores agrícolas, porém a investigação sobre a sua eficácia ainda é limitada. Este estudo avaliou a viabilidade da água ozonizada (OW) para o controlo de doenças da videira e o seu impacto na microbiota da planta. Seis estratégias de aplicação foram testadas em duas vinhas da cultivar 'Albariño', avaliando-se a severidade da doença e as comunidades microbianas em folhas e bagos. A incidência de doenças foi geralmente baixa, exceto para o míldio, que prevaleceu. Consequentemente, a eficácia da OW foi avaliada apenas contra esta doença. Enquanto os tratamentos convencionais controlaram eficazmente o míldio, os tratamentos com OW falharam, resultando em perdas significativas de rendimento. A análise microbiana permitiu identificar 400 géneros fúngicos e 270 bacterianos. De modo geral, as plantas tratadas com produtos convencionais apresentaram menor abundância microbiana, evidenciando o impacto prejudicial dos produtos químicos na biodiversidade. Em conclusão, embora a OW seja benéfica para as comunidades microbianas em comparação com os produtos convencionais, não é recomendada para o controlo do míldio em regiões com elevada pressão da doença.

Keywords: Chemical pesticides, grapevine, microbiota, ozone, *Plasmopara viticola*.
Palavras-chave: Pesticidas químicos, videira, microbiota, ozono, *Plasmopara viticola*.

INTRODUCTION

Legislation on conventional plant protection products is becoming increasingly restrictive due to their adverse effects on the environment and human health. Continuous application contributes to soil and water contamination (Manjarres-López *et al*., 2021) and disrupts soil microbiota (Walder *et al*., 2022). Copper, one of the most widely used products in viticulture, remains the primary control agent for downy mildew in organic farming.

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However, due to its accumulation and associated soil contamination risks (Fernández-Calviño *et al*., 2009), European regulations have imposed restrictions on the maximum allowable use (Tamm *et al*., 2022).

In addition to legislative changes, there is a growing shift in mindset amongst farmers and consumers, with viticulturists increasingly adopting sustainable practices. Concerns over contamination and regulatory restrictions have driven the search for safer alternatives to conventional plant protection products. In response, numerous studies in recent years have explored potential solutions, including biocontrol agents and low-dose copper formulations (Cabús *et al*., 2017; Leal and Gramaje, 2024).

Ozone (O_3) is an unstable, partially water-soluble form of oxygen with a high redox potential, making it a powerful chemical oxidant. This property accounts for its strong germicidal action and rapid decomposition into oxygen (Khadre *et al*., 2001). Due to its short half-life, ozone cannot be stored or distributed like other industrial gases and must be generated on-site from air or pure oxygen using high-voltage electrical discharge. When dissolved in water, ozone is completely harmless, with a half-life ranging from 20 to 160 minutes at neutral pH and 20 ºC, decreasing further as pH increases. Lower water temperatures increase ozone solubility and prolong its half-life, favoring longer contact time, whereas higher temperatures speed up ozone reactions with pathogens but also increase its decomposition rate. Water quality and composition also affect the disinfection process, as organic substances consume ozone through competition for oxidation, decreasing the residual ozone available for disinfection (Galdeano *et al*., 2018; Lim *et al*., 2022).

The germicidal properties of ozone are well established, making it an effective agent against fungi, bacteria, and viruses without any toxic residues (Glowacz *et al*., 2015; Prigigallo *et al*., 2019). This has led to its widespread use in the agri-food industry, particularly for water disinfection (Białoszewski *et al*., 2010; Çetinkaya *et al*., 2022). In the United States of America, ozone is recognized as a GRAS (Generally Recognized As Safe) product for post-harvest treatment, and its demand in the agri-food sector continues to grow due to increasing interest in environment-friendly treatments (Barthwal *et al*., 2024). For instance, its efficiency in reducing *Phaeoacremonium minimum* (syn. *Phaeoacremonium aleophilum*) in grapevine has been demonstrated (Pierron *et al*., 2015). However, while ozone treatments effectively reduce pathogen loads, they may also impact the balance of plant microbiota (Sun *et al*., 2022).

Microorganisms play a fundamental role in plant health, with hundreds of thousands of species and strains interacting directly or indirectly with plants. The collective genetic system of a microbial community is referred to as the plant microbiome, which is essential for plant nutrition and vegetative development. Microbial communities differ across plant tissues, with plant organ being the primary factor influencing microbial community structure (Zarraonaindia *et al*., 2015). Together, the plant genome and its microbiome form the plant holobiome, a selective genetic unit. In a stable ecosystem, such as the plant rhizosphere, microbiome communities adapt to the environmental conditions, interact symbiotically, and collaborate with the host plant for mutual benefit. This symbiosis allows plants to provide nutrients to the microbiota, which in turn enhances plant resilience to biotic and abiotic stresses (Bettenfeld *et al*., 2022). A well-balanced microbiome acts as a natural barrier against pathogens by limiting their establishment through microbial antagonism (Li *et al*., 2021).

The objective of this study was to evaluate the potential of ozonated water (OW) as a sustainable alternative to conventional fungicides, assessing its efficacy in the control of grapevine diseases, as well as its impact on plant microbiota.

MATERIALS AND METHODS

Plant material and phytosanitary treatments

The experiment was conducted in 2021 in the Rías Baixas wine Appellation (Galicia, Spain, 42°32'20.9"N, 8°44'11.9"W), in two adjacent vineyards established in 2006 with the 'Albariño' variety trained in a Scott Henry system. Plantation frame was 1.5 x 3 m, with a permanent cover crop between the rows. The study followed a randomized block design with three replications per treatment and a total of 252 plants per block. Each replication consisted of three rows, with data collected from the plants in the middle of the central row. The experimental design was repeated in both vineyards. Six treatment application strategies were evaluated for the control of diseases:

- **T1:** OW to control downy mildew and chemical treatments for other diseases.

– **T2:** OW to control powdery mildew and chemical treatments for other diseases.

- **T3:** Only OW.

- **T4:** Chemical treatments during the most sensitive phenological stages, and the remaining treatments with OW.

- **T5:** Chemical products only in high-risk moments, following the recommendations of the advisory platform, and OW in the other treatments.

- **T6:** Standard chemical treatment.

For the OW treatment, an ozone (O3) generator (ONDINA - 20, Trasvap S.L., Valencia, Spain) was connected to a water container and a tractor sprayer pump.

Ozone was generated from atmospheric air and injected into the water at the time of application until an Oxidation-Reduction Potential (ORP) of 900 mV was reached. ORP is an indicator of microbial inactivation that does not depend on temperature or water quality (Suslow *et al*., 2004). This ORP value was selected based on previous studies in grapevine and other crops (Martínez-Sánchez and Aguayo, 2019; Campayo *et al*., 2021; Díaz-López *et al*., 2022).

