***Verticillium dahliae* inoculation and *in vitro* propagation modify the xylem microbiome and disease reaction to *Verticillium* wilt in a wild olive genotype**

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

MA-M and BBL: conceived research, performed statistical and bioinformatics analyses, interpret results and wrote the manuscript; MA-M, JLT, CO-G, DR-R and EP-R: prepare materials and equipment and performed the experiments; JAN-C and RMJ-D: contributed to reviewing the manuscript and interpret results. All authors viewed the draft of manuscript.

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33 **Data Availability Statement**

34 The raw sequence data have been deposited in the Sequence Read Archive (SRA)
35 database at the NCBI under BioProject accession number PRJNA679263.

36 Abstract

37 Host resistance is the most practical, long-term and economically efficient disease
 38 control measure for Verticillium wilt in olive caused by the xylem-invading
 39 fungus *Verticillium dahliae* (Vd), and it is at the core of the integrated disease
 40 management. Plant's microbiome at the site of infection may have an influence on the
 41 host reaction to pathogens; however, the role of xylem microbial communities in the
 42 olive resistance to Vd has been overlooked and remain unexplored to date. This
 43 research was focused on elucidating whether *in vitro* olive propagation may alter the
 44 diversity and composition of the xylem-inhabiting microbiome and if those changes
 45 may modify the resistance response that a wild olive clone shows to the highly virulent
 46 defoliating (D) pathotype of Vd. Results indicated that although there were differences
 47 in microbial communities among the different propagation methodologies, most
 48 substantial changes occurred when plants were inoculated with Vd, regardless whether
 49 the infection process took place, with a significant increase in the diversity of bacterial
 50 communities when the pathogen was present in the soil. Furthermore, it was noticeable
 51 that olive plants multiplied under *in vitro* conditions developed a susceptible reaction to
 52 D Vd, characterized by severe wilting symptoms and 100% vascular infection.
 53 Moreover, those *in vitro* propagated plants showed an altered xylem microbiome with a
 54 decrease in total OTU numbers as compared to that of plants multiplied under non-
 55 aseptic conditions. Overall, 10 keystone bacterial genera were detected in olive xylem
 56 regardless infection by Vd and the propagation procedure of plants (*in vitro* vs nursery),
 57 with *Cutibacterium* (36.85%), *Pseudomonas* (20.93%), *Anoxybacillus* (6.28%),
 58 *Staphylococcus* (4.95%), *Methylobacterium-Methylorubrum* (3.91%), and
 59 *Bradyrhizobium* (3.54%) being the most abundant. *Pseudomonas* spp. appeared as the
 60 most predominant bacterial group in micropropagated plants and *Anoxybacillus*
 61 appeared as a keystone bacterium in Vd-inoculated plants irrespective of their
 62 propagation process. Our results are first to show a breakdown of resistance to Vd in a
 63 wild olive that potentially maybe related to a modification of its xylem microbiome, and
 64 will help to expand our knowledge of the role of indigenous xylem microbiome on host
 65 resistance which can be of use to fight against main vascular diseases of olive.

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68 Introduction

69 Verticillium wilt, caused by the vascular-colonizing, soil-borne fungus, *Verticillium*
70 *dahliae* (Vd), is one of the main diseases threatening the health and growth of olive
71 (*Olea europaea* subsp. *europaea* var. *europaea*) production worldwide. This disease,
72 first reported in Italy in 1946, has steadily increase in prevalence and incidence to
73 become an actual threat to olive cultivation in the Mediterranean Basin due to the high
74 rates of tree mortality and important reductions in yields (Jiménez-Díaz et al., 2011;
75 Landa et al., 2019). Infection of olive plants by Vd takes place through the root system;
76 then the pathogen colonizes the xylem vessels and impairs the sap flow by means of
77 mycelial proliferation, the formation of occlusions and tyloses that ultimately cause the
78 tree death (Báidez et al., 2007; Jiménez-Díaz et al., 2011). Two pathotypes have been
79 identified among Vd isolates in olive, namely defoliating (D) and non-defoliating (ND),
80 which differ much in virulence and determine severity of Verticillium wilt. The ND
81 pathotype induces moderately severe branch die back and leaf necrosis, whereas the
82 highly virulent D pathotype induces severe falling of green leaves, necrosis of entire
83 plant canopy sectors and eventually tree death (Navas-Cortés et al., 2008; Jiménez-Díaz
84 et al., 2011). In Andalusia, the world's largest producer of olive oil, circa 39% of old
85 and newly planted olive orchards were reported affected by Verticillium wilt, but
86 inspections of 90 arbitrarily chosen orchards in the three main olive-growing provinces
87 of the region indicated 71% disease prevalence with a mean incidence of 20% in the
88 affected orchards (Jiménez-Díaz et al., 2011).

89 The most practical and economically efficient method for the management of
90 Verticillium wilt is the use of resistant cultivars. However, most of olive cultivars
91 widely grown in Spain, are moderately to highly susceptible to D Vd (López-Escudero
92 et al., 2010; Jiménez-Díaz et al., 2011; Ostos et al., 2020). Recently, a few wild olive
93 genotypes were identified as highly resistant to D Vd that may have a valuable potential
94 as rootstocks for the management of Verticillium wilt (Jiménez-Fernández et al., 2016).
95 Nevertheless, use of a single control measure may not be fully effective for the
96 management of Verticillium wilt in olives, as shown for other wilt diseases. Thus, an
97 integrated management strategy it is advisable, combining the use of resistant olive
98 cultivars, or of tolerant ones grafted onto resistant rootstocks, with adequate irrigation
99 management and agricultural practices that prevent the spread of inoculum of the
100 pathogen (Jiménez-Díaz et al., 2011; Jiménez-Fernández et al., 2016).

101 Although plants have evolved their own adaptations to alleviate most biotic and
102 abiotic stresses in nature, they also rely on their microbial partners to survive and
103 defend themselves against microbial invaders and pathogens (Hassani et al., 2018). In
104 nature, healthy plants live in association and interact with a myriad of microorganisms,
105 collectively called the plant microbiome, which is now known to bear important roles in
106 plant health. Endophytes are bacteria and fungi that live within plants where they
107 establish nonpathogenic relationships with their hosts (Azevedo et al., 2000).
108 Endophytic microbial communities are dynamic and are influenced by several factors.
109 These communities promote plant growth directly by phytostimulation and
110 biofertilization, and/or indirectly by inducing stress tolerance and disease suppression
111 (Compant et al., 2016; Hassani et al., 2018). Therefore, a thorough knowledge of the
112 microbial communities residing within the xylem vessels of olive trees may be crucial
113 for understanding their potential influence on the healthy growth of this plant as well as
114 on the resistance shown by specific olive genotypes against *D Vd* or other vascular plant
115 pathogens (Hong and Park, 2016). Thus, certain members of the microbial community
116 may play a crucial role in the expression of disease resistance in plants to plant
117 pathogens by setting on diverse mechanisms that mainly include a via for nutrient
118 mobilization, direct antagonism, niche exclusion and induction of systemic and
119 localized resistance (Müller et al., 2015; Hassani et al., 2018; Wille et al., 2019).
120 However, although it can be considered that most of the olive xylem microbiota should
121 be assembled by microorganisms with neutral or positive effects, their mechanistic role
122 in defense against vascular pathogens has not yet been addressed.