To determine the optimal water volume for plant coverage in each application, the Tree Row Volume (TRV) was calculated (Byers *et al*., 1971), considering canopy dimensions at each phenological stage. A correction factor was applied to enhance wetting while avoiding runoff. Prior to each application, preliminary tests were conducted using different atomizer calibration configuration settings, adjusting working pressure, nozzle type and flow rate, air flow, and spraying speed. The proper distribution of the spray within the canopy was verified using hydrosensitive paper. The first treatment applied 300 L/ ha, gradually increasing to 1500 L/ha in the last four treatments, when the canopy had reached full development.

During the first three applications, four nozzles were used simultaneously, while six nozzles were employed in subsequent applications, always with hollow cone sprays. The working pressure was maintained at approximately 3 bar to generate fine droplets (150-200 microns). An axial fan, operating at 15,000 m3/h, was used to ensure efficient foliage coverage. The spray programme and the pesticides employed are detailed in Table I. The active substances of the pesticides and the target pathogens are specified in Table II.

All treatments were applied at manufacturer-recommended doses.

Table I

Spray programme and pesticides used for the control of grapevine diseases

Date	T1	T2	T3	T4	T5	T6
9 April	OW* Collis	OW Electis CX	OW	Collis Electis CX	Collis Electis CX	Collis Electis CX
12 April	OW	OW	OW			
22 April	OW Microthiol	OW Profiler	OW	OW	OW	Microthiol Profiler
23 April	OW		OW			
26 April	OW	OW	OW			
7 May	OW	Zorvec	OW	OW	OW	Zorvec
14 May	OW Collis	OW Enervin	OW	Collis Enervin	Collis Enervin	Collis Enervin
18 May	OW	OW	OW			
20 May	OW Flint Max	OW Milraz Pro	OW	Flint Max Milraz Pro	Flint Max Milraz Pro	Flint Max Milraz Pro
23 May	OW		OW			
31 May		OW	OW			
02 June	OW Dynali	OW Ampexio	OW	Ampexio Dynali	OW	Ampexio Dynali
08 June		OW	OW			
11 June	OW Flint Max	OW Milraz Pro	OW	Flint Max Milraz Pro	Flint Max Milraz Pro	Flint Max Milraz Pro
14 June		OW	OW			
16 June		OW	OW			
22 June	OW Microthiol	OW Ampexio	OW	OW	OW	Ampexio Microthiol
02 July	**	Mildicut	**	OW	Mildicut	Mildicut
26 July	**	Ampexio C	**	OW	OW	Ampexio C Microthiol

* OW: Ozonated water ** No treatments applied due to high plant damage.

Table II

Composition of commercial pesticides used for the control of grapevine fungal diseases

Product (Company)	Active substance	Causal agent
Ampexio® (SYNGENTA)	25 % Mandipropamid 24 % Zoxamide	*P. viticola*
Ampexio C® (SYNGENTA)	2.5 % Mandipropamid 13.95 % Copper oxychloride	*P. viticola*
Collis® (BASF)	20 % Boscalid 10 % Kresoxim-methyl	*E. necator*
Dynali® (SYNGENTA)	5.57 % Difenoconazole 2.79 % Cyflufenamid	*E. necator, P. ampelicida*
Electis® CX (GOWAN)	33 % Cymoxanil 33 % Zoxamide	*P. viticola*
Enervin® Duo (BASF)	25 % Ametoctradin 22.5 % Dimethomorph	*P. viticola*
Flint® Max (BAYER)	50 % Tebuconazole 25 % Trifloxystrobin	*E. necator, P. ampelicida*
Microthiol Special Disperss® (KENOGARD)	80 % Sulfur	*E. necator*
Mildicut® (SYNGENTA)	2.5 % Cyazofamid	*P. viticola*
Milraz® Pro (BAYER)	33 % Cymoxanil 24 % Zoxamide	*P. viticola*
Profiler® (BAYER)	4.44 % Fluopicolide 66.67 % Fosetil-Al	*P. viticola*
Zorvec® (CORTEVA)	50% Folpet 1% Oxathiapiprolin	*P. viticola*

Disease severity assessment

The effectiveness of the treatment strategies was evaluated thorough continuous monitoring of disease pressure throughout the growing season. Disease severity was evaluated following the guidelines of the European and Mediterranean Plant Protection Organization (EPPO). Five assessments of foliar and three of bunch infection were conducted between April 28 and July 8. On each assessment date, 100 randomly selected plant organs (leaves and bunches) were examined from ten plants in each repetition across each vineyard. Disease severity was quantified using the standardized scales EPPO Standard PP 1/31(3) and PP 1/17(3), which classify infection severity based on the percentage of affected tissue (Pennington *et al.*, 2017). The degree of pathogen damage was calculated with the Townsend-Heuberger formula (Townsend and Heuberger, 1943) – Equation 1.

$$DS\ (\%) = (\sum (nv)/NV) \times 100 \quad \textbf{Eq. 1}$$

In this formula, DS represents disease severity, n is the number of evaluated organs (leaves or bunches), v denotes the class value, N is the total number of assessed organs, and V corresponds to the maximum class value.

Microbial population analysis

Microbial populations were assessed in leaves and bunches across all application strategies in both vineyards, with five and three sampling dates, respectively. For each replicate, application strategy, date, and vineyard, six leaves (from the middle third of the shoot) and 20 berries were collected per plant.

Sampling was conducted on three plants per replicate, consistently using the same plants across all sampling times. Samples were stored at -70 ºC after collection until DNA extraction, which was carried out up to one week.

The DNA isolation procedure varied depending on the sample type:

For leaf samples, small random fragments were collected using sterilized forceps, and placed in 2 mL

round-bottom tubes containing 27 mm stainless steel beads. Tissue homogenization was performed using the TissueLyser LT (Qiagen) at 50 Hz for 1 min. DNA was then isolated using the Isolate II Plant DNA Kit (Bioline), following the manufacturer's protocol. The final DNA elution volume was 50 µL.

For berry samples, small random pieces, including skin and pulp, were collected using sterilized forceps and disposable blades and placed in 2 mL round-bottom tubes containing 27 mm stainless steel beads and 1% polyvinylpolypyrrolidone (PVPP). The tubes were flash-frozen in liquid nitrogen and then processed with a TissueLyser LT (Qiagen) at 50 Hz for 30 s. This incubation and homogenization process was repeated three times to ensure complete tissue disruption while preventing thawing. DNA isolation and elution were performed following the same procedure as for the leaves. In both cases (leaves and berries), the DNA of the three samples of each replicate was extracted individually, and then pooled into a single composite sample for the microbial analysis, as pooling mitigates sample-to-sample variability, resulting in a total of six composite samples per treatment (two vineyards x three replicates).