123 Knowledge of the olive tree xylem microbiome is limited and very fragmentary,
124 despite progress made on understanding the structure and functions of the plant
125 microbiome in recent years. Possibly, technical difficulties for isolation of xylem
126 microbiome have diffculted its study, and only a few have focused on microorganisms
127 inhabiting the olive xylem. However, different methodological approaches, including
128 culture-dependent procedures complemented with next-generation sequencing (NGS)
129 technologies, have recently made possible to characterize microbial communities
130 associated with olive xylem tissue (Hardoim et al., 2015; Fausto et al., 2018; Anguita-
131 Maeso et al., 2020; Zicca et al., 2020).

132 The modification or attenuation of the diversity and composition of xylem
133 microbial communities might result in different responses from the plant host to cope

134 with vascular pathogens. The transmission of microbiota to the progeny in plants
135 vegetatively propagated represents a way to ensure the presence of beneficial symbionts
136 within the plant (Vannier et al., 2018; Liu et al., 2019). However, it is unknown how
137 xylem endophytic microbiota in olive may be transmitted from shoot tips to explants, as
138 well as to mature plants, and how stable would that be during this process.
139 Micropropagation has become an important tool to reproduce selected olive genotypes
140 and guarantees true to type and pathogen free plants (Fabbri et al., 2009). Olive
141 micropropagation, through tissue culture, which is conducted *in vitro* under aseptic
142 conditions, for at least a certain period of time, can potentially induce changes and alter
143 the composition of the xylem microbiome. Thus, by producing olive plants by tissue
144 culture, some beneficial and non-pathogenic endophytes may be excluded, since tissue
145 cultures are initiated from shoots after extensive surface sterilization and then plants are
146 maintained under aseptic conditions. Consequently, this propagation procedure
147 represents an ideal experimental approach to test whether the xylem microbiome has a
148 role on the resistance shown by specific olive genotypes to vascular pathogens.

149 This present research was conceived to elucidate how plants protect themselves
150 by shaping their xylem microbiome in a resistant wild olive genotype as a first step to
151 assess the complex plant–microbe interactions in the xylem that can contribute to
152 maintain olive health and its resistance response against vascular pathogens. The
153 specific objectives of this work were to determine whether: i) *in vitro* propagation
154 methodology can modify the diversity and composition of the xylem microbiome in
155 olive; ii) those changes may alter host resistance response to the vascular pathogen *Vd*;
156 and iii) inoculation and vascular infection of olive by *Vd* may induce changes in the
157 olive xylem microbiome. Determining those effects may be essential to identify
158 microbial communities that are triggered after pathogen infection and could act as
159 antagonists against *Vd*. Furthermore, understanding the tight relationships between
160 xylem-inhabiting microorganisms and vascular pathogens will help to reveal
161 determinant microbial players that may contribute to produce olive plants more resilient
162 to infection by vascular pathogens.

163

164

165 **Material and methods**

166 **Olive Plant material**

167 A wild olive (*Olea europaea* var. *sylvestris*) clone ‘Ac-18’ highly resistant to the D
168 pathotype of *Vd* (Jiménez-Fernández et al., 2016) was used in this study. The highly
169 resistance of ‘Ac-18’ plants to D *Vd* is characterized by symptomless infection, together
170 with plugging of xylem vessels, no re-isolation of the fungus from stem vascular tissue
171 and the plant’s ability to quantitatively reduce the extent of stem colonization by the
172 pathogen (Jiménez-Fernández et al., 2016). Also, olive cv. ‘Picual’ highly susceptible to
173 D and susceptible to ND *Vd* pathotypes (Calderon et al., 2014), and a wild olive clone
174 ‘Ac-15’ highly susceptible to D *Vd* (Narváez et al., 2019) were used in the pathogenicity
175 experiments as controls of disease reaction.

176 Three types of ‘Ac-18’ plant material were used in the study, which were
177 derived from shoots of a same mother adult plant, named: i) *in vitro*-standard plants:
178 plants micropropagated using the standard olive methodology of axillary shoot
179 elongation; ii) *in vitro*-adapted plants: plants micropropagated and subsequently adapted
180 to greenhouse conditions; and iii) nursery propagated: plants propagated following
181 standard semi-woody stacking procedure at a commercial olive nursery.

182 For *in vitro*-standard propagation of ‘Ac-18’ and ‘Ac-15’ plants, 1.5 to 2.0 cm
183 long shoots bearing two nodes were multiplied as in Narváez et al. (2020) using RP
184 medium [DKW macro- and micronutrients and vitamins as modified by Roussos and
185 Pontikis (2002)] supplemented with 2 mg/L zeatin riboside (Vidoy-Mercado et al.,
186 2012). For rooting, 2-cm long shoots were cultured for 3 days in basal RP liquid
187 medium supplemented with 10 mg/L IBA and subsequently transferred to basal solid
188 RP medium supplemented with 1 g/L activated charcoal. Plants were incubated under a
189 16 h photoperiod at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ at $25 \pm 2^\circ\text{C}$ for 8 weeks until ensuring at least three
190 to four roots. Adaptation to *ex vitro* conditions was carried out initially using 35x24x18
191 cm germination boxes under high levels of sterility. Propagated ‘Ac-18’ rootlets were
192 transplanted to 300 ml pots filled with a sterilized (121°C for 20 min) perlite and
193 vermiculite (1:1) mixture. Plants were sprayed with sterile water, and the cover was
194 sealed with transparent film, and incubated at $25 \pm 2^\circ\text{C}$ in darkness in a growth chamber.
195 After 2 days, a 2-h cycle of indirect fluorescent light of $360 \mu\text{mol m}^{-2} \text{s}^{-1}$ was provided,
196 which duration was increased daily until reaching a 12-h light cycle within 1 week. The

starting high relative humidity provided by the closed environment made unnecessary watering the plants for 3 weeks. Afterwards, plants were watered (3 ml per pot) weekly with sterile water using a 10-ml syringe and fertilized once per month with Hoagland's nutrient solution (Hoagland and Arnon, 1950). After 8 weeks, plants were transplanted to 500 ml pots filled with a sterile peat:perlite (3:1) mixture. Plants grew for additional 6 months in a growth chamber adjusted to $22\pm 2^{\circ}\text{C}$, 60–80% relative humidity and a 14 h photoperiod of fluorescent light of $360\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$. Plants were watered as needed and fertilized weekly with 100 mL Hoagland's nutrient solution.

In vitro-adapted 'Ac-18' plants were produced using the same methodology than for *in vitro*-standard plants, with the exception that plants were grown for additional 12 months in a glass greenhouse at a fluctuating minimum/maximum temperature of 15 ± 5 and $25\pm 5^{\circ}\text{C}$ across the entire growing period and daylight conditions. Plants were watered using tap water as needed and fertilized weekly as described before.

Finally, nursery propagated 'Ac-18' and 'Picual' plants followed standard semi-woody stacking procedure at a commercial olive nursery (Plantas Continental S.A, Córdoba, Spain). Briefly, semi-hard stem cuttings with two active leaves on the top were dipped in an indole butyric acid solution to stimulate rooting and planted on peat:coconut fiber (1:5) pellets under mist conditions in plastic tunnels (Caballero and Del Río, 2010). Once callus was formed and roots appeared (three to four roots) plants were transplanted to 500 ml pots containing a perlite:coconut fiber:peat (1.5:5:3.5) mixture amended with $4\ \text{g l}^{-1}$ of slow release fertilizer (Osmocote® Exact standard 15-9-12+2MgO; ICL Specialty Fertilizers, The Netherlands). Plants were incubated under natural environmental conditions for 6 months in a plastic greenhouse. During this time, plants received water as needed but no additional fertilizers.

At the time of inoculation with the pathogen *in vitro*-standard, nursery propagated and *in vitro*-adapted 'Ac-18' plants were 10-, 10- and 18-months old, respectively. Apparently, plants from all type of propagation procedures had a similar degree of bark lignification of the main stem and root development (**Supplementary Fig. 1**).

Pathogenicity experiment

228 A monoconidial *Vd* isolate (V138I) from defoliated ‘Coker 310’ cotton plants at
 229 Córdoba (Spain), and representative of the D pathotype was used in the experiment.
 230 This isolate proved highly virulent on olive in previous work (Jiménez-Fernández et al.,
 231 2016). Inoculum consisted of an infested cornmeal-sand mixture (CMS;
 232 sand:cornmeal:deionized water, 9:1:2, w/w) produced as described by Jiménez-
 233 Fernández et al. (2016). The infested CMS was homogenized, allowed to desiccate in an
 234 incubator adjusted to 33°C for 3 days, and thoroughly mixed with a pasteurized soil
 235 mixture (clay loam:peat, 2:1, v/v) at a rate of approximately 1:20 (w/w) to reach an
 236 inoculum density of 5×10^7 CFU g⁻¹ soil of *Vd* as determined by dilution-plating on
 237 chlortetracycline-amended water agar (CWA; 1 L distilled water, 20 g agar, 30 mg
 238 chlortetracycline) (Jiménez-Fernández et al., 2016).

258 Plants of ‘Ac-18’ clone, representative of each propagation procedure, and
 259 susceptible ‘Picual’ and ‘Ac-15’ plants, were then transplanted to 1500 ml pots filled
 260 with the D *Vd*-infested soil mixture. Before transplanting, plants were uprooted from the
 261 potting substrate, gently shaken to retain only the rhizosphere soil and placed in pots
 262 filled with the infested soil mixture. Non-inoculated plants were treated similarly and
 263 transplanted to the pasteurized soil mixture mixed with non-infested CMS at the same
 264 rate as infested CMS. Inoculated and control plants were incubated in a growth chamber
 265 adjusted to 22±2°C, 60–80% relative humidity and a 14-h photoperiod of fluorescent
 266 light of 360 μmol m⁻² s⁻¹ for 3 months. During this time, plants were watered as needed
 267 with tap water and fertilized weekly as previously described. There were six replicated
 268 pots (one plant per pot) for inoculated and non-inoculated plants of each plant genotype,
 269 respectively, in a completely randomized design.

270 Disease reaction was assessed by the incidence (percentage of plants showing
 271 disease symptoms) and severity of foliar symptoms. Symptoms were assessed on
 272 individual plants on a 0 to 4 rating scale according to the percentage of affected leaves
 273 and twigs at 2- to 3-day intervals throughout the duration of the experiment (Jiménez-
 274 Fernández et al., 2016). Upon termination of the experiment, the extent of colonization
 275 by *Vd* was determined by isolations of the fungus in CWA (Jiménez-Fernández et al.,
 276 2016) from 6-cm-long stem pieces sampled from the main stem at the same time than
 277 similar samples were processed for extraction of xylem microbiome (see below). Data
 278 of pathogen isolation from the stem were used to calculate the intensity of stem vascular

colonization for each individual plant, according to a stem colonization index (SCI) as described before (Jiménez-Fernandez et al., 2016).

Additionally, the amount of *Vd* present in ‘Ac-18’ stem samples was determined by using the TaqMan qPCR assay developed by Bilodeau et al. (2012) as described in Gramaje et al. (2013). The same DNA samples used for the xylem microbiome characterization were used for pathogen quantification, with each sample being analyzed in duplicate. All qPCR assays were performed in a LightCycler480 (Roche Diagnostics) apparatus. The cycle threshold (Ct) values for each qPCR reaction was calculated using the default estimation criteria in the manufacturer’s software. The quantification limit of the TaqMan qPCR assay was fixed at a Ct of 36 (0.1 pg of *Vd* DNA μl^{-1}) (Gramaje et al., 2013).

DNA xylem microbiome extraction and sequencing

The xylem microbiome was extracted following the procedure described by Anguita-Maeso et al. (2020). Briefly, a number of three 6-cm-long pieces from the bottom, middle and upper stem of each ‘Ac-18’ plant were debarked and xylem chips were obtained by scraping the most external layer of the debarked woody pieces with a sterile scalpel. Xylem chips from a ‘Ac-18’ plant were mixed together, and a 0.5-g sample was placed in a Bioreba bag containing 5 ml of sterile phosphate-buffered saline (PBS), the bags were closed with a thermal sealer and the content was macerated with a hand homogenizer (BIOREBA, Reinach, Switzerland). Extracts were stored at -80°C until DNA extraction. All the processes described above took place under sterile conditions within a flow hood chamber (Anguita-Maeso et al., 2020). Aliquots (0.5 ml) of macerated xylem chips in PowerBead tubes (DNeasy PowerSoil Kit, QIAGEN) were homogenized 7 min at 50 pulses s^{-1} with the TissueLyser LT (QIAGEN) prior to incubation in lysis buffer for 1 h at 60°C for increasing cell lysis, and then processed following the DNeasy PowerSoil Kit manufacturer's protocol.

Extracted DNA was used directly to amplify the V5-V6 rRNA region with the primers 799F (5’-AACMGGATTAGATACCCCKG-3’) and 1115R (5’-AGGGTTGCGCTCGTTG-3’). PCR products were purified using Agencourt AMPure XP (Beckman Coulter) following manufacture instructions. Purified PCR products were quantified using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific)

311 and a Tecan Safire microplate reader (Tecan Group, Männedorf, Switzerland).
 312 Equimolecular amounts from each individual sample in 10 mM of Tris were combined
 313 and the pooled library was sequenced by the Genomics Unit at 'Fundación Parque
 314 Científico de Madrid', Madrid, Spain using the Illumina MiSeq platform (Nano-V2; PE
 315 2x 250 bp). The ZymoBIOMICS microbial standard (Zymo Research Corp., Irvine, CA,
 316 USA) and water (no template DNA) were used as internal positive and negative
 317 controls, respectively, for library construction and sequencing. Raw sequence data have
 318 been deposited in the Sequence Read Archive (SRA) database at the NCBI under
 319 BioProject accession number PRJNA679263.