DNA concentration in each sample was quantified using the Qubit High Sensitivity dsDNA Assay (Thermo Fisher Scientific). For DNA library preparation and sequencing, distinct libraries were constructed for each taxon of interest, using specific primer pairs and conditions as follows.

For bacterial library preparation, a fragment of the 16S rRNA gene (V4 region) of approximately 300 bp was amplified using the following primers, with Illumina sequencing primer sequences included at their 5' ends:

Forward - 515F-Y (5' GTG YCA GCM GCC GCG GTA A 3') (Parada *et al*., 2016)

Reverse - 806R (5' GGA CTA CNV GGG TWT CTA AT 3') (Apprill *et al*., 2015)

In the first amplification step, PCR was conducted in a final volume of 12.5 µL, which contained 1.25 µL of template DNA, 0.5 µM of primers, 3.13 µL of Supreme NZYTaq 2x Green Master Mix (NZYTech), and ultrapure water to reach 12.5 µL. The reaction mixture was incubated as follows: an initial denaturation step at 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 s, 47 °C for 45 s, 72 °C for 45 s, and a final extension step at 72 °C for 7 min.

For fungal library preparation, a fragment of the ITS2 region of approximately 300 bp was amplified using the following primers, with Illumina sequencing primer sequences included at their 5' ends:

Forward - ITS86F (5' GTG AAT CAT CGA ATC TTT GAA 3') (Turenne *et al*., 1999)

Reverse - ITS4R (5' TCC TCC GCT TAT TGA TAT GC 3') (White *et al*., 1990)

In this case, PCR reaction mixture of the first amplification step has the same composition as above. Incubation cycles were as follows: an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 49 °C for 45 s, 72 °C for 45 s, and a final extension step at 72 °C for 7 min.

In a second amplification step, tailed primers containing indices and adapters were added for multiplexing different libraries in the same sequencing pool. This step was performed under identical conditions but with only 5 cycles and at 60 °C. A negative control without DNA (BPCR) was included in each PCR round to detect potential contamination during library preparation.

The size of the libraries was verified by running them on 2% agarose gels stained with GreenSafe (NZYTech) and imaging under UV light. The libraries were then purified using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek), following the manufacturer's instructions.

The finished libraries were pooled in equimolar amounts based on the results from a Qubit dsDNA HS Assay (Thermo Fisher Scientific). The pool was sequenced on a portion of a NovaSeq PE250 flow cell (Illumina) with the goal of achieving a total output of 16 gigabases.

Bioinformatic and Statistical analysis

Differences in disease severity in leaf and bunch were assessed using Mann-Whitney test and statistical significance was considered at $p<0.05$ using R Statistical Software v4.3.3 (2024).

ITS2 sequence quality was visualized using FastQC-0.10.1 (Andrews, 2010), followed by data processing in SEED v2.0.3 (Větrovský *et al*., 2018), raw paired-end reads joined by usage of fastq-join (Aronesty, 2013).

Full length ITS2 region was covered by extraction using ITSx v1.1.2 (Bengtsson-Palme *et al*., 2013). Fungal diversity was analyzed by clustering of all ITS2 sequences into operational taxonomic units (OTUs) (Blaxter *et al*., 2005) at similarity 97%, by USEARCH v11.0.667, and removing of chimeric sequences by UPARSE method (Edgar, 2013). The most abundant sequences were selected to represent each OTU for identification by BLASTn search against UNITE version 8.1, released 2.2.2019 (Nilsson *et al*., 2019).

Sequences with non-fungal hits, without hits or with e-values BLASTn search result > e−50 were excluded for increasing chances for obtaining only taxa belonging into the fungal kingdom (Tedersoo *et al*., 2014; Baldrian *et al*., 2022).

Processing of 16S rRNA gene sequences followed a similar workflow. Raw paired-end reads were processed in SEED v2.0.3, with read merging performed using the integrated fastq-join tool. Low-quality sequences (quality score < 30) and reads

shorter than 200 bp were removed using UPARSE. The remaining sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using USEARCH v11.0.667, followed by denoising and chimera removal implemented with the built-in MAFFT v7.222.64 (Katoh *et al*., 2009). Representative sequences were taxonomically assigned using BLASTn against the SILVA database v138.1 (Quast *et al*., 2013). To ensure taxonomic reliability, sequences with non-bacterial matches, no significant hits, or BLASTn e-values higher than 1×10^{-50} were excluded from further analysis.

A total of 126 leaf samples and 54 berry samples were analyzed in the microbial population analysis using the Microbiomeanalyst.ca software. Abundance and alpha-diversity were determined. Alpha-diversity was assessed through the calculation of Chao1 and Shannon indexes via the Phyloseq package (Dhariwal *et al*., 2017). Linear discriminant analysis Effect (LEfSe) was applied for the taxa identification (at the genus level) that differed in relative abundance between treatments.

RESULTS AND DISCUSSION

Effective disease control is essential for profitable grapevine cultivation. Increasingly restrictive regulations on phytosanitary products, along with the shift towards more sustainable practices, have driven the evaluation of environmentally friendly alternatives (García *et al*., 2015). In this respect, the present work has consisted of the evaluation of OW as an alternative product for the diseases control in an area with a high downy mildew pressure.

Ozone is a greenhouse gas that, at high concentrations, has harmful effects on humans and the environment. Its phytotoxicity has been reported by several authors (Fumagalli *et al*., 2019; Agathokleous *et al*., 2020). Nevertheless, due to its strong oxidizing power and absence of residues, ozone is widely employed as a sanitizing agent in the agri-food industry (Glowacz *et al*., 2015). *In vitro* studies have demonstrated its efficacy in gaseous form against *Botrytis cinerea* (Sharpe *et al*., 2009). Gaseous ozone has been shown to be more effective than aqueous ozone in post-harvest grape treatments, reducing "Brett character" in wines, as well as in controlling seedborne fungal and bacterial pathogens such as *Fusarium oxysporum*, *Clavibacter michiganensis* and *Pseudomonas syringae* in tomatoes (Cravero *et al*., 2018; Çetinkaya *et al*., 2022). An inhibitory effect of OW on *Botrytis cinerea in vitro* has also been described (Tanuwidjaja and Fuka, 2022). Additionally, OW has demonstrated effectiveness against soil nematodes and viruses, including *Pratylenchus penetrans* (Kanfra *et al*., 2021), *Meloidogyne incognita* and Tomato spotted wilt virus (Prigigallo *et al*., 2019).