320

321 **Statistical and bioinformatics analyses**

322 Quality control and adapter trimming of demultiplexed raw fastq 16S rRNA sequences
 323 obtained from MiSeq output was performed with FastQC and TrimGalore tools.
 324 Truncation length in 225 bp of the forward and reverse reads was needed to increase the
 325 Phred quality ($Q > 30$) score visualized in MultiQC tool. No trimming length was
 326 needed. Quality reads were then analyzed using the Quantitative Insights into Microbial
 327 Ecology bioinformatics pipeline, QIIME2 (version 2020.2; [https:// view.qiime2.org/](https://view.qiime2.org/))
 328 (Caporaso et al., 2010; Bolyen et al., 2019) with default parameters unless otherwise
 329 noted. DADA2 pipeline were used for denoising fastq paired-end sequences along with
 330 filtering chimeras. Operational taxonomic units (OTUs) were obtained at 99% of
 331 similarity and were taxonomically classified using VSEARCH consensus taxonomy
 332 classifier (Rognes et al., 2016) against Silva SSU v.138 database. Singletons were
 333 discarded for taxonomy and statistical analysis.

334 Differences in bacterial communities were calculated in QIIME2 using
 335 rarefaction curves of alpha-diversity indexes (Richness, Shannon, and Simpson) at OTU
 336 level. Alpha and beta diversity as well as alpha rarefaction curves were conducted
 337 rarefying all samples to the minimum number of reads found. The nonparametric
 338 Scheirer–Ray–Hare test ($P < 0.05$) was used to assess the effects of the inoculation
 339 treatment, propagation method and their interaction in alpha diversity indexes, using the
 340 rcompanion package (Mangiafico, 2020) in R. Dunn's Kruskal–Wallis multiple
 341 comparisons were performed for post hoc analysis. The P -value was adjusted with the
 342 Benjamini–Hochberg method (Benjamini and Hochberg, 1995). Venn diagrams were

generated using Venn package (Dusa, 2018) in R were used to identify shared (core microbiome) or unique taxa according to the inoculation treatment and propagation methods. Linear discriminant analysis effect size (LEfSe) method to identify differentially abundant bacterial taxa associated to inoculation treatments and propagation methods (Segata et al., 2011).

Additionally, weighted UniFrac distances were estimated at OTU level taking into account the phylogenetic distance among bacterial communities (Lozupone and Knight, 2005). Principal coordinates analysis (PCoA) of weighted UniFrac distance matrix was used to evaluate similarities among the bacterial communities according to the inoculation treatment or propagation procedure. Additionally, the *adonis* function within the vegan package in R (999 permutations) was performed to test the effects ($P < 0.05$) of the inoculation treatment, the plant propagation method and their interaction.

Results

Pathogenicity experiment

Typical Verticillium wilt symptoms consisting on early dropping of green leaves, and necrosis and death of some branches, characteristics of infection by *D. Vd*, started to develop in ‘Ac-15’ and ‘Picual’ plants, as expected, by 21 and 27 days after pathogen inoculation, respectively (**Supplementary Figure S1**). The mean incubation period was of 21.5 ± 1.7 and 29.5 ± 1.7 days for ‘Ac-15’ and ‘Picual’, respectively. All ‘Picual’ and ‘Ac-15’ plants were dead by 2 months after inoculation. On the contrary, no symptoms developed on nursery and *in vitro*-adapted propagated ‘Ac-18’ plants as expected (Jiménez-Fernández et al., 2016). Surprisingly, plants of ‘Ac-18’ that underwent *in vitro* propagation under aseptic conditions (i.e., *in vitro*-standard) started to develop disease symptoms 29 after inoculation (mean incubation period of 33.8 ± 1.8 days), reaching a disease incidence of 100% and a final disease severity of 1.95 ± 0.6 on a 0-4 rating scale at the end of the experiment (**Figure 1; Supplementary Figure S1**). *Vd* was not re-isolated from any of the stem zones sampled from ‘Ac-18’ nursery and *in vitro*-adapted propagated plants, but was isolated from all ‘Ac-18’ *in vitro*-standard propagated plants, with a mean SCI value of $80.95 \pm 6.55\%$. No symptoms developed on non-inoculated control plants (**Supplementary Figure S1**).

In parallel with the *Vd* isolations, the pathogen was detected in DNA samples from all *in vitro*-standard propagated plants, with mean Ct values of 28.84 ± 0.18 . In contrast, *Vd* was not detected in DNA samples of any of the nursery propagated plants; and only in three samples from the *in vitro*-adapted propagated plants, that showed Ct values slightly above the detection limit (i.e. 37.0 ± 0.30).

Alpha and beta diversity analysis

Sequencing analysis resulted in a total of 19,936 good quality reads with a mean length of 333 bp after removal of chimeras, unassigned or mitochondrial reads. No chloroplasts reads were detected in our samples. A total of 118 OTUs were identified for all treatments, with 18 OTUs being retained for alpha and beta diversity analysis after rarefying all data to the minimum number of reads obtained and singleton removal. High values of Good's coverage were obtained for all samples (*data not shown*) indicating enough sequencing depth.

Rarefaction curves of observed OTUs clearly showed a higher number of OTUs on *Vd*-inoculated plants when compared to that on non-inoculated plants, with lower differences among propagation methods within them (**Supplementary Figure S2**). Similarly, the Scheirer–Ray–Hare test indicated significant differences ($P < 0.05$) for all alpha-diversity indexes (Richness, Shannon, and Simpson) according to the inoculation treatment ($H > 9.94$, $P < 0.002$), with no significant differences ($P \geq 0.05$) among propagation methods ($H < 5.14$, $P > 0.076$) or its interaction with the inoculation treatment ($H < 1.08$, $P > 0.581$) (**Figure 2; Supplementary Table S1**). Interestingly, inoculation with *Vd* significantly increased the number of OTUs identified in all types of propagation (**Figure 1; Supplementary Figure S2**).

PCoA of weighted UniFrac distances differentiated xylem bacterial communities according to the inoculation treatment. Thus, with a few exceptions, there was a clear trend to group the bacterial communities firstly by the presence of *Vd* in the soil mixture, and then according to the propagation method, with *in vitro*-adapted and nursery propagated plants being more similar between them (**Figure 3**). ADONIS analysis indicated a significant effect of the propagation method ($R^2 = 0.268$, $P < 0.001$) followed by the inoculation treatment ($R^2 = 0.112$, $P = 0.004$), with no interaction effect ($R^2 = 0.062$, $P = 0.175$) (**Supplementary Table S2**).

406

407 **Composition of olive xylem bacterial communities**

408 A total of 10 phyla, 15 classes, 45 orders, 67 families, 103 genera and 118 OTUs were
 409 identified considering all treatments, with four phyla, five classes, 11 orders, 11
 410 families, 10 genera and five OTUs being shared among all of them (**Supplementary**
 411 **Figure S3**). When analyzing the samples according to the inoculation procedure, a
 412 higher number of OTUs was found in 'Ac-18' plants inoculated with *Vd* (94), while 61
 413 were found on non-inoculated plants. This trend of detecting higher number of taxa on
 414 *Vd*-inoculated plants was observed at all the taxonomic levels. Interestingly, the lowest
 415 number of OTUs from all taxonomy ranks was found in non-inoculated plants
 416 propagated under *in vitro* conditions. Additionally, *in vitro*-adapted plants shared higher
 417 number of OTUs with nursery than with *in vitro*-standard propagated plants
 418 (**Supplementary Figure S3**).

419 At genus level, a total of 18 genera formed the core microbiome of *Vd* -
 420 inoculated plants when considering all propagation methods jointly, whereas 10 genera
 421 were shared within non-inoculated plants. Those same 10 genera were shared between
 422 *Vd*-inoculated and non-inoculated treatments (*Acidibacter*, *Anoxybacillus*,
 423 *Bradyrhizobium*, *Corynebacterium*, *Cutibacterium*, *Methylobacterium-Methylobacterium*,
 424 *Paenibacillus*, *Pseudomonas*, *Sphingomonas*, *Staphylococcus*), while eight genera
 425 (*Acinetobacter*, *Caulobacter*, *Comamonadaceae*, *Dermacoccus*, *Flavisolibacter*,
 426 *Massilia*, *Paracoccus*, *Sericytochromatia*) were detected exclusively in *Vd*-inoculated
 427 plants. In addition, a higher number of genera was found in *Vd*-inoculated plants (82)
 428 compared to those in non-inoculated plants (53) (**Figure 4A**).

429 When analyzing only non-inoculated plants, 25 and 27 genera were identified in
 430 *in vitro*-standard and *in vitro*-adapted propagated plants, respectively, while 29 genera
 431 were identified in nursery propagated plants. Additionally, a high number of unique
 432 genera were found in each propagation method. A total of 12 unique genera were
 433 identified for *in vitro*-standard propagated plants (*Adhaeribacter*, *Bacillus*,
 434 *Bifidobacterium*, *Burkholderia-Caballeronia-Paraburkholderia*, *Cupriavidus*,
 435 *Curtobacterium*, *Eremococcus*, *Escherichia-Shigella*, *Frigoribacterium*, *Kocuria*,
 436 *Sphingopyxis*, *Variovorax*), 12 for nursery propagated plants (*Bergeyella*,
 437 *Brevibacillus*, *Caulobacteraceae*, *Deinococcus*, *Hymenobacter*, *Nevskia*, *Phreatobacter*,

438 *Propionibacteriaceae*, *Pseudokineococcus*, *Psychrobacter*, *Sediminibacterium*,
 439 *Spirosoma*) and 11 for *in vitro* adapted plants (*Aeromonas*, *Dialister*, *Haemophilus*,
 440 *Lawsonella*, *Leptotrichia*, *Micrococcus*, *Paracoccus*, *Peptoniphilus*, *Prevotella*,
 441 *Saccharimonadales*, *Sphingobium*) (**Figure 4B**). On the other hand, when analyzing
 442 each propagation methodology for Vd-inoculated plants 45, 42 and 42 genera were
 443 identified in *in vitro*-standard, *in vitro*-adapted and nursery propagated plants,
 444 respectively. Unique genera differed according to the propagation approach. Thus, a
 445 total of 19 unique genera were found in *in vitro*-standard propagated plants (*CAG-352*,
 446 *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Azospirillum*, *Blautia*,
 447 *Eremococcus*, *Faecalibacterium*, *Frigoribacterium*, *Leuconostoc*, *Modestobacter*,
 448 *Mucilaginibacter*, *Niastella*, *Pediococcus*, *Raoultella*, *Solirubrobacter*,
 449 *Stenotrophomonas*, *unc.-Chitinophagales*, *unc.-Oxalobacteraceae*, *unc.-*
 450 *Solimonadaceae*, *Variovorax*), 16 genera were exclusive from *in vitro*-adapted
 451 propagated plants (*Aeromonas*, *Atopobium*, *Brevibacillus*, *Candidatus_Nitrotoga*,
 452 *Carnobacterium*, *Cloacibacterium*, *Dialister*, *Fimbriimonadaceae*, *Gardnerella*,
 453 *Gemella*, *Haemophilus*, *hgcI_clade*, *Hyphomicrobium*, *Pelistega*, *Streptococcus*, *Vibrio*)
 454 and 18 from nursery propagated plants (*Actinomyces*, *Aeromicrobium*, *Agromyces*,
 455 *Aureimonas*, *Bosea*, *Burkholderia-Caballeronia-Paraburkholderia*, *Caulobacteraceae*,
 456 *Empedobacter*, *Enhydrobacter*, *Exiguobacterium*, *Fusobacterium*, *Klenkia*,
 457 *Lachnospiraceae*, *Marmoricola* *Neisseria*, *Pseudokineococcus*, *Ralstonia*, *Rothia*)
 458 (**Figure 4B**).

459

460 **Bacterial abundance**

461 At phylum level, *Actinobacteriota* presented the highest relative abundance considering
 462 all experimental treatments together (43.62%), followed by *Proteobacteria* (38.72%),
 463 *Firmicutes* (15.24%) and *Bacteroidota* (1.63%). However, these relative abundances
 464 varied within each treatment tested. *Actinobacteria* was more abundant in non-
 465 inoculated plants, with a proportion of 46.41%, decreasing to 40.82% for Vd-inoculated
 466 plants. For non-inoculated plants, this Phylum was the most abundant for *in vitro*-
 467 adapted propagated plants (70.98%), followed by nursery (52.21%) and *in vitro*-
 468 standard (16.04%) propagation methods; showing the same trend in Vd-inoculated
 469 plants, in which reached 58.56, 39.16 and 24.73%, for these same propagation methods,

470 respectively. *Proteobacteria* were present at similar percentage in non-inoculated
471 (40.77%) and *Vd*-inoculated (36.66%) plants, but varied within propagation methods.
472 Thus, it was the most abundant phylum for the *in vitro*-standard plants both for non-
473 inoculated (75.86%) and *Vd*-inoculated plants (49.94%), being lowest for *in vitro*-
474 adapted propagated plants with 18.79 and 22.12%, for non- and *Vd*-inoculated plants,
475 respectively. Finally, *Firmicutes*, showed a different response to both, inoculation
476 treatment and propagation methods compared to the two previous Phyla. Firstly, it
477 showed the highest global abundance in *Vd*-inoculated plants with 18.92%, that
478 decreased to 11.56% in non-inoculated plants. Secondly, while in non-inoculated plants,
479 the highest abundance was estimated in nursery propagated plants (17.53%), it
480 represented ca. 8.57% in both *in vitro* propagation methods; but similar abundance
481 values were reached in *Vd*-inoculated plants (17.61 to 20.34%), irrespective of the plant
482 propagation method (**Figure 5A**).