Few field studies have investigated the use of ozone treatments in grapevines. It has been suggested that ozone could effectively control *Erysiphe necator*, which develops on the plant surface, but would be ineffective against pathogens such as *Plasmopara viticola*, *Phyllosticta ampelicida* and *Greeneria uvicola*, which develop within plant tissues (Bhadra, 2015). However, a recent laboratory and field study found that OW was ineffective against *Erysiphe necator* and *Pseudococcus maritimus* (McDaniel *et al*., 2024).

OW is being used in some regions, such as Castilla-La Mancha (Spain), to control fungal diseases in vineyard (García-Martínez *et al*., 2021). Given the high cost of the machinery involved and the limited number of studies on its effectiveness in field, this study was conducted in an area with a high incidence of fungal diseases with the aim of providing grape growers with clear recommendations on the use of this product.

In this study, no occurrence of powdery mildew (*Erysiphe necator* Schwein) or botrytis (*Botrytis cinerea* Pers.) was detected on leaves or bunches, and the incidence of black rot (*Phyllosticta ampelicida* (Engelm.) was very low. Therefore, the results presented below focus on assessing the severity of downy mildew (*Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni). To evaluate treatment effectiveness, comparisons were made against treatment T6, which involved the use of chemical plant protection products for controlling downy mildew, powdery mildew and black rot.

No significant differences between vineyards in disease severity for each treatment were observed; therefore, the data were analyzed together. This approach increases statistical power and improves the robustness of the results by enlarging the sample size, while maintaining biological consistency across treatments. Regarding disease severity evaluations on the grapevine leaves (Figure 1):

- April 28: no significant differences between treatments were observed, with a maximum average disease severity of 1 %.

- May 7: disease severity in T3 (OW only) reached 12 %, while in all other treatments it did not exceed 6 %. Significant differences were found between T3 and treatments T1, T2, and T6.

- May 19: disease severity in T3 exceeded 15 %, confirming that OW alone is ineffective for downy mildew control. Treatment T6 exhibited the lowest severity (1 %). T2 and T6 (both treated with chemical products against downy mildew) showed significant differences compared to T3 and T4.

- June 1: Disease severity reached nearly 40 % in T1 and exceeded 70 % in T3, indicating that both strategies were ineffective. Severity was also high in T4 (32 %), whereas T5 (11 %) and T2 (3 %) maintained levels closer to T6 (2 %).

- July 8: Treatments T1 and T3 could no longer be evaluated due to severe leaf damage. Amongst the remaining treatments, T4 approached 30 %, while T5 showed lower severity (21 %). T2 and T6 maintained the lowest disease severity.

Evaluations conducted on grape bunches confirmed the results observed in leaves (Figure 1):

- May 19: Significant differences between treatments were observed, though disease severity remained low, except for T3, which exceeded 5 %.

- June 1: Disease severity in T3 reached 70 %, confirming its ineffectiveness. Significant differences were observed between T3 and treatments T2 and T6.

- July 8: Treatments T1 and T3 could no longer be assessed due to severe bunch damage, while T4 exceeded 50 %. No significant differences were observed between T2 and T5, both with less than 20 % severity, compared to T6, which had the lowest severity, below 5 %.

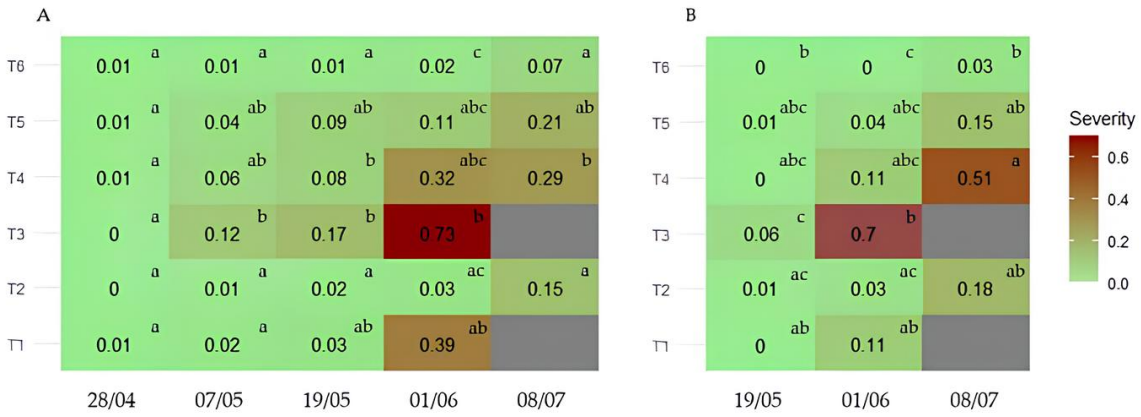

Figure 1. Heatmap and significant differences between treatments in disease severity in leaf (A) and bunch (B) on the different dates of EPPO assessments (28/04, 07/05, 19/05, 01/06, and 08/07).

Considering the results, it can be inferred that the exclusive use of OW for downy mildew control was ineffective under the high disease pressure recorded during the year of study, as the plants without chemical fungicides for this disease (T1 and T3 treatments) suffered severe damage. Notably, T1 (OW for downy mildew and chemical pesticides for other diseases) and T3 (OW only) differed significantly at the second leaf and the first bunch assessment dates. While no significant differences were observed in later assessments, disease severity remained much higher in T3 than in T1 for both leaves and berries. This suggests that the pesticides used in T1 contributed to downy mildew control. T1 had been previously treated with Collis and Microthiol Special Disperss. Collis, a preventive fungicide registered for powdery mildew control, contains boscalid and kresoxim-methyl as active ingredients. While boscalid is ineffective against downy mildew (Clippinger *et al*., 2024), kresoxim-methyl activity has reported to provide satisfactory preventive control of the disease (Ypema and Gold, 1999). This likely explains the differences in severity levels between T1 and T3. On the other hand, a phytotoxic effect was observed for the treatment with only OW (T3), consisting of a slight yellowing of the leaves.

The most effective was the conventional treatment (T6). Although T2 and T5 showed higher levels of infection in leaves and berries, no significant differences were observed in berry infection compared to T6 at the end of the evaluation period. However, from a crop profitability perspective, the associated yield loss could still be relevant. T2 received chemical treatments for all diseases except powdery mildew, which was managed with OW. This likely explains the lower infection levels observed in T2 comparted to the other OW-based strategies, as downy mildew was controlled with conventional fungicides. Until June 2, T4 and T5 were treated with the same products, and no significant differences in downy mildew severity were observed between them. However, T4 showed slightly higher leaf infection on June 1 (severity index: 0.32 for T4 vs. 0.11 for T5), which may be attributed to natural variability in field conditions. In the following assessment (July 8), T4

exhibited greater berry damage compared to T5 (0.51 vs. 0.15), though the difference was not statistically significant. This may again reflect field variability; however, another plausible explanation is the application of a systemic fungicide (Mildicut) to T5 on July 2, which may have successfully halted new downy mildew infections emerging in late June. In contrast, T4 was treated with OW on the same day, which may have failed to prevent disease progression.

Contamination of water and soil due to chemical applications has been widely documented (Manjarres-López *et al*., 2021), along with their detrimental effects on microbial communities. To evaluate the impact of the different treatments on plant microbiota, a microbial population analysis was performed. Results from both vineyards are presented collectively, as no significant differences were detected in fungal or bacterial diversity between them.

A total of 400 fungal genera were identified. Leaf sampling began in April, revealing low overall microorganism abundance, with T1 exhibiting the highest levels. In May, microbial abundance increased across all treatments, and a greater diversity of genera emerged during this period. In June, berry sampling began alongside leaf sampling; however, T1, T3, and T4 were no longer assessed due to severe downy mildew infection. From this point onwards, the microbiota monitoring continued only in T2, T5 and T6. In the berries, genera were more evenly distributed this month, while T6 showed notably lower microbial abundance in leaves. By August, T6 had the lowest abundance in both berries and leaves. In September, overall abundance peaked, with T6 exhibiting increased microbial presence in berries, along with greater genus diversity (Figure 2A and 2B).

Ascomycota and Basidiomycota were the dominant fungal phyla, consistent with previous reports (Bettenfeld *et al*., 2022). The remaining three detected phyla contained only one genus each. The most abundant genera in leaves were *Cladosporium*, *Aureobasidium* and *Alternaria*, with the highest number of genera recorded in September. *Cladosporium* and *Aureobasidium* remained abundant throughout the season, while *Alternaria* was particularly prevalent in leaves in May for T6, in June and August in T5, and predominantly in T2 in September. *Cladosporium* is one of the most widespread fungal genera globally, comprising over 40 identified species. Indeed, this genus has been identified as dominant, together with *Fusarium*, in Australia, Denmark, Germany and Portugal, in a global study performed in 13 countries on four continents (Gobbi *et al*., 2022). *C. herbarum* and *C. cladosporioides* are commonly found in diverse ecological niches, including grapevines. These species typically increase in abundance as berries ripen and are associated with green rot, which can cause berry desiccation both in the vineyard or during storage (Briceño and Latorre, 2008). *Aureobasidium pullulans* is a widely distributed yeast-like fungus present in diverse ecological niches. Organic vineyards typically exhibit higher abundances of *A. pullulans* compared to conventionally managed ones (Schmid *et al*., 2011). Several *A. pullulans* strains have demonstrated biological potential against pathogenic fungi (Bozoudi and Tsaltas, 2018), such as *Botrytis cinerea* (Galli *et al*., 2021), *Aspergillus niger* and *Rhizopus stolonifer* (Castoria *et al*., 2001). However, a recent study has detected a promoting effect of this species on the pathogenesis of Esca disease (Karácsony *et al.*, 2023). In the present study, the use of OW did not appear to affect the presence of this genus.

A notable increase in *Botrytis* abundance was observed in the T2 leaves in September, and this genus was also among the most abundant fungal genera in berries that month, alongside *Cladosporium* (Figure 2). While *Cladosporium* has been reported to be predominant from the early stage of berry development until harvest, *Botrytis*, a mould genus, peaks at harvest, mainly in humid temperate climates (Destanque *et al*., 2024). The Venn diagram illustrates the fungal genera shared amongst treatments. The conventional chemical strategy (T6) exhibited the lowest number of fungal genera, except in berry samples from June. It also shared fewer genera with the other two treatments in leaf samples, particularly towards the end of the season (Figure 3). Maante-Kuljus *et al*. (2025) reported that OW significantly decreased fungal colonies on grapevine leaves and berries, with variable results depending on the phenological stage; however, it should be noted that they compared the results with a control without chemical applications.

Figure 2. Actual abundance and genera of the most frequent fungi in leaf (A) and berry (B) samples.

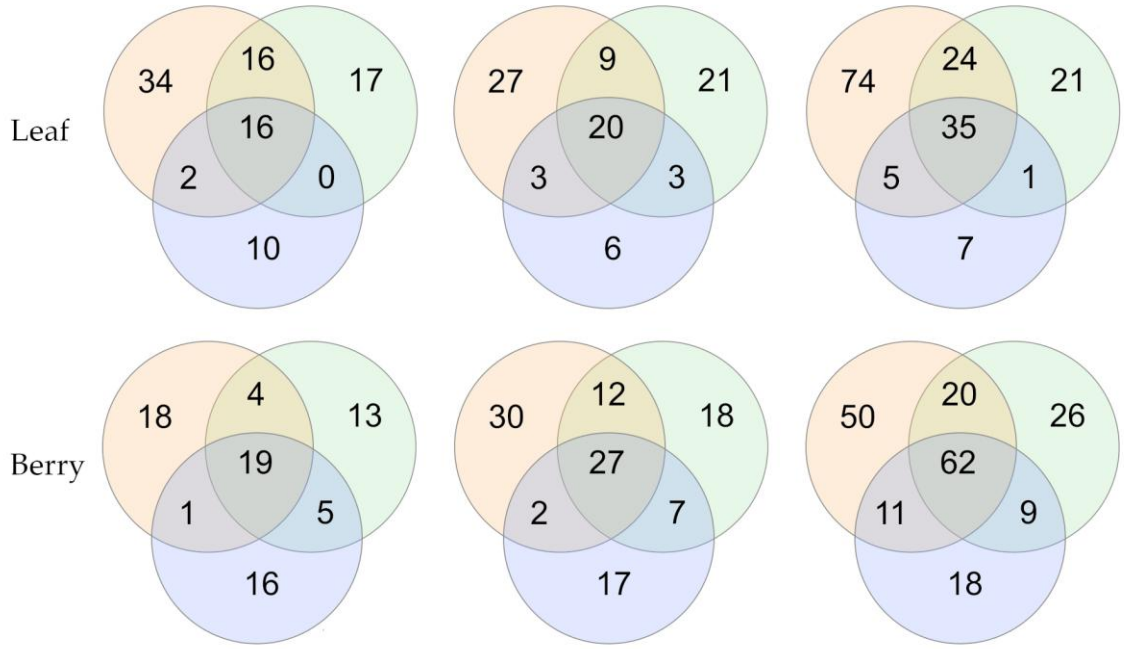

Figure 3. Venn diagrams of fungi genera counts in June (left), August (middle) and September (right) samplings. Colors corresponding to the treatments are orange (T2), green (T5) and blue (T6).