483 In line with these results, Least discriminant analysis effect size (LEfSe) was
484 used to identify the key phylotypes that could be differentially associated to the
485 different experimental treatments. When comparing the three propagation methods
486 within each inoculation treatment, Proteobacteria-Gammaproteobacteria was the most
487 significant Phylum-Class for non-inoculated *in vitro*-standard propagated plants. For
488 plants growing in *Vd*-infested soils, more diversity was observed among propagation
489 methods, with Proteobacteria-Gammaproteobacteria and Bacteroidota-Bacteroidia, and
490 Actinobacteriota-Actinobacteria being the most prevalent Phylum-Class in *in vitro*-
491 standard and in *in vitro*-adapted propagated plants, respectively, while the Class-Family
492 Proteobacteria-Alphaproteobacteria was the most prominent in nursery propagated
493 plants. On the other hand, when comparing the effect of the inoculation with the
494 pathogen within each propagation method, Firmicutes-Bacilli was a significant Phylum-
495 Class for *Vd*-inoculated plants, in both, *in vitro*-standard and *in vitro*-adapted
496 propagated plants, whereas Proteobacteria-Gammaproteobacteria and Bacteroidota-
497 Bacteroidia were also the prevalent Phyla-Class for *in vitro*-standard and nursery
498 propagated plants, respectively. For non-inoculated plants only a Phylum-Class
499 (Actinobacteriota-Actinobacteria) appeared as the most prominent and only for *in vitro*-
500 adapted plants (**Figure 6**).

501 The most abundant genera identified among all treatments were *Cutibacterium*
502 (36.85%), *Pseudomonas* (20.93%), *Anoxybacillus* (6.28%), *Staphylococcus* (4.95%),

503 *Methylobacterium-Methylobacterium* (3.91%), *Bradyrhizobium* (3.54%),
 504 *Corynebacterium* (2.53%), *Acinetobacter* (1.77%), *Pseudokineococcus* (1.59%) and
 505 *Flavisolibacter* (1.07%) (**Figure 5B**). *Cutibacterium* was the genus with the highest
 506 relative abundance, reaching maximum frequencies in *in vitro*-adapted plants [both in
 507 non-inoculated (67.81%), and *Vd*-inoculated plants (53.04%)]. Other predominant
 508 genera varied in proportion depending upon the treatment combination. *Pseudomonas*
 509 (54.25%) was the most representative genera in non-inoculated plants, propagated by
 510 the *in vitro*-standard method, whereas similar abundances were found for the remaining
 511 treatments (14.66%), with the exception of *in vitro*-adapted non-inoculated plants
 512 (5.77%). A noticeable lower abundance of *Anoxybacillus* was found in non-inoculated
 513 plants from both *in vitro* propagation methods (standard and adapted with 2.42% and
 514 1.06%, respectively) compared with the nursery propagation (7.32%). Also, it was
 515 remarkable the small proportion of *Staphylococcus* found in *in vitro*-standard
 516 propagated and non-inoculated plants (0.52%) compared with the rest of the treatments
 517 (5.84%). Also, *Acinetobacter* showed a high proportion (3.34%) in *in vitro* propagated
 518 plants, but were much less abundant in nursery propagated plants (0.04%) (**Figure 5B**).

519 In line with these results, when comparing the propagation methods within each
 520 inoculation treatment, LEfSe displayed *Cutibacterium* as the only significant genus
 521 when plants were not inoculated, but only for *in vitro*-adapted propagated plants;
 522 whereas for *Vd*-inoculated plants *Anoxybacillus* appeared as a significant genus for all
 523 propagation methods (**Figure 6**). Furthermore, other distinct genera appeared as the
 524 most dominant within each propagation method when *Vd* was infesting the soil
 525 including *Flavisolibacter*, *Methylobacterium-Methylobacterium*, and *Pseudomonas* in
 526 nursery propagated plants; *Corynebacterium*, *Staphylococcus* and *Methylobacterium-*
 527 *Methylobacterium* in *in vitro*-standard propagated plants and *Gardnerella* in *in vitro*-
 528 adapted plants. Finally, when comparing the three propagation methods *Pseudomonas*
 529 appeared as the most dominant genera for *in vitro*-standard propagated plants infected
 530 by *Vd* (**Figure 6**).

531

532 Discussion

533 Olive is a crop of utmost ecological, economic and cultural significance which must be
 534 preserved for next generations (Besnard et al., 2013). However, old and emerging

535 xylem-inhabiting pathogens, such as *Vd* or *Xylella fastidiosa* endanger its health status
 536 nowadays (Jiménez-Díaz et al., 2011; Schneider et al., 2020). The use of host resistance
 537 is the most practical, long-term and economically efficient disease control measure for
 538 Verticillium wilt in olive, and it is at the core of integrated disease strategies that must
 539 be practiced for the efficient management of the disease. Use of wild olive rootstocks
 540 highly resistant to D *Vd* can provide an improved means for the management of
 541 Verticillium wilt, especially for grafting susceptible olive cultivars that are
 542 agronomically adapted, commercially desirable or used in protected designation of
 543 origin extra virgin olive oils (Jiménez-Díaz et al., 2011; Trapero et al., 2012, 2013;
 544 Jiménez-Fernández et al., 2016; Ostos et al., 2020). In addition to that, use of
 545 endophytic plant-associated microorganisms with a specific beneficial interaction with
 546 the host plant could help to improve olive health and productivity providing a potential
 547 perspective for sustainable plant protection (Ryan et al., 2008; Berg, 2009; Berg et al.,
 548 2014; Müller et al., 2015).

549 In olive, several defense mechanisms, including both biochemical responses and
 550 plant structural characteristics, have been proposed as factors contributing to the
 551 resistance shown by different genotypes against its vascular pathogens *Vd* and/or *X.*
 552 *fastidiosa*. Those mechanisms should operate within the xylem tissues contributing to
 553 reduce systemic colonization by the pathogen, and may include build-up of vessel
 554 occlusions by gums, gels or tyloses, phenolics and lignin content or their accumulation,
 555 induction of pathogenesis-related proteins, antioxidant-related enzymes, and ionome
 556 content (Báidez et al., 2007; Markakis et al., 2010; Jiménez-Fernández et al., 2016;
 557 Gharbi et al., 2017a, 2017b; Leyva-Pérez et al., 2017; Luvisi et al., 2017; Sabella et al.,
 558 2017, 2019; D'Attoma et al., 2019).

559 Although some cultivated and wild olive clones, including the 'Ac-18' used in
 560 this study, have been described as resistant to D *Vd* based on symptomless resistance;
 561 for most of them the pathogen could be detected by molecular methods or re-isolated
 562 from stem tissues (Bubici and Cirulli, 2012; Colella et al., 2008; Gramaje et al., 2013;
 563 Jiménez-Fernández et al., 2016) indicating the plant's ability to reduce the extent of
 564 stem colonization or other pathogenesis mechanisms that result in the absence of visible
 565 disease symptoms. However, the role that xylem microbial communities may play in
 566 that resistant response to *Vd* has been overlooked and remains unexplored to date. In
 567 this study, we tested the hypothesis that xylem microbiome may have a functional role

on plant resistance. With that purpose, we explored whether or not *in vitro* propagation of ‘Ac-18’ plants can alter the diversity and composition of xylem-inhabiting bacteria, and to which extent this could result in a modification of the high resistance response of that wild olive clone to the highly virulent D pathotype of *Vd*. Surprisingly, plants that underwent *in vitro* propagation under aseptic conditions lost the high resistance phenotype characteristic of the ‘Ac-18’ clone. Actually, those plants developed wilting symptoms similar to those reported for other olive cultivars with a moderate-susceptible reaction to D *Vd* such as ‘Frantoio’ ‘Oblonga’ ‘Koroneiki’ ‘Empeltre’ or ‘Leccino’ in similar inoculation experiments using olive plants of age similar to that in our study (López-Escudero et al., 2004; Martos-Moreno et al., 2006; Trapero et al., 2013).