Linear discriminant analysis effect size (LEfSe) identified significant differences in fungal genera between treatments on certain dates (Figure 4). In May, *Dioszegia* (p=0.00059) and *Itersonilia* (p=0.00026) were found in low abundance in T6 (conventional treatment) but were more abundant in T3 leaves (OW) (Figure 4A). Species from both genera have been associated with low downy mildew incidence in a study on a network of 1200 untreated controls performed in France (Fournier *et al*., 2025). In the present study, they show the opposite trend, as they presented low abundance in T6, which had the lowest downy mildew severity. This suggests that these genera may be sensitive to one or more of the chemical products used in T6, as T3 was treated exclusively with OW. *Dioszegia* spp. has also been reported as dominant in an older vineyard compared to a younger one in a study on grapevine trunk diseases (Langa-Lomba *et al*., 2023), and to show preference for grapes from cold areas (Drumonde-Neves *et al*., 2021).

In August, several genera showed significant differences between treatments (Figure 4B): *Filobasidium* was more abundant in T2 and less in T5. Since both treatments received OW application, differences in pesticide use and timing of OW application may explain the observed variation in fungal abundance. This genus has been established, based on a wide literature review, as one of the genera that make up the core group of vineyard basidiomycete yeasts (Bunbury-Blanchette *et al*., 2025). *Symmetrospora* was less abundant in T6 (conventional treatment) and more in T2. This aligns with the study of Bunbury-Blanchette *et al*. (2024) that reported *Symmetrospora* as indicator species of organic vineyards. *Saccharomyces* was more abundant in T6 and less in T5, whereas *Cladosporium* showed an inverse pattern to *Filobasidium*, with higher abundance in T5 and lower in T2. Additionally, a higher abundance of unidentified genera was observed in T6, whereas T2 had the lowest. Another noteworthy genus, *Ramularia*, was consistently detected in leaf samples from treatment T2 across all sampling dates. It was also observed in T6 leaves in April, in berries from T6 in May, and in berries from T2 and T5 in September. While a previous study reported the absence of *Ramularia* in leaves of *Vitis vinifera* L., detecting it only in wild *V. riparia* (Kernaghan *et al*., 2017), other studies documented its presence in *V. vinifera* leaves (Pinto *et al*., 2014; Videira *et al*., 2016) and canes (Del Frari *et al*., 2019).

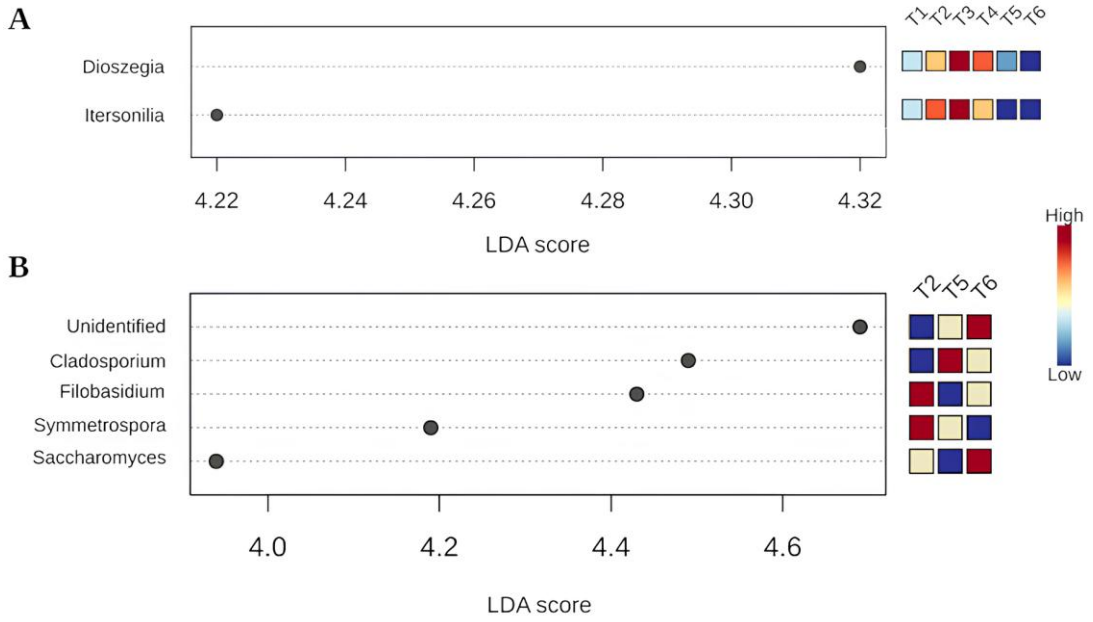

Figure 4. LEfSe dot plot showing differences for some fungal genera in leaf samples collected in May (A) and August (B).

In terms of fungal alpha-diversity, the Chao1 index showed significant differences in leaf samples in May, June, and September (Figure 5A to 5D). In June, T2 differed significantly from T5 and T6 in leaves, whereas in September, T2 differed only from T6. In berries, significant differences were observed only in August, with T6 differing from T2 and T5. The Shannon index showed significant differences in September for leaves and in both August and September for berries (Figure 5E to 5G). This index distinguished T6 from T2 in leaves (September) and berries (August). In September, the conventional treatment (T6) was significantly different in berries compared to the other two treatments. In almost all these cases, conventional treatment (T6) presented lower values than the other treatments, indicating the impact of conventional fungicides in fungal diversity.

A total of 270 bacterial genera were identified, with abundance increasing as September approached, following a trend similar to that observed in fungi. This increase was evident in both leaf and berry samples, particularly in T5 treatment. T6 (conventional treatment) generally exhibited the lowest abundance (Figure 6). Indeed, some of the fungicides used in the study have shown antibacterial activity *in vitro*. For instance, cymoxanil has been effective against a wide range of bacterial species, and Firmicutes was found to be particularly sensitive to tebuconazole. In contrast, boscalid did not exhibit negative effects on bacterial populations (Andreolli *et al*., 2023). Qiu *et al.* (2025) reported, in a compilation study of 88 experiments performed in agricultural and non-agricultural soils, that an increase in the risk of pesticide use decreased beneficial bacteria functions, affecting soil health. OW has also demonstrated bactericidal effects in previous studies (Białoszewski *et al*., 2010).