Resistance to fungal vascular wilts may change during plant growth and development. Some authors found that disease severity in olive cultivars susceptible to *Vd* decreases with host age (López-Escudero et al., 2010; Trapero et al., 2013). In this present study, the loss of resistance shown by ‘Ac-18’ *in vitro*-standard propagated plants cannot be associated to a more juvenile stage since those plants were of the same age than that of nursery-propagated plants, and showed a similar growth (i.e., similar bark lignification and root development). However, ‘Ac-18’ plants showed distinct xylem microbiome profiles according to the propagation procedure. The most significant change associated to *in vitro*-standard propagation was a decrease in the total number of OTUs detected, and a significantly higher number of Gammaproteobacteria (mainly *Pseudomonas*) and lower number of Actinobacteria (mainly *Cutibacterium*). In parallel, alpha- and beta-diversity indexes of xylem microbiome differed among propagation procedures, with plants that were initially propagated under *in vitro*-standard conditions and then challenged to a less restricted aseptic environmental conditions (i.e., *in vitro*-adapted plants) showing a xylem microbiome more similar with the commercial nursery propagated plants. The olive explants from tissue culture may contain a genotype-specific core xylem microbiome that is transmitted from shoot tips of last generation (Liu et al., 2019). In our study, the explants grew under aseptic conditions and roots that differentiated from them did not contact with outside microbes, at least until the challenge with the pathogen. Thus, most of the differences found in *in vitro*-adapted or nursery propagated plants may be attributed to bacteria that were present at very low level, below the detection limit, at the beginning of micropropagation procedure and could not be detected by NGS. Alternatively, those

601 bacteria might have been acquired by roots after recruitment when plants grew on a
602 non-sterile substrate under less aseptic environmental conditions as proposed for other
603 woody crops, including olive (Antoniou et al., 2017; Fausto et al., 2018; Deyett and
604 Rolshausen, 2019).

605 Plant core microorganism are considered to be consistently established in plants
606 not being influenced by differences in space, time or plant organs (Vorholt et al., 2017).
607 In our study, 10 keystone bacterial genera could be considered the core microbiome
608 being transmitted from generation to generation in olive, since they were detected in all
609 samples regardless plant propagation procedure (*in vitro* vs nursery) or inoculation with
610 the pathogen; with *Cutibacterium*, *Pseudomonas*, *Anoxybacillus*, *Staphylococcus*,
611 *Methylobacterium-Methylorubrum*, and *Bradyrhizobium* being the most abundant, in
612 that order. These bacterial taxa have also been identified in olive xylem in other works
613 in which olive trees of different ages, belonging to different cultivars or growing under
614 different environments were evaluated (Müller et al. 2015; Fausto et al., 2018; Sofo et
615 al., 2019; Anguita-Maeso et al., 2020; Giampetruzzi et al., 2020) which strength the
616 hypothesis that those genera may represent keystone olive xylem bacteria. More
617 interestingly, some of these genera have been already reported or used as plant growth
618 promoting bacteria (Otieno et al., 2015; Subramanian et al., 2015) or as biological
619 control agents against *V. dahliae* (Berg et al., 2002, 2006). Interestingly, the ratio
620 *Cutibacterium/Pseudomonas* seemed to be an important factor associated to the plant
621 propagation procedure. Thus, *Pseudomonas* spp. and *Cutibacterium* were present at
622 high and low abundance, respectively, for *in vitro*-standard propagated plants, that lost
623 resistance to D *Vd*, whereas for *in vitro*-adapted and nursery propagated plants the
624 opposite trend occurred. However, little is known about the role of the genus
625 *Cutibacterium* as a component of plant microbiome, whereas the beneficial functions of
626 *Pseudomonas* spp. in plants have been widely reported for several crops, including olive
627 (Mercado-Blanco et al., 2004; Weller, 2007; Loper et al., 2012).

628 The role of microorganisms in the biocontrol of Verticillium wilt diseases has
629 been reported mostly on non-woody plant species such as cotton, potato, strawberry or
630 tomato (Azad et al., 1985; Nallanchakravarthula et al., 2014; Cao et al., 2016; Wei et
631 al., 2019; Snelders et al., 2020) with few studies focused on woody hosts including
632 olive (Mercado-Blanco et al., 2004; Aranda et al., 2011; Gómez-Lama Cabanás et al.,
633 2018). However, the characterization of microbial communities inhabiting xylem

vessels colonized by *Vd* has not been studied to date, despite some work done on other tree species or other vascular pathogens (Martín et al., 2015; Pérez-Martínez et al., 2018; Giampetruzzi et al., 2020; Vergine et al., 2020). To the best of our knowledge, this present study is the first to address this knowledge gap, by determining changes in xylem bacterial communities of a resistant olive clone after challenge inoculation with *D Vd*. Our results indicated that a significantly higher diversity and number of OTUs occurs in *Vd*-inoculated plants regardless of the plant propagation method and success of stem vascular infection by the pathogen. Additionally, several genera appeared as unique in *Vd*-inoculated plants including *Acitenobacter*, *Caulobacter*, an uncultured Comamonadaceae, *Dermacoccus*, *Flavisobacter*, *Massilia*, *Paracoccus* and *Sericytochromatia*. Also, *Anoxybacillus*; represented a keystone bacterial taxa that significantly increased its frequency after challenge inoculation with the pathogen in all treatments.

The significantly higher xylem microbial diversity in *Vd*-inoculated plants is in line with results from other studies involving vascular pathogens on woody crops. For instance, Deyett and Rolshausen (2019) found higher diversity in *X. fastidiosa*-infected vines as compared to healthier ones. Several of the unique or keystone bacterial genera (e.g., *Acinetobacter*, *Comamonas*, *Caulobacter*, *Massilia*, *Methylobacterium*) detected in our study in the xylem of *Vd* inoculated plants were also found in the xylem of other plant species such as banana, citrus, grapevine and olive, so that those bacteria may be biomarkers of plant infection by vascular-plant pathogens (Araújo et al., 2002; Deyett and Rolshausen, 2019; Liu et al., 2019). The role of these bacterial genera in *Vd*-infected olive plants remains unknown, although several studies suggested that the antifungal activity of *Acinetobacter* or *Dermacoccus* might be involved in pathogen suppression (Liu et al., 2007; AlMatar et al., 2017). *Methylobacterium* isolates have been shown to induce resistance against attack by diverse plant-pathogenic fungi (Rajendran et al., 2009; Ardanov et al., 2012) and modify the response of citrus to the xylem-inhabiting bacterial pathogen *X. fastidiosa*, resulting in a reduction of Citrus Variegated Chlorosis symptoms (Azevedo et al., 2016). *Anoxybacillus*, a keystone bacteria that increased its abundance in pathogen inoculated-plants, can produce several cellulose-, hemicellulose-, lignin- and starch-degrading enzymes, whose activity might have been induced following infection by *Vd* in olive vascular tissue, thus facilitating its population increase (Goh et al., 2013).