Amongst bacterial phyla, Proteobacteria contained the highest number of genera, followed by Actinobacteriota and Firmicutes. At the beginning of sampling, *Acinetobacter* was the predominant genus in leaves and remained dominant through May, when *Pseudomonas* and *Sphingomonas* also appeared. By June, *Sphingomonas* and *Pseudomonas* were the most abundant genera in leaves, though *Pseudomonas* declined in subsequent samplings as *Methylobacterium* and *Hymenobacter* increased. In berries, *Psychrobacter* and *Rhizobium* were dominant in June and August, whereas *Sphingomonas*, *Methylobacterium* and *Gluconobacter* became the most abundant genera in September (Figure 6). The high abundance of *Gluconobacter* at harvest suggests that it thrives during the later stages of berry development.

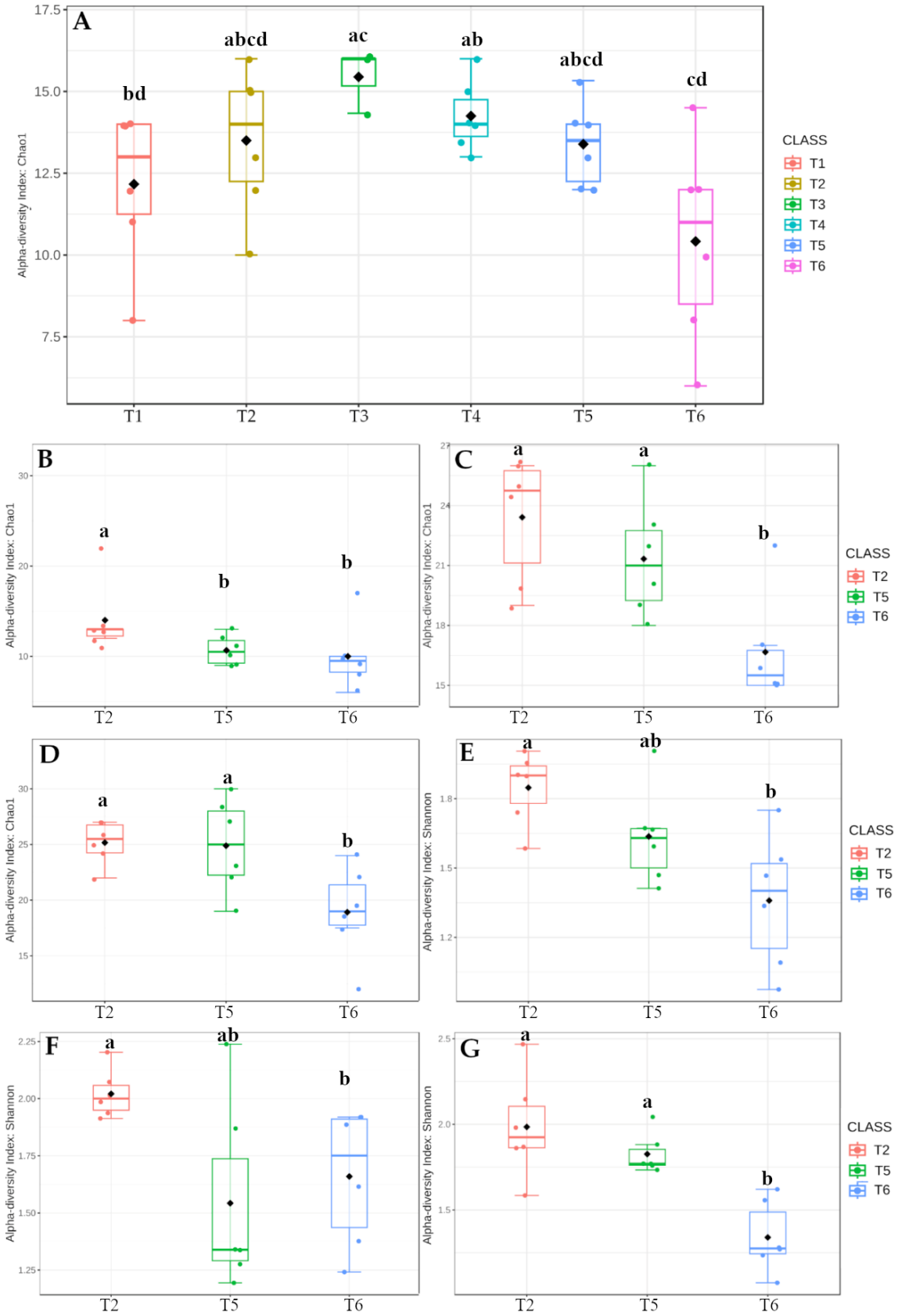

Figure 5. Boxplots illustrating the differences in Chao1 (A - leaf 31 May; B - leaf 30 June; C - berry 9 August; D - leaf 16 September) and Shannon (E - berry 9 August, F - leaf 16 September, G - berry 16 September) diversity measures of the fungal communities among treatments. Different letters indicate significant differences ($p < 0.05$).

This genus is commonly found in damaged berries and contributes to sugar degradation, producing acetic acid (Dwivedi, 2020). Significant differences between treatments were detected in only one genus, namely *Kineococcus*, which was more abundant in T2 and T5 than in T6 in leaf samples from August. Belonging to the class Actinobacteria, *Kineococcus*, together with *Sphingomonas*, *Hymenobacter* and others, were considered as hub taxa, that implies they have a strong influence on the connectivity of the microbiome network, in a study with healthy grapevine tissues (Aleynova *et al*., 2023). That is, these hub taxa have a paramount effect on shaping microbial communities, affecting the growth and diversity of other microbes.

Figure 6. Actual abundance and genera of the most frequent bacteria in leaf (A) and berry (B) samples.

When an external pressure perturbs these taxa, the effect on the microbial community of this disturbance will be stronger (Agler *et al*., 2016), and their reduction and/or elimination, as seen for T6 in the case of *Kineococcus*, can disrupt the microbiome network. Indeed, from 204 bacterial genera identified in a study on 37 cultivars in a grapevine collection, only eleven were present in all the cultivars, these being considered as a core microbiome. *Kineococcus*, *Sphingomonas*, and *Hymenobacter* were included in this group (Awad *et al*., 2020). The Venn diagram (Figure 7) shows a lower number of bacterial genera shared with T6 in almost all samplings, especially in September, both in leaves and berries, compared to the shared between T2 and T5. Notably, T6 had the lowest number of genera in both sample types during this month.