667 The general increase in alpha diversity and abundance of specific bacterial taxa
668 observed in our study after challenging with *D. Vd* may be explained by several
669 hypotheses, including: i) a passive entry or direct recruitment of new bacterial species
670 from the plant rhizosphere or soil, taking advantage of injuries caused during root
671 infection and colonization by the pathogen (Ardanov et al., 2012; Liu et al., 2019); ii)
672 the secretion of specific molecules by the pathogen (such as effector proteins) with
673 antimicrobial activity that modify host microbiome to facilitate host colonization
674 (Snelders et al., 2020); and/or iii) the pathogen provokes a series of host physiological
675 responses that trigger multiplication of a specific microbiome to cope with the pathogen
676 infection in order to mitigate its effect (Ardanov et al., 2012; Hassani et al., 2018;
677 Carrión et al., 2019). These hypotheses emphasize the need for better understanding of
678 the changes occurring in xylem microbial communities in response to vascular infection
679 by pathogens, in order to determine specifically activated disease-suppressive and/or
680 plant-protecting microbiome-mediated activities in olive.

681 Our study provides new insights for the characterization of changes occurring in
682 the xylem microbial communities of a wild olive genotype following inoculation with
683 the vascular plant pathogen *Vd*. Also, it provides a quantitative and qualitative
684 assessment of the effect of specific propagation methods where the attenuation and
685 reduction of olive xylem microbiome is a unique approach described to date. We are
686 aware of limitations in our study, in part because only some propagation methods and a
687 single host genotype were evaluated. However, this present research is relevant for
688 future studies on the olive xylem microbiome that may lead to identification of xylem-
689 inhabiting bacteria potentially involved in host resistance and plant defense by acting as
690 biocontrol agents against xylem-inhabiting pathogens. Deciphering the core olive xylem
691 microbiome and their correlation with the host plant and its pathogens is a first critical
692 step for exploiting the microbiome in order to enhance olive growth and health.

693

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1006 **Figure legends**

1007 **Figure 1.** *Verticillium* wilt disease progression in ‘Ac-18’ *in vitro* (standard and
1008 adapted) and nursery propagated olive plants infested with the defoliating pathotype of
1009 *Verticillium dahliae*. ‘Picual’ and ‘Ac-15’ olive genotypes were used as positive control
1010 to determine the inoculation success and development of the disease. Each point
1011 represents the mean disease severity (0–4 scale: 0= healthy, 4= dead plant) of data
1012 and error bars shows the standard error from six plants per treatment.

1013 **Figure 2.** Boxplots of Richness, Simpson and Shannon alpha diversity indices at OTU
1014 taxonomic level in olive xylem from *Verticillium dahliae* (Vd)-inoculated and non-
1015 inoculated ‘Ac-18’ plants following *in vitro* (standard and adapted) and nursery
1016 propagation methods. Boxes represent the interquartile range while the horizontal line
1017 inside the box defines the median and whiskers represent the lowest and highest values
1018 of six values for each treatment combination. For all three indexes and propagation
1019 methods, values on Vd-inoculated plants were significantly higher compared to that on
1020 non-inoculated treatments according to the Scheirer–Ray–Hare test at $P < 0.05$.

1021 **Figure 3.** Principal coordinates plot of weighted UniFrac distances of bacterial
1022 communities at OTUs taxonomic level in olive xylem from *Verticillium dahliae* (Vd)-
1023 inoculated and non-inoculated ‘Ac-18’ plants following *in vitro* (standard and adapted)
1024 and nursery propagation methods. Points are colored by plant inoculation treatment and
1025 shaped by propagation methods.

1026 **Figure 4.** Prevalence Venn diagram showing the unique and shared bacterial genera in
1027 olive xylem from the core microbiome (A) or by each propagation approach (B)
1028 obtained from *Verticillium dahliae* (Vd)-inoculated and non-inoculated ‘Ac-18’ plants
1029 following *in vitro* (standard and adapted) and nursery propagation methods.

1030 **Figure 5.** Barplots showing the relative bacterial abundance taxa at phylum (A) and
1031 genera (B) level present in olive xylem from *Verticillium dahliae* (Vd)-inoculated and
1032 non-inoculated ‘Ac-18’ plants following *in vitro* (standard and adapted) and nursery
1033 propagation methods.

1034 **Figure 6.** Cladogram representation from LEfSe analysis showing the taxonomic ranks
1035 from innermost phylum ring to outermost genera ring. Each point is a member within
1036 each taxonomic rank. Significant taxa ($P < 0.05$) by inoculation treatment (Vd:

1037 *Verticillium dahliae* (Vd)-inoculated; NI: non-inoculated plants) or by propagation
1038 method (*in vitro* standard and adapted, and nursery propagated plants) are shown in
1039 different colors.

1040

1041

1042 **Supplementary Material**

1043 **Figure S1.** Effect of inoculation with the defoliating pathotype of *Verticillium dahliae*
1044 in ‘Picual’, ‘Ac-15’ and ‘Ac-18’, plants obtained using *in vitro*-standard, *in vitro*-
1045 adapted and nursery propagation methods. ‘Picual’ and ‘Ac-15’ were used as positive
1046 control to determine the inoculation success and the development of the disease. Note
1047 the defoliation of green leaves observed for *in vitro*-standard propagated ‘Ac-18’ plants.

1048 **Figure S2.** Richness rarefaction curves at OTU taxonomic level in olive xylem from
1049 *Verticillium dahliae* (Vd)-inoculated and non-inoculated (NI) ‘Ac-18’ plants following
1050 *in vitro* (standard and adapted) and nursery propagation methods. Error bars represent
1051 standard error of six values.

1052 **Figure S3.** General prevalence Venn diagram showing the unique and shared bacteria at
1053 different taxonomy ranks in olive xylem from *Verticillium dahliae* (Vd)-inoculated and
1054 non-inoculated (NI) ‘Ac-18’ plants following *in vitro* (standard and adapted) and
1055 nursery propagation methods.

A

B

A **B**

By inoculation treatment

NI-Adapted
NI-Standard

Non-Inoculated

a: Cutibacterium
b: Propionibacteriaceae
c: Propionibacteriales

Vd-Inoculated

a: Cutibacterium
b: Propionibacteriaceae
c: Propionibacteriales
d: Rhizobiales
e: Pseudomonas
f: Pseudomonadaceae

Vd-Adapted
Vd-Standard
Vd-Nursery

By propagation method

Standard

a: Corynebacterium
b: Corynebacteriaceae
c: Corynebacteriales
d: Chitinophagaceae
e: Chitinophagales
f: Anoxybacillus
g: Staphylococcus
h: Staphylococcaceae
i: Staphylococcales
j: Methylobacterium_Methyl
k: Beijerinckiaceae

Adapted

a: Gardnerella
b: Bifidobacteriaceae
c: Bifidobacteriales
d: Cutibacterium
e: Propionibacteriaceae
f: Propionibacteriales
g: Anoxybacillus
h: Bacillaceae
i: Bacillales

Nursery

a: Flavobacter
b: Chitinophagaceae
c: Chitinophagales
d: Anoxybacillus
e: Bacillaceae
f: Bacillales
g: Methylobacterium_Methyl
h: Beijerinckiaceae
i: Rhizobiales
j: Pseudomonas
k: Pseudomonadaceae
l: Pseudomonadales