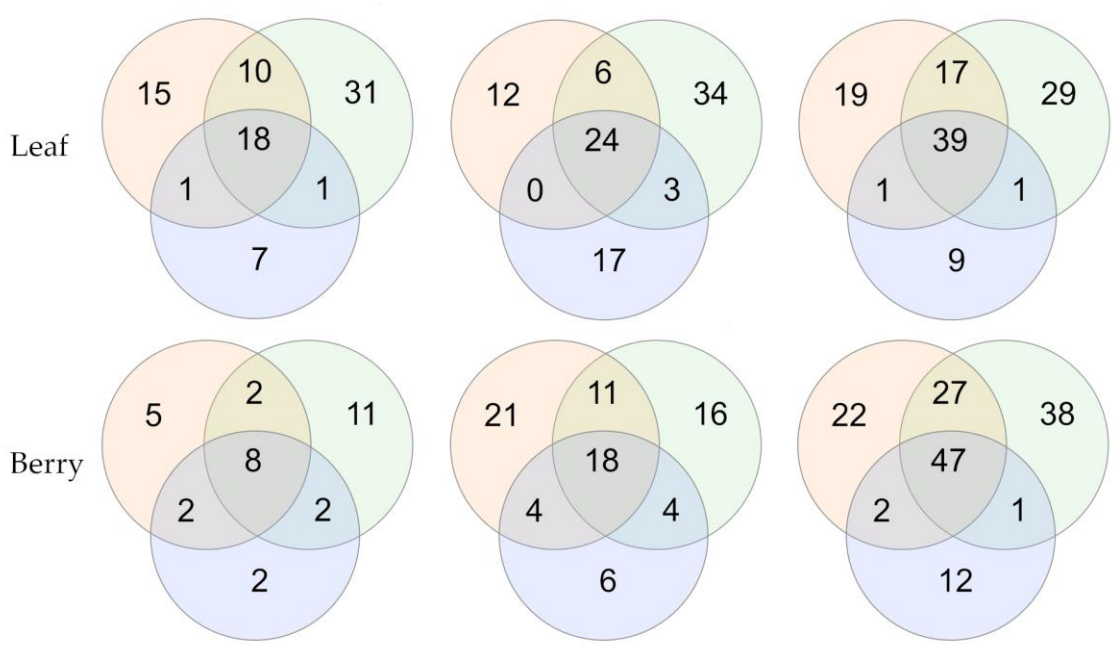

Figure 7. Venn diagrams of bacterial genera counts in June (left), August (middle) and September (right) samplings. Colors corresponding to the treatments are orange (T2), green (T5) and blue (T6).

Bacterial alpha-diversity in leaf samples showed no significant differences between treatments until September, when T2 and T5 exhibited significantly greater diversity than T6. In berries, T2 had lower diversity than T5 in June, but by August, T6 had the lowest diversity. In September significant differences were observed between T6 and the other two treatments (Figure 8). Overall, T6 showed lower bacterial abundance and diversity throughout the season, suggesting that the use of fungicides may suppress bacterial communities more effectively than OW.

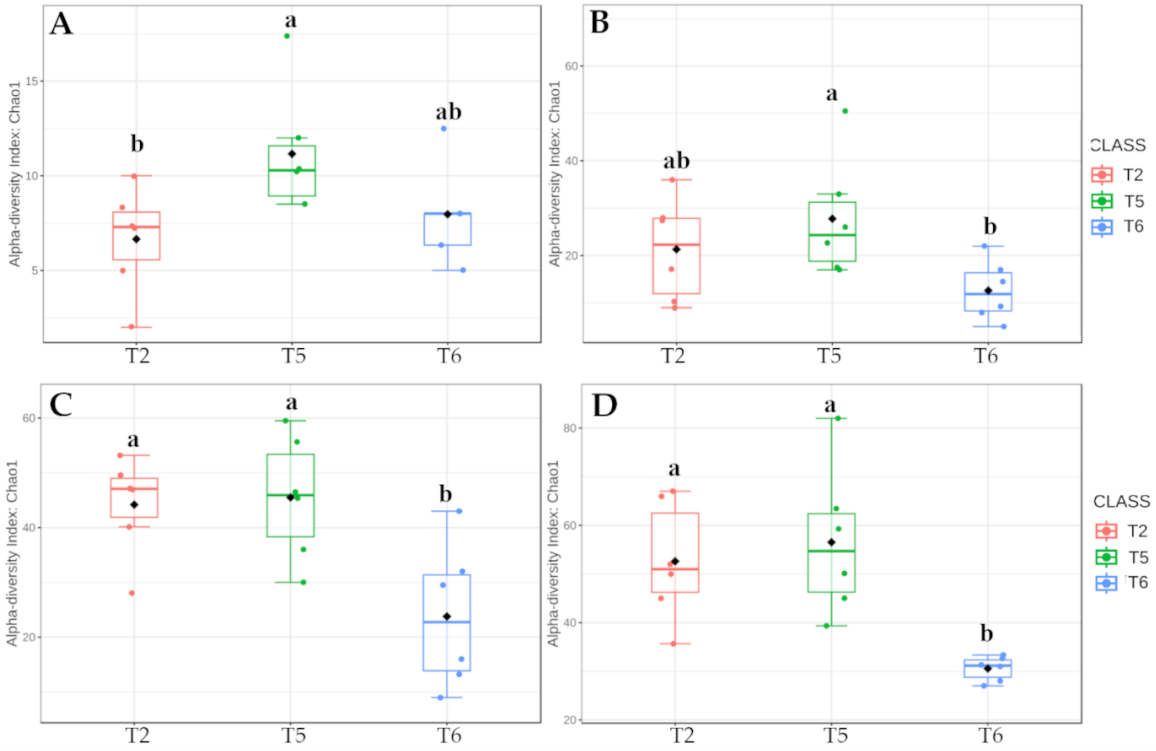

Figure 8. Boxplots illustrating the differences in Chao1 diversity measures on the bacterial communities among treatments (A – berry 30 June, B – berry 9 August, C – leaf 16 September, D – berry 16 September). Different letters indicate significant differences ($p < 0.05$).

CONCLUSIONS

The use of OW did not demonstrate biocidal effect against downy mildew in the year of study, characterized by a high disease pressure, leading to significant yield losses that rendered the crop unprofitable. Therefore, OW should not be considered as a sustainable alternative to chemical pesticides in regions with high downy mildew incidence. The effectiveness of OW against other fungal diseases could not be assessed, as their incidence was nearly absent during the studied period. However, the reduction of chemical active substances in OW-based treatments lead to an overall increase in the abundance and richness of fungal and bacterial communities, regardless of the plant organ. An exception was observed at harvest, when berries from the conventional treatment (T6) exhibited the highest fungal abundance, indicating a possible shift in microbial dynamics late in the season. The fungal communities were more affected by the various treatments than the bacterial ones. *Dioszegia* and *Itersonilia*, two fungal genera linked to a low incidence of downy mildew, showed low abundance in the conventional treatment in the May sampling but high abundance in the OW treatment, suggesting that they might be susceptible to one or more of the chemical products used earlier.

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The Use of Artificial Intelligence (AI)-Assisted Technology was restricted to improve English spelling and language clarity.

